Molecular cloning and functional expression of a recombinant 72.5 kDa fragment of the 110 kDa regulatory subunit of smooth muscle protein phosphatase 1M

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Received 11 October 1995

Abstract **We have cloned a partial rat kidney cDNA that encodes a 72.5 kDa N terminal fragment of a third isoform of the M110 subunit of phosphatase 1. This new isoform contains an insert in the 542–597 position not present in the M110 previously cloned (Chen et al. (1994) FEBS Lett. 356, 51–55) from the same species. The encoded cDNA was expressed as a soluble GST-fusion protein in *E. coli*, and its ability to interact with native PP-1C was measured both in vitro and in permeabilized smooth muscle. In vitro, the fusion protein was capable of selectively binding PP-1C and increasing the substrate specificity of the phosphatase towards myosin 13.2 ± 3.5-fold (S.E. of the mean, $n = 3$). In permeabilized smooth muscle pretreated with microcystin, the recombinant protein alone (1.0 μM) did not cause relaxation, but did significantly enhance the ability of PP-1C (0.3 μM) to relax the muscle. These findings show that the N terminal domain of the M110 subunit is the primary site for both PP-1C and myosin binding, and thereby determines myosin specificity. The presence of isoformic variation within this sequence may permit organ/cell specific regulation of phosphorylation sites.**

Key words: Smooth muscle; Protein phosphatase; Myosin; cDNA sequence; Microcystin

1. Introduction

Phosphorylation of the myosin P light chain (MLC_{20}) on serine 19 is the key event that triggers contraction in smooth muscle. Much has been learned about the regulation of the enzyme that catalyzes this reaction, the Ca^{2+}/Calmodulin-sensitive protein kinase, myosin light chain kinase (MLCK) (for review see [1,2]). However, the mechanisms that regulate myosin light chain phosphatase activity and their physiological significance have only recently been recognized (for review see [3,4] and refs. therein). At least three laboratories have purified to homogeneity the major form of avian and mammalian smooth muscle myosin phosphatase, SMPP-1M [5–7]. By SDS-PAGE analysis, SMPP-1M consists of a heterotrimer of subunits of 130 kDa, 37 kDa and 20 kDa respectively [5,6]. The 37 kDa subunit has been identified by amino acid sequencing as the catalytic subunit of protein phosphatase 1 [5,6]. The 130 kDa and 20 kDa subunits can be separated from PP-1C using high concentrations of chaotropic agents. Once separated from these subunits, PP-1C dramatically loses specificity for myosin, regaining it when the subunits are remixed [5,6]. Since, the 130 kDa and 20 kDa subunits can not be readily separated from each other under non-denaturing conditions, it is not clear whether both subunits are required for myosin and PP-1C binding or if they can act independently of one another. Indication of the role of the 130 kDa subunit has come from data showing that a 58 kDa proteolytic fragment has both myosin and PP-1C binding properties in vitro [5,7]. In addition, digoxigenin-labeled PP-1C was demonstrated to bind exclusively to the 130 kDa subunit in Western blotting studies [5].

The primary structures of rat aorta and chicken gizzard SMPP-1M 130 kDa [7,8] and 20 kDa [8] subunits have been determined by cDNA cloning. The full length nucleotide sequences were obtained from partial overlapping cDNA's and predicted to encode proteins in both species with molecular masses of ~110 kDa (designated M110 here on) for the 130 kDa subunit and 21 kDa (designated M21 here on) for the 20 kDa subunit. Alignment of the nucleotide sequence and encoded amino acid sequence shows that the chicken and rat M110 subunits are highly conserved in their N-terminal regions. Greatest divergence between the two species occurs in the C terminal domain, which in the case of the rat M110 appears to contain the entire M21 chicken sequence. Chicken M21 was also isolated as a separate cDNA, leading to the suggestion that the rat M21 subunit may be derived from alternate splicing of the M110 [8].

In order to determine the functional role of the M110 subunit and explore the existence of isoforms we have obtained in the current study a partial cDNA clone from a rat kidney cDNA library and show that it encodes a third isoform of M110. This new isoform contains an insert in the 542–597 position that is not present in the M110 previously cloned from the same species [8]. The clone was expressed as a 101 kDa soluble GST-fusion protein in *E. coli*. The ability of the recombinant M subunit to bind PP-1C and alter the substrate specificity of the phosphatase towards myosin was established both in vitro and in permeabilized smooth muscle.

