Interactions of the Subunits of Smooth Muscle Myosin Phosphatase*

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Myosin phosphatase from smooth muscle consists of a catalytic subunit (PP1c) and two non-catalytic subunits, M130 and M20. Interactions among PP1c, M20, and various mutants of M130 were investigated. Using the yeast two-hybrid system, PP1c was shown to bind to the NH2-terminal sequence of M130, 1–511. Other interactions were detected, i.e. PP1c to PP1c, M20 to the COOH-terminal fragment of M130, and dimerization of the COOH-terminal fragment of M130. Mutants of M130 were constructed to localize the PP1c and light chain binding regions. Results from the two-hybrid system indicated two binding sites for PP1c on M130: one site in the NH2-terminal 38 residues and a weaker site(s) in the ankyrin repeats region. Inhibition of PP1c activity with phosphorylated light chain required binding sites for PP1c and substrate, plus an additional sequence COOH-terminal to the ankyrin repeats. Activation of PP1c with phosphorylated light chain required binding sites for PP1c and substrate, plus an additional sequence COOH-terminal to the ankyrin repeats. Thus, activation of phosphatase and binding of PP1c and substrate are properties of the NH2-terminal one-third of M130.

Phosphorylation of the two regulatory light chains of myosin is an important mechanism in controlling the contractile activity of smooth muscle (1). The extent of phosphorylation depends on the balance of activities of two enzymes: the Ca2+/calmodulin-dependent myosin light chain kinase and a myosin phosphatase (MP). Under some conditions, for example stimulation with certain agonists, the extent of phosphorylation at suboptimal Ca2+ concentrations can increase, and this has been traced to an inhibition of MP activity (2). The pathway from receptor occupancy to phosphatase inhibition is not established but is thought to involve trimeric (2) and monomeric (2–6) G proteins. In addition it has been suggested that MP inhibition is caused by dissociation of the subunits by arachidonic acid (2, 7) or by phosphorylation of the large MP subunit (8, 9).

*A classification of protein phosphatases which is used widely was based on substrate preferences and inhibition by inhibitors 2 and 10 (10). Two classes were identified, 1 (PP1) and 2 (PP2) with the latter divided into three subgroups, A, B, and C. Several years ago it was suggested that the smooth muscle MP was a type 1 enzyme (11). Subsequently three laboratories have reported that MP from gizzard (12, 13) and pig bladder (14) consists of three subunits: a catalytic subunit of about 38 kDa; a large subunit of 110–130 kDa (based on mobilities on SDS-PAGE), termed M130; and a subunit of about 20 kDa, M20. The cDNAs for each subunit have been sequenced. The PP1c from gizzard is the δ isoform (13), also referred to as the β isoform (12). In gizzard, two isoforms of the large subunit exist, M130 and M133 (13); these are similar to, but not identical with, the large subunit from rat aorta, termed M110 (15). Another isoform of this subunit was detected from a rat kidney cDNA library and represented the NH2-terminal 72.5-kDa fragment (16). The derived sequence of M20 from gizzard (15) indicates several leucine zipper sequences at the COOH-terminal end. This structure is similar to the COOH terminus of rat aorta M110 (15) but different from the two gizzard isoforms (13) which lack leucine zippers.

A possible regulation mechanism for phosphatases, and kinases, involves targeting (12) or anchoring subunits (17). These bind to the catalytic subunit and to the substrate or other specific target. In addition to localizing the phosphatases they may also modify the activity of the catalytic subunit. Thus the two non-catalytic subunits of MP could act as targeting subunits (12). In support of this idea it is known that the holoenzyme has enhanced activity toward myosin, compared with PP1c (12, 14), and also that the holoenzyme binds to myosin (13). The sites for interactions among subunits are not defined, but it has been shown that the NH2-terminal portion of M130 probably contains the PP1c site(s) and myosin binding site(s) (16, 18). An interesting feature of the M130 molecule is that it contains several ankyrin repeat sequences (13, 15) close to the NH2 terminus which could provide a platform for multiple interactions.

