Molecular Identification of I_1^{PP2A}, a Novel Potent Heat-Stable Inhibitor Protein of Protein Phosphatase 2A†

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Received March 8, 1996; Revised Manuscript Received April 17, 1996⊗

ABSTRACT: The amino acid sequences of two tryptic peptides derived from purified preparations of I_1^{PP2A} indicated that this potent heat-stable protein inhibitor of protein phosphatase 2A (PP2A) may be equivalent to putative histocompatibility leukocyte antigens class II-associated protein I (PHAP-I). Experiments using purified preparations of recombinant human PHAP-I confirmed that this protein inhibited PP2A. Half-maximal inhibition of the phosphatase occurred at about 4 nM PHAP-I, similar to the half-maximal inhibition obtained with purified preparations of bovine kidney I_1^{PP2A}. In addition, PHAP-I did not affect the activities of protein phosphatase 1, 2B, and 2C in a manner analogous to that of I_1^{PP2A}. Together, the results establish the identity of I_1^{PP2A} on a firm basis.

Protein phosphatase 2A (PP2A)[1] is a major mammalian protein serine/threonine phosphatase involved in the regulation of diverse cellular processes (Cohen, 1989; Shenolikar & Nairn, 1991; Mumby & Walter, 1993; Wera & Hemmings, 1994). Two forms of PP2A, termed $PP2A_1$ and $PP2A_2$, have been isolated from numerous sources (Cohen, 1989; Shenolikar & Nairn, 1991; Mumby & Walter, 1993; Wera & Hemmings, 1994), although more recent molecular cloning and tissue distribution studies established that another form termed $PP2A_0$ (Tung et al., 1985) is also ubiquitous (Csortos et al., 1996). $PP2A_1$, $PP2A_2$, and $PP2A_0$ contain A and C subunits exhibiting apparent M_r values of approximately 65 000 and 36 000, respectively. In addition, $PP2A_1$ and $PP2A_0$ contain several distinct B (B, B′, and B″) subunits with apparent M_r values of approximately 54 000, 55 000, 56 000, 72 000, and 130 000 [e.g. Hemmings et al. (1990), Hendrix et al. (1993), Zolnierowicz et al. (1994), McCright and Virshup (1995), and Csortos et al. (1996)]. By contrast, only two forms of each of the A and C subunits exhibiting 86% (Hemmings et al., 1990; Mayer et al., 1991) and 97% identity (Da Cruz e Silva & Cohen, 1987; Da Cruz e Silva et al., 1987; Stone et al., 1987; Arino et al., 1988; Sneddon et al., 1990) in their predicted amino acid sequences, respectively, have been identified by molecular cloning methods. Although the significance of the different A, B, and C subunits is not well-understood, the distinct substrate specificities of the various $PP2A_1$ and $PP2A_0$ forms appear to be conferred, at least in part, by the variable B subunit (Cohen, 1989; Shenolikar & Nairn, 1991; Mumby & Walter, 1993; Wera & Hemmings, 1994).

However, little information is available on the regulation of PP2A. Nevertheless, recent studies revealed the existence of two heat-stable inhibitor proteins of this phosphatase (Li et al., 1995). Purified preparations of these inhibitor proteins from bovine kidney consisted of single polypeptides with apparent M_r values of approximately 30 000 and 20 000 as determined by SDS−PAGE, which were designated I_1^{PP2A} and I_2^{PP2A}, respectively (Li et al., 1995). The purified preparations of I_1^{PP2A} and I_2^{PP2A} inhibited PP2A in a manner noncompetitive with the substrate and exhibited apparent K_i values in the nanomolar range (Li et al., 1995). Because I_1^{PP2A} and I_2^{PP2A} did not affect the activities of $PP1_C$, PP2B, and PP2C, the other three major protein serine/threonine phosphatases (Cohen, 1989; Shenolikar & Nairn, 1991; Mumby & Walter, 1993; Wera & Hemmings, 1994), as well as the activities of 11 different protein kinases (Li et al., 1995), the inhibitor proteins are considered PP2A-specific. Because the purified preparations exhibited distinct peptide patterns following cleavage with *Staphylococcus aureus* V8 protease, I_1^{PP2A} and I_2^{PP2A} appeared to be products of distinct genes, although direct evidence for this possibility was not provided (Li et al., 1995).

