# Interaction of Myosin Phosphatase Target Subunit 1 with the Catalytic Subunit of Type 1 Protein Phosphatase†

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*Received April 7, 1998; Revised Manuscript Received August 17, 1998*

ABSTRACT: In the investigation of the sequences of myosin phosphatase target subunit 1 (MYPT1) involved in binding the substrate and catalytic subunit of protein phosphatase type 1 (PP1c), fragments of MYPT1 were prepared and characterized. The shortest fragment capable of full activation of PP1c contained the sequence of residues 1−295. Within this fragment, the N-terminal sequence of residues 1−38 is involved in activation of PP1c ($k_{cat}$) and the ankyrin repeats (residues 39−295) were involved in substrate binding ($K_m$). The ankyrin repeats alone (residues 39−295) and the C-terminal fragment of residues 667−1004 did not activate PP1c. Using gel filtration, an interaction with PP1c was detected for the sequences of residues 1−295, 17−295, and 1−170. Affinity columns were prepared with various fragments to assess binding of PP1c. Binding to the column with residues 1−295 was strongest, followed by the binding to the column with residues 1−170. A weak interaction was observed with the column with residues 1−38. The column with residues 1−295 was used to isolate PP1c from gizzard. The purified PP1c was activated by MYPT1 and fragments to a greater extent than previous preparations. These results suggest that the N-terminal sequence (residues 1−38) and the ankyrin repeats are involved in binding PP1c. The C-terminal ankyrin repeats appear to be dominant, but there is an interaction of PP1c with the N-terminal ankyrin repeats. The N-terminal peptide has two apparent functions, the binding of PP1c via the consensus binding sequence and activation of PP1c by the sequence of residues 1−16.

Phosphorylation of the 20 kDa light chain of myosin (MLC20)[1] is an important regulatory mechanism in smooth muscle and in many nonmuscle cells (*1*). The level of phosphorylation depends on the relative activities of MLCK and myosin phosphatase (MP). Previously, it was assumed that since MLCK is dependent on $Ca^{2+}$−calmodulin the phosphorylation level followed the $Ca^{2+}$ transients (*1*, *2*). However, it has been shown that under certain conditions the level of myosin phosphorylation for a given $Ca^{2+}$ concentration can vary. The shifts can lead to increased or decreased phosphorylation levels, referred to as increased or decreased $Ca^{2+}$ sensitivity, respectively (*2*). Increased levels of phosphorylation of myosin at a constant $Ca^{2+}$ concentration could reflect either an activation of MLCK (the $Ca^{2+}$−calmodulin−MLCK complex or the MLCK apoenzyme) or an inhibition of MP. The opposite may be suggested to account for a decreased level of phosphorylation of myosin. Increased $Ca^{2+}$ sensitivity, as frequently occurs on agonist stimulation of smooth muscle, was found to be due to an inhibition of MP (*2*). This finding established the important point that MP could be regulated. The mechanism(s) underlying inhibition of MP is not established, but several suggestions have been made, including arachidonic acid (*3*), protein kinase C (*4*), and phosphorylation of MP (*5*) by either an endogenous kinase (*6*) or Rho-associated kinase (*7*). In addition, a phosphorylation-dependent inhibitor of MP has been isolated from smooth muscle (*8*). The molecular mechanism responsible for inhibition, or activation, of MP activity is not established but is assumed to involve changes in subunit interactions.

Smooth muscle MP is composed of three subunits (*9*−*11*): the $\delta$ isoform of the catalytic subunit, PP1c$\delta$ (*10*) [also referred to as the $\beta$ isoform (*9*)]; and large (approximately 110 kDa) and small (approximately 20 kDa) noncatalytic subunits. The large subunit binds to myosin and to PP1c and effects an activation of PP1c activity using P-myosin or P-MLC20 as the substrate (*10*, *12*−*16*). For these reasons, it has been termed the myosin phosphatase target (or targeting) subunit, MYPT1 (*17*). MYPT1 also binds the 20

† This work was supported in part by grants-in aid for Scientific Research, for Cancer Research (to M.I. and T.N.), and for International Scientific Research (to M.I., T.N., and D.J.H.) from the Ministry of Education, Science, Sports and Culture, Japan, by grants from the Naito Foundation, the Mie Medical Research Foundation (to M.I.), the Kanae Foundation of Research for New Medicine (to K.I.), and by grants HL 23615 and HL 20984 from the National Institutes of Health (to D.J.H.).

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[1] Abbreviations: PP1, type 1 protein phosphatase; PP1c, catalytic subunit of PP1; PP1c$\delta$, $\delta$ isoform of PP1c; MP, myosin phosphatase; MYPT1, myosin phosphatase target subunit 1; M130 and M133, 130 000 and 133 000 Da chicken MYPT1, respectively; M20, 20 000 Da subunit of myosin phosphatase; GST, glutathione *S*-transferase; P-myosin, phosphorylated myosin; MLC20, 20 000 Da myosin light chain; P-MLC20, phosphorylated MLC20; MLCK, myosin light chain kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