To investigate interactions of the subunits of MP the yeast two-hybrid system was employed. This powerful technique to study protein-protein interactions was developed by Fields and Song (19) and subsequently has been used for many systems, including screening of a gizzard cDNA library for PP1c-binding proteins (20, 21). In this present application various subunits, or fragments thereof, were inserted as bait or prey with the objectives of obtaining an outline of the subunit interactions of the holoenzyme. Where applicable the interactions were also monitored by enzyme assays or by overlay techniques.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized at the Macromolecular Structure Facility at the University of Arizona and by National Biosciences Inc. Plymouth, MN. The antibody to the hexahistidine tag (Ni2+NTA-agarose) was from Qiagen. Enzymes and media for bacterial and yeast cultures and radionucleotides were as listed previously (20).
Bacteria and Yeast Strains—Escherichia coli DH5α was used as the transformation recipient for plasmid constructions in the two-hybrid system. Saccharomyces cerevisiae strain Y190 (20) and Y187 (Clontech) were used for the two-hybrid assay, as a recipient of bait and prey plasmid, respectively. E. coli JM109 and M15[RecP4] were used as transformation recipient and as expression host for the pQE expression system (Qiagen), respectively.

**Plasmid Construction for the Two-hybrid Assay—** Plasmid pAS1 was used to construct the bait plasmid encoding the GAL4 DNA binding domain hybrid protein (20). Plasmid pACT2 (Clontech) or pGAD424 (20) was used to construct the prey plasmid encoding the GAL4 activation domain hybrid protein. All cDNA plasmids were obtained by PCR amplification with Taq DNA polymerase (Boehringer Mannheim). A cDNA of the full-length coding region of the catalytic subunit of type 1 protein phosphatase (PP1α) was obtained as described (20). The cDNAs for some of the plasmids were obtained by PCR amplification directly from a chicken gizzard Uni-Zap cDNA library (13). These included the cDNAs for two NH2-terminal fragments of M130 containing residues 1–511 and 1–633 (referred to as M1301–511 and M1301–633, respectively; a COOH-terminal fragment of M130 containing residues 512–963; a COOH-terminal fragment of M130 containing residues 512–963; and the coding region of M20, minus the COOH-terminal 28 residues (M201–159). In some plasmid constructions the insert was transferred from a preexisting plasmid to another, using PCR amplification of the insert DNA. All primers were designed to contain restriction sites for ligating the insert, to which a stop codon was inserted in the primer. All plasmids thus obtained were pAS1-PP1α, pAS1-PP1α1–150, pAS1-M20, pAS1-M1301–511, and pAS1-M1301–633. The prey plasmids were pACT2-PP1α, pACT2-M1301–511, pACT2-M1301–633, pGAD424-PP1α, pGAD424-M20, and pGAD424-M1301–511. The DNA sequences of all plasmids were determined.

**Construction of Truncation Mutants—** Various truncation mutants of M130 were constructed for use in the two-hybrid system and for expression as hexahistidine-tagged proteins (18). All cDNAs for the truncation mutants were obtained by PCR amplification with pACT2-M1301–633 as the template DNA. The sense and antisense primers were designed to contain BamHI and SalI sites for ligation to the vector plasmid, respectively. Pfu DNA polymerase (Stratagene) was used for the PCR. The BamHI and SalI digested PCR products were ligated to the BamHI- and XhoI-digested pACT2 for the prey constructs in the two-hybrid system and to BamHI- and SalI-digested pQE32 for expression as hexahistidine-tagged proteins. Truncation mutants obtained were M1301–38, M1301–69, M1301–104, M1301–137, M1301–171, M1301–196, M1301–207, M1301–231, M1301–255, and M1301–271. The DNA sequences of all plasmids were determined.

