Interactions and Properties of Smooth Muscle Myosin Phosphatase†

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ABSTRACT: Interactions of the type 1 phosphatase catalytic subunit (PP1c) and the myosin phosphatase holoenzyme (MBP) were compared using affinity columns. In the absence of ATP, MBP bound to dephosphorylated myosin, heavy meromyosin (HMM), and subfragment 1. In contrast, PP1c was not bound. In the presence of ATP, the binding of MBP occurred only with phosphorylated protein. The interaction of MBP with phosphorylated proteins also was demonstrated using thiophosphorylated proteins as competitive inhibitors. Kinetics parameters were determined. With phosphorylated light chains (P-LC20), the major difference between PP1c and MBP was a lower K_m for the latter. With myosin, MBP showed a marked increase in k_{cat}, compared to PP1c. ATP did not affect these parameters. To investigate the role of the large phosphatase subunit, two recombinant proteins representing the N-terminal two-thirds of the molecule were expressed. These activated PP1c, and activation was maximum at approximately an equimolar ratio. The equimolar mixture of recombinant fragment and PP1c exhibited K_m values similar to MBP and increased k_{cat} values, compared to PP1c alone. An affinity column was prepared using the recombinant fragment. Phosphorylated HMM and P-LC20 were bound in the presence and absence of ATP. The interaction of P-LC20 was not ATP-dependent. Dephosphorylated HMM did not bind in the presence of ATP. The N-terminal fragment of the large subunit also contained a binding site for PP1c. These results indicate that the N-terminal portion of the large subunit of MBP contained binding sites for P-LC20 and PP1c.

Contraction of smooth muscle is regulated primarily by phosphorylation of myosin (Hartshorne, 1987). The level of myosin phosphorylation is controlled by a balance of the activities of two key enzymes, namely, myosin light chain kinase (MLCK)[1] and myosin light chain phosphatase. MLCK is activated by the Ca^{2+}−calmodulin complex, and this interaction provides the link between phosphorylation and the intracellular Ca^{2+} transients. Initially it was assumed, since there was no evidence to indicate otherwise, that phosphatase activity was not regulated. However, more recent data have indicated that under certain conditions phosphatase activity can be inhibited. In general, stimulation by certain agonists or application of GTPγS to permeabilized smooth muscle preparations activates a G protein-linked mechanism that ultimately results in inhibition of phosphatase activity [see review by Somlyo and Somlyo (1994)]. This inhibition of phosphatase activity increases the Ca^{2+} sensitivity of phosphorylation and increases the extent of myosin phosphorylation at a given Ca^{2+} level. The effect obviously is more dramatic at submaximal levels of activating Ca^{2+}. The increase in Ca^{2+} sensitivity is induced by AlF_4 (Kawase & van Breemen, 1992), and this suggests a role for trimeric G proteins. In addition, several studies have suggested that the monomeric G proteins, rho (Hirata *et al*., 1992; Fujita *et al*., 1995; Noda *et al*., 1995) or *ras* (Satoh *et al*., 1993), may be involved. Incubation of α-toxin-permeabilized portal vein with ATPγS also reduced phosphatase activity, and several proteins were thiophosphorylated, including the large phosphatase subunit (Trinkle-Mulcahy *et al*., 1995). The mechanism of Ca^{2+} sensitization, or inhibition of phosphatase activity, is not established, but may utilize arachidonic acid as a second messenger (Gong *et al*., 1992; 1995) and/or activation of protein kinase C (Masuo *et al*., 1994).

In order to define this pathway, a prerequisite is identification and characterization of the phosphatase and associated subunits. Several preparations have been reported that can dephosphorylate smooth muscle myosin or light chains [see review by Erdödi *et al*. (1996)]. In order to limit the number of possibilities, the following restrictions were imposed, *i.e.*, that the phosphatase binds to myosin or the contractile apparatus and that the phosphatase is effective with intact phosphorylated myosin as substrate. Three similar preparations were isolated (Alessi *et al*., 1992; Shimizu *et al*., 1994; Shirazi *et al*., 1994), and the phosphatase was found to be composed of three subunits: ∼130, 38, and 20 kD. The 38 kDa subunit is the PP1δ [or PP1β (Alessi *et al*., 1992)] catalytic subunit (Okubo *et al*., 1993; Shimizu *et al*., 1994), and the two other subunits are putative regulatory subunits or target molecules. In our laboratory, the trimeric phosphatase was isolated from a crude gizzard smooth muscle myosin preparation and is referred to as myosin-bound

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[1] Abbreviations: MLCK, myosin light chain kinase; PP1c, catalytic subunit of type 1 phosphatase; PP1δ, the δ isoform of PP1c; MBP, myosin-bound phosphatase; LC20, the 20000 dalton myosin light chain; P-LC20, phosphorylated LC20; P-myosin, phosphorylated myosin; HMM, heavy meromyosin; P-HMM, phosphorylated HMM; S1, heavy meromyosin subfragment 1; ATPγS, adenosine 5′-O-(3-thiotriphosphate); $rN130^{1-633}$ and $rN133^{1-674}$, the recombinant N-terminal fragments of the 130 and 133 kD subunits, respectively.

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phosphatase (MBP). Primary structures, derived from cDNAs, have been obtained for the gizzard 130/133 kDa subunits (Shimizu *et al*., 1994); the large subunit from rat phosphatase, M110, and the gizzard 20 kDa (Chen *et al*., 1994); and the PP1δ from rat (Sasaki *et al*., 1990) and gizzard (Shimizu *et al*., 1994).