2. Materials and Methods

2.1. Purification of Pig Bladder SMPP-1M

SMPP-1M was purified from whole pig bladders using a modification of procedures described previously by Shirazi et al. [6]. Briefly, following the polyethylene glycol precipitation step, the pellet was resuspended in buffer A (50 mM Tris-HCl pH 7.4 4°C, 100 mM NaCl,

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Abbreviations: Smooth muscle protein phosphatase 1M, SMPP-1M; catalytic subunit of protein phosphatase 1, PP-1C; 130 kDa myosin binding subunit, M110; 20 kDa myosin binding subunit, M21; glutathione-S-transferase, GST; microcystin, MC.

SSDI 0014-5793(95)01318-0

1 mM EGTA, 1 mM EDTA, 0.03% Brij (w/v), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 6 μg/ml leupeptin, 1 mM Benzamidine), centrifuged at 100,000 × *g* for 30 min and applied directly to a 1.0 ml column of microcystin-Sepharose [9]. Following washing with buffer A plus 1 M salt, SMPP-1M was eluted with buffer A plus 3 M sodium isothiocynate following methods previously described by Moorehead et al. [9]. The eluted protein was separated into its component subunits (37 kDa catalytic subunit and complex of 110 kDa and 20 kDa M subunits) by applying the sodium isothiocynate eluate directly to an SW300 (Waters) gel filtration column equilibrated with buffer A plus 150 mM NaCl [6]. The purified catalytic subunit was dialyzed into buffer B [buffer A plus 50% glycerol] and stored at −20°C. To obtain amino acid sequence, the 110 kDa and 20 kDa subunit complex was dialyzed into 50 mM sodium bicarbonate pH 8.0 and digested for 18 hours with trypsin (1:50, w/w). Individual tryptic peptides were isolated by reverse phase HPLC and their amino acid sequence determined in an Applied Biosystems gas phase sequencer.

2.2. Molecular cloning and expression of rat kidney isoform of the 110 kDa subunit of SMPP-1M

To isolate cDNA clones encoding rat kidney SMPP1M 110 kDa subunit a rat cDNA λgt 10 library was screened with two distinct oligonucleotide probes (5′GGAGTTGCAAAAAAAACAAAATCTGCTCCATAGTGAAAAGCGGATAAGAAA3′ and 5′GAACCAGAGAGAACAAGAAAACGAAGAAAAAG3′) derived both from tryptic peptide fragments of pig bladder SMPP-1M, and previously published cDNA sequences of rat aorta [8] and chicken gizzard [7] M110 subunits. Screening of 1×10^6 recombinants yielded 4 positive clones. A 2.0 kb clone was subcloned into the *Eco*RI site of pKS⁻, and sequence analysis was carried out on an Applied Biosystems 377 automated DNA sequencer. Sequencing was performed at least twice in both directions. The cloned cDNA encoded an open reading frame of 1975 bases corresponding to bp 153 (chicken)/bp 123 (rat aorta) and bp 2299 (chicken)/bp 2101 (rat aorta) in the previously published nucleotide sequences of Chen et al. [8] and Shimizu et al. [7] (Fig.1). For expression as a recombinant protein, the partial cDNA clone was subcloned into the *Eco*RI site of the bacterial expression vector pGEX-4T and transformed into *E. coli* strain DH5a. Individual colonies were grown at 30°C in LB containing ampicillin (100 μg/ml) until an absorbance of 0.8 was reached at 600 nms. Isopropyl-β-D-thiogalactopyranoside (0.1 M final) was added to induce expression of the cloned subunit. After 16 hours, cells were harvested by centrifugation at 6000 × *g* for 10 min. Following freezing at −20°C, the cells were thawed and lysed in 20 mls of buffer C (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 6 μg/ml leupeptin, 1 mM benzamidine) containing 0.5 mg/ml lysozyme. Following complete lysis, NP40 (10 μl/litre) was added and the lysate centrifuged at 30,000 × *g* for 30 min. The supernatant was mixed with 1.0 ml of a 1:1 slurry of GST-Sepharose (Pharmacia) for 30 minutes. The Sepharose was collected by mild centrifugation (500 × *g* for 2 min), washed with buffer C plus 1 M NaCl, and the protein eluted with 4.0 ml of C plus 5 mM glutathione. The protein was applied to an AP1Q (Waters) anion-exchange chromatography column (0.5 × 10 cm) equilibrated in buffer A. The column was developed (1.0 ml/min) with a linear salt gradient to 1 M NaCl in buffer A over 100 minutes. The purified fusion protein was identified in column fractions (1.0 ml) by SDS-PAGE and Coomassie blue staining, then dialyzed into buffer B and stored at −20°C.