**The Two-hybrid Assay Using Yeast Mating—** The principle of the two-hybrid assay system was to observe protein-protein interactions described elsewhere (22). In the standard two-hybrid assay, haploid yeast cells are cotransformed with two plasmids, bait and prey. This standard assay was used to examine interactions between the phosphatase subunits with pAS1 and pGAD424 as bait and prey vectors, respectively. However, the sensitivitiy of this assay was low. To increase sensitivity the two-hybrid assay for protein-protein interaction was described elsewhere (22). In the standard two-hybrid assay, haploid yeast cells are transformed with two plasmids, bait and prey. This standard assay was semi-quantitatively evaluated by examining growth of yeast colonies on SC-Try-Leu-His containing various concentrations of 3-aminotriazole. Therefore, the level of His selection can be modulated by varying the concentrations of 3-aminotriazole.


1 through 511 (M1301–511), and the COOH-terminal part of M130 (M130512–963). The prey plasmids included PP16 and M1301–511 plus an NH2-terminal fragment of PP16 (PP161–108), a larger NH2-terminal fragment of M130 (M1301–633), and a COOH-terminal portion of M130 (M130514–963). Results from the two-hybrid assays are shown in Table I. With PP16 as bait, the interacting bait-prey pairs were PP16-PP16, PP16-M1301–633, and PP16-M1301–633. The interaction between the catalytic subunit and the NH2-terminal fragment of M130 was bidirectional and was shown when M1301–511 was used as bait and PP16 as prey (Table I). A PP16 mutant containing the NH2-terminal third of PP16 (PP161–108) did not show this interaction (Table I). Other interactions also were detected and were: binding of M20 (as bait) and the COOH-terminal fragment of M130 (M130512–963); interaction among the COOH-terminal fragments of M130; and a weaker interaction between PP16 and the COOH-terminal fragment of M130.

The key point from the results shown in Table I was that PP16 bound to the NH2-terminal fragment of M130. However, the smallest NH2-terminal fragment used was still relatively large, i.e. 511 residues, and it was important to define more precisely the PP16 binding site(s). To achieve this a variety of mutants was constructed as shown in Fig. 1. These include progressive COOH-terminal deletions from Asn-511 and NH2-terminal deletions with Asn-511 fixed as the COOH terminus. These mutants were used in the two-hybrid system as prey proteins with PP16 as bait and were also expressed as hexahistidine-tagged fusion proteins.

The results of the two-hybrid assays using both the filter lift and β-galactosidase measurements are shown in Table II. All of the mutants that contained sequences initiated at Met-1 bound PP16. The shortest NH2-terminal segment was Met-1 to Phe-38, and this also bound to PP16. This sequence precedes the ankyrin repeats that for the gizzard M130133 isoforms begin at Asp-39 (13). Although the interaction between M1301–38 and PP16 appeared weaker than for longer segments of M130 (Table II), it represented a critical interaction since those mutants lacking the NH2-terminal 39 residues did not bind PP16. This is shown in Table II for the mutant M1301–38. Other mutants lacking longer NH2-terminal sequences also did not bind PP16 (Table II).

The 3-amino triazole resistance (an assay of the HIS3 reporter gene) of these bait-prey pairs also was estimated. In general, the same pattern was observed as shown in Table II. The strongest interactions, i.e. PP16-M1301–633 and PP16-M1301–69, showed vigorous growth at 30 mM 3-amino triazole and positive but reduced growth at 50 mM. The weaker interactions had a reduced tolerance of 3-amino triazole (data not shown).

The Effect of M130 Mutants on Phosphatase Activity—It was shown previously that the MP holoenzyme is more active with phosphorylated myosin or P-LC20 than the isolated catalytic subunit (12, 14, 18) and that this activation is carried by an NH2-terminal fragment, i.e. M1331–674 (18). It is assumed that activation of phosphatase activity requires the presence of at least two sites on M130, a binding site for PP16 and one for the substrate, P-LC20. Thus the M130 mutants, expressed as hexahistidine fusion proteins, were assayed for their effect on PP1c activity using 32P-LC20 as substrate (Fig. 2). Three of the mutants containing the NH2-terminal region of M130 activated PP1c activity. These were M1301–633, M1301–511, and M1301–374. However, the smallest mutant was less effective, and activation required higher concentrations. Further truncation of the NH2-terminal segment caused loss of activation, as shown for M1301–296. The M130 fragment in which the first 39 residues were deleted, M13040–511, also did not activate phosphatase activity (Fig. 2).

