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Rapid Identification of Protein Phosphatase 1-binding Proteins by Mixed Peptide Sequencing and Data Base Searching

CHARACTERIZATION OF A NOVEL HOLOENZYMIC FORM OF PROTEIN PHOSPHATASE 1*

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Microcystin-affinity chromatography was used to purify 15 protein phosphatase 1 (PP1)-binding proteins from the myofibrillar fraction of rabbit skeletal muscle. To reduce the time and amount of material required to identify these proteins, proteome analysis by mixed peptide sequencing was developed. Proteins are resolved by SDS-polyacrylamide gel electrophoresis, electroblotted to polyvinylidene fluoride membrane, and stained. Bands are sliced from the membrane, cleaved briefly with CnBr, and applied without further purification to an automated Edman sequencer. The mixed peptide sequences generated are sorted and matched against the GenBank using two new programs, FASTF and TFASTF. This technology offers a simple alternative to mass spectrometry for the subpicomolar identification of proteins in polyacrylamide gels. Using this technology, all 15 proteins recovered in PP-1C affinity chromatography were sequenced. One of the proteins, PP-1bp55, was homologous to human myosin phosphatase, MYPT2. A second, PP-1bp80, identified in the EST data bases, contained a putative PP-1C binding site and a nucleotide binding motif. Further affinity purification over ATP-Sepharose isolated PP-1bp80 in a quaternary complex with PP-1C and two other proteins, PP-1bp29 and human p20. Recombinant PP-1bp80 also bound PP-1C and suppressed its activity toward a variety of substrates, suggesting that the protein is a novel regulatory subunit of PP-1.

Protein phosphatase 1 (PP-1,[1] EC 3.13.16) dephosphorylates serine and threonine residues on proteins that control a diverse range of cellular processes from metabolism and muscle contraction to the cell cycle and gene expression (1). The involvement of PP-1 in all of these events raises the question as to how all of these processes can be regulated independently of one another? This question is even more apparent when one considers that the catalytic subunit of PP-1 (PP-1C) is expressed at micromolar concentrations in cells and shares 49% sequence homology within its catalytic core with at least 3 other highly expressed phosphatases, PP-2A, PP-2B, and PP-5 (2–5). The key to this paradox is the finding that the functions of PP-1 are closely linked to its subcellular localization with regulatory targeting subunits that confer substrate specificity (1). At the time of writing, 10 regulatory subunits have been sequenced at the protein and DNA level in mammalian tissues, and at least 10 others identified in *Saccharomyces cerevisiae* (6). Five of the mammalian subunits potently inhibit the enzyme's activity toward all substrates, whereas the others target the activity of PP-1C toward myosin, glycogen synthase, p53, or the nucleus (6). The small number of PP-1 regulatory proteins discovered thus far is unlikely to account for all the intracellular actions of the phosphatase, suggesting that many others must exist.

One of the mechanisms by which PP-1C recognizes its regulatory subunits was recently characterized (6). Amino acid sequence alignments of nine of the mammalian regulatory subunits identified the motif (K/R)(V/I)*X*F as common in these proteins. In yeast, alignment of 10 PP-1C-binding proteins identified a similar arrangement, (K/R)*X*(V/I)*X*F. Co-crystallization studies involving synthetic peptides containing the sequence RRVSF with PP-1C showed that the peptide bound to a hydrophobic channel on the protein surface at the interface of two β sheets opposite to the catalytic cleft. Although (R/K)(V/I)*X*F appears to specify a PP-1C binding site, a data base search reveals this motif is in over 10% of all proteins present in the GenBank. Various explanations can be put forward to reduce the possible number of proteins that might bind PP-1C *in vivo*. First, not all amino acids in the *X* position of the (R/K)(V/I)*X*F sequence or immediately N- or C-terminal to the motif are likely to be tolerated. Second, the (R/K)(V/I)*X*F motif may be buried within the folded protein. However, because of its high frequency of occurrence in protein sequences, a data base search using the (R/K)(V/I)*X*F motif alone is unlikely to be fruitful in identifying additional PP-1C targeting subunits.

Recently, we utilized a combination of microcystin and PP-1C-affinity chromatography to purify 36 proteins that specifically bound to PP-1C in the cytosolic and particulate fractions of rabbit skeletal muscle (7). Although the large number of proteins recovered by this method supported the hypothesis that the major mechanism of regulation of PP-1 *in vivo* involves localization of phosphatase catalytic subunit with regulatory proteins that target the enzyme to specific substrates, the identity of most of these proteins was unknown. In this report, we have used a novel sequencing technology (mixed peptide sequencing) to rapidly characterize 15 proteins that were recovered from the particulate fraction of rabbit skeletal muscle. One of the proteins identified, PP-1bp55, is related to the recently cloned human myosin phosphatase, MYPT2 (11). Analysis of the full-length sequence of a second protein, PP-1bp80, identified a PP-1C binding site and a nucleotide binding motif. Further affinity purification over γ-phosphate-linked ATP-Sepharose isolated PP-1bp80 in a quaternary complex

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[1] The abbreviations used are: PP, protein phosphatase; MBP, myelin basic protein; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis; PVM, polyvinylidene fluoride membrane.

with PP-1C and two other proteins of 29 and 20 kDa. Recombinant PP-1bp80 binds PP-1C and suppresses its activity toward a variety of nonspecific substrates, suggesting that the protein is a novel regulatory subunit of PP-1.

MATERIALS AND METHODS

Native PP-1Cδ was purified from rabbit skeletal muscle following the protocol of Tung *et al.* (8), except that microcystin-Sepharose was used to obtain a homogeneous preparation following the polylysine step. Microcystin derivatives were prepared as described previously (7). ATP-Sepharose (10) was prepared as described (9). All EST clones were obtain from the American Tissue Culture Collection. All phosphoprotein substrates were prepared as described previously (13).

Purification of PP-1-binding Proteins—MC-Sepharose affinity chromatography was used to prepare an isothiocyanate eluate from the particulate fraction of rabbit skeletal muscle (500 g) that is enriched in PP-1C- and PP-2A-binding proteins, but not their catalytic subunits as described previously (7). To specifically isolate proteins from this complex mixture that bound to PP-1C, following extensive dialysis against buffer A (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride), the isothiocyanate eluate was first passed over an avidin-Sepharose column (5 × 10 cm). This step ensured that any proteins that were biotinylated endogenously were removed. The eluate was then passed over a second avidin-Sepharose column (5 × 10 cm) that had been previously saturated with purified PP-1C bound to MC-biotin (8). To remove proteins that bound nonspecifically either through ionic or hydrophobic interactions, the column was washed first with 40 volumes of buffer A containing 1 M NaCl, and then with 40 volumes of buffer A containing no NaCl. The column was re-equilibrated into buffer A, and then one column volume of buffer A containing 10 mM biotin applied and the flow stopped. After 60 min, the flow was resumed. The eluted proteins were characterized by SDS-PAGE and silver staining.