The functions of the two noncatalytic subunits are not established. One possibility is that they act as targeting subunits to colocalize the substrate, myosin, and the PP1δ catalytic subunit (Alessi *et al*., 1992). In support of this, it was shown that addition of the 130 and 20 kDa subunits to PP1δ increased activity toward P-LC20 and P-HMM (Alessi *et al*., 1992). Similar findings were reported by Shirazi *et al*. (1994), who in addition found that the trimeric phosphatase was more effective in relaxing permeabilized smooth muscle preparations than the isolated PP1c. However, more detailed studies have not been carried out. Our objectives in this presentation are to establish basic kinetic parameters, with an emphasis on activities measured in the presence of ATP, and to begin an analysis of the function(s) of the large subunit.

MATERIALS AND METHODS

Materials. Chemicals and vendors were as follows: ATPγS (Boehringer Mannheim); [γ-^{32}P]ATP (DuPont NEN); ATP, A grade, and cyanogen bromide-activated Sepharose 4B (Sigma); *Staphylococcus aureus* protease (Pierce); microcystin-LR (LC Laboratories); *Taq* DNA polymerases (Perkin Elmer Cetus); bacterial culture media (Difco). Oligonucleotides were synthesized at the Macromolecular Structure Facility (University of Arizona).

Protein Preparations. The myosin-bound phosphatase (MBP) was purified from frozen turkey gizzards (Shimizu *et al*., 1994) and was used after the Mono Q HR 5/5 column. Myosin from frozen turkey gizzards (Ikebe & Hartshorne, 1985a) was used for the preparation of HMM and S1 (Ikebe & Hartshorne, 1985b). LC20 was isolated from gizzard actomyosin (Hathaway & Haeberle, 1983) and phosphorylated with [γ-^{32}P]ATP (Ishihara *et al*., 1989). For the preparation of ^{32}P-labeled, or thiophosphorylated, myosin, the purified myosin was incubated at 0 °C with 1 μM microcystin L-R and then dialyzed against 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 1 mM benzamidine, and 0.2 μM 4-amido-phenylmethanesulfonyl fluoride. Myosin, approximately 2.5 mg/mL, was then incubated at 25 °C for 15 min with 10 μg/mL MLCK and 10 μg/mL calmodulin in 0.1 M KCl, 30 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and either 0.5 mM [γ-^{32}P] ATP or 0.5 mM ATPγS. The myosin was dialyzed against 0.3 M KCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.2 mM dithiothreitol. The stoichiometry of phosphorylation, or thiophosphorylation, was 1.8−1.9 mol of P/mol of myosin. The level of phosphorylation remained constant for over 30 min when the myosin was incubated at 25 °C. Phosphorylated, or thiophosphorylated, HMM and S1 were prepared similarly.

Other protein preparations were as follows: PP1c from rabbit skeletal muscle and frozen turkey gizzards (Martin *et al*., 1994); the 58 + 38 kD phosphatase from turkey gizzards (Okubo *et al*., 1993); MLCK and calmodulin from frozen turkey gizzards and bovine testes, respectively (Walsh *et al*., 1983). The MBP holoenzyme was digested with α-chymotrypsin (1:1000 w/w α-chymotrypsin:MBP ratio) at 25 °C for 3 min in 30 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and the reaction was stopped by addition of diisopropyl fluorophosphate to 1 mM. The proteolyzed MBP was applied to a Superose 12 HR 10/30 column, and fractions with phosphatase activity was collected and concentrated by Centricon 50 concentrators (Amicon). The major species after 3 min digestion were 58 + 38 kDa.

Preparation of Affinity Columns. Myosin (40 mg), thiophosphorylated myosin (30 mg), HMM (30 mg), S1 (70 mg), thiophosphorylated S1 (20 mg), and thiophosphorylated LC-20 (20 mg) were dialyzed against 0.5 M KCl, 0.1 M $NaHCO_3$ (pH 8.5) and coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. Approximately 1 mL of resin was used for 5 mg of protein. The coupling reaction was carried out overnight at 4 °C and was terminated by filtration on a sintered glass funnel and washing with the KCl−$NaHCO_3$ buffer. Unreacted groups were blocked by incubation of the resin with 0.2 M glycine−NaOH (pH 8.0) at room temperature for 2 h. The resin was washed thoroughly with the KCl−$NaHCO_3$ buffer and equilibrated in buffer A [10 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.2 mM dithiothreitol, and 0.2 μM 4-amidophenylmethanesulfonyl fluoride]. Coupling efficiency was greater than 80% for each of these columns.

An affinity column was prepared using rN130$^{1-633}$ and Affi-gel 15 (Bio-Rad). Approximately 2 mg of rN130$^{1-633}$ was coupled, following the manufacturer's recommendations. The coupling efficiency was 68%. The column was equilibrated in buffer A. Proteins were applied in this solvent and eluted with a linear NaCl gradient. The proteins were ^{32}P-HMM (1.65 mol of P/mol of HMM), dephosphorylated HMM, ^{32}P-LC20 (0.8 mol of P/mol of LC20), and PP1c from rabbit skeletal muscle and turkey gizzard.

Phosphatase Assays. Activities were measured at 30 °C using either 5 μM ^{32}P-LC20 or 1.1 μM ^{32}P-myosin in 0.1 M KCl, 30 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP (or as indicated), 0.2 mg/mL bovine serum albumin, and the presence (holoenzyme) or absence (PP1c) of 0.3 mM $CoCl_2$. The reactions were started by addition of substrate and terminated by addition of trichloroacetic acid and bovine serum albumin to final concentrations of 10% and 4 mg/ml, respectively. Precipitated protein was sedimented by centrifugation in an Eppendorf benchtop centrifuge (Model 5414), and the radioactivity of the supernatant determined by Cerenkov counting. Phosphatase rates were estimated from the linear portion of time courses, with points taken every 30 s. Any inhibition due to phosphorylation of the holoenzyme (Ichikawa *et al*., 1996) resulted in deviation from linearity. For the longest assay times involved (about 3 min), less than 10% inhibition was observed. For practical reasons, the highest concentrations of ^{32}P-myosin and ^{32}P-LC20 used were 5 and 60μM, respectively. K_m values were estimated by extrapolation.