3. Results

3.1. Nucleotide and predicted amino acid sequence

Fig. 1 shows the nucleotide and corresponding predicted amino acid sequence of the cloned rat kidney M subunit cDNA. The cloned nucleotide sequence encodes a protein of predicted molecular mass of 72557 Da. Amino acid alignment with the previously published rat aorta [8] and two chicken gizzard [7] M subunits shows that the clone is truncated at residue 658 in the C-terminal domain and lacks completely the M21 subunit sequence found in the M110 rat aorta sequence. In addition, at the N terminus, the clone lacks the initiating methionine and

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   1 CGCGAGCAGCTGAAGCGCTGGATCGGCTCCGAGACGGACCTCGAGCCTCCCGTGGTGAAGCGCCAG   66
   1 R  E  Q  L  K  R  W  I  G  S  E  T  D  L  E  P  P  V  V  K  R  Q    22
  67 AAGACCAAGGTGAAGTTCGACGATGGCGCCGTCTTCCTCGCCGCCTGCTCCAGCGGCGACACGGAC  132
  23 K  T  K  V  K  F  D  D  G  A  V  F  L  A  A  C  S  S  G  D  T  D    44
 133 GAGGTCCTCAAGCTGCTGCACCGCGGCGCCGACATCAATTACGCCAATGTGGACGGACTCACCGCC  198
  45 E  V  L  K  L  L  H  R  G  A  D  I  N  Y  A  N  V  D  G  L  T  A    66
 199 CTGCACCAGGCTTGCATTGATGACAATGTTGATATGGTGAAGTTTCTGGTAGAAAATGGAGCAAAT  264
  67 L  H  Q  A  C  I  D  D  N  V  D  M  V  K  F  L  V  E  N  G  A  N    88
 265 ATCAATCAACCTGACAATGAAGGCTGGATTCCACTCCATGCAGCCGCTTCCTGTGGATATCTGGAT  330
  89 I  N  Q  P  D  N  E  G  W  I  P  L  H  A  A  A  S  C  G  Y  L  D   110
 331 ATTGCAGAATTTTTGATTGGTCAAGGAGCACATGTAGGAGCTGTCAACAGTGAAGGTGACACACCT  396
 111 I  A  E  F  L  I  G  Q  G  A  H  V  G  A  V  N  S  E  G  D  T  P   132
 397 TTAGATATTGCAGAGGAGGAAGCAATGGAAGAGCTACTTCAAAATGAGGTTAATCGGCAAGGTGTT  462
 133 L  D  I  A  E  E  E  A  M  E  E  L  L  Q  N  E  V  N  R  Q  G  V   154
 463 GATATAGAAGCAGCTCGAAAAGAAGAGGAACGCATAATGCTTAGAGACGCGAGGCAGTGGTTGAAC  528
 155 D  I  E  A  A  R  K  E  E  E  R  I  M  L  R  D  A  R  Q  W  L  N   176
 529 AGTGGTCACATCAGTGACGTCCGGCATGCAAAGTCCGGAGGCACAGCACTCCACGTGGCAGCGGCC  594
 177 S  G  H  I  S  D  V  R  H  A  K  S  G  G  T  A  L  H  V  A  A  A   198
 595 AAAGGGTATACAGAAGTTTTAAAACTTTTAATACAGGCAGGCTATGATGTTAATATTAAAGATTAT  660
 199 K  G  Y  T  E  V  L  K  L  L  I  Q  A  G  Y  D  V  N  I  K  D  Y   220
 661 GATGGCTGGACACCTCTTCATGCTGCAGCTCACTGGGGTAAAGAAGAAGCATGTCGGATTTTAGTG  726
 221 D  G  W  T  P  L  H  A  A  A  H  W  G  K  E  E  A  C  R  I  L  V   242
 727 GACAATCTGTGTGATATGGAGACGGTCAACAAAGTGGGCCAAACAGCCTTTGATGTAGCAGATGAA  792
 243 D  N  L  C  D  M  E  T  V  N  K  V  G  Q  T  A  F  D  V  A  D  E   264
 793 GACATTTTGGGATATCTAGAGGAGTTGCAAAAAAAACAAAATCTGCTCCATAGTGAAAAGCGGGAT  858
 265 D  I  L  G  Y  L  E  E  L  Q  K  R  Q  N  L  L  H  S  E  K  R  D   286
 859 AAGAAATCTCCACTGATTGAATCAACAGCAAATATGGAAAATAATCAACCACAGAAGAATTTTAAA  924