Mixed Peptide Sequencing—Samples (100 μl) representing 1/50th of the isothiocynate and biotin eluates were mixed with SDS-PAGE sample buffer (1:5, v/v) separated on 12% polyacrylamide (6 × 10 cm × 0.5 mm) gels. The gels were equilibrated into transfer buffer (3 g/liter Tris base, 14.25 g/liter glycine, 0.1 g/liter SDS, 200 ml/liter MeOH) for 20 min at room temperature. The separated proteins were electroblotted overnight at 35 V to PVM membrane that had previously been wetted with sequencer-grade methanol. The transferred proteins were stained with Amido Black (1 mg/ml in 5% acetic acid, 10% methanol) for 1 min, washed once with 5% acetic acid, 10% methanol, and then with several changes of water. The membrane was air-dried and individual stained bands cut out and placed in Eppendorf tubes. The pieces were washed three times with water (1.0 ml) and then methanol. CnBr solution (100 μl, 500 mg/ml in 70% formic acid) was added and digestion carried out for 90 min at room temperature. The reactions were stopped by removal of the membrane and three alternate washes with 1.0 ml of water followed by methanol. The pieces were placed without further purification into an Applied Biosystems 494 automated sequenator. A single run of Edman sequencing (8–18 cycles of pulsed liquid chemistry) was carried out, and the mixed peptide sequence data generated were sorted and matched to the public data bases with the FASTF or TFASTF algorithms.[2,3]

Expression and Purification of Recombinant PP-1bp80—A data base search with the full-length sequence of PP-1bp80 identified 13 overlapping human EST sequences covering approximately two thirds of the entire molecule from residue 1 to 484 (Fig. 1*A*). No EST sequences were identified that matched PP-1bp80 beyond residue 484 in the translated protein. The exact insert size of the reported EST sequences was unknown; therefore, three of the largest reported clones (clones 232705, 318291, and 933493) were obtained from the ATCC for characterization. Bacterial cultures of the clones where plated out onto LB Amp plates and grown overnight at 37 °C. Single colonies were isolated from these plates and 5-ml overnight cultures grown. The plasmid DNA was then purified via Wizard Miniprep (Promega) and sequenced using M13 forward and reverse primers. The sequence was analyzed using NCBI blast search and the appropriate reading frames for each clone determined (Fig. 1*A*). This analysis indicated that clone 232705 was truncated at residue 484 in the full-length PP-1bp80 sequence (Figs. 1*A* and 5). Clones 318291 and 933493 were somewhat smaller, encoding between residues 101 and 484 and between 205 and 376, respectively.

For insertion into GST fusion vectors, the plasmid DNA from each clone was incubated with the appropriate restriction enzymes in order to remove the insert DNA from the phagemid. Clones 232705 and 31829, as well as the GST fusion vectors pGEX 4T-1 and 4T-3 (Amersham Pharmacia Biotech), were digested with restriction enzymes *Eco*RI and *Xho*I. Clone 933493 and pGEX 4T-1 were digested overnight at 37 °C with restriction enzymes *Eco*RI and *Not*I. The clones were separated on a 1% agarose gel via electrophoresis, excised, and purified. The restriction-digested GST fusion vectors were purified via phenol chloroform extraction. The clones were ligated into the pGEX 4T-1 GST fusion vector, which yielded the correct reading frame using T4 DNA ligase (Promega). The ligation reactions were performed at 25 °C for 4 h using a 3:1 ratio of insert to vector DNA, 2 μl of ligation buffer (10×), 1 μl of T4 DNA ligase, and water to bring the final volume to 20 μl. Following ligation, the clones transformed into competent cells. Water (20 μl) was added to each ligation reaction, and 20 μl of this mixture combined with 100 μl of *Escherichia coli* strain BL21 and incubated on ice for 20 min. The mixture was heat-shocked at 50 °C for 90 s and placed back on ice for 2 min. The cells were added to 1 ml of LB in a 15-ml culture tube and incubated for 1 h at 37 °C. The BL21 cells containing the GST fusion protein plasmid DNA were plated onto LB Amp plates in 10-, 50-, and 100-μl aliquots and incubated at 37 °C overnight. Single colonies were isolated from these plates, from which 5-ml overnight cultures were grown and lysed with 2× crack buffer (100 mM NaOH, 10 mM EDTA pH 8.0, 1% SDS, 10% glycerol, 5 mg of bromcresol green). The plasmid DNA was visualized by electrophoresis on a 1% agarose gel (Fig. 1*B*). Fig. 1*B* shows that clone 232705 was ~2000 base pairs, clone 318291 was ~1600 base pairs, and clone 933493 ~1200 base pairs. This analysis demonstrated that each of the clones contained the GST fusion vector and were of the predicted insert size.

For the expression and purification of the recombinant proteins, individual colonies of each clone were grown to an optical density of 0.8 (600 nm) at 30 °C in LB containing ampicillin (100 μg/ml). Isopropyl-1-thio-β-D-galactopyranoside (0.1 mM final) was added to induce expression of the cloned subunit. After 16 h, cells were harvested by centrifugation at 6000 × *g* for 10 min. Following freezing at −20 °C, the cells were thawed and lysed in 20 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5) containing 0.5 mg/ml lysozyme. Following complete lysis, Nonidet P-40 (10 μl/liter) 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 (Amersham Pharmacia Biotech) for 30 min. The Sepharose was collected by mild centrifugation (500 × *g* for 2 min), and washed with PBS plus 1 M NaCl. The Sepharose was packed into a glass column (1 × 5 cm) and re-equilibrated into PBS. One column volume of this buffer containing 1 unit of thrombin was allowed to flow into the column and the flow stopped for 60 min. Flow was resumed and column fractions collected (1.0 ml). In order to remove the thrombin, the eluted proteins were diluted 20-fold into column buffer A (25 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM benzamidine) and applied directly to an AP-1Q (Waters) anion-exchange column equilibrated in this buffer. The column was developed (1.0 ml) in buffer A over 60 min with a linear salt gradient to 600 mM NaCl. The purified recombinant proteins were identified in column fractions by SDS-PAGE and Coomassie Blue staining. Consistent with their respective insert size, following removal of the GST fusion protein, each clone produced recombinant proteins of the predicted molecular masses of 54, 42, and 20 kDa (Fig. 1*C*). The identity of each protein was confirmed by N-terminal amino acid sequencing following transfer of the proteins to PVM (Fig. 1*C*). Approximately 1–2 mg of purified recombinant protein per liter of bacterial culture was obtained for each clone following the outlined protocol.

RESULTS

Identification of Protein Phosphatase-binding Proteins by Mixed Peptide Sequencing and the FASTF and TFASTF Algorithms—Previously, we had shown that microcystin affinity chromatography can be utilized to isolate fractions from crude cell extracts that are highly enriched in PP-1- and PP-2A-binding proteins, but not their catalytic subunits, which remain largely associated with the covalently linked microcystin (7). Using this procedure, over 100 distinct proteins were recovered from the particulate and cytosolic fractions of rabbit skeletal muscle. Additional purification of these complex mixtures over a PP-1C affinity column isolated 20 distinct proteins

[2] W. R. Pearson and T. A. J. Haystead, manuscript in preparation.

[3] The FASTF and TFASTF programs can be obtained via FTP (ftp://ftp.virginia.edu/pub/fasta) or by contacting the authors.