Expression of N-Terminal Fragments of the Large Subunit. Two N-terminal fragments of the 130/133 kDa subunits were expressed. One encoded the N-terminal 633 residues of the 130 kDa subunit, ending at D633, termed rN130$^{1-633}$, and the other the N-terminal 674 residues of the 133 kDa subunit, ending at D674, termed rN133$^{1-674}$.

The cDNA clone for rN130$^{1-633}$ was obtained by PCR amplification of a Uni-Zap cDNA library (Shimizu *et al*., 1994). The sense and anti-sense primers corresponded to

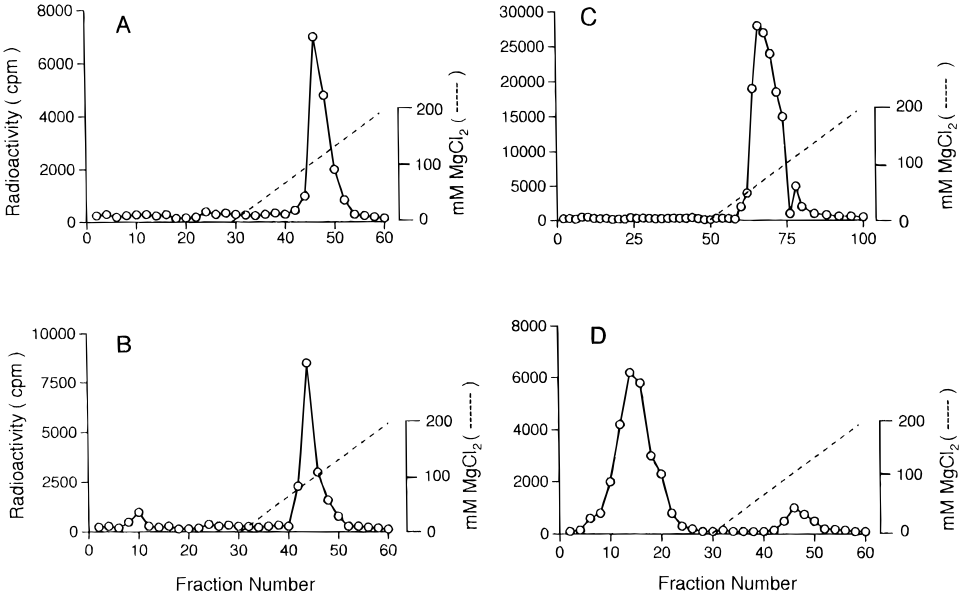

FIGURE 1: Binding of MBP holoenzyme or PP1c to affinity columns in the absence of ATP. Myosin, HMM, and S1 affinity columns were prepared as described and equilibrated with buffer A (Materials and Methods). (A) MBP (10 μg) applied to myosin column. (B) MBP (10 μg) applied to HMM column. (C) MBP (30 μg) applied to S1 column. (D) PP1c (10 μg) from rabbit skeletal muscle applied to myosin column. Proteins were eluted by a linear $MgCl_2$ gradient (0−200 mM) in buffer A. Phosphatase activity (expressed as cpm) was determined in each fraction using ^{32}P-LC20 as substrate.

nucleotides 109−135 and 2131−2160 of the 133 kDa sequence and contained *BamH*I and *Sal*I sites for ligation, respectively. The anti-sense primer also was designed to introduce a stop codon after D633. The rN130 clone did not contain the insert sequence. Partial nucleotide sequences around the 5′ and 3′ cloning sites were identical to the 130/133 kDa sequence. The cDNA clone was ligated to the expression vector pQE30 (Qiagen) at *Bam*HI and *Sal*I sites. Expression in *E. coli* M15 [pREP4] and purification of the expressed protein using a metal affinity column (TALON, Clontech) were as described previously (Hirano *et al*., 1995). SDS−PAGE of the expressed protein showed a band of the expected size (approximately 79 kDa) and greater than 80% purity. This protein cross-reacted with the monoclonal antibody to the 58 kDa fragment (Okubo *et al*., 1994). The construct encoded 633 residues of the 130 kDa subunit plus an N-terminal 12 residue peptide containing the hexahistidine tag plus additional residues from the vector (M-R-G-S-$[H]_6$-G-S-).

The $rN133^{1-674}$ fragment was expressed in pET5a (Novagen). The cDNA used was clone Z-1 (Shimizu *et al*., 1994), and this was amplified by PCR. The two primers were the following: for 5′, GCGGCATATGAAGATG-GCGGACGC (underlined residues show a *Nde*I site at the initiating M codon); for 3′, GACTCTGGATCCCGTACTG-GAGTG (underlined residues show a *Bam*HI site for insertion into the pET5a vector). After digestion with *Nde*I and *Bam*HI, the PCR product was ligated into the pET5a vector. This construct was used to transform competent *E. coli* HMS 174 and BL 21 (DE 3). Colonies of the latter cells, containing the construct, were used to inoculate initially a 5 mL culture (LB medium) containing ampicillin and subsequently a 3 L culture. The culture was grown at 37 °C until the $OD_{600\ nm}$ reached 1.0, isopropyl β-D-thiogalactopyranoside was then added to 0.4 mM, and the culture was grown an additional 4 h. Cells were collected by centrifugation at 5000*g* for 5 min. The pellet was washed with 1 L of phosphate-buffered saline and extracted using a dounce tissue grinder (Wheaton) with 200 mL of solvent B [30 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM diisopropyl fluorophosphate, 1 mM benzamidine, and 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride]. The mixture was sonicated (4 × 30 s bursts; Branson sonifier) and centrifuged at 100000*g* for 1 h. The pellet was reextracted with 100 mL of solvent B and centrifuged. Combined supernatants were dialyzed against 30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 mM dithiothreitol, 10 μM (*p*-amidinophenyl) methanesulfonyl fluoride, and 1 mM benzamidine. After clarification at 10 000*g* for 20 min, the supernatant was applied to SP-Sepharose (1.6 × 11 cm) followed by Mono Q HR 5/5. Columns were equilibrated with the dialysis buffer and developed with linear gradients of 0−0.5 M KCl. The $rN133^{1-674}$ was detected by the apparent molecular weight on SDS−PAGE and by Western blots using the anti-58 kD monoclonal antibody (Okubo *et al*., 1994). Approximately 10 mg of purified $rN133^{1-674}$ (∼90% purity) was obtained from 3 L of culture. The $rN133^{1-674}$ contained residues 1 through 674 of the 133 kDa subunit plus additional C-terminal residues, P-N-S.