10.1021/bi980782x CCC: $15.00 
Published on Web 11/04/1998

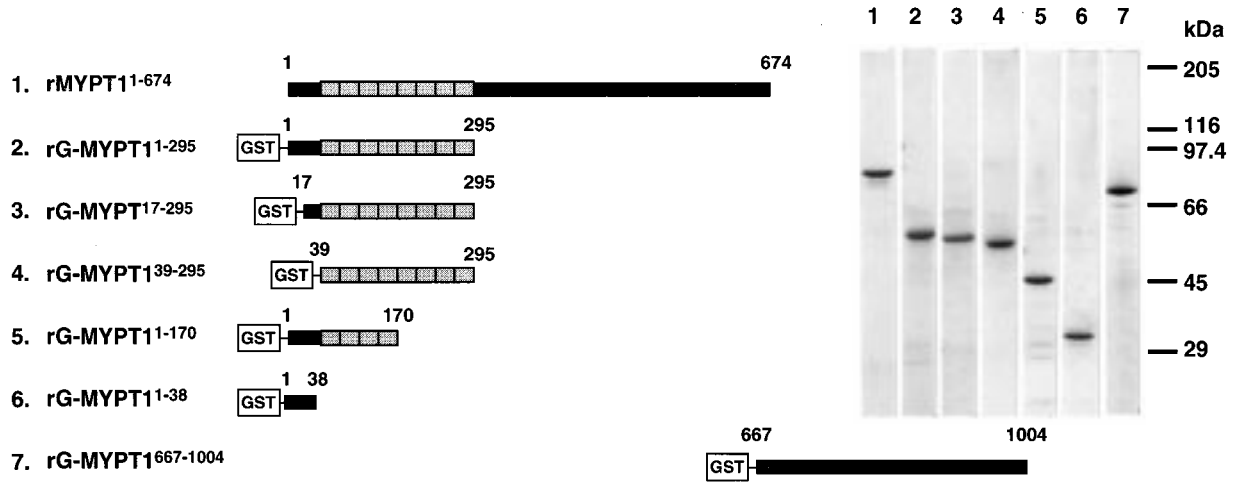


FIGURE 1: Schematic representation of the MYPT1 mutants. The sequences refer to the chicken gizzard M133 isoform (*10*). With the exception of rMYPT1$^{1-674}$, the other mutants were expressed as GST fusion protein. The shaded boxes indicate the eight ankyrin repeats. The inset shows SDS−PAGE of each purified recombinant protein: lane 1, rMYPT1$^{1-674}$; lane 2, rG−MYPT1$^{1-295}$; lane 3, rG−MYPT1$^{17-295}$; lane 4, rG−MYPT1$^{39-295}$; lane 5, rG−MYPT1$^{1-170}$; lane 6, rG−MYPT1$^{1-38}$; and lane 7, rG−MYPT1$^{667-1004}$.

kDa subunit (*10*, *16*, *18*), but the functional significance of this interaction is not known. MYPT1 has been cloned from chicken gizzard (*10*), rat kidney (12), rat aorta and uterus (*18*, *19*), and human aorta and brain (*17*), and a related protein has been described in *Caenorhabditis elegans* (*20*). All of these isoforms possess some similar structural characteristics (reviewed in ref *21*), and dominant among these is an N-terminal series of ankyrin repeats. Since ankyrin repeats are thought be involved in protein−protein interactions (*22*), this region of MYPT1 has been investigated with respect to PP1c and myosin binding (*16*). Both interactions are thought to be necessary for activation of phosphatase activity; however, the regions of MYPT1 involved in this effect have not been established, and reports about them are controversial (*14*−*16*).

In this paper, the activation of PP1c activity and interactions of MYPT1 with PP1c are investigated using a variety of mutants spanning the N-terminal part of MYPT1 and a synthetic peptide corresponding to the sequence of residues 1−38. The results obtained differ from previous reports in that it is shown that the N-terminal sequence (residues 1−38) and the ankyrin repeats are required for full activation of PP1c. In addition, it was found that an affinity column prepared with the fragment containing the sequence of residues 1−295 provided an effective method for purifying PP1c.

## MATERIALS AND METHODS

*Materials.* Chemicals and vendors were as follows: [$\gamma$-$^{32}$P]ATP (NEN Life Science Products); ATP and microcystin-LR (Sigma); DEAE-Sepharose Fast Flow, gluthatione−Sepharose 4B, and pGEX4T-1 vector and thrombin (Pharmacia Biotech, Uppsala, Sweden); bacterial culture media (Difco); LA Taq polymerase (Takara Shuzou); Ligation kit (Nippon Gene); TA cloning kit (Invitrogen); and Affi-gel 15 and Affi-gel 10 (Bio-Rad). Oligonucleotides were synthesized at Japan Bio Service Co. The peptide corresponding to the MYPT1 sequence of residues 1−38 was synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO). All other chemicals were of the highest grade commercially available.