 287 K  K  S  P  L  I  E  S  T  A  N  M  E  N  N  Q  P  Q  K  N  F  K   308
 925 AACAAGGAAACGTTGATTATTGAGCCAGAGAAAAATGCATCTCGAATCGACGTCTCTGGACAAGAA  990
 309 N  K  E  T  L  I  I  E  P  E  K  N  A  S  R  I  E  S  L  E  Q  E   330
 991 AAGGCTGATGAGGAGGAGGAAGGCAAGAAGGATGAGTCCAGCTGCTCCAGTGAGGAGGATGAGGAG 1056
 331 K  A  D  E  E  E  E  G  K  K  D  E  S  S  C  S  S  E  E  D  E  E   352
1057 GATGACTCCGAGTCCGAAGCGGAGACAGATAAGACAAAACCCATGGCTTCTGTAACTAATGCTCAC 1122
 353 D  D  S  E  S  E  A  E  T  D  K  T  K  P  M  A  S  V  T  N  A  H   374
1123 ACTGCCAGCACTCAGGCAGCTCCTGCCGCTGTGACAACACCTACTCTGTCTTCCAACCAGGGGACC 1188
 375 T  A  S  T  Q  A  A  P  A  A  V  T  T  P  T  L  S  S  N  Q  G  T   396
1189 CCTACATCACCTGTTAAAAAGTTTCCTACATCAACTACAAAAATTTCTCCCGAAGAAGAAGAAAGA 1254
 397 P  T  S  P  V  K  K  F  P  T  S  T  T  K  I  S  P  E  E  E  E  R   418
1255 AAAGATGAATCTCCTGCATCCTGGAGGTTAGGACTTAGAAAGACTGGCAGTTATGGTGCCCTGGCT 1320
 419 K  D  E  S  P  A  S  W  R  L  G  L  R  K  T  G  S  Y  G  A  L  A   440
1321 GAGATCACTGCATCTAAAGAAGCTCAGAAGGAGAAAGACACTGCAGGCGTGATACGTTCAGCTTCG 1386
 441 E  I  T  A  S  K  E  A  Q  K  E  K  D  T  A  G  V  I  R  S  A  S   462
1387 AGTCCCAGACTCTCGTCCTCTTTGGATAATAAAGAAAAGGAGAAAGACAATAAAGGAACAAGACTT 1452
 463 S  P  R  L  S  S  S  L  D  N  K  E  K  E  K  D  N  K  G  T  R  L   484
1453 GCATATGTCGCCCCTACAATCCCAAGGCGACTAGGCAGTACGTCTGACATTGAAGAGAAGGAAAAC 1518
 485 A  Y  V  A  P  T  I  P  R  R  L  G  S  T  S  D  I  E  E  K  E  N   506
1519 AGAGAGTCTTCAAATTTGCGAACAAGTAGTTCTTACACAAGAAGAAATGGGAAGATGATCTTAAA 1584
 507 R  E  S  N  L  R  T  S  S  S  Y  T  R  R  K  W  E  D  D  L  K   528
1585 AAAAATAGTTCAATCAATGAAGGATCTACTTACCATAGAAGTTGCTCCTTTGGTAGAAGACAAGAT 1650
 529 K  N  S  S  I  N  E  G  S  T  Y  H  R  S  C  S  F  G  R  R  Q  D   550
1651 GATTTGATTAGTTGTAGTGTTCCAAGCACCACATCAACACCAACAGTTACCTCTCCAGCTGGGCTT 1716
 551 D  L  I  S  C  S  V  P  S  T  T  S  T  P  T  V  T  S  P  A  G  L   572
1717 CAGAAAAGCTTCCTTTCCAGCACAAGCACTACTGCAAAGACTCCCCCGGGTTCCTCCCCAGCAGGC 1782
 573 Q  K  S  F  L  S  S  T  S  T  T  A  K  T  P  P  G  S  S  P  A  G   594
1783 ACACAGAGCAGGTCATACCTCACTCCTGTTAGGGATGAAGAGTCTGAATCCCACAGGAAAGCAAGA 1848
 595 T  Q  S  R  S  Y  L  T  P  V  R  D  E  E  S  E  S  H  R  K  A  R   616
1849 TCTAGACAAGCAAGACAGTCTAGACGGTCAACACAGGGGGTGACACTGACTGACCTCCAGGAAGCC 1914
 617 S  R  Q  A  R  Q  S  R  R  S  T  Q  G  V  T  L  T  D  L  Q  E  A   638
1915 GAAAAGACAATAGGAAGAAGCCGTTCTACGAGAACCAGAGAACAAGAAAACGAAGAAAAAG 1975
 639 E  K  T  I  G  R  S  R  S  T  R  T  R  E  Q  E  N  E  E  K    658
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Fig. 1. Nucleotide and predicted amino acid sequences of the cloned rat kidney M110 subunit.