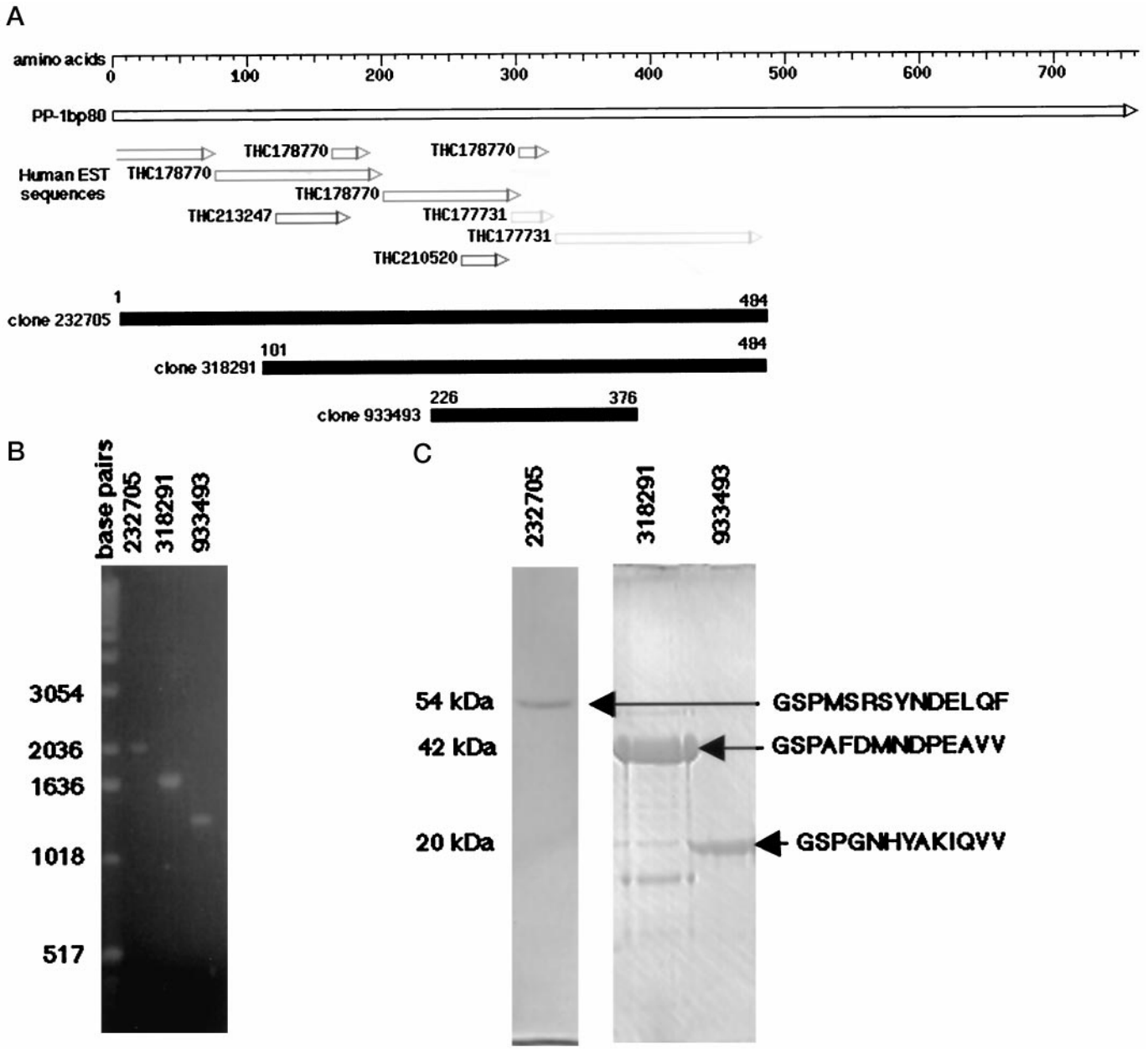

FIG. 1. **Characterization of PP-1bp80 clones and purification of recombinant proteins.** *A*, identification of overlapping THC sequences identified by BLAST search and their alignment against the full-length sequence of PP-1bp80. *B*, crack screen showing the insert size of clones 232705, 318291, and 933493. *C*, recombinant GST fusion proteins were produced and isolated from glutathione-Sepharose following treatment with thrombin. The cleaved fusion proteins were purified by anion-exchange chromatography and peak fractions analyzed by SDS-PAGE and Coomassie Blue staining. Each protein was verified by N-terminal sequencing on PVM membrane.

from the cytosolic fraction and another 16 from the particulate fraction. Although the recovery of so many proteins by these methods supported the hypothesis that the catalytic subunits of PP-1 and PP-2A are regulated by multiple and distinct regulatory subunits that target the phosphatases toward specific substrates, the identity of most of these proteins was unknown.

In this present study, we report the development of a novel sequencing technology, mixed peptide sequencing, and the FASTF and TFASTF algorithms, which enabled the rapid identification of proteins in the MC-Sepharose and PP-1C affinity column eluates from the particulate fraction. Initially, the affinity column eluates (1/50th) were characterized by SDS-PAGE and silver staining (Fig. 2, *A* and *B*). For mixed peptide sequencing, the eluates (1/50th) were first separated by SDS-PAGE, then electroblotted to PVM. Following staining with Amido Black, the stained proteins were sliced from the membrane, and the slices placed in Eppendorf tubes for treatment with CnBr. After washing, the slices were placed directly into an automated Edman sequencer. Mixed peptide sequences are defined as PTH amino acids that are simultaneously recovered during Edman degradation chemistry of N-terminally blocked proteins that have been cleaved internally at multiple sites. Fig. 3 shows an example of a mixed peptide sequencing run performed on a 16-kDa silver staining protein present in the MC-Sepharose eluate (Fig. 2*A*). In the first Edman cycle, three major PTH amino acids are recovered: threonine (T), isoleucine (I), and lysine (K). These are written in tabular form in order of picomolar amount recovered. In the second cycle, two major PTH amino acids appeared, glutamic acid (D) and aspartic acid (N). The amount of aspartic acid recovered in this cycle was approximately 1.5-fold greater than the glutamic acid residue,