*Expression and Purification of Recombinant Proteins.* Various fragments of the chicken gizzard MYPT1 (M133 isoform) were expressed in *Escherichia coli.* These are referred to as rMYPT1$^{x}$, with *x* denoting the relevant sequence. GST fusion proteins are designated rG−MYPT1$^{x}$. The preparation of rMYPT1$^{1-674}$ and rG−MYPT1$^{667-1004}$, formerly termed rN133$^{1-674}$ (*13*) and rG−M133[667−1004] (*23*), respectively, was described previously. Other mutants (see Figure 1) were expressed as GST fusion proteins. All cDNAs for these truncation mutants were obtained by PCR amplification from clone Z-1 (*10*) as a template. The primers used were as follows. The forward primer for rG−MYPT1$^{1-295}$, rG−MYPT1$^{1-170}$, and rG−MYPT1$^{1-38}$ was 5′-AGGCGGGGAGGGATCCATGAAGATGGCGGACGC-3′ (underlined residues show the *Bam*HI site). The forward primers for rG−MYPT1$^{17-295}$ and rG−MYPT1$^{39-295}$ were 5′-CAGCTGGGATCCTGGATCGGCTCC-3′ and 5′-AAGGTGGGATCCGACGACGGCGCC-3′ (underlined residues show the *Bam*HI site), respectively. The reverse primer for rG−MYPT1$^{1-295}$, rG−MYPT1$^{17-295}$, and rG−MYPT1$^{39-295}$ was 5′-GGGAGATTTCTTTTCACGTTTTTAACTGGTTGGAAG-3′. The reverse primer for rG−MYPT1$^{1-170}$ was 5′-TATTTCCGCTCGAATTCTTATCGAGCTGCCTCTATATC-3′ (underlined residues show the *Eco*RI site). The reverse primer for rG−MYPT1$^{1-38}$ was 5′-CAGGAAGACGGGCGAATTCCTAGAACTTCACCTTGGT-3′ (underlined residues show the *Eco*RI site). Conditions for PCR amplification were 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min for 30 cycles. The fragments subcloned into the pCRII vector were excised by digestion with *Bam*HI and *Eco*RI. The DNA fragments were then ligated into the pGEX4T-1 vector, which had been previously digested with *Bam*HI and *Eco*RI and purified by agarose gel electrophoresis. Each mutant was sequenced to confirm that the PCR-amplified cDNA was identical to the original sequence.

The constructs were used to transform competent *E. coli* BL21(DE3) cells on Luria-Bertani (LB) plates containing 0.1 mg/mL ampicillin, and the plates were incubated at 37 °C for 12 h. Colonies of the cells containing the construct were used to inoculate initially a 5 mL culture containing

ampicillin and subsequently a 2 L culture. The culture was grown at 37 °C until the $OD_{600nm}$ reached 1.0, and then isopropyl $\beta$-D-thiogalactopyranoside was added to 0.1 mM and the culture grown for an additional 3 h at 30 °C. Cells were collected by centrifugation at 4000*g* for 10 min. The pellet was washed with 500 mL of phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.4 mM $KH_2PO_4$ (pH 7.4)] and extracted using a dounce tissue grinder with 50 mL of a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM EDTA, 1 mM dithiothreitol, 10 mM (*p*-amidinophenyl)methanesulfonyl fluoride, and 10 $\mu$M leupeptin. The mixture was sonicated (4 × 30 s bursts with a Branson sonifier). After centrifugation at 100000*g* for 30 min, the GST fusion proteins were purified from the supernatant using glutathione−Sepharose 4B according to the manufacturer's instructions. The GST-free mutants were obtained by cleavage with thrombin. The purified mutants (1 mg/mL) were digested at 25 °C for 25 min with thrombin (1 unit/mg of mutant) in buffer A [30 mM Tris-HCl (pH 7.5) and 30 mM KCl] and 150 mM NaCl and 2.5 mM $CaCl_2$. The digested samples were applied to a Mono Q HR 5/5 column equilibrated with buffer A, and the GST- and thrombin-free mutants were eluted with a linear gradient of 0.2 to 0.6 M NaCl and dialyzed against buffer A.

*Protein Preparations*. Smooth muscle MLC20 (*24*) and P-MLC20 (*6*) were prepared as described previously. PP1c was purified as described in the text using the $rMYPT1^{1-295}$ affinity column. Other protein preparations were as follows: MLCK from frozen chicken gizzard (*25*) and calmodulin from bovine brain (*26*).

*Phosphatase Assay*. Activities were measured at 30 °C using 5 $\mu$M P-MLC20 as the substrate in a buffer containing 30 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 0.5% (v/v) glycerol, and 0.2 mg/mL BSA, unless otherwise indicated. The final volume was 50 $\mu$L. The reactions were initiated by the addition of substrate and terminated by the addition of trichloroacetic acid to 12.5%. After centrifugation at approximately 15000*g* for 3 min, the radioactivity of the supernatant was determined by Cerenkov counting. Phosphatase rates were estimated from the linear portion of the time courses. For practical reasons, the highest concentration of P-MLC20 used was 20 $\mu$M, and $K_m$ values were estimated by extrapolation.

*Binding Assays by Gel Filtration*. Isolated PP1c (0.6−0.7 $\mu$g) was incubated with a 10-fold molar excess of each recombinant protein at 25 °C for 10 min in buffer B [20 mM Tris-HCl (pH 7.5), 150 mM KCl, and 1 mM dithiothreitol]. The final volume was 70 $\mu$L. The amount of PP1c incubated without recombinant proteins was 0.7 $\mu$g. After centrifugation at 15000*g* for 10 min, 50 $\mu$L of the supernatant was applied to a Superdex 200 PC 3.2/1.6 column equilibrated with buffer B (40 $\mu$L/min) attached to a SMART system (Pharmacia Biotech). Each fraction was assayed for phosphatase activity.

*Preparation of Affinity Columns*. The synthetic peptide (residues 1−38, 8 mg), $rMYPT1^{1-295}$ (10 mg), and $rMYPT1^{1-170}$ (8 mg) were dialyzed overnight against 100 mM Hepes (pH 7.5) at 4 °C and coupled to 3 mL of either Affi-gel 15 (for recombinant proteins) or Affi-gel 10 (for peptide) according to the manufacturer's instructions. The coupling reaction was carried out overnight at 4 °C and was terminated by filtration on a sintered glass funnnel followed by washing with Hepes buffer. Unreacted groups were blocked by incubation with 1 mM monoethanolamine at 4 °C for 12 h. The gel was washed thoroughly with Hepes buffer and equilibrated in buffer C [20 mM Tris-HCl (pH 7.5), 0.2 mM EGTA, 2 mM $MgCl_2$, 0.1 mM dithiothreitol, and 5% (v/v) glycerol]. Coupling efficiency was greater than 90% for each of these columns. Control columns were prepared from 3 mL of Affi-gel 15 or Affi-gel 10, and both were blocked with 1 mM monoethanolamine.