subsequent 9 amino acids. The truncations in the clone appear to be due to the insert size of the cDNA library which was estimated <2.2 kb. At the nucleotide level, the cloned cDNA is 94% homologous with rat aorta M110 [8] and 84% homologous with the two chicken clones [7,8]. Significant nucleotide sequence divergence is confined to a single region, between bp 1622 and 1790 when compared with rat aorta M, and in two distinct regions, bp 1114–1154 and bp 1528–1790, when compared with chicken M. Comparison at the amino acid level, reveals the encoded protein to be 91% homologous to the N terminal domain of the rat aorta sequence and 90% homologous to the N terminal domain of chicken M subunit. Importantly, the potential phosphorylation sites [7] for the cyclic AMP-dependent protein kinase and protein kinase C are conserved.

Consistent with the single region of divergence at the nucleotide level, when the cloned rat kidney M110 is aligned with the amino acid sequence of rat aorta M subunit, a distinct region of divergence occurs between residues 542–597 (Fig. 2A). When aligned with the two chicken M subunit sequences, two distinct divergent regions occur at residues 364–393 and 537–597. Residues 364–393, are however, completely conserved in the rat aorta sequence. Interestingly, part of the amino acid sequence (RSXSFGRRQDDLXSXXVPSTXSTXT) present in the rat kidney M110 insert is also present in the two chicken M110 cDNAs (residues 552–576, chicken M) isolated by Shimizu et al. [7] (Fig. 2B). Notably, as discussed earlier, this region is absent in the rat aorta sequence [8]. The functional significance of the inserts in all the M isoforms cloned thus far is not clear,

A. Rat kidney insert compared with rat aorta.

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       542                                                            598
Kidney SCSFGRRQDDLISCSVPSTTSTPTVTSPAGLQKSFLSSTSTTAKTPPGSSPAGTQSR
       S S      +D        S  + T+       +  + SST+    T  G+   + ++ R
Aorta  STSNRLWA EDSTEKEKDSAPTAATILVAPT VVSAAASSTTALTTTTAGTLSST S EVR
       552                                                            608
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B. Rat kidney insert compared with chicken gizzard.