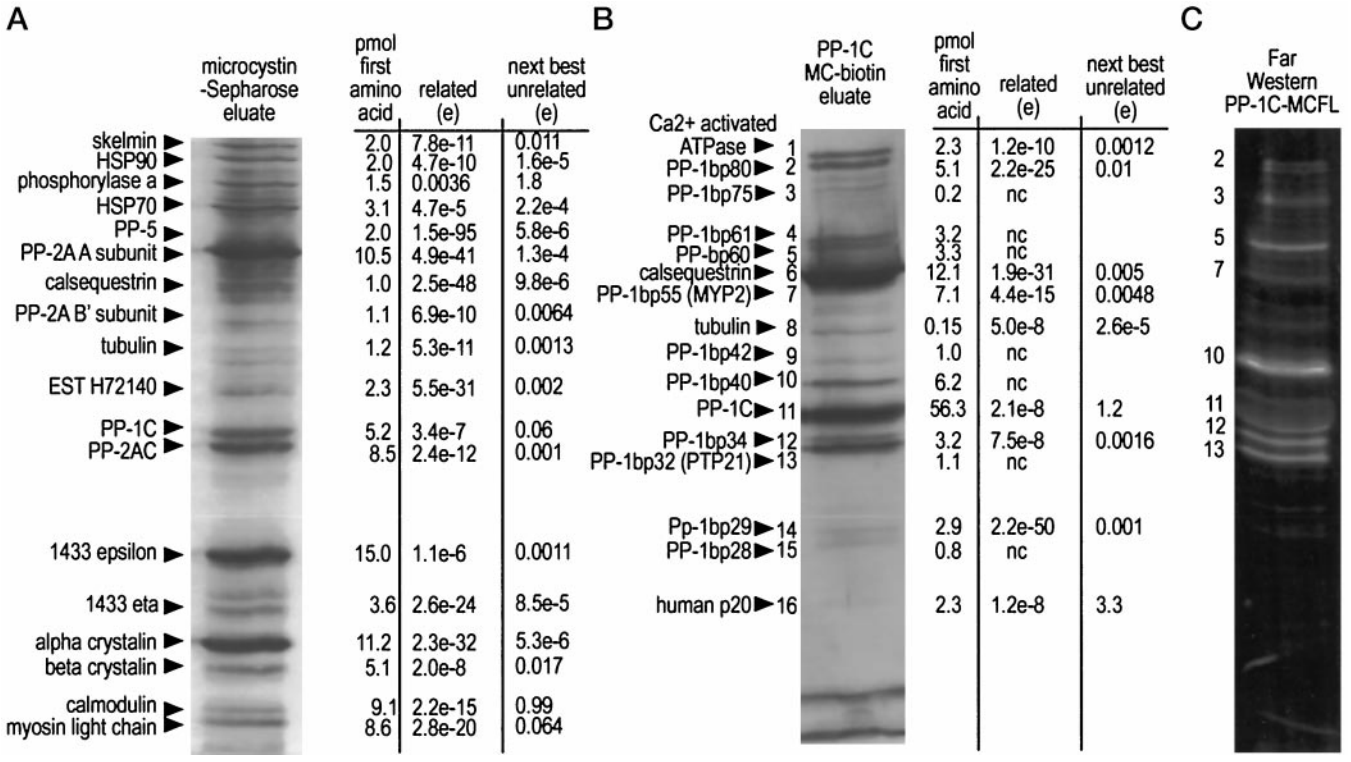

FIG. 2. **Identification of phosphatase-binding proteins in SDS gels by mixed peptide sequencing.** *Gels A* and *B* are silver stains of proteins present in the isothiocyanate (*A*) and biotin (*B*) eluates following MC-affinity chromatography. *Gel C* shows the results of Far Western analysis of gel B with PP-1C bound to MC-fluorescein (8). The number assignments in *B* and *C* indicate proteins that were recovered in PP-1C affinity chromatography and also bound PP-1C MC-fluorescein in Far Western analysis. Proteins were identified in *A* and *B* by mixed peptide sequencing. The picomole yield of the highest recovered PTH-amino acid in the first Edman cycle is given for each protein sequenced. The expectation values (*e*) for identified proteins are compared with the expectation values for the next highest scoring unrelated protein. Generally, data bases searched in the above examples were SwissProt, NBRF, human EST, *C. elegans*, and yeast.

indicating that it was derived from two separate peptides. Thus, D, N, and N is recorded for the second cycle. One then proceeds to the third cycle recording amino acids that are recovered and so on until the end of the sequencing run is reached. An *X* is recorded in cases where an amino acid is not identified. Methionine is added to the beginning of the list of each set of amino acids, as CnBr was used to cleave the protein. The list of amino acids identified in Fig. 3 is written as shown, using a semicolon to separate each set: Call 1, mkdldded; Call 2, minegeae; Call 3, mtntversusnl.

The data derived from Fig. 3 suggests that CnBr digestion cleaved the protein internally in three places, as each cycle showed the appearance of three new PTH amino acids. Inasmuch as the membrane slice was derived from a portion of the gel containing a protein that was relatively pure (>90%), one would therefore predict that the mixed sequences were derived from at least three separate internal peptides. The list of amino acids in the format written above are entered into the FASTF (searches the protein data bases) and TFASTF (searches the DNA data bases in three frames in both directions) algorithms. The algorithms are designed to sort and match mixed peptide sequences against the protein and DNA data bases. Details of the programs' design are given elsewhere.[2] When the programs identify a match in the data base, an expectation score (*e*) is assigned along with a sequence alignment showing the position of the sorted peptides in the identified protein (Fig. 4). Fig. 4*A* shows the deconvoluted data from Fig. 3 using the FASTF algorithm and identifies the 16-kDa protein unambiguously in the NBRF data base as calmodulin (predicted molecular mass 15.6 kDa). The expectation score (*e*) for the identified protein was $2.9e^{-38}$ as compared with a score of 0.001 for presenilin-β from African clawed frog, which was the next highest scoring protein identified by FASTF (Fig. 4*B*). Consistent with a significantly lower expectation score ($e = 0.001$), and in contrast to calmodulin, presenilin shows poor alignment with any combination of the sorted amino acids. Furthermore, the predicted molecular mass of 44.7 kDa for presenilin is significantly greater than that of the 16-kDa protein excised from the PVM membrane.

To determine the sensitivity of the methodology, three other proteins of varying staining intensity at 80 kDa (PP-1bp80), 29 kDa (PP-1bp29), and 20 kDa (MLC20) were selected from the gels in Fig. 2 (*A* and *B*) for mixed peptide sequencing. Table I shows that TFASTF identified the 80- and 29-kDa proteins as novel proteins that were reconstructed from expressed sequence tags (ESTs) in the DNA data base (see below and Fig. 5). FASTF identified the 20-kDa protein as myosin light chain 20. Recovery of individual PTH amino acids (in pmol) after each cycle was used to determine both the amount of protein present on the membrane following CnBr treatment, as well as the repetitive yield during sequencing. The data in Table I shows that mixed peptide sequencing can be used to identify proteins on PVM over a range from 40 to 0.1 pmol of total protein. Repetitive yield, using the aligned sequences shown in Table I was 95.2 ± 3.2% (S.D.) for the 4 proteins. The average background noise due to nonspecific contaminating amino acids varied from 3 ± 2 fmol (S.D.; $n = 12$) for the 29-kDa protein to 20 ± 5 fmol (S.D.; $n = 6$) for MLC20. These data demonstrate that mixed peptide sequencing can unambiguously identify proteins in polyacrylamide gels with a sensitivity that rivals state of the art MALDI time of flight or nano-electrospray mass spectrometry.

To further characterize proteins in the affinity column eluates, mixed peptide sequencing was used to rapidly identify 18