Other Procedures. SDS−PAGE on 7.5−20% acrylamide gradient gels was carried out with the discontinuous buffer system of Laemmli (1970). Protein concentrations were determined with the bicinchoninic acid (Pierce) or Bradford (Bio-Rad) procedures. The stoichiometries of phosphorylation were determined using [γ-^{32}P]ATP and the funnel assay (Walsh *et al*., 1983). The levels of thiophosphorylation were estimated by back-titration of available sites with [γ-^{32}P] ATP following the reaction with ATPγS.

RESULTS

Interactions of Phosphatase. In previous experiments, it has been established that the phosphatase holoenzyme binds to myosin in the absence of ATP (Okubo *et al*., 1993; Shimizu *et al*., 1994). These findings are confirmed using a different approach by the experiments shown in Figure 1.

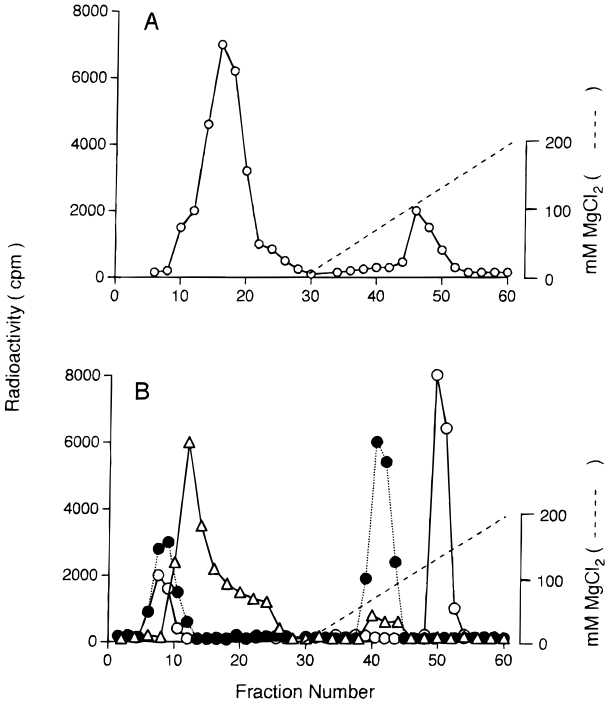

FIGURE 2: Binding of MBP and PP1c to myosin affinity columns in the presence of ATP. Columns were prepared with dephosphorylated myosin (A) and thiophosphorylated myosin (B) and equilibrated with buffer A plus 1 mM ATP and 2 mM $MgCl_2$. MBP (10 μg) was applied to each column and elution carried out with a $MgCl_2$ gradient in buffer A plus 1 mM ATP (○). For the thiophosphorylated myosin column (B), PP1c (10 μg) from gizzard was applied in buffer A plus 1 mM ATP (△), and MBP (10 μg) was applied in buffer A plus 1 mM ATP and 0.1 M NaCl (●). Other conditions as in Figure 1.

Affinity columns were prepared for myosin, HMM, and S1. In each case, dephosphorylated proteins were used. The holoenzyme was applied and the bound protein eluted with a $MgCl_2$ gradient. Binding of the phosphatase holoenzyme to myosin was strongest (Figure 1A) and was eluted at approximately 90 mM $MgCl_2$, compared to 80 and 50 mM $MgCl_2$ for HMM and S1, respectively (Figure 1B,C). The binding profile of the holoenzyme to the myosin-affinity column was not affected by the inclusion of 1 mg/mL bovine serum albumin. The bound phosphatase could also be eluted by NaCl and for the myosin-affinity column, the holoenzyme was eluted at approximately 0.5 M NaCl. In contrast, the catalytic subunit from rabbit skeletal muscle and gizzard did not bind to these affinity columns. This is illustrated in Figure 1D with PP1c from rabbit skeletal muscle and the myosin-affinity column.

Similar experiments were carried out in the presence of 1 mM ATP and 2 mM $MgCl_2$. Under these conditions, the binding of the holoenzyme to dephosphorylated myosin was reduced, and most of the phosphatase activity was recovered in the flow-through (Figure 2A). Binding of the holoenzyme, however, was observed using a thiophosphorylated myosin-affinity column (Figure 2B) and was eluted by $MgCl_2$. In the presence of 0.1 M NaCl, MBP also was bound to the thiophosphorylated myosin column (Figure 2B) and was eluted at a lower $MgCl_2$ concentration. The presence of ATP was the critical factor, and in the same solvents minus ATP, binding of holoenzyme occurred with dephosphorylated myosin. The catalytic subunit from turkey gizzard did not bind to the thiophosphorylated myosin as effectively as the holoenzyme and was eluted by the equilibration buffer (*i.e.*,