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        537                                                               597
Rat     STYHRSCSFGRRQDDLISCSVPSTTSTPTVTSPAGLQKSFLSSTSTTAKTPPGSSPAGTQS
        ++Y RS SFGRRQDDL+S +VPST ST T ++              ++T+T + T    S+   + S
Chicken TSYQRSGSFGRRQDDLVSSNVPSTASTVTSSAGLQKTLPASANTTTKSTTGSTSAGVQSSS
        548                                                               608
```

Fig. 2. Amino acid sequences of the insert region of rat kidney M110 compared with the insert regions of rat aorta and chicken gizzard M110. Amino acid sequence alignment was performed by Gene Bank BLAST analysis software [18]. Common sequences are indicated with single amino acid letter code, + indicates conservative substitutions.

since none of them appear to have significant homology with other proteins in the data base. One possibility is that the inserts may target the subunit for selective interaction with other molecules, such as different isoforms (smooth and non-muscle) of myosins. Notably, 42% of the kidney insert is made up of serine (25%) or threonine (17%) residues, suggesting the insert may be the target for multiple phosphorylation events. Indeed, number of consensus phosphorylation sites are present in the insert sites in all the clones and include those for MAPK and protein kinase C. Importantly, the overall degree of conservation in the N terminal domains of the M110 cDNA clones suggests that M110 isoforms arise as the result of alternate splicing of a single gene.

3.2. Characterization of the expressed cDNA

In order to further characterize the role of the M110 subunit in the regulation of smooth muscle contraction the cloned rat kidney cDNA was expressed as a soluble GST-fusion protein in *E. coli* (Fig. 3). Following purification from the induced *E. coli*, approximately 2–3 mg of purified fusion protein was obtained per litre of culture. On SDS-PAGE, the purified GST-protein migrated with a putative molecular weight of ~101 kDa, consistent with an encoded M subunit sequence of ~73 kDa combined with 28 kDa fusion protein. Western blotting analysis with a monoclonal antibody to the 58 kDa fragment of chicken gizzard M subunit, confirmed the identity of the expressed protein as M subunit (Fig. 3B). To test whether the expressed protein was functional, the ability of the protein to bind and alter the specificity of PP-1C towards ^{32}P-labeled myosin and ^{32}P-labeled phosphorylase *a* was measured in gel filtration studies (Fig 4). Fig. 4, shows that in the absence of the recombinant M subunit, PP-1C elutes from the gel filtration column with a putative molecular weight of ~37 kDa. As a free catalytic subunit, PP-1C displayed low activity towards myosin relative to phosphorylase *a*. In contrast, when PP-1C was incubated with a 2 fold molar excess of recombinant M subunit prior to gel filtration, >90% of the phosphorylase phosphatase activity eluting at 37 kDa was lost. A new peak, displaying high myosin phosphatase activity and low phosphorylase phosphatase activity was recovered eluting at ~150 kDa (Fig. 4A). The recombinant protein interacted specifically with PP-1C, since

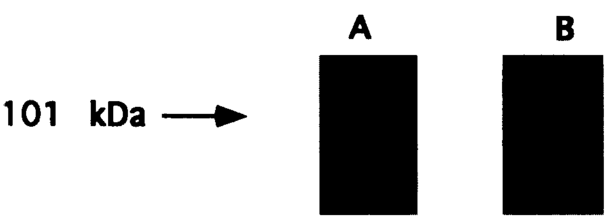

Fig. 3. Characterization of the purified GST-fusion M subunit by SDS-PAGE and Western blotting. (A) Coomassie blue staining of the GST-fusion protein following purification from GST-Sepharose and anion-exchange chromatography. (B) The purified GST-fusion protein was immunoblotted with monoclonal antibody (gift from D. Hartshorne, U. Arizona, Tucson) raised to the 58 kDa fragment of chicken gizzard M110.