Subsequent studies (Li et al., 1996) showed that I_2^{PP2A} was equivalent to a largely nuclear myeloid leukemia-associated protein with a calculated molecular mass of 32 100 and apparent M_r of approximately 39 000 as estimated by SDS−PAGE (von Lindern et al., 1992). Thus, the original bovine kidney I_2^{PP2A} preparations (Li et al., 1995) were partially proteolyzed most likely during the isolation procedure. The aim of this study was to determine the identity of I_1^{PP2A} and its relationship, if any, to I_2^{PP2A}. In this communication, we show that I_1^{PP2A} is distinct from I_2^{PP2A} but that it is equivalent to PHAP-I, a protein of hitherto unknown function (Vaesen et al., 1994). Previously, it was suggested that this protein may be involved in the immune response, possibly by interacting with the cytoplasmic C-terminal region of the DR2α chain of human major histocompatibility complex (MHC) class II receptors (Vaesen et al., 1994).

† This work was supported by American Cancer Society Grant BE-247.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: PP2A, protein phosphatase 2A; $PP2A_C$, purified catalytic subunit of PP2A; $PP1_C$, purified catalytic subunit of protein phosphatase 1; PP2B, protein phosphatase 2B; PP2C, protein phosphatase 2C; MBP, myelin basic protein; PHAP-I, putative class II human histocompatibility leukocyte-associated protein I; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

S0006-2960(96)00581-8 CCC: $12.00

EXPERIMENTAL PROCEDURES

Materials. Bovine brain MBP (Deibler et al., 1984), $PP1_C$ (Amick et al., 1992), and PP2B (Amick et al., 1992) and bovine kidney I_2^{PP2A} (Li et al., 1995), $PP2A_1$ (Amick et al., 1992), $PP2A_2$ (Amick et al., 1992), and PP2C (Amick et al., 1992) were purified to apparent homogeneity as described. Purification of I_1^{PP2A} was also as described except that additional gel permeation chromatography on Sephacryl S-200 was performed (Li et al., 1995). Synthetic oligonucleotides were synthesized at GIBCO Life Technologies. Other materials are given elsewhere (Reddy et al., 1990, 1993; Guo et al., 1993; Makkinje et al., 1995).

^{32}P-labeled MBP was prepared, and assay of $PP2A_2$, $PP1_C$, PP2B, and PP2C was performed as described (Amick et al., 1992; Li et al., 1995). One unit of phosphatase activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of phosphoryl groups per minute from ^{32}P-labeled MBP. To ensure linearity, the extent of phosphoryl group release was limited to <10%. Inhibitor protein activity was measured as described (Li et al., 1995). One unit of inhibitor protein activity was defined as the amount of protein that inhibited 1 unit of $PP2A_2$ by 50% in the standard assay.

SDS−PAGE was performed in slab gels (12% acrylamide) with 0.1% SDS and Tris-glycine buffer (pH 8.3) (Laemmli, 1970). Protein bands were detected by staining with Coomassie blue. Protein was determined as described (Bradford, 1976). Amino acid sequencing was performed using standard reagents for gas-phase chemistry on an automated protein sequencer (Applied Biosystems) equipped with an on-line UV detector to identify phenylthiohydantoin derivatives.

Generation of PHAP-I cDNA. First strand PHAP-I cDNA was generated at 42 °C for 30 min in 0.02 mL of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 5 mM $MgCl_2$, 20 units of RNase inhibitor, 0.5 μg of human kidney polyadenylated RNA (Clontech), 50 pmol of the 3′ antisense PHAP-I-based oligonucleotide (GGATTCCACTTAGTCATCATCTT), 2.5 units of MuLV reverse transcriptase (Perkin-Elmer), and 1 mM of each dNTP. After heat denaturation at 99 °C for 5 min, a 0.005 mL aliquot of the mixture was subjected to amplification by PCR in 0.1 mL of 20 mM Tris-HCl (pH 8.3) containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 100 pmol of the 3′ and 5′ (GCGAGCCATGGAGATGGGCAGAC) PHAP-I-based oligonucleotides, 0.2 mM of each dNTP, and 5 units of recombinant *pfu* DNA polymerase (Stratagene). After heat denaturation for 5 min at 94 °C, PCR amplification for 30 cycles was performed in a Temptronic Series 669 thermocycler (Thermolyne) as follows: 30 s at 94 °C, 30 s at 55 °C, and 3 min at 72 °C, except that, in the last cycle, extension was carried out for 7 min. The amplified cDNA (766 base pairs) was subcloned into the *Sma*I site of pUC18 and used to transform *Escherichia coli* ONE SHOT as recommended by the manufacturer (Invitrogen). After growth at 37 °C in Luria-Bertani medium (Sambrook et al., 1989) containing 100 μg/mL ampicillin, the pUC18 containing PHAP-I cDNA was purified from the transformed *E. coli* ONE SHOT by the alkaline lysis method (Sambrook et al., 1989). Both cDNA strands were then sequenced using M13 sequencing primers by the dideoxynucleotide chain termination method (United States Biochemicals TAquence sequencing kit and DuPont-New England Nuclear [α-^{35}S]dATP).