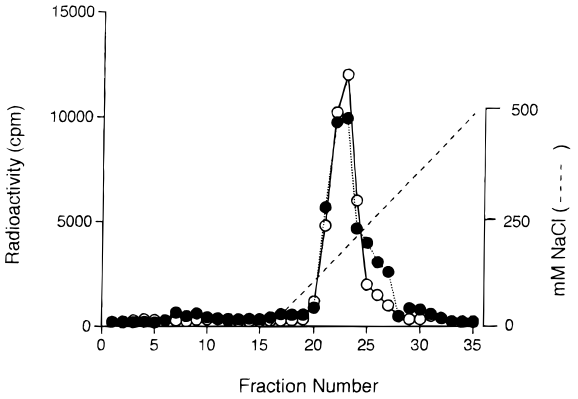

FIGURE 3: Binding of MBP to a thiophosphorylated LC20 column. The LC20 affinity column was prepared and equilibrated in buffer A with 1 mM ATP (●) and without ATP (○). MBP (10 μg) was applied. A linear NaCl gradient (0−0 .5 M) in buffer A ± ATP was used. Other conditions as in Figure 1.

the $MgCl_2$ gradient was not necessary). However, the activity profile was retarded compared to the void volume and was asymmetric (Figure 2B). This may have indicated a weak interaction.

Some experiments were carried out using a thiophosphorylated LC20 column, since this is used frequently in phosphatase preparations. PP1c, from both rabbit muscle and gizzard, did not bind to this column (data not shown). In contrast, the MBP was bound, both in the presence and in the absence of ATP, and was eluted at approximately 0.13 M NaCl (Figure 3).

From the experiments carried out with the substrate affinity columns, the following points should be emphasized. In the presence of ATP, MBP bound only to phosphorylated proteins (*i.e.*, substrates). In the absence of ATP, MBP bound to the dephosphorylated proteins. PP1c did not bind effectively to any of the columns, although a weak interaction may be indicated with the thiophosphorylated myosin column. Thus, one potential function for the noncatalytic subunits could be to increase the affinity of PP1c for its substrate, P-LC20.

Competition Assays. Binding of myosin and its fragments to the holoenzyme also can be assessed by determining if these proteins act as competitive inhibitors of phosphatase activity. Using P-myosin as substrate (1.1 μM), the effect of various proteins on phosphatase activity in the presence of ATP was determined. Each of the thiophosphorylated proteins (myosin, S1, and LC20) inhibited the activity of the holoenzyme (Figure 4). On the basis of the concentration of myosin heads (or light chain), the thiophosphorylated myosin and S1 were slightly more effective inhibitors than the thiophosphorylated LC20. It appears from these curves that the thiophosphorylated substrates bind the phosphatase holoenzyme more strongly than the phosphorylated substrates, since 50% inhibition would be expected at equimolar concentrations of ^{32}P-labeled myosin and thiophosphorylated proteins. These proteins in the dephosphorylated state did not affect the activity of the holoenzyme (Figure 4). The activities of PP1c from rabbit skeletal muscle and gizzard were not affected by the thiophosphorylated proteins over the concentration range used in Figure 4.

These results confirm those obtained with the affinity columns and indicate that the dominant recognition site for binding of MBP to myosin was the phosphorylated light chain.

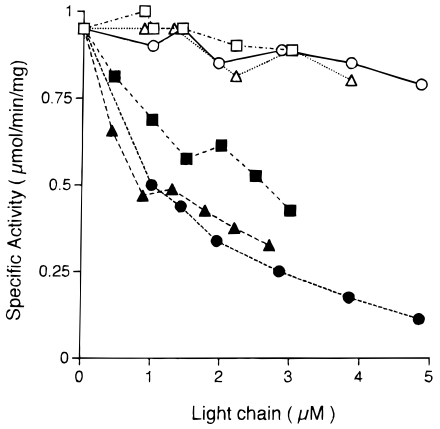

FIGURE 4: Effect of thiophosphorylated and dephosphorylated proteins on phosphatase activity. Assays were carried out at 30 °C in the presence of 1 mM ATP and 2 mM $MgCl_2$ with ^{32}P-myosin (1.1 μM) and MBP (see Materials and Methods) and dephosphorylated (□) and thiophosphorylated (0.9 mol of thiophosphate/mol) LC20 (■); dephosphorylated (△) and thiophosphorylated (0.8 mol of thiophosphate/mol) S1 (▲); and dephosphorylated (○) and thiophosphorylated (1.8 mol of thiophosphate/mol) myosin (●). Added protein shown as concentration of LC20.

Table 1: Kinetic Parameters ± ATP for PP1c and MBP for P-LC20 and P-Myosin[a]

	P-LC20		P-myosin	
	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
PP1c[b]				
35 kDa + ATP	314 ± 20	188 ± 10	35.7 ± 5.0	16.1 ± 1.4
38 kDa + ATP	228 ± 10	274 ± 11	51.9 ± 1.9	27.8 ± 2.7
35 kDa − ATP	93 ± 7	269 ± 17	31.5 ± 1.5	28.3 ± 0.7
MBP + ATP	13.4 ± 1.5	993 ± 66	14.3 ± 3.1	679 ± 33
MBP − ATP	17.2 ± 1.8	1424 ± 116	8.3 ± 2.2	1109 ± 33

[a] Values are means ± SD (*n* not less than 3). [b] The PP1c preparations were from rabbit skeletal muscle (35 kDa) and from turkey gizzard (38 kDa).