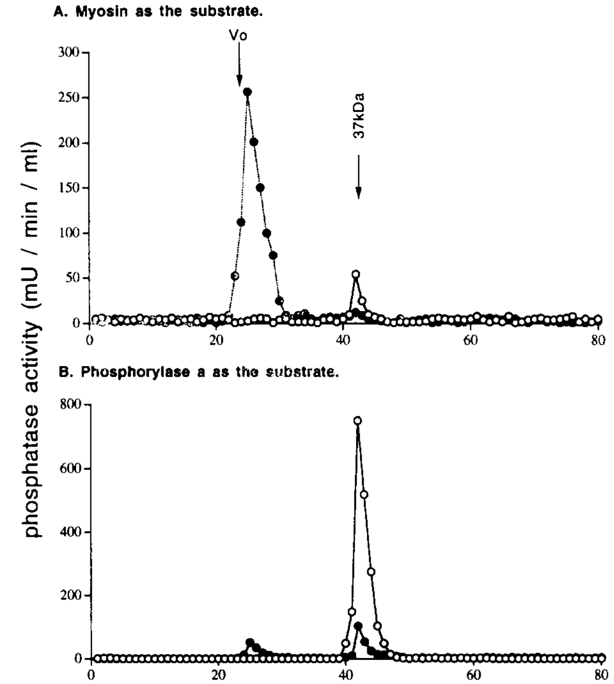

Fig. 4. Analysis of the interaction of GST-fusion M subunit with PP-1C by gel filtration chromatography. Purified pig bladder PP-1C (0.3 μM) was incubated for 30 min on ice in the presence (●) or absence (○) of recombinant M subunit (0.6 μM). The proteins were applied (100 μl) to an SW300 column equilibrated (1.0/min) in buffer A containing 150 mM NaCl. Column fractions (0.2 ml) were assayed either with ^{32}P-labeled myosin (A) or ^{32}P-labeled phosphorylase *a* (B). Methods for phosphatase assays are described [6].

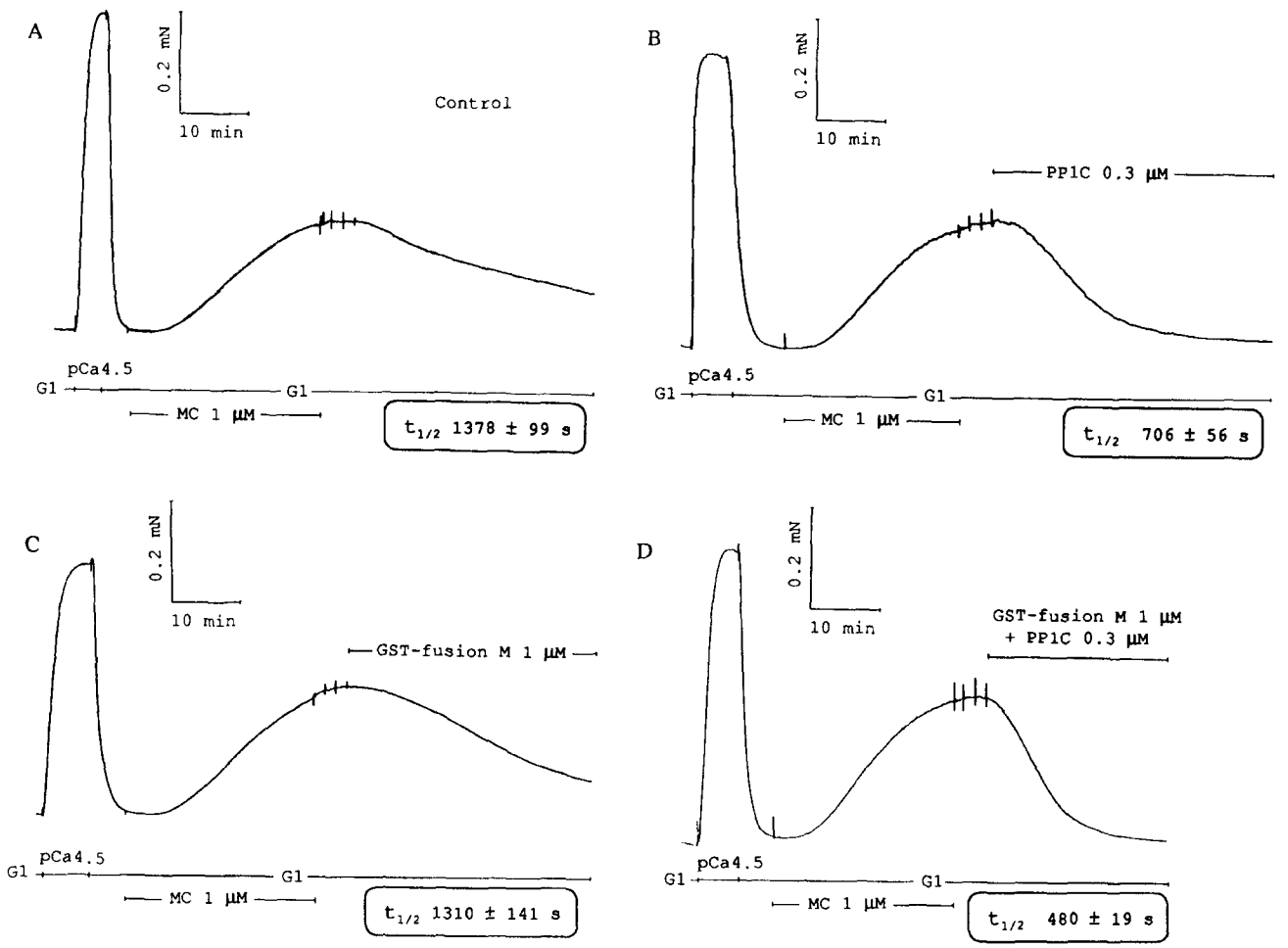

Fig. 5. Effects of GST-fusion M subunit on the ability of PP-1C to relax microcystin treated permeabilized rabbit portal vein. (A) Spontaneous rate of relaxation, (B) Effects of PP-1C alone, (C) effects of GST-fusion M subunit, (D) effects of the addition of PP-1C in the presence of GST-fusion M subunit. Methods for the preparation and treatment of permeabilized smooth muscle are detailed elsewhere [6]. G1: EGTA 1 mM containing solution. $t_{1/2}$ = half time of relaxation. Results shown are mean (±S.E.) of five separate experiments.

no increase in molecular weight or effect on myosin and phosphorylase phosphatase activity was observed when the gel filtration experiments were repeated with purified PP-2AC (data not shown). These results suggest that the recombinant, like the native protein [5,6], is capable of both selectively binding PP-1C and altering its specificity towards myosin. Since the recombinant protein lacks the M21 sequence, the present result also indicates that the M21 subunit is not required for binding PP-1C to or conferring specificity for myosin.

3.3. *The effects of the recombinant protein on smooth muscle contraction*

To further examine the role of the M110 subunit and the functional properties of the recombined protein in regulating smooth muscle contraction, we determined its effects on relaxation of microcystin-treated permeabilized smooth muscle in the presence and absence of purified PP-1C (Fig. 5). As shown previously [6,10], microcystin caused a sustained Ca^{2+}-independent contraction that reached a plateau within 20 minutes (Fig. 5A). Following removal of the unbound microcystin, the contractile response was sustained for 10–15 min and then spontaneously relaxed slowly and incompletely. Addition of the recombinant protein (1.0 μM) to the microcystin treated muscles in the absence of PP-1C, caused no significant change in the rate of relaxation from control values (Fig. 5C, Table 1). Purified PP-1C (0.3 μM) caused a significant increase in the rate of relaxation (Fig. 5B) that was further accelerated when purified PP-1C was added in combination with recombinant M110 (Fig. 5D). These data are consistent with the gel filtration studies (Fig. 3), showing that recombinant M110 was capable of enhancing the ability of PP-1C to selectively dephosphorylate ^{32}P-labeled myosin. The half time of relaxation achieved with the combination of recombinant M110 (1.0 μM) and PP-1C (0.3 μM) compares well with previous studies [6] using a similar concentration of native SMPP-1M or native 130 kDa/20 kDa (PP-1C + fusion protein $t_{1/2}$ = 480±19 s (n = 5 ± S.E.M.) this study; native pig bladder SMPP-1M $t_{1/2}$ = 395 ± 40 s (n = 5 ± S.E.M.) previous work; 130 kDa/20 kDa M subunit + PP-1C $t_{1/2}$ = 427 ± 24 s (n = 2, ± S.E.M.) previous work).