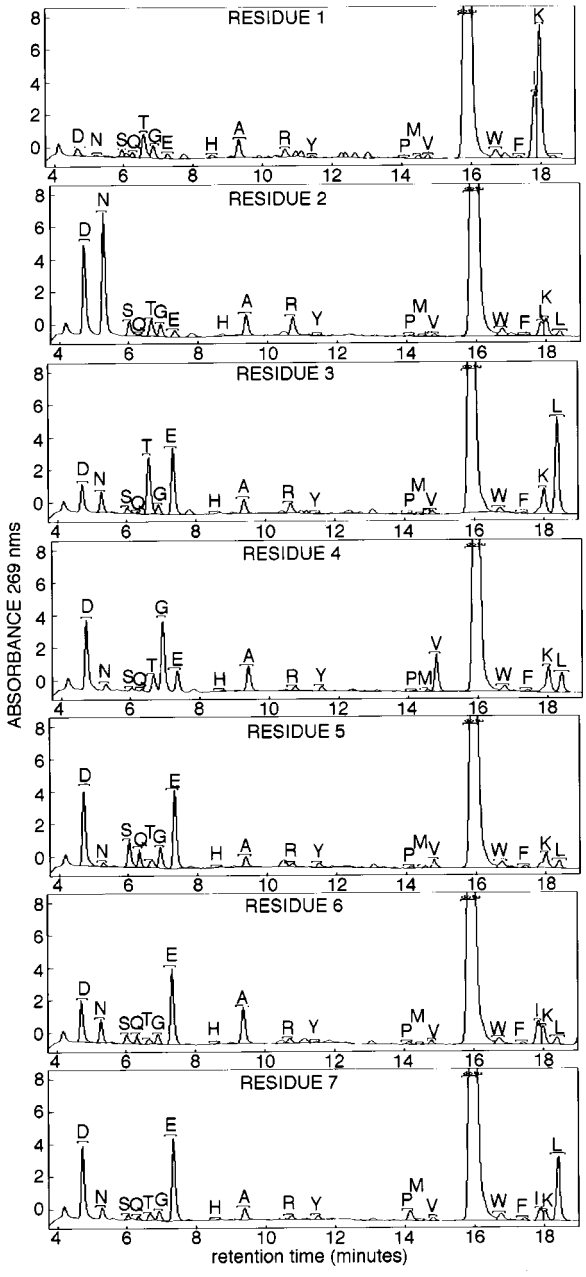

FIG. 3. **Mixed peptide sequencing of calmodulin.** A 16-kDa silver staining protein from Fig. 2*A* was cut from PVM and digested with CnBr. The PVM piece was placed in the protein sequencer and seven cycles of Edman sequencing carried out. The figure shows the reverse phase high performance liquid chromatograms of the PTH amino acids recovered for each cycle.

prominently staining proteins in the MC-Sepharose eluate and 16 proteins recovered from PP-1C affinity chromatography. Mixed peptide sequencing data derived from these proteins indicated that on average between 6 and 12 cycles of Edman sequencing was sufficient to unambiguously identify any given protein, provided it was present in the GenBank. The expectation scores for the 36 proteins identified in Fig. 2 ranged from $1.1e^{-6}$ to $1.5e^{-95}$. This contrasts with expectation scores for the next highest scoring unrelated proteins, which ranged from 3.3 to $9.8e^{-6}$ (Fig. 2). In each case the amount of protein that was sequenced was determined by the highest recovered PTH amino in the first cycle and ranged from 60 pmol (PP-1C) to 0.15 pmol (tubulin) (Fig. 2). In most cases, CnBr digestion yielded between three and five simultaneous sequences. This finding is consistent with the frequency of occurrence of methionine in any given protein. In a sample of over 30,000 proteins in the Swiss Prot and PIR1 protein data bases, we determined that 4% of all proteins contain no methionines, 9% of all proteins contain 1 methionine, and 50% of all proteins contain 6 methionines. This suggests that approximately 1:20 proteins are unlikely to cut with CnBr.

The 18 proteins identified in the MC-Sepharose eluate included the catalytic subunits of PP-1C, PP-2AC, and PP-5; several of their regulatory subunits; an uncharacterized EST; as well as a several potential substrate and/or chaperone proteins (Fig. 2*A*). From previous work (7), the proteins identified in this fraction represent approximately 15–20% of actual number known to be present in the eluate. Therefore, to isolate proteins that bound specifically to phosphatase 1, the MC-Sepharose eluate was repurified by PP-1C-MC-biotin affinity chromatography. Fig. 2*B* shows that mixed peptide sequencing of the PP-1C affinity column eluate unambiguously identified 9 of the 16 proteins visible in the gel. Two of these proteins were identified as a potential PP-1 regulatory subunits based on their primary amino acid sequence. FASTF aligned mixed peptide sequence data from PP-1bp55 with the N terminus of the myosin phosphatase, MYPT2, which was recently cloned from a human brain library (11). MYPT2 shows approximately 80% homology with the M110 regulatory subunit of smooth muscle myosin phosphatase, SMPP-1M (11). In addition, Northern analysis demonstrated that MYPT2 is highly expressed in skeletal and heart muscle (11). These findings therefore suggest that PP-1bp55 is the skeletal muscle isoform of M110 regulatory subunit of PP-1. The 55-kDa molecular mass of PP-1bp55 is somewhat smaller than the reported 110-kDa mass for full-length sequence of MYPT2, suggesting it is a proteolytic fragment. This finding is reminiscent of the smooth muscle M110 subunit, which shows spontaneous degradation to a stable 58-kDa N-terminal fragment upon purification (12–15). PP-1bp80 was identified by TFASTF as several uncharacterized EST sequences in the mammalian DNA data bases. Because EST sequences are generally derived from short sequences of cDNA (300–400 base pairs), mixed peptide sequences obtained for these proteins enabled three full-length sequences to be constructed from overlapping EST sequences in the human, mouse, and *Caenorhabditis elegans* data bases (Figs. 1 and 5). Inspection of the derived full-length sequence of PP-1bp80 revealed the presence of a conserved PP-1C binding domain in the C-terminal domain and a nucleotide binding motif in its N-terminal half (Fig. 5). Consistent with the presence of a PP-1C binding motif, PP-1bp80 also showed cross-reactivity with the catalytic subunit in Far Western analysis (Fig. 2*C*). Taken together with its recovery in PP-1C affinity chromatography, these results tentatively identify the protein as a new regulatory subunit of PP-1.

The remaining 7 of the 9 proteins identified in the PP-1C affinity column eluate had strong similarity with protein sequences in the NBRF, SwissProt, or EST data bases and included calsequestrin (sp}caqs_human), tubulin (sp}tbb2_caeel), PP-1Cδ (sp}pp1b_human), a Ca^{2+}-activated ATPase (sp}atcd_rabit), PP-1bp32 (sp}pt21_stypl), PP-1bp29 (sp}AA108607_mouse), and human p20 (pir}B53814). The Ca^{2+}-activated ATPase partial sequence also showed strong similarity with a human EST sequence (AA462167, expectation score (e) $<10^{-22}$), which clearly encodes a Ca^{2+} ATPase. Interestingly, PP-1bp32 showed significant similarity to the protochordate *Styela plicata* protein-tyrosine phosphatase PTP21 (16). PP-1bp32 is of larger molecular mass than PTP21 (32 kDa), suggesting it may be a previously undescribed mammalian tyrosine phosphatase that associates with PP-1C. PP-1bp29 is present as several uncharacterized EST sequences in

A Related calmodulin - rabbit

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QUERY                                                MINEVDAD-----------------MKDTDSEE-------
                                                     ::::::::                 ::::::::
MCRB   EFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFR(90)

QUERY  ------------------MTNLGENL
                         :::::: :
MCRB   VFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREANIDGDGQVNYEEFVQMMTAK(140)
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B Next best non-related presenilin-beta - African clawed frog

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QUERY  MINLGDNE------------------------------------------------------------------MKNEDE
       :: : : :                                                                  :::.:
JC5391 MIKLSDSEDEECNERTSLITSESPPLPSYQDGVQASEGLETSYHRERQPDSTQNNEDVPNGRTSGADAYNSETTVENEEE(80)
JC5391 ELTLKYGARHVIMLFVPVTLCMVVVVATIKSVSFYTEKDGQLIYTPFSEDTTSVGERLLNSVLNTLIMISVILVMTIFLV(160)
JC5391 LLYKYRCYKFIHGWLILSSLMLLFMFTYIYLSEVFKTYNIAMDYPTLFMVIWNFGAVGMICIHWKGPLQLQQAYLIMISA(240)
Query  ------------------------------------------------------------MTDTVSAD
                                                                    : :   ::
JC5391 LMALVFIKYLPEWSAWVILGAISVYDLLAVLCPKGPLRMLVETAQERNEPIFPALIYSSAMMWTVGMADSATADGRMNQQ(320)
JC5391 VQHIDRNTPEGANSTVEDAAETRIQTQSNLSSEDPDEERGVKLGLGDFIFYSVLVGKAAATASGDWNTTLACFVAILIGL(400)
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FIG. 4. **Alignment of sorted mixed peptide sequence data against the identified protein and the next non-related sequence.** Mixed peptide sequence data derived from Fig. 3 was applied to the FASTF algorithm. *A* shows the sorted data aligned to calmodulin. *B* shows the next best alignment to a non-related protein presenilin. Note: in the experiment shown above, over 100 calmodulins were identified in a search of the NBRF with expectation scores of $>e^{-48}$ before FASTF attempted to align the sequence to presenilin sequence.