*Isolation of PP1c from Chicken Gizzard*. All procedures were performed at 4 °C except the acetone treatment. The procedure through the DEAE-Sepharose Fast Flow chromatography was described previously (*10*). After elution from DEAE-Sepharose, the fractions containing phosphatase activity were adjusted to 50% (v/v) acetone at room temperature. After centrifugation at 10000*g* for 20 min, the pellet was extracted with 10 mL of buffer C and 1 M NaCl and recentrifuged under the above conditions. The supernatant was separated and the pellet re-extracted as above. The combined supernatants were dialyzed against buffer C and loaded onto the $rMYPT1^{1-295}$ Affi-gel 15 column (0.5 cm × 5 cm) at a flow rate of 0.2 mL/min. The column was washed with buffer C, and the bound proteins were eluted in two steps using 0.6 and 3.0 M LiBr in buffer C. Fractions (0.2 mL) were assayed for phosphatase activity. The purified PP1c was dialyzed against 30 mM Tris-HCl (pH 7.5), 30 mM KCl, and 50% (v/v) glycerol and stored at −30 °C. On the basis of the previous data (*10*), the PP1c isolated was predominantly the $\delta$ isoform.

*Immunological Techniques*. The preparation of polyclonal antibodies to PP1c (*27*) and to chicken MYPT1 (*7*) was as described previously. Western analyses were carried out as follows. After SDS−PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 5% (w/v) nonfat dry milk and 0.05% (v/v) Tween 20 for 1 h and then incubated with the polyclonal rabbit antibodies for an additional 1 h at room temperature. The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h, and the immunocomplex was detected by enhanced chemiluminescence (ECL, Amersham).

*Other Procedures*. SDS−PAGE on 7.5 to 20% acrylamide gradient gels was carried out with the discontinuous buffer system of Laemmli (*29*). Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) procedures, using bovine serum albumin or $\gamma$-globulin as the standard.

## RESULTS

*Effect of MYPT1 Fragments on Phosphatase Activity*. The mutants used in this study are shown diagrammatically in Figure 1. The GST moiety was removed (see Materials and Methods) to avoid any influence of GST. The two longer N-terminal fragments ($rMYPT1^{1-674}$ and $rMYPT1^{1-295}$) both activated PP1c activity toward P-MLC20 about 15-fold (Figure 2A). This extent of activation is considerably higher than in previous reports (*13*, *14*, *16*). Maximum activation occurred at about a 4-fold molar excess (mutant to PP1c), although there was considerable activation at a 1:1 molar ratio. The activity of the reconstituted phosphtatase is similar to that of the holoenzyme. For example, the specific activity

Table 1: Kinetic Parameters for PP1c Activity and MYPT1 Fragments[a]

| | rMYPT1$^{1-674}$ | rMYPT1$^{1-295}$ | rMYPT1$^{17-295}$ | rMYPT1$^{1-170}$ | peptide$^{1-38}$ | PP1c$\delta$ |
|---|---|---|---|---|---|---|
| $K_m$ ($\mu$M) | 1.22 ± 0.09 | 1.27 ± 0.22 | 1.63 ± 0.32 | 5.96 ± 3.93 | 11.6 ± 3.3 | 12.7 ± 2.8 |
| $k_{cat}$ (min$^{-1}$)[b] | 2301 ± 250 | 2180 ± 438 | 618 ± 100 | 1866 ± 613 | 1936 ± 445 | 497 ± 151 |

[a] For the mutants, rMYPT1$^{1-674}$, rMYPT1$^{1-295}$, and rMYPT1$^{1-170}$, a molar ratio of 10:1 (mutant:PP1c) was used. For the other mutant, rMYPT1$^{17-295}$, and peptide, a molar ratio of 300:1 was used. The values are mean ± SD ($n \geq 3$). [b] $k_{cat}$ was calculated using a molecular mass of 38 kDa for PP1c.

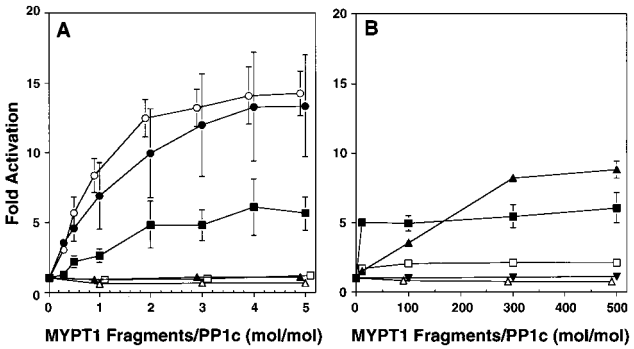


FIGURE 2: Effect of MYPT1 fragments on PP1c activity. Each fragment at the indicated molar ratio with respect to PP1c was incubated at 30 °C for 10 min, and the phosphatase activity of the mixture was determined (see Materials and Methods). The results are presented as the relative activity with respect to PP1c alone. The following GST-free mutants were assayed: rMYPT1$^{1-674}$ (●), rMYPT1$^{1-295}$ (○), rMYPT1$^{17-295}$ (▲), rMYPT1$^{39-295}$ (△), rMYPT1$^{1-170}$ (■), and rG−MYPT1$^{667-1004}$ (▼). The peptide of residues 1−38 (□) also was assayed. The data points represent the mean ± the standard error of the mean ($n = 3$ or 4).