Experession of PHAP-I. The PHAP-I cDNA was excised from pUC18 by incubation with *Bam*HI and *Nco*I and then isolated with GLASSMILK (Gene Clean II, Bio 101 Inc.) from low-melting agarose following gel electrophoresis. pET-3d DNA (50 ng) (Novagen) was linearized with *Bam*HI and *Nco*I and dephosphorylated with calf intestinal alkaline phosphatase (Stratagene). This DNA was then incubated overnight at 16 °C with 10 ng of the isolated cDNA in 0.01 mL of 20 mM Tris-HCl (pH 7.6) containing 1 mM ATP, 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, and 4 units of T4 DNA ligase (Invitrogen) as recommended (Sambrook et al., 1989). The mixture was then used to transform *E. coli* BL21(DE3)pLysS as recommended by the manufacturer (Novagen). A 5 mL overnight culture of the transformed cells was inoculated into 500 mL of Terrific Broth (Sambrook et al., 1989) containing 100 μg/mL ampicillin. After growth at 37 °C to log phase ($A_{600\ nm} \sim 0.4$), IPTG was added to a final concentration of 0.5 mM, and the cells were grown for an additional 2 h.

Purification of IPTG-Induced Inhibitor. Unless indicated otherwise, all operations were performed at 4 °C. After centrifugation for 15 min at 3000*g* in a Beckman JA-10 rotor, cells were resuspended in buffer A [25 mM Tris-HCl (pH 7.4) containing 10% glycerol, 4 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, and 14 mM β-mercaptoethanol] and lysed at 1200 psi with a French press. After centrifugation at 39000*g* for 30 min in a Beckman JA-20 rotor, pellets were discarded, and to the supernatant was added 30 volumes of buffer B (buffer A containing 1 mM instead of 4 mM EDTA). The mixture was applied onto a column (2.5 × 8.5 cm) of poly(L-lysine)-agarose equilibrated with buffer B. After being washed with 500 mL of buffer B, the column was developed with a 600 mL linear gradient from 0 to 1.0 M NaCl. Inhibitor activity was recovered at about 0.65 M NaCl. Active fractions were pooled and then mixed at room temperature with 2 volumes of a 99% ethanol solution. After centrifugation at 30000*g* for 10 min in a Beckman JA-14 rotor, pellets were resuspended in buffer B and subjected to gel permeation chromatography on a calibrated column (2.5 × 95 cm) of Sephacryl S-200 equilibrated with buffer B containing 0.1 M NaCl and 0.01% Brij 35. A major peak of inhibitor protein activity emerged at V_0 (∼220 mL) from Sephacryl S-200. Fractions containing this inhibitor protein activity were pooled, and 20 g/L trichloroacetic acid was added with stirring for 10 min. After centrifugation, the supernatant was discarded, and pellets were resuspended in a solution of 70% ethanol. The mixture was centrifuged, and the supernatant was discarded. This procedure was repeated three times. The final pellets were resuspended in buffer C (buffer A containing 0.1 mM instead of 4 mM EDTA) and dialyzed, with three changes, against 20 volumes of this buffer in 16 h. The preparations were aliquotted and stored at −70 °C. A second minor peak of unidentified inhibitor activity emerged with an apparent M_r of approximately 50 000 from Sephacryl S-200. This peak was discarded because it was detected following gel permeation chromatography of extracts from mock-transformed and transformed cells incubated in the absence or presence of IPTG and was therefore considered not to be a product of the introduced cDNA.