the mouse DNA data base (Fig. 5). The protein shows no known homologies with any other protein in the data base. Examination of the primary sequences of the 7 proteins does not, with the exception of PP-1Cδ, readily explain their recovery from the PP-1C affinity column. None of them contain the putative PP-1 binding motif (K/R)(V/I)*X*F (6). Furthermore, as shown previously (7), none of these proteins showed cross-reactivity with PP-1C in Far Western analysis (Fig. 2*C*). Because of the stringent column washing conditions that were employed during affinity purification, their recovery is therefore more likely to be due to a strong interaction with one or more of the other proteins identified in Fig. 2*B*, rather than PP-1C itself. This finding suggests that these proteins may be physiological targets of PP-1. Ca^{2+}-dependent ATPase, calsequestrin, and tubulin have all been identified as phosphoproteins in skeletal muscle (17–20). Human p20 may also be a target substrate, as it is related to the crystallin family of phosphoproteins (21).

The identity and physiological function of 7 proteins (PP-1bp75, PP-1bp61, PP-1bp60, PP-1bp42, PP-1bp40, PP-1bp34, and PP-1bp28) identified in Fig. 2*B* remains unknown. All 7 proteins produced strong mixed peptide sequence data, but at the time of writing neither FASTF or TFASTF were able to match them to any known proteins in the GenBank. As shown previously (7), Far Western analysis of the PP-1C affinity column eluate suggests all 7 proteins may bind the catalytic subunit directly (Fig. 2*C*). This finding, coupled with their recovery from the PP-1C-MC-biotin affinity column, suggests that they may be novel regulatory subunits of PP-1. This hypothesis is currently being investigated. Importantly, the finding that none of the proteins sequenced in the biotin eluate were known PP-2A regulatory subunits, despite the relative abundance of these proteins in the MC-Sepharose eluate, demonstrates that PP-1C-MC-biotin affinity chromatography specifically recovers proteins that interact with PP-1C or its regulatory subunits.

PP-1bp80 Is an ATP-binding Protein That Forms a Holoenzymic Complex with PP-1C, PP-1bp29, and Human p20—Inspection of the predicted amino acid sequence of PP-1bp80 identifies, in addition to a PP-1C binding site, several motifs that tentatively suggest its is either a member of the protein kinase family or an ATP-binding protein (Fig. 5). Alignment of the full-length sequence of PP-1bp80 with cyclic AMP-dependent protein kinase identifies the sequence G*X*G*XX*GVI between residues 154 and 161. This motif is conserved in subdomain I of all protein kinases and forms the essential phosphate binding loop that coordinates orientation of the α and β phosphates of MgATP. At residues 171–173, there is an APE sequence which is 11 amino acids C-terminal of a DMG*X*A*X*RV sequence. This would be equivalent to subdomains VII and VIII in all protein kinases. The substitution of a hydrophobic methionine residue in place of a phenylalanine at DMG does distinguish PP-1bp80 from true members of the protein kinase family, although this is not without precedence. In CKII, phenylalanine in the motif DFG is substituted with a bulky tryptophan residue (22). To explore the possibility that PP-1bp80 is a protein kinase, the PP-1C-affinity column eluate was incubated with [γ-^{32}P]ATP or [γ-^{32}P]GTP and tested for the incorporation of phosphate into the protein itself or one or more of the other proteins present in the extract. The presence of an -S-P- sequence between the APE and DMG sequences suggested a likely autophosphorylation site. This would be equivalent to threonine 197 in the activation loop of cyclic A and other protein kinases. Following incubation with [γ-^{32}P]ATP, the reactions were separated by SDS-PAGE and characterized by autoradiography. Results from these experiments, however, failed to demonstrate conclusively that PP-1bp80 has protein kinase activity (data not shown). Although these findings may suggest PP-1bp80 is not a protein kinase, the lack of apparent activity could also be explained by the autophosphorylation site being already fully phosphorylated or the correct substrate not being present.

To explore the possibility that PP-1bp80 binds Mg^{2+} ATP, the PP-1C affinity column eluate was passed over γ-phosphate-linked ATP-Sepharose (Fig. 6). This resin specifically binds proteins belonging to the classical dinucleotide or protein kinase class of adenosine-binding proteins (9). Elution of the column with ATP recovered four proteins of 80, 37, 29, and 20 kDa in molecular mass (Fig. 6*A*). Densitometry of the gels indicated that these proteins were recovered in a stoichiometric ratio of 1:1:1:1. Mixed peptide sequencing of the four proteins identified them as PP-1bp80, PP-1C, PP-1bp29, and human p20, suggesting that they were recovered in the ATP eluate as a novel holoenzymic form of PP-1. Subsequent gel filtration analysis of the PP-1bp80 complex also recovered all four pro-

TABLE I

Mixed peptide sequence data and cycle yield for four proteins in the microcystin column affinity eluates ranging from 40 pmol to 0.15 pmol

Data shown are representative examples of four typical proteins that were identified in Fig. 2 by mixed peptide sequencing. Each protein was excised from PVM, treated with CNBr as described under "Materials and Methods," and placed in an Applied Biosystems 494 automated Edman sequencer linked to an on-line microbore HPLC system for the separation of PTH amino acids. Column 2 shows the major PTH amino acids in pmols that were detected after each cycle of Edman degradation. PTH amino acids that did not change during each cycle were not reported and generally represented a background of <1–20 fmol. Individual PTH amino acids were identified by their retention times relative to purified standards. Column 3 shows the resulting translated sequence using the FASTF or TFASTF algorithms. Column 4 shows the identification of the protein and the data base used. Expectation values for each example are given in Fig. 1. Methionine is included in the translated sequences because CNBr was used to cleave the protein.