Activities of PP1c and Holoenzyme. Kinetic parameters were determined using PP1c and the holoenzyme for two substrates, P-LC20 and P-myosin. Activities were determined in the presence and absence of ATP, and the respective values are compared (Table 1). The following points should be made: (1) with P-LC20 as substrate, the major effect of the noncatalytic subunits (*i.e.*, comparing PP1c and holoenzyme) was to decrease K_m (about 20-fold). The k_{cat} value was also increased, but only about 4-fold. (2) With P-myosin as substrate, the situation was reversed, and the major effect was on k_{cat}, with a marked increase in activity for the holoenzyme (Table 1). The K_m value for the holoenzyme was reduced slightly. (3) In the absence of ATP, the kinetic parameters were similar to those obtained in the presence of ATP. There were slight decreases for the K_m values (see Table 1), the 3-fold decrease in K_m for PP1c and P-LC20 being the most marked. There was also a slight increase in k_{cat} values in the absence of ATP. Thus, the major effect of ATP was not on phosphatase kinetics but was to reduce binding of the holoenzyme to dephosphorylated substrate.

Effect of Recombinant Fragments. There are no data on the role(s) of the two noncatalytic MBP subunits. To begin such an investigation, the effect of the recombinant 130/133 kDa fragments on the activity of PP1c was determined. As shown in Figure 5, rN133$^{1-674}$ (see Materials and Methods) activated the activity of PP1c from rabbit skeletal muscle and turkey gizzard. This effect was more pronounced

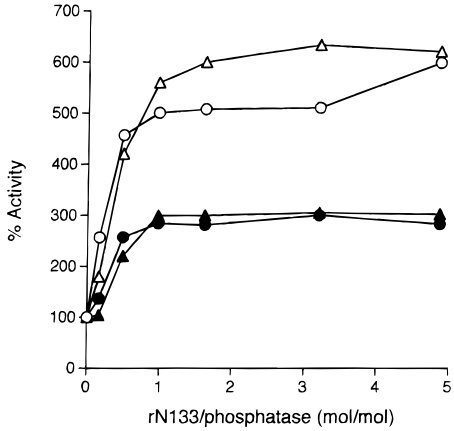

FIGURE 5: Effect of rN133 fragment on PP1c activity. Assays carried out in the presence of 1 mM ATP with ^{32}P-LC20 (●, ▲) or ^{32}P-myosin (○, △) as substrate. PP1c from rabbit skeletal muscle (○, ●) and from turkey gizzard (△, ▲) were used.

Table 2: Effect of rN133 on Kinetics[a]

	P-LC20		P-myosin	
	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min$^{-1)}$
PP1c rabbit[b]	314 ± 20	188 ± 10.5	35.7 ± 5.0	16.1 ± 1.4
PP1c plus rN133	23.7 ± 7.8	228 ± 17.5	7.0 ± 3.9	94.7 ± 17.5
PP1c gizzard[b]	228 ± 10	274 ± 11.4	51.9 ± 1.9	27.8 ± 2.7
PP1c plus rN133	27.0 ± 3.0	264 ± 30.4	12.3 ± 2.3	247 ± 45.6
holoenzyme	13.4 ± 1.5	993 ± 66.2	14.3 ± 3.1	679 ± 33.1
58 + 38 MBP[c]	20.8	500	20.2	356
digested MBP[d]	30.1	481	20	298

[a] Values are means ± SD (*n* at least 3). <sen Other values are means of two determinations. [b] The two catalytic subunits used were isolated from rabbit skeletal muscle (predominantly 35 kDa) and from turkey gizzard (predominantly 38 kDa). [c] The phosphatase isolated by the method of Okubo *et al*. (1993) was composed of two subunits, 58 and 38 kDa. [d] This preparation was obtained by digestion of MBP and gel filtration. It was composed of two major subunits, 58 and 38 kDa.

for P-myosin compared to P-LC20. The activation was maximum at a stoichiometry of approximately 1 (PP1c:rN133 molar ratio). Similar results were obtained for rN130$^{1-633}$ (data not shown). The holoenzyme, MBP, was not activated by rN133$^{1-674}$ (at a molar ratio of 1) with either substrate.

An equimolar mixture of PP1c (from rabbit skeletal muscle and gizzard) and rN133$^{1-674}$ was prepared and used for kinetic measurements. The basic kinetic parameters are shown in Table 2 and compared with the holoenzyme and two preparations of the 58 plus 38 kDa phosphatase complex. (The data for the PP1c preparations and holoenzyme are taken from Table 1.) Addition of the recombinant fragment to PP1c reduced the K_m values for both P-LC20 and P-myosin. The effect of rN133$^{1-674}$ was more marked when P-LC20 was substrate. The K_m values for the 58 + 38 kDa phosphatase preparations were similar to those obtained in the presence of rN133$^{1-674}$. The k_{cat} values, using P-LC20, were not affected by rN133$^{1-674}$, but were increased when P-myosin was substrate. For the 58 + 38 kDa complexes, k_{cat} was slightly higher than for the PP1c and rN133$^{1-674}$ mixtures, but the values were in the same range. The k_{cat} values for the holoenzyme were consistently higher with either of the two substrates (Table 2). Thus, the recombinant N-terminal fragment restored the K_m values, and was partially effective in activation of enzymatic activity.