One possibility is that the interaction of PP1c and M130 induced a conformational change in PP1c which resulted in activation of phosphatase activity. It would be predicted that this effect is independent of substrate. To test if the activation of PP1c is dependent only on interaction with M130 and is not specific with respect to substrate, the effect of various mutants on phosphatase activity was assayed using phosphorylase α as substrate. The effect of three representative M130 mutants is shown in Fig. 3. Each mutant inhibited phosphatase activity, but the potency of inhibition varied markedly. The most effective inhibitor was M1301–511, whereas M13040–511 was considerably less potent. To categorize this effect each of the mutants was assayed for its effect on the PP1c-phosphorylase α system, and IC50 values (i.e. the concentration of mutant required for 50% inhibition) were calculated. These are given in Table III. The relative value for each mutant compared with M1301–633 also is given. These values can be divided into three groups. For the mutants including the sequence from 1–633 down to 1–296 (top four mutants of Table III) the IC50 values were in the subnanomolar range. On further truncation at the COOH-terminal end of M130, i.e. those mutants including 1–171 to 1–38, the IC50 values were approximately 10-fold higher. Deletion of the NH2-terminal sequences, notably the first 39 residues, caused a further increase in IC50 values (bottom three mutants of Table III).

Light Chain Overlays—To obtain an independent assessment of P-LC20 binding an overlay procedure was used. Each of the M130 mutants was screened for binding to thiophosphorylated, biotinylated LC20. In Fig. 4 are shown the SDS-PAGE patterns of the mutants and the light chain overlays. For the larger mutants many of the lower molecular mass bands were proteolysis products since they retained the hexahistidine tag (as shown by the MRG8His antibody). For the smaller mutants (M1301–171 and below) proteolysis apparently was not a problem. Binding of light chain was detected for the NH2-terminal fragments containing a complete ankyrin repeat region M1301–296 and larger and also for mutants M13040–511 and M1301–511. The latter two mutants did not activate PP1c. The M1301–511 mutant contains the COOH-terminal half of the ankyrin repeats, and this suggests that this part of the molecule is involved in light chain interaction.

### Table I

| Bait (pAS1) | Empty | PP16 | PP161–108 | M1301–511 | M1301–633 | M1301–963 |
|------------|-------|------|-----------|-----------|-----------|-----------|
| PP16       | 0.03 ± 0.03 | 1.0 ± 0.4 | 0.05 ± 0.002 | 3.1 ± 0.6 | 2.2 ± 0.6 | 0.14 ± 0.02 |
| M20        | 0.04 ± 0.01 | 1.20 ± 0.04 | 0.04 ± 0.01 | 0.09 ± 0.03 | 0.07 ± 0.01 | 12.2 ± 2.1 |
| M1301–511  | 0.04 ± 0.03 | 5.5 ± 0.6 | 0.04 ± 0.002 | 0.06 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.002 |
| M130512–963| 0.01 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.02 | 1.3 ± 0.1 |

β-Galactosidase activity of mated yeast cells expressing subunits and mutants of myosin phosphatase as bait and prey

β-Galactosidase activity of the yeast cell lysate was determined using chlorophenol red-β-D-galactopyranoside as substrate. Unit activity is defined as in Bartel and Fields (22). The underlined values showed significant difference (p < 0.01) from that of the empty prey vector with the same bait protein, as assessed by Student’s t test for unpaired data. Data are mean ± S.E. (n = 3).
DISCUSSION

From the initial two-hybrid screen a rough plan of the holoenzyme can be obtained. The NH$_2$-terminal third of M130 is involved in the interaction with PP1c, and M20 binds to the COOH-terminal part of M130. The function of M20 is not known. The results of the two-hybrid assays indicate that it does not bind to PP1d. Earlier results showed that the complex of PP1c and an NH$_2$-terminal fragment of M130, i.e. the 58-kDa component, was similar to the trimeric MP holoenzyme in terms of myosin dephosphorylation and binding to myosin (33). This complex did not include M20. Thus a role for M20 in activation of PP1c or in binding to myosin is unlikely. It has not been determined if M20 is required for regulation of MP activity. In addition, it is possible that M20 does not influence phosphatase activity but may serve an auxiliary function such as targeting MP to other proteins and indirectly modifying activity or determining cellular localization.