Cycle no.	Called amino acid 1	2	3	4	Translation and alignments	Protein and data base
					MINEVDAD	
1	**K** 37.1	**I** 27.7	**T** 10.1		::::::::	
2	**D** 18.5	**N** 34.2	(N)		(27)MINEVDAD(34)	
3	**L** 24.6	**E** 16.2	**T** 23.9		MKDTDSEE	
4	**D** 14.2	**G** 28.2	**V** 13.5		::::::::	Calmodulin
5	**D** 16.0	**E** 19.1	**S** 11.1		(52)MKDTDSEE(58)	
6	**E** 18.8	**A** 13.4	**N** 8.5		MTNLGENL	NBRF
7	**D** 15.1	**E** 20.5	**L** 23.6		::::::::	
					(85)MTNLGEKL(92)	
1	**K** 5.1	**N** 5.0	**A** 2.2	**T** 1.0	MAVALVFYVGKVL	
2	**E** 4.2	**V** 4.2	**Q** 2.0	**A** 1.5	: : :::::::::	
3	**P** 3.9	**L** 5.2	**A** 3.1	**G** 2.1	(7)MQVEGVFYVNDAL(19)	
4	**G** 5.2	**L** 5.0	**P** 2.0	**F** 0.6	MKQLGNVAALPGI	
5	**N** 4.5	**R** 2.1	**V** 2.0	**D** 1.0	:::.:::::::::	PP1bp80
6	**V** 6.3	**F** 3.2	(V)	**A** 1.0	(43)MKQIGNVAALPGI(55)	
7	**A** 4.2	**V** 4.0	**K** 3.0	**Y** 0.8	MNEPPVVVTPGTV	EST
8	**V** 5.2	**K** 0.9	**A** 2.1	**T** 0.5	::: .::.:::.:	
9	**L** 3.2	**V** 2.1	**P** 1.8	**G** 1.6	(81)MNDPEAVVSPGSV(93)	
10	**K** 1.6	**P** 1.8	**G** 2.0	*X*	(724)MTAGFDAKKXRF(736)	
11	**G** 1.0	**R** 0.7	**V** 0.8	**T** 0.2	::: ::: :	
12	**I** 0.5	**V** 1.0	**F** 1.0	**L** 1.0	MTAIFDAIPFLF	
1	**G** 2.0	**A** 1.8	**T** 0.8			
2	**K** 2.2	**L** 1.5	**G** 1.8		MTKRKLTALDYFN	
3	**A** 1.9	**R** 0.8	**S** 0.9		: .::::::::: :	
4	**N** 1.5	**K** 2.0	**I** 1.1		(12)MFRRKLTALDYHN(24)	
5	**L** 2.3	**R** 0.6	(L)		MALANLLQIQKHD	PP1bp29
6	**T** 0.5	**I** 1.1	**L** 1.1		::::::::::::	
7	**A** 1.1	**Q** 0.5	**K** 0.8		(146)MALANLLQIQRHD(158)	EST
8	**V** 0.9	**I** 0.6	**L** 1.0		MGGSIRIKVEEXL	
9	**Q** 0.6	**D** 0.7	**E** 0.5		: .::: :.: :	
10	**Y** 0.6	**E** 0.4	**K** 0.4		(163)MLKAIRILVQERL(175)	
11	**H** 0.2	**F** 0.4	*X*			
12	**L** 0.6	**D** 0.3	**N** 0.3			
					MFDQTQ	
					::::::	
					(21)MFDQTQ(26)	
1	**F** 40.1	**L** 38.2	**F** 29.3	**G** 50.2	MKEASG	
2	**G** 45.2	**A** 32.1	**E** 24.5	**D** 22.1	::::::	Myosin light chain
3	**Q** 22.1	**A** 40.6	(A)	**E** 17.2	(71)MKEASG(76)	
4	**S** 15.2	**T** 11.2	**F** 14.5	**K** 6.2	MGGEK	
5	**Q** 21.3	**P** 18.3	**G** 17.5	*X*	:.:::	
					(86)MFGEK(90)	NBRF
					MFAAFP	
					:.::::	
					(139)MWAAFP(144)	

teins together with a putative molecular mass of >200 kDa (Fig. 6*B*). Recovery of PP-1bp80 from ATP-Sepharose demonstrates that the protein is an ATP-binding protein. This hypothesis is supported by the finding that elution of the ATP-Sepharose with 10 mM NADH, AMP, or ADP failed to elute any of the proteins identified in Fig. 6. PP-1bp80 is therefore the first example of a PP-1 regulatory subunit that is also a nucleotide-binding protein. Recovery of PP-1bp29 and human p20 in the complex suggest that these proteins bind PP-1bp80 directly, since neither protein interacts with PP-1C in Far Western analysis (Fig. 2*C*). In addition, neither protein contain motifs that would characterize them as nucleotide-binding proteins.

PP-1bp80 Is a Regulatory Subunit of PP-1C—Far Western analysis of the PP-1C-affinity column eluate with PP-1C bound to microcystin fluorescein indicated that PP-1bp80 binds the catalytic subunit of PP-1 directly (Fig. 2*C*). To test this hypothesis further, recombinant fragments of PP-1bp80 were passed separately over avidin-Sepharose (100 μl) that had previously been saturated with PP-1C bound to MC-biotin. The Sepharose was washed extensively with buffer containing 1 M NaCl, and then the bound proteins eluted with 10 mM biotin. Fig. 6*C* shows that recombinant protein derived from clone 232705 was recovered in the biotin eluate associated with PP-1C. In contrast, none of the proteins expressed by clones 318291 or 933493 were recovered in the biotin eluate (data not shown). These data suggest that recombinant protein expressed by 232705 contains a domain that binds PP-1C that is absent in the proteins expressed by 318291 and 933493. Inspection of the encoded sequences of each of the clones indicates that the putative PP-1C binding domain in 232705 lies within the first 101 amino acids of the N terminus of PP-1bp80. Significantly,

FIG. 5. **Amino acid sequence of PP-1bp80, PP-1bp55, and PP-1bp29.** The sequences shown were identified after a data base search with the TFASTF algorithm from peptide sequences shown in Table I. For PP-1bp80 the full-length sequence (AA1763991) was derived from a 2.2-Mb contiguous nucleotide sequence from chromosome III of *C. elegans* (23). The human and mouse alignments were derived from overlaps in the GenBank from human EST sequences AA308403, AA324858, AA278985, and AA144032 and mouse EST sequences AA544724, W45835, W171374, W89881, and AA409546. For PP-1bp29 the full-length sequence was derived from chicken EST sequence U46756. Overlapping sequences for human and mouse were derived from human ESTs AA310450, AA148660, and AA363054 and mouse ESTs AA109802, AA108607, and AA114777. The mixed peptide sequence from PP-1bp55 is matched to the N-terminal domain of human MYPT2 (AB003062) (11). *Periods* denote amino acid identities; gaps are denoted by substituted amino acids.

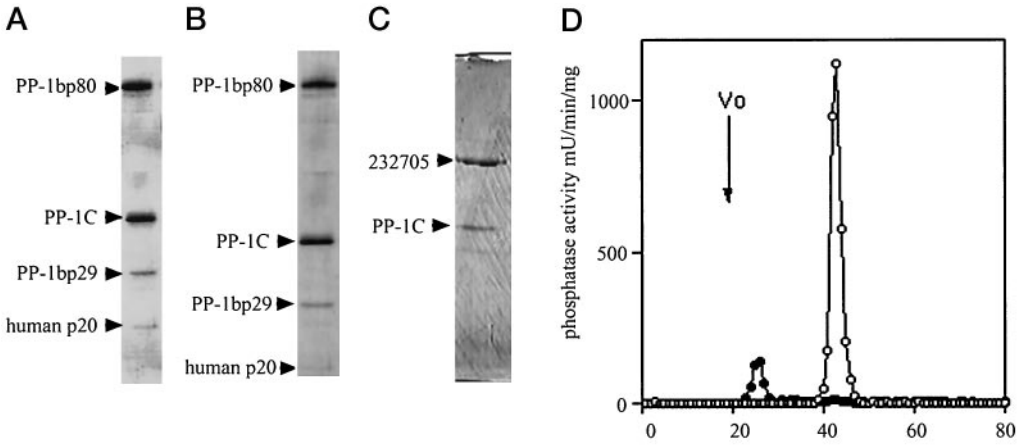

FIG. 6. **Purification and characterization of PP-1bp80.** The MC-biotin eluate was passed over γ-phosphate-linked ATP-Sepharose (2.0 ml) previously equilibrated in buffer B (25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 60 mM $MgCl_2$). The column was washed extensively with buffer B containing 1 M NaCl, and then the bound proteins eluted in buffer B containing 10 mM ATP. The peak fractions were characterized by SDS-PAGE and silver staining (*A*). The ATP eluate was applied to a G75 superfine column equilibrated in buffer A. *Gel B* shows a silver stain of the eluted protein from a single peak fraction. The proteins shown in *A* and *B* were identified by mixed peptide amino acid sequencing. *C*, recombinant protein purified from clone 232705 was passed over an avidin-Sepharose column that had previously been saturated with PP-1C bound to MC-biotin. The column was washed extensively and eluted with biotin. Peak column fractions were analyzed by SDS-PAGE and Coomassie Blue staining. *D*, PP-1C was mixed in the presence (●) and absence (○) of a 2-fold (mol/mol) excess of recombinant PP-1bp80 clone 232705. The mixture was applied to a Waters SW300 gel filtration column and column fractions assayed for phosphorylase phosphatase activity.

as the C-terminal portion of PP-1bp80 containing the (K/R)(I/V)*X*F motif is missing in 232705, its ability to bind PP-1C suggests a second PP-1 binding site in the protein.

To test the effects of the recombinant proteins on PP-1C activity, each was mixed in a 2-fold molar excess with purified PP-1C. The mixtures were then applied to a Waters SW300 gel filtration column equilibrated in buffer A containing 150 mM NaCl. Column fractions were then assayed for nonspecific phosphatase activity using phosphorylase *a*, myelin basic protein (MBP), and myosin light chain (MLC20) as the substrates (Fig. 6*D*). Fig. 6*D* shows that in the absence of any recombinant protein phosphorylase phosphatase activity is detected between fractions 40 and 50. Elution of the catalytic subunit in these fractions is consistent with its molecular mass of 37 kDa. Similar data were obtained if MBP or MLC20 were used as the substrate (data not shown). Gel filtration analysis of a mixture of 232705 protein and PP-1C produced a second phosphorylase phosphatase peak (fractions 24–30, ~100 kDa) eluting close to the void volume of the column. No phosphorylase phosphatase activity was detectable eluting between fractions 40 and 50, suggesting the recombinant protein had bound the catalytic subunit, causing it to elute as a higher molecular weight complex. Significantly, the amount of phosphatase activity recovered in the early eluting peak was reduced by a factor of 10-fold compared with that measured in the absence of the PP-1bp80 clone. Similar data were obtained if MBP or MLC20 were used as the substrate (data not shown). Repeating the experiment using higher concentrations of the recombinant protein, or adding additional protein to the assay, failed to suppress phosphatase activity further. These findings suggest that the recombinant protein binds PP-1C, but does not completely inhibit its activity. This observation is reminiscent of the effects of the regulatory M110 subunit of SMPP-1M, which also suppresses but does not completely inhibit the nonspecific phosphatase activity of PP-1C *in vitro* (12–14). Consistent with the binding experiments described above, neither the 318291 or 933493 proteins altered the elution position or activity of PP-1C (data not shown). These findings suggest that binding of PP-1C by PP-1bp80 is necessary to suppress nonspecific phosphatase activity.

DISCUSSION

In this present study, we have utilized a novel sequencing method, mixed peptide sequencing, and two new algorithms, FASTF and TFASTF, to rapidly unambiguously identify 36 proteins recovered from microcystin affinity chromatography. The approach identified at least two *bona fide* PP-1 regulatory subunits that were recovered in PP-1C affinity chromatography. One of these proteins, PP-1bp55, is homologous to human MYPT2, indicating it is the skeletal muscle isoform of M110 subunit of SMPP-1M. A second protein, PP-1bp80, is a previously undescribed PP-1 regulatory subunit that was identified in the DNA data bases as several uncharacterized EST sequences. Inspection of the full-length reconstructed sequence of PP-1bp80 identified the (K/R)(V/I)*X*F motif, which is conserved in 10 other mammalian and yeast PP-1 regulatory subunits (6). Two additional lines of evidence suggest PP-1bp80 is a PP-1 regulatory subunit. First, PP-1bp80 was recovered as a quaternary complex with PP-1C, PP-1bp29, and human p20 following ATP-Sepharose affinity chromatography. Densitometry of the purified complex indicated that all 4 proteins were recovered in the complex with a stoichiometry of 1:1:1:1. Second, a truncated form of recombinant PP-1bp80 bound PP-1C and altered its specific activity toward three phosphoprotein substrates. PP-1bp80 is therefore the second example of a PP-1 regulatory subunit that alters the specific activity of PP-1C toward nonspecific substrates. This phenomenon was first observed for the M110 subunit that targets PP-1C toward smooth muscle myosin (12–14). In this case, the M110 subunit functions to reduce nonspecific phosphatase activity while enhancing smooth muscle myosin phosphatase activity 10–20-fold (12–14). Although the physiological substrate for the PP-1bp80 complex has yet to be identified, by analogy with smooth muscle M110, one might predict that the 80-kDa subunit will greatly enhance the ability of PP-1C to bring about its dephosphorylation. Human p20 and PP-1bp29 are currently being investigated as candidate substrates for the PP-1bp80 complex. Human p20 is related to the crystallin family of phosphoproteins (21). The crystallins are ubiquitously expressed proteins, which, in addition to forming the lens material of the eye (21), have also been implicated as stress response proteins. The crystallins are also unique in that they can convert from a liquid to gel like state when phospho-

rylated (21). The function PP-1bp29 is unclear, since this was identified as several undescribed EST sequences in the data base. The protein contains several phosphorylation sites for numerous protein kinases, but has no significant homology with any known protein in the data base. The finding that native PP-1bp80 also binds ATP suggests a potential mechanism of regulation in which phosphatase activity could be controlled in response to changes in intracellular ATP concentration. Cellular stress is one scenario in which intracellular ATP concentrations could be altered. The co-purification of human p20 with PP-1bp80, PP-1bp29, and PP-1C points to a possible function for PP-1bp80 in controlling the phosphorylation state of chaperone proteins like the crystallins in stress situations. In addition to identifying two *bona fide* PP-1 regulatory subunits, seven others were tentatively identified in this present study by their recovery in PP-1C affinity chromatography and far Western analysis. All of these proteins appear to be novel, because at the time of writing neither FASTF or TFASTF were able to match the mixed peptide sequence derived from these proteins to any sequences present in the GenBank.

The development of mixed peptide sequencing in this present study offers a simple alternative to investigators interested in the identification of individual proteins in complex mixtures. All that is required is to transfer the separated proteins from SDS-PAGE (one- or two-dimensional) gels to PVM. No further purification is necessary. When identifying proteins in polyacrylamide gels, we have shown that the method rivals state of the art mass spectrometry, since several of the proteins sequenced were well below 1 pmol. Indeed, in some cases, sequence data were obtained with <10 ng of protein. The methodology is rapid; in the present study, all of the proteins were identified in less than 5 days. Importantly, the technology described is not limited only to the identification of proteins *per se*, but, as shown in the case of PP-1, enables meaningful functional assignments to be made when combined with Far Western analysis or screening for binding partners with a purified protein.

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