To investigate the binding of myosin and its subfragments, the rN130$^{1-633}$ affinity column was used. In the absence of

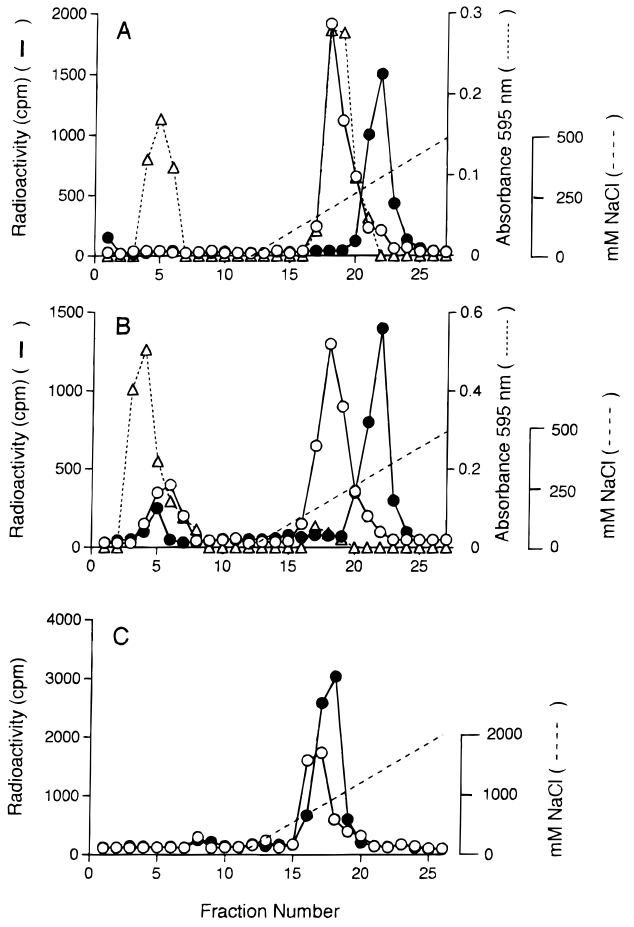

FIGURE 6: Binding of HMM, LC20, and PP1c to rN130 affinity column. (A) In the absence of ATP, dephosphorylated HMM (△), ^{32}P-HMM (●), ^{32}P-LC20 (○) applied and bound proteins eluted with a linear NaCl gradient (0−0.5 M) in buffer A minus ATP. 50 μg of HMM and 10 μg of ^{32}P-LC20 were applied. Fractions monitored either by ^{32}P content or by Bradford protein assay ($A_{595\ nm}$). (B) In the presence of 1 mM ATP, the same procedure as in (A) was followed. (C) PP1c (10 μg) from rabbit skeletal muscle (○) or, turkey gizzard (●) applied in buffer A minus ATP, and a linear NaCl gradient (0−2 M) was applied. Fractions were assayed for phosphatase activity.

ATP (Figure 6A), P-HMM and P-LC20 both bound to the rN130$^{1-633}$ column. The binding of P-HMM was stronger and was eluted at approximately 0.3 M NaCl, compared to 0.2 M NaCl for P-LC20. About 70% of dephosphorylated HMM was bound, and the bound fraction showed an apparent affinity similar to P-LC20. In the presence of ATP, the major difference was that dephosphorylated HMM did not bind (Figure 6B). A major fraction of the P-LC20 and P-HMM preparations bound (Figure 6B), and the elution profiles in the presence and absence of ATP were similar. In the presence or absence of ATP the dephosphorylated LC20 did not bind to the rN130$^{1-633}$ column (data not shown). These results indicated that the N-terminal fragment of the 130/133 kD subunit contains an independent site for interaction with the substrate, P-LC20. The interaction of rN130$^{1-633}$ and dephosphorylated HMM was sensitive to ATP.

The binding of PP1c to this column also was monitored. As shown in Figure 6C, the two preparations of PP1c both bound to rN130$^{1-633}$. The binding was relatively strong (compared to substrate), and PP1c was eluted only at high NaCl concentrations (0.6−0.7 M). There was little difference between the 35 kDa rabbit muscle PP1c and the 38 kDa gizzard PP1c, although a slightly reduced affinity may be noted for the 35 kDa species. These interactions were not sensitive to ATP (data not shown).

DISCUSSION

Effect of ATP. Most of the previous studies indicating an interaction of myosin phosphatase with myosin were carried out in the absence of ATP. These include binding of the proteolyzed MBP (Okubo *et al*., 1993) and the trimeric MBP (Shimizu *et al*., 1994). In the studies presented above, it is shown that in the presence of ATP, and therefore under conditions approaching physiological, binding occurred only with phosphorylated proteins. One of the partners in this interaction appears to be P-LC20, and there was no evidence to indicate a direct interaction of MBP with myosin heavy chain, although this cannot be eliminated. The simplest interpretation is that in the absence of ATP binding of MBP to dephosphorylated myosin can occur, to either LC20 or the myosin heavy chain. In the presence of ATP, there is a conformational change, presumably induced by ATP binding to the active site, that prevents this interaction. The distinction between the conformations of dephosphorylated myosin plus and minus ATP is not known. However, it is known that the conformation of smooth muscle myosin is sensitive to both ATP and phosphorylation. For example, in the presence of ATP, the 10S conformation is favored by dephosphorylated myosin and the 6S conformation by P-myosin (Ikebe *et al*., 1983). The discrimination of MBP binding to P-myosin represents a simple regulatory mechanism in which the phosphatase would be recruited only by the phosphorylated cross bridges. Once the myosin is dephosphorylated, the MBP would become available for further reactions. This mechanism would eliminate the problem of sequestration of phosphatase by dephosphorylated myosin. In this context, it should be noted that the total myosin concentration is about 50 μM (Hartshorne, 1987) compared to the phosphatase concentration of approximately 0.7 μM (Alessi *et al*., 1992). Recently it was found (Ichikawa *et al*., 1996) that phosphorylation of the 130/133 kDa subunit inhibited phosphatase activity and this would provide an additional regulatory component. It is not known whether phosphorylation of the large phosphatase subunit affects the affinity of MBP for P-myosin.