Various interactions with PP1c (PP1δ) were indicated. The initial two-hybrid screen showed that PP1δ could self-associate. Dimer formation of PP1c has been demonstrated (34), and it was also proposed that myosin light chain phosphatases isolated from gizzard consisted of a tetramer of catalytic subunits (35, 36). Thus, the association of PP1c subunits is consistent with earlier results, but the physiological significance of dimer or tetramer formation is not known. It is unlikely that a PP1c dimer or tetramer would have a high affinity for myosin since PP1c in solution does not bind to dephosphorylated or phosphorylated myosin (18).

Interaction of PP1c and M130 forms an important component of the function of the MP holoenzyme. The two-hybrid assays for various M130 mutants as prey and PP1δ as bait indicated that PP1δ binds to the NH$_2$-terminal part of M130. The surprising observation was that PP1δ bound to the NH$_2$-terminal segment of 38 residues. This precedes the ankyrin repeats that begin at Asp-39. The binding of PP1δ to this segment was weaker than for the longer NH$_2$-terminal sequences but was an important component of overall binding since the mutants lacking this sequence, e.g. M130$_{40-511}$, did not bind to PP1δ as strongly as the wild-type M130.

**TABLE II**

β-Galactosidase activity of mated yeast cells expressing truncation mutants of M130$^{1-633}$ and PP1δ as prey and bait

Expression of β-galactosidase in the mated yeast cells expressing PP1δ as bait and various truncations of M130$^{1-633}$ as prey was assessed by the filter lift assay and β-galactosidase activity assay (see Table I). The criteria used for the filter lift assay were: ++++, turned blue ≤1 h; +++, 1–3 h; ++, 3–8 h; +, >8 h; −, no blue color development after overnight incubation. The significant difference (*, p < 0.01; **, p < 0.05) in activity from that of the empty vector was determined by Student’s t test. Activity data are mean ± S.E.

| Prey            | Filter lift assay | β-Galactosidase activity (Unit n) |
|-----------------|------------------|----------------------------------|
| pACT2 vector    | −                | 0.03 ± 0.03 (5)                  |
| M130$^{1-633}$  | ++++             | 2.2 ± 0.6* (6)                   |
| M130$^{1-511}$  | ++++             | 3.1 ± 0.5* (15)                  |
| M130$^{1-374}$  | ++++             | 5.5 ± 0.5* (3)                   |
| M130$^{1-296}$  | ++++             | 14.6 ± 2.7* (4)                  |
| M130$^{1-171}$  | ++++             | 5.0 ± 0.2* (3)                   |
| M130$^{1-137}$  | ++++             | 4.4 ± 0.7* (4)                   |
| M130$^{1-104}$  | ++++             | 9.7 ± 4.9* (7)                   |
| M130$^{1-69}$   | ++++             | 11.8 ± 3.6* (15)                 |
| M130$^{1-38}$   | +                | 0.25 ± 0.10** (4)                |
| M130$^{40-511}$ | −                | 0.06 ± 0.03 (3)                  |
| M130$^{168-511}$| −                | 0.03 ± 0.02 (3)                  |
| M130$^{304-511}$| −                | 0.03 ± 0.01 (3)                  |

**FIG. 1.** Schematic representation of the M130 mutants. The NH$_2$- and COOH-terminal residues are indicated, and the abbreviation used for each is given. The shaded box areas indicate the eight ankyrin repeats, and the open box area is the acidic cluster.