of PP1c$\delta$ and rMYPT1$^{1-674}$ (4-fold molar excess of the mutant) was 7.44 ± 0.96 [mean ± standard deviation (SD), $n = 7$] $\mu$mol of $P_i$ min$^{-1}$ (mg of PP1c$\delta$)$^{-1}$ compared to the specific activities of the holoenzyme of 1.26 ± 0.20 ($n = 4$) and 10.37 ± 1.25 ($n = 3$) $\mu$mol of $P_i$ min$^{-1}$ (mg of PP1c$\delta$)$^{-1}$ (assuming that PP1c$\delta$ is 23% of the mass of the holoenzyme) assayed in the absence and presence of $Co^{2+}$, respectively. The rMYPT1$^{17-295}$ mutant did not activate over the range of concentrations used in Figure 1A, but at high concentrations (about 300-fold molar excess) achieved an 8-fold activation (Figure 2B). These results indicate the importance of the sequence of residues 1−16 of MYPT1 in activation of PP1c. The mutant in which the N-terminal sequence was further shortened, rMYPT1$^{39-295}$, did not activate PP1c even at 500-fold molar excess (Figure 2B). This mutant contained just the ankyrin repeats. The fragment containing the N-terminal half of the ankyrin repeats and the N-terminal peptide, rMYPT1$^{1-170}$, activated PP1c at a reasonable stoichiometry (2−4-fold molar excess) but achieved only a 5-fold activation (Figure 2A). The extent of activation remained constant even at high molar ratios (Figure 2B). The C-terminal fragment, rMYPT1$^{667-1004}$, did not activate PP1c (Figure 2A,B). The N-terminal peptide (residues 1−38) showed a slight activation (about 2-fold), but only at high molar ratios of peptide:PP1c (Figure 2B). Some of the GST fusion fragments were assayed for activation. rG−MYPT1$^{1-295}$ and rG−MYPT1$^{1-170}$ activated PP1c, but the extent of activation was 30−50% less than that of the GST-free mutants (data not shown). For rG−MYPT1$^{17-295}$ and rG−MYPT1$^{1-38}$, no activation was observed even at high mutant concentrations.

Kinetic parameters were determined for PP1c and various mutants and the N-terminal peptide. A 10-fold molar excess of mutant with respect to PP1c was used for rMYPT1$^{1-674}$, rMYPT1$^{1-295}$, and rMYPT1$^{1-170}$, and a 300-fold molar excess was used for rMYPT1$^{17-295}$ and the peptide of residues 1−38. The results are shown in Table 1. The two mutants that achieved the most effective activation of PP1c, rMYPT1$^{1-674}$ and rMYPT1$^{1-295}$, induced a decrease in $K_m$ (about 10-fold) and an increase in $k_{cat}$ (about 4−5-fold). The mutant that caused only restricted activation of about 5-fold, rMYPT1$^{1-170}$, induced a less pronounced decrease in $K_m$ and about a 4-fold increase in $k_{cat}$. An interesting finding was that the mutant lacking the N-terminal 16 residues, rMYPT1$^{17-295}$, caused a marked decrease in $K_m$ but had little effect on $k_{cat}$. For the N-terminal peptide (residues 1−38), the opposite effect was observed, namely, an increase in $k_{cat}$ and no effect on $K_m$.

*Interaction of MYPT1 Fragments with PP1c.* Gel filtration was used to detect interactions between PP1c and the mutants and peptide. PP1c was mixed with a 10-fold molar excess of the putative interactive species. The recombinant proteins used were GST fusion proteins. Several of the mutants caused a shift in the elution profile toward apparent higher molecular masses (phosphatase activity was monitored in the eluate). These were rG−MYPT1$^{1-295}$, rG−MYPT1$^{17-295}$, and rG−MYPT1$^{1-170}$, as shown in panels B, C, and E of Figure 3, respectively. The elution positions of the peaks corresponded to apparent molecular masses of 110 kDa for rG−MYPT1$^{1-170}$ and 130 kDa for the other two mutants. The complex of PP1c and GST-free rMYPT1$^{1-295}$ gave an approximate molecular mass of 60 kDa, and tentatively, this is consistent with a stoichiometry of 1:1 (data not shown). In contrast, the mixtures of rG−MYPT1$^{39-295}$, rG−MYPT1$^{1-38}$, and rG−MYPT1$^{667-1004}$ eluted at the same position as PP1c alone (Figure 3A), i.e., corresponding to about 40 kDa (panels D, F, and G of Figure 3, respectively). A mixture of GST (alone) and PP1c also eluted at the position of the isolated catalytic subunit (compare panels A and H of Figure 3). These results suggest that a complex can be detected between PP1c and rG−MYPT1$^{1-295}$, rG−MYPT1$^{1-170}$, and rG−MYPT1$^{17-295}$.

A second method for investigating the interactions of PP1c was to prepare affinity columns using various mutants or peptide, i.e., rMYPT1$^{1-295}$, rMYPT1$^{1-170}$, and the peptide of residues 1−38 (see Materials and Methods). PP1c (approximately 100 $\mu$g) was loaded onto each column, and after washing, the column was subjected to a linear gradient of LiBr (0 to 3 M). Control columns using just Affi-gel 10 and Affi-gel 15 were also prepared. As shown in Figure 4, PP1c bound to each column, but was eluted at different concentrations of LiBr. PP1c was eluted from the rMYPT1$^{1-295}$, rMYPT1$^{1-170}$, and peptide columns at 2.3, 1.6, and 0.8 M LiBr, respectively. (Phosphatase activity was determined directly in these fractions. After removal of LiBr by dialysis, the total activity in each fraction was equal.) Some nonspecific binding of PP1c was observed with the control Affi-gel columns, and PP1c was eluted at about the

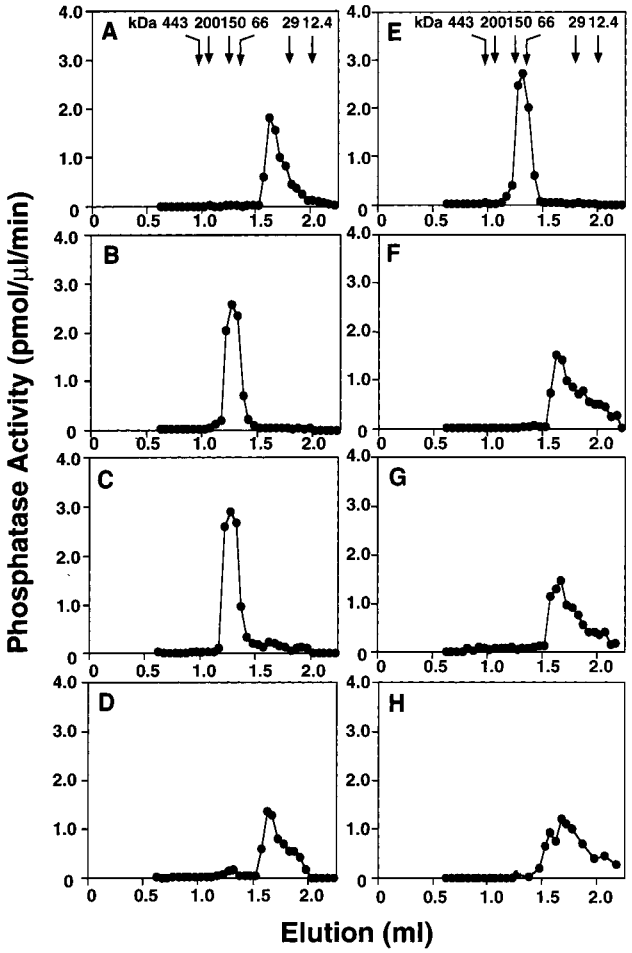


FIGURE 3: Gel filtration of PP1c and the mutants of MYPT1. PP1c was mixed with a 10-fold molar excess of each GST fusion protein and the mixture subjected to gel filtration (see Materials and Methods). Fractions were assayed for phosphatase activity using P-MLC20 as the substrate. Arrows in panel A denote elution positions of the marker proteins apoferritin (443 kDa), $\beta$-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). (A) PP1c alone, (B) rG−MYPT1$^{1-295}$, (C) rG−MYPT1$^{17-295}$, (D) rG−MYPT1$^{39-295}$, (E) rG−MYPT1$^{1-170}$, (F) rG−MYPT1$^{1-38}$, (G) rG−MYPT1$^{667-1004}$, and (H) GST alone.

same concentration of LiBr as required for the peptide affinity column. However, the capacity of the control column appeared to be smaller than that of the peptide affinity column (about 60% of the applied PP1c was absorbed to the control column), and although not conclusive, this suggests a weak interaction between PP1c and the peptide of residues 1−38. Judging from the concentration of LiBr required for elution of phosphatase activity, the strongest interaction with PP1c occurred with rMYPT1$^{1-295}$.

*Purification of PP1c Using an Affinity Column.* The relatively tight binding of PP1c to the rMYPT1$^{1-295}$ affinity column suggested that it might be useful for the purification of PP1c. Following the acetone treatment (initial procedure outlined in Materials and Methods), the crude PP1c fraction was applied to the affinity column. The column was washed, and step elutions at 0.6 and 3 M LiBr were applied. The flow through and both eluted fractions contained phosphatase activity (Figure 5A), and each contained PP1c$\delta$, as shown by Western blots using anti-PP1c$\delta$ antibody (Figure 5B). Western blots using an anti-MYPT1 polyclonal antibody

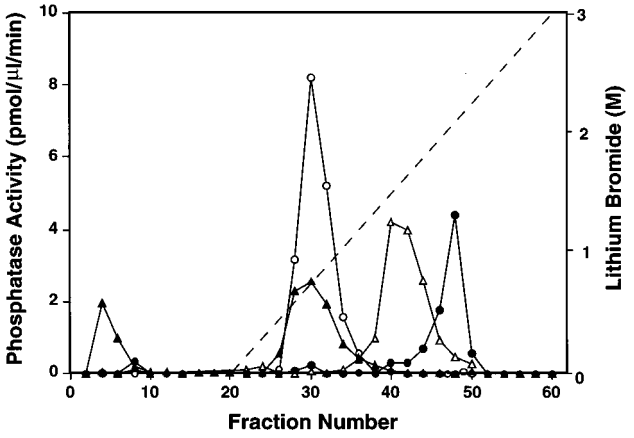


FIGURE 4: Binding of PP1c to affinity columns prepared with fragments of MYPT1. Isolated PP1c (100 $\mu$g) was applied to each column (5 cm × 0.5 cm) equilibrated with buffer C. After washing with 10 bed volumes of buffer C, a linear gradient of LiBr from 0 to 3 M was applied (total volume of 20 bed volumes) at a flow rate of 0.2 mL/min. Each fraction was assayed for phosphatase activity with P-MLC20 (see Materials and Methods). The concentration of LiBr is indicated by the dashed line. The affinity columns used were prepared with rMYPT1$^{1-295}$ and Affi-gel 15 (●), rMYPT1$^{1-170}$ and Affi-gel 15 (△), and the peptide of residues 1−38 and Affi-gel 10 (○). The results from a control Affi-gel 10 column are also shown (▲). The profile from the Affi-gel 15 control column was the same as that from the Affi-gel 10 column (data not shown).

(Figure 5B) showed the presence of MYPT1 (both the M130 and M133 isoforms and their degradation fragments) in the flow through and the 0.6 M LiBr fraction. However, the fraction eluted at 3 M LiBr did not contain these subunits. The purity of PP1c$\delta$ in this fraction was greater than 90%, as judged from SDS−PAGE (Figure 5B). The yield of PP1c was approximately 50 $\mu$g from 200 g of chicken gizzard.

## DISCUSSION

The objective of this study was to investigate interactions of various fragments of MYPT1 with PP1c. Activation of phosphatase activity and direct interactions were monitored. With respect to the activation, it was found that the shortest fragment of MYPT1 that achieved full activation (using the activation by rMYPT1$^{1-674}$ for comparison) was rMYPT1$^{1-295}$. Activation was due to both a reduction of $K_m$ and an increase in $k_{cat}$. A similar trend was observed previously for rMYPT1$^{1-674}$ (*13*). Fragments shorter than residues 1−295 caused a decrease in the extent of activation. These results are consistent with two general effects, namely, that the N-terminal sequence (residues 1−38) is involved in activation, i.e., $k_{cat}$, and the ankyrin repeats are involved in binding to substrate and thus $K_m$.

The importance of the N-terminal sequence (residues 1−38) has been noted previously. Johnson et al. (*14*) reported that this peptide activated PP1c activity and accelerated the rate of relaxation of permeabilized portal vein (*15*). However, both effects required relatively high concentrations of the peptide of residues 1−38. An interaction between the peptide of residues 1−38, expressed as a hexahistidine fusion protein, and PP1c$\delta$ was detected by the yeast two-hybrid system, although this interaction was weaker than for longer N-terminal sequences (*16*). It appears that at least part of the function of the peptide of residues 1−38 reflects the presence of a PP1c binding motif. Endo

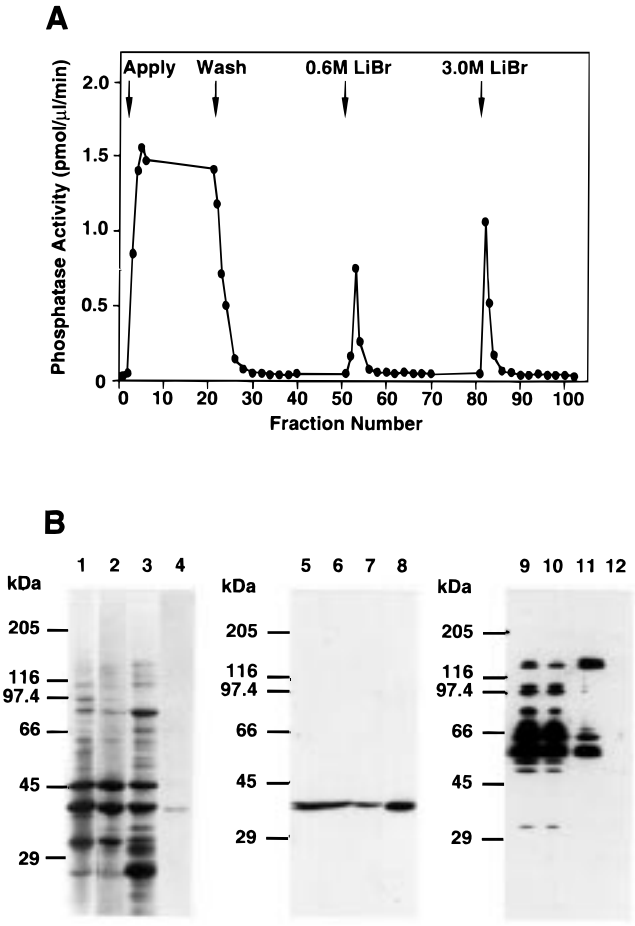


FIGURE 5: Purification of PP1c using the rMYPT$1^{1-295}$ affinity column. (A) A crude extract was prepared (see Materials and Methods) and applied to the column. The column was washed, and steps of 0.6 and 3 M LiBr were applied. Phosphatase activity was measured in the eluate. (B) Lanes 1−4 show SDS−PAGE patterns. Lanes 5−8 show Western blots using the anti-PP1c$\delta$ antibody. Lanes 9−12 show Western blots using the anti-MYPT1 antibody. The applied sample is in lanes 1, 5, and 9. The flow through fraction is in lanes 2, 6, and 10. The 0.6 M LiBr fraction is in lanes 3, 7, and 11. The 3 M LiBr fraction is in lanes 4, 8, and 12.

et al. (*30*) suggested that a tetrapeptide, KIQF, was required for full inhibition of PP1c by inhibitor 1. Subsequently, Egloff et al. (*31*) proposed a consensus sequence for the binding motif of R/K-V/I-X-F and using a 13-residue peptide from the glycogen-binding subunit reported the structure of a 1:1 complex of PP1c and the peptide. Zhao and Lee (*32*) screened a random peptide library and determined consensus binding motifs of VXF or VXW, and these sequences commonly were preceded by two to five basic residues and followed by one acidic residue. For MYPT1, the relevant sequence (residues 30−40) is KRKKTKVKFDD, and this clearly contains the necessary elements of the PP1c binding motif. In this study, only a weak interaction between the peptide of residues 1−38 and PP1c was detected, and this could probably be attributed to the C-terminal end of the peptide. A novel and surprising feature was that the N-terminal part of the peptide also appeared to have a function, namely, in activation of PP1c activity. This is illustrated by the effects of the rMYPT$1^{17-295}$ mutant that induced a decrease in $K_m$ but had little effect on $k_{cat}$ compared to PP1c alone. Activation of PP1c by rMYPT$1^{17-295}$ was markedly less effective than that by rMYPT$1^{1-295}$ and was observed only at a high molar excess. Another finding consistent with the idea that the N-terminal part of this peptide is involved in activation of PP1c comes from the results obtained with MYPT2. This isoform is a distinct gene product and has a sequence different from the N-terminal side of the PP1c-binding motif (*33*). Activation of PP1c by MYPT2 is less effective compared to that by MYPT1, and we speculate that this property reflects the difference in the N-terminal sequence. Finally, the overall importance of residues 1−38 was demonstrated by the finding that mutants lacking this sequence, e.g., rMYPT$1^{39-295}$, neither activated phosphatase activity nor bound to PP1c.

The shift in $K_m$ involved the ankyrin repeats region. With respect to substrate binding, it has been suggested that the C-terminal ankyrin repeats (repeats 6−8) interact with the phosphorylated light chain (*16*). However, from this study, we suggest that repeats 1−4 may also be involved. The rMYPT$1^{1-170}$ mutant caused a shift in $K_m$, although it was not as marked as the shifts for those mutants containing the full complement of ankyrin repeats. Either the N-terminal repeats (1−4) have a secondary role in binding to substrate, or they can assume this role if the C-terminal repeats (6−8) are absent. The binding of substrate to MYPT1 is a controversial topic, and it has also been claimed that the C-terminal 291 residues bind myosin (*18*). It was suggested (*18*) that the N-terminal 38 residues of MYPT1 stimulate dephosphorylation of myosin and the C-terminal sequence provides the targeting capability. This interpretation is not consistent with our data, and we propose that binding sites for PP1c and substrate are both present in the sequence required for full activation of PP1c, i.e., residues 1−295. With respect to the binding of PP1c, it is clear that the PP1c binding motif is important (see above discussion), but it is possible that a second PP1c binding site(s) exists in the ankyrin repeats region. From the two-hybrid screen (*16*), it was suggested that both the N-terminal peptide (residues 1−38) and the ankyrin repeats were involved in binding to PP1c. This is consistent with this study, and it appears that the N-terminal repeats (1−4) can interact with PP1c since a direct interaction of PP1c with rG−MYPT$1^{1-170}$ was observed on gel filtration. How the binding of PP1c to MYPT1 might effect $K_m$ is not clear. It is apparent, however, that both the N-terminal peptide (residues 1−38) and the ankyrin repeats are required for full activation of PP1c at a reasonable ratio of mutant:PP1c. From our results, the sequence of residues 1−295 was the minimum sequence needed to achieve full activation. Previously, it was suggested (*16*) that the sequence of residues 1−374 of MYPT1 was essential. This includes an acidic cluster at residues 326−372 (*10*). For the studies of Hirano et al. (*16*), the mutants were expressed as fusion proteins with the hexahistidine tag. Since the latter is basic, it is possible that the acidic cluster was required to neutralize the added charge.

The strong interaction of PP1c with the fragment of residues 1−295 was utilized in an affinity column for the purification of PP1c. Previous affinity purification procedures have used inhibitor 2 (*34*) and microcystin (*35*, *36*). These are valuable techniques, but their application differs from that of the rMYPT$1^{1-295}$ column. The various PP1c inhibitors bind to both isolated PP1c and to complexes containing PP1c and are therefore useful in isolating PP1c-binding subunits. Indeed, Campos et al. (*36*), using bioti-

nylated microcystin, identified 29 distinct PP1c-binding proteins in skeletal muscle. In contrast, the benefit of the rMYPT1$^{1-295}$ column is for isolation of PP1c free from binding proteins or their fragments. Since there is competition between the mutant of residues 1−295 and native MYPT (and its fragments), the PP1c in complex with binding proteins elutes at a lower LiBr concentration (0.6 M) than homogeneous PP1c (3 M). The PP1c isolated by this procedure could be activated about 15-fold by MYPT1 and its mutants using P-MLC20 as the substrate. Activation of PP1c activity using P-myosin as the substrate was equally marked (data not shown). With other procedures, a much lower extent of activation was obtained, presumably because of the copurification of PP1c-binding proteins. The wider range of activation levels described above provided a more sensitive assay of the effects of the various mutants.

In summary, these results add information about the interactions of the MYPT1 subunit. With respect to binding of PP1c, it is suggested that two regions of MYPT1 are involved. The first region is the N-terminal peptide of MYPT1 (residues 1−38). The C-terminal part of this sequence contains the PP1c-binding motif and is probably targeted for interaction with PP1c. The N-terminal part of the sequence has an additional function in that it is required for activation of PP1c activity, as evidenced from measurements of $k_{cat}$. The second region incorporates the ankyrin repeats. These are thought to be involved in binding to substrate (and hence reduced $K_m$ values) and also binding to PP1c. The minimum sequence required for full activation of PP1c is residues 1−295, and this includes both of the two regions outlined above. A strong interaction between PP1c and the fragment of residues 1−295 was demonstrated, and this was utilized to improve the purification procedure for PP1c.

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BI980782X