4. Discussion

In this study we have cloned a rat kidney cDNA that encodes a 72.5 kDa N-terminal fragment of a third isoform of the M110 subunit of phosphatase 1. The encoded cDNA was expressed as a soluble GST-fusion protein in *E. coli* and its ability to

interact with native PP-1C measured both in vitro and in permeabilized smooth muscle. In vitro, the fusion protein was capable of selectively binding PP-1C and altering the substrate specificity of the phosphatase towards myosin. In permeabilized smooth muscle pretreated with microcystin, the recombinant protein (1 μM) alone did not cause relaxation, but did significantly enhance the ability of PP-1C to relax the muscle. These findings demonstrate that the N-terminal domain of the M110 subunit is the primary site of both PP-1C and myosin binding, and therefore, determines myosin specificity. Indeed, the 58 kDa fragment of M110 binds the catalytic subunit, and this complex, in turn, binds to myosin, albeit with reduced efficiency compared to the holocomplex [7]. The M21 subunit is not required for any of these functions, since this sequence was absent in the recombinant protein.

The finding that rat kidney M subunit contains an insert region that is completely distinct from other previously cloned rat (aortic) protein suggests that the activity of isoforms of M may be regulated selectively. The function and nature of this regulation is not clear, although, given its size (~53 amino acids compared with rat aorta) the insert may form a selective site for interaction with other proteins or factors. The presence of the M110 subunit(s) in many tissues [7] in addition to smooth muscle and the multiple forms of non-muscle myosins regulated by phosphorylation/ dephosphorylation, suggests a general regulatory mechanism based on modulation of myosin phosphatase activity [4]. Evidence of regulated SMPP-1M activity has already been obtained in smooth muscles in which GTPγS and G-protein-coupled agonists can induce an increase in MLC_{20} of myosin and, consequently, force at constant Ca^{2+} [11–13]. The increase in MLC_{20} phosphorylation is due to inhibition of SMPP-1M activity [14]. Two potential mechanisms of such inhibition have already been described: arachidonic acid, released by GTPγS and Ca^{2+} sensitizing agonists [15] dissociates the SMPP-1M holoenzyme and selectively inhibits its activity toward myosin [10,15]. This suggests a regulatory mechanism analogous to that proposed for regulation of glycogen synthase phosphorylation through association/dissociation between the targeting G subunit and the catalytic subunit [16]. A second mechanism of inhibition is suggested by the correlation between thiophosphorylation of the M subunit and inhibition of light chain dephosphorylation in vivo [17]. Either or both of the above mechanisms represent potential pathways of regulation, perhaps selectively modulated by M subunit isoforms (present study) within the same species.

Acknowledgements: Thanks to Mr. Zhiding Qian for technical assistance. Thanks to Kevin Lynch (University of Virginia) for supplying the rat cDNA λgt 10 library. We are grateful to Dr. David Hartshorne for his generosity in providing the monoclonal antibody to chicken gizzard M110. This work was support by a grant from the National Institutes of Health PO1 HL19242–20.

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