**FIG. 2.** Activation of phosphatase by M130 mutants with phosphorylated light chain as substrate. See “Experimental Procedures” for assay conditions. The following mutants are shown: M130$^{1-633}$ (●), M130$^{1-511}$ (■), M130$^{1-374}$ (▲), M130$^{1-296}$ (▼), M130$^{1-171}$ (△), M130$^{1-38}$ (●) and M130$^{40-511}$ (○).
not give a positive signal. Recently, Endo et al. (37) have shown that an NH$_2$-terminal peptide of inhibitor-1, KIQF, was required for full inhibition by phosphorylated inhibitor-1. One possibility, suggested by these authors, was that the tetrapeptide represented part of a PP1c binding site distinct from the catalytic site occupied by the phosphorylated Thr-35 of inhibitor-1. A similar sequence, KVKF, is found only in one position in M130/133, namely at residues 35–38 (13). This NH$_2$-terminal sequence also is present in rat aorta M110 (15) and rat kidney M110 (16). Thus it is possible that this sequence forms at least part of the PP1c binding site present in the sequence 1–38.

While this manuscript was in preparation, Johnson et al. (38) also noted the importance of the NH$_2$-terminal sequence of M130 in binding PP1c. However, they reported that the sequence 1–38 activated PP1c (38) and facilitated relaxation in skinned fibers (39), although at relatively high concentrations. This was not observed in our studies.

The inhibition data from the PP1c-phosphorylase a assays can also be used to assess PP1c binding to the M130 mutants. If it is assumed that there is no specific interaction between M130 and phosphorylase a, then inhibition of phosphatase activity by M130 and its mutants would reflect competitive binding of PP1c to M130 and phosphorylase a. In addition, it is required that the binding sites(s) on PP1c for M130 and phosphorylase a is similar, or the sites overlap. This has recently been shown (38). The most effective inhibitors, therefore, would possess a higher affinity for PP1c. These are represented (see Table III) by those mutants possessing the NH$_2$-terminal 38 residues plus a longer NH$_2$-terminal segment, possibly the ankyrin repeats. Truncation of these mutants at the COOH-terminal end reduces the inhibitory potency. Removal of the four COOH-terminal ankyrin repeats (in M130$^{1–171}$) produces a less effective inhibitor. The predicted PP1c binding site for this second group of mutants is the NH$_2$-terminal sequence 1–38. The loss of this NH$_2$-terminal sequence generates the third group of inhibitory mutants. Here the inhibitory potency is low, indicating a reduced affinity of binding, and it is difficult to assign the location of the additional PP1c binding site. The very high IC$_{50}$ for M130$^{40–511}$ compared with the other two mutants in this group cannot be explained but was a reproducible observation.

The results from the light chain overlays (using the biotinylated thiophosphorylated LC20 as a probe) indicate that the ankyrin repeats are required for light chain binding. The NH$_2$-terminal segment of 39 residues is not involved. Further, it is suggested that the COOH-terminal half of the ankyrin repeats is important, since binding was detected for M130$^{1–296}$ but not M130$^{1–171}$. The fifth ankyrin repeat shows a considerably lower homology than the other repeat sequences (13) and in fact was

![FIG. 3. Inhibition of phosphatase activity by M130 mutants with phosphorylase a as substrate. See “Experimental Procedures” for assay conditions. The following mutants are shown: M130$^{1–511}$ (●), M130$^{1–38}$ (■), and M130$^{40–511}$ (○).](image)

![FIG. 4. SDS-PAGE profiles (panel A) and light chain overlays (panel B) of the M130 mutants. Lanes 1, M130$^{1–633}$; lanes 2, M130$^{1–511}$; lanes 3, M130$^{1–374}$; lanes 4, M130$^{1–296}$; lanes 5, M130$^{1–171}$; lanes 6, M130$^{1–137}$; lanes 7, M130$^{1–104}$; lanes 8, M130$^{1–69}$; lanes 9, M130$^{1–38}$; lanes 10, M130$^{40–511}$; lanes 11, M130$^{168–511}$. Overlay assays were carried out with biotinylated thiophosphorylated LC20, as described under “Experimental Procedures.” Molecular mass markers are indicated.](image)

### Table III

| Mutants    | IC$_{50}$ $^a$ (nM) | Relative value$^b$ |
|------------|---------------------|-------------------|
| M130$^{1–633}$ | 0.32 ± 0.11       | 1.0               |
| M130$^{1–511}$ | 0.46 ± 0.26       | 1.4               |
| M130$^{1–374}$ | 0.21 ± 0.09       | 0.7               |
| M130$^{1–296}$ | 0.22 ± 0.11       | 0.7               |
| M130$^{1–171}$ | 4.0 ± 0.6         | 13                |
| M130$^{1–137}$ | 3.6 ± 3.3         | 11                |
| M130$^{1–104}$ | 1.9 ± 2.5         | 6                 |
| M130$^{1–69}$  | 3.2 ± 1.9         | 10                |
| M130$^{1–38}$  | 6.0 ± 4.4         | 19                |
| M130$^{40–511}$ | 1.068 ± 0.273     | 3,338             |
| M130$^{168–511}$ | 18.3 ± 4.0       | 57                |
| M130$^{104–511}$ | 22.0 ± 7.0       | 69                |

$^a$ Data are mean ± S.E. ($n = 3$).

$^b$ Values compared with IC$_{50}$ for M130$^{1–633}$. 

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not considered as an ankyrin repeat in the rat M110 (15). Thus it is suggested that repeats 6, 7, and 8 may play a more crucial role.

The smallest NH2-terminal fragment that could activate PP1c (using 32P-LC20 as substrate) was M1301–374. This mutant contains in addition to the ankyrin repeats another 78 residues, and in this sequence the notable feature is an acidic cluster, residues 326–372 (13). It is not known if this sequence contains an additional binding site for PP1c or P-LC20 or if it is necessary for correct folding or orientation of the ankyrin repeats.

In summary, a tentative plan of the M130 molecule can be assembled from the above data, with the emphasis on the NH2-terminal portion. For PP1c at least two sites are indicated: a relatively strong site in the NH2-terminal 38 residue sequence and a second weaker site in the ankyrin repeats, possibly in the COOH-terminal half of the repeats. If such is the case then the NH2-terminal portion of M130 may wrap around PP1c. The binding of P-LC20 is indicated in the ankyrin repeats, and again the COOH-terminal repeats are suggested to be involved. Activation of PP1c by M130 is assumed to require binding to both PP1c and substrate, P-LC20. Thus, theoretically the sequence 1–296 should be sufficient for activation. However, the situation is more complex, and additional COOH-terminal sequence (297–374) was required for activation. Whether this sequence contains additional sites for interaction or is needed to stabilize the NH2-terminal segment is not known. Another possibility is suggested by earlier results (9), namely, that if inhibition of PP1c by phosphorylated M130 results from binding of Thr-654 to the active site of PP1c then the M130 molecule must fold to accommodate this interaction.

Acknowledgments—We are grateful for the expert technical assistance of M. Hirano and C. Dudas and to Dr. T. Butler (Jefferson Medical College) for many helpful discussions and assistance with the manuscript.

REFERENCES
1. Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract (Johnson, L. R., ed) Vol. 2, pp. 423–482, Raven Press, New York
2. Somlyo, A. P., and Somlyo, A. V. (1994) Nature 372, 231–236
3. Hirata, K., Kikutchi, A., Sasaki, T., Kuroda, S., Kibauchi, K., Matsuzura, Y., Seki, H., Saida, K., and Takai, Y. (1992) J. Biol. Chem. 267, 8719–8722
4. Fujita, A., Takeuchi, T., Nakajima, H., Nishio, H., and Hata, P. (1995) J. Pharmacol. Exp. Ther. 274, 555–561
5. Noda, M., Yasuda-Fukazawa, C., Moriishi, K., Kato, T., Okuda, T., Kurokawa, K., and Takawa, Y. (1995) FEBS Lett. 367, 246–250
6. Gong, M. C., Iizuka, K., Nixon, G., Browne, J. P., Hall, A., Eccleston, J. F., Sugai, M., Kobayashi, S., Somlyo, A. V., and Somlyo, A. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1340–1345
7. Gong, M. C., Kintner, M. T., Somlyo, A. V., and Somlyo, A. P. (1995) J. Physiol. (Lond.) 486, 113–122
8. Trinkle-Mulcahy, L., Ichikawa, K., Hartshorne, D. J., Siegmam, M. J., and Butler, T. M. (1995) J. Biol. Chem. 270, 18191–18194
9. Ichikawa, K., Ito, M., and Hartshorne, D. J. (1996) J. Biol. Chem. 271, 4735–4740
10. Ingebritsen, T. S., and Cohen, P. (1983) Eur. J. Biochem. 132, 255–261
11. Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D., and Hartshorne, D. J. (1989) Biochem. Biophys. Res. Commun. 159, 871–877
12. Alessi, D., MacDougall, L. K., Sola, M. M., Beke, M., and Cohen, P. (1992) Eur. J. Biochem. 209, 1023–1035
13. Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) J. Biol. Chem. 269, 30407–30411
14. Shirazi, A., Iizuka, K., Fadden, P., Mose, C., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1994) J. Biol. Chem. 269, 31598–31606
15. Chen, Y. H., Chen, M. X., Alessi, D. R., Campbell, D. G., Shanahan, C., Cohen, P., and Cohen, P. T. W. (1994) FEBS Lett. 356, 51–55
16. Haystead, C. M. M., Gaillly, P., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1995) FEBS Lett. 377, 123–127
17. Faux, M. C., and Scott, J. D. (1996) Cell 85, 9–12
18. Ichikawa, K., Hirano, K., Ito, M., Tanaka, T., Nakano, T., and Hartshorne, D. J. (1996) Biochemistry 35, 6313–6320
19. Fields, S., and Song, O. (1989) Nature 340, 245–247
20. Hirano, K., Ito, M., and Hartshorne, D. J. (1995) J. Biol. Chem. 270, 19786–19790
21. Hirano, K., Erdof, F., Patton, J. G., and Hartshorne, D. J. (1996) FEBS Lett. 389, 191–194
22. Bartel, P., and Fiedls, S. (1995) Methods Enzymol. 254, 241–263
23. Shiestl, R. H., and Gietz, R. D. (1989) Curr. Genet. 16, 339–346
24. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H., and Elledge, S. J. (1993) Genes Dev. 7, 555–569
25. Cohen, P., Aernany, S., Hemmings, H. A., Resink, T. J., Stafvel, D., and Tang, H. Y. L. (1988) Methods Enzymol. 159, 390–408
26. Ichikawa, K., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 13146–13153
27. Hachow, D. R., and Haeberle, J. R. (1985) Anal. Biochem. 135, 37–43
28. Ichikawa, K., Kurokawa, K., and Miyahara, M. (1996) Methods Enzymol. 5, 211–217
29. Ozaki, H., Ichikawa, K., Hirano, K., Kato, Y., Karaki, H., Kurokawa, K., and Hartshorne, D. J. (1988) Biochemistry 27, 16727–16735
30. Yoshida, M., and Yagi, K. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4350–4354
31. Martin, B. L., Shriner, C. L., and Brautigam, D. L. (1994) Protein Expression Purif. 5, 211–217
32. Deleted in proof
33. Okubo, S., Erdof, F., Ito, M., Ichikawa, K., Konishi, T., Nakano, T., Kawamura, T., Brautigam, D. L., and Hartshorne, D. J. (1993) Adv. Protein Research Phosphatases 7, 295–314
34. Brautigam, D. L., and Shriner, C. L. (1989) Arch. Biochem. Biophy. 275, 44–52
35. Mitsu, T., Inagaki, M., and Ibeke, M. (1992) J. Biol. Chem. 267, 16727–16735
36. Yoshida, M., and Yagi, R. (1988) J. Biochem. (Tokyo) 105, 380–383
37. Endo, Y., Zhou, X., Connor, J., Wang, B., and Shenolikar, S. (1996) Biochemistry 35, 5220–5228
38. Johnson, D. F., Moorhead, G., Caudwell, F. B., Cohen, P., Chen, Y. H., Chen, M. X., and Cohen, P. T. W. (1996) Eur. J. Biochem. 239, 317–325
39. Gaillly, P., Wu, X., Haystead, T. A. J., Somlyo, A. P., Cohen, P. T. W., Cohen, P., and Somlyo, A. V. (1996) Eur. J. Biochem. 254, 326–332