Kinetic Data. A comparison of the kinetics for MBP and PP1c indicated that MBP is more effective with either P-LC20 or P-myosin as substrate. This confirms earlier observations (Alessi *et al*., 1993; Shirazi *et al*., 1994). There appeared to be two trends. With P-LC20 as substrate, the major difference between PP1c and MBP was on K_m, and the K_m for MBP plus P-LC20 was about 20-fold lower than with the catalytic subunit. A slight increase in k_{cat} also was observed with MBP. With P-myosin as substrate, the major difference between PP1c and MBP was on k_{cat}. The k_{cat} for the holoenzyme showed a marked increase. There was a slight decrease in K_m, but this was probably not significant. Thus, MBP is more effective with both substrates than PP1c. It is interesting that the kinetic parameters for MBP plus either substrate are similar, but the mechanisms involved in achieving these ends may be different. For the isolated light chain, the major influence of the noncatalytic subunits seems to be in stabilizing the PP1c/P-LC20 complex, and with P-myosin, their function is to increase the turnover rate. Several phosphatases have been isolated from smooth muscle that will dephosphorylate the isolated light chain, but not

intact myosin [see review by Erdödi *et al*. (1996)]. This tendency is shown also by PP1c, since it was considerably less effective with P-myosin than with P-LC20. The difference could reflect steric hindrance at the phosphorylated serine 19, or a different conformation around the phosphorylation site. The noncatalytic subunits of MBP increased the phosphatase rate (compared to PP1c) and overcame whatever conformational restrictions were imposed. Simply, this could involve independent binding of one of the noncatalytic subunits to myosin or substrate, and/or an alteration of the active site of PP1c by the other subunits.

Interactions of the 130/133 kDa Subunit. In this discussion, the interactions of the N-terminal fragment of the large subunit will be considered. The two recombinant proteins represented about two-thirds of the 130/133 kDa subunit, and they contained the ankyrin repeat region (Chen *et al*., 1994; Shimizu *et al*., 1994). It is known that the 58 kDa fragment represents the N-terminal part of the 130/133 kDa subunit (Okubo *et al*., 1994; Shimizu *et al*., 1994) and also that the 58 + 38 kDa complex binds to myosin. In addition, it was suggested that the N-terminal domain contained a binding site for PP1c (Moorhead *et al*., 1994). Thus, the initial studies to investigate subunit interactions focused on the N-terminal region of the large subunit.

The two recombinant proteins, $rN130^{1-633}$ and $rN133^{1-674}$, behaved identically. Both activated the phosphatase activity of PP1c toward P-LC20 and P-myosin, and both modified kinetic parameters of PP1c. A critical question was whether an independent binding site existed for substrate on either of the two non-catalytic subunits. This is answered by the experiments done with the $rN130^{1-633}$ affinity column. These showed that $rN130^{1-633}$ contained a binding site for P-LC20 (or P-HMM). The stronger binding to P-HMM could have reflected the presence of two light chains and/or an altered conformation of the "intact" light chain. In the absence of ATP, an interaction was observed for dephosphorylated HMM but not dephosphorylated LC20. A more precise location for the P-LC20 binding site is not available, and deletion mutants are being tested to achieve this.

In addition to the P-LC20 binding site, the $rN130^{1-633}$ fragment contained a site for PP1c. This confirms earlier results (Moorhead *et al*., 1994) and predictions (Shimizu *et al*., 1994). The binding to PP1c was relatively strong, compared to P-LC20, as judged by its elution at higher ionic strengths. However, the holoenzyme is not dissociated under conditions where PP1c was eluted, and it is possible that additional interactions influence the binding of PP1c to the 130/133 kDa subunit.

Recently, Haystead *et al*. (1995) cloned and expressed a fragment of the large phosphatase subunit from a rat kidney cDNA library. This fragment represented approximately the N-terminal two-thirds of the molecule and showed some differences in sequence between the chicken (Shimizu *et al*., 1994) and rat (Chen *et al*., 1994) isoforms. However, its properties were similar to those described above for the 130/133 kDa fragments. It bound PP1c and increased the specificity of PP1c towards myosin as substrate. In addition, the rat kidney fragment enhanced the ability of PP1c to relax permeabilized rabbit portal vein fibers.

Conclusions. It is difficult to provide simple interpretations that will account for all of the observed effects. However, two assumptions appear to be necessary for an initial working hypothesis. These are: (1) that the catalytic subunit is modified by interaction with the other subunits, principally the 130/133 kDa subunit; and (2) that the holoenzyme has two binding sites for P-LC20. One of these is the enzyme−substrate complex, *i.e.*, PP1c to P-LC20, and the second is the interaction of the N-terminal fragment of the 130/133 kDa subunit with P-LC20. This interaction occurs with dephosphorylated HMM in the absence of ATP and probably accounts for the binding of MBP to dephosphorylated myosin and its subfragments in the absence of ATP.

Several observations support the first point. PP1c is altered by combination with the glycogen-binding subunit (Cohen & Hardie, 1991; Doherty *et al*., 1995) and by the M-subunit in skeletal muscle (Moorhead *et al*., 1994). With the smooth muscle phosphatase, the holoenzyme had a reduced sensitivity to inhibitor 1 and inhibitor 2 (Alessi *et al*., 1992) compared to PP1c, and the holoenzyme was sensitive to Co^{2+} and PP1c was not (Okubo *et al*., 1993; Hirano *et al*., 1995). For the second point, there is direct evidence based on the binding of P-LC20 to the $rN130^{1-633}$ affinity column.

Alteration of PP1c by the 130/133 kDa subunit may be manifested by changes in k_{cat}, and in general, the k_{cat} values were higher for the holoenzyme compared to PP1c. Binding of the N-terminal region of the 130/133 kDa subunit to P-LC20 should stabilize the MBP−substrate complex and lead to a decrease in K_m. This trend was observed but was not marked with P-myosin as substrate. Thus, although this scheme may be used as an initial hypothesis, the situation appears to be more complex, and other factors could be involved. One example is that the binding of PP1c appears to be much stronger for P-myosin compared to the isolated light chain and this might reflect a difference in light chain conformations under the two situations. These and other factors should be considered when additional data are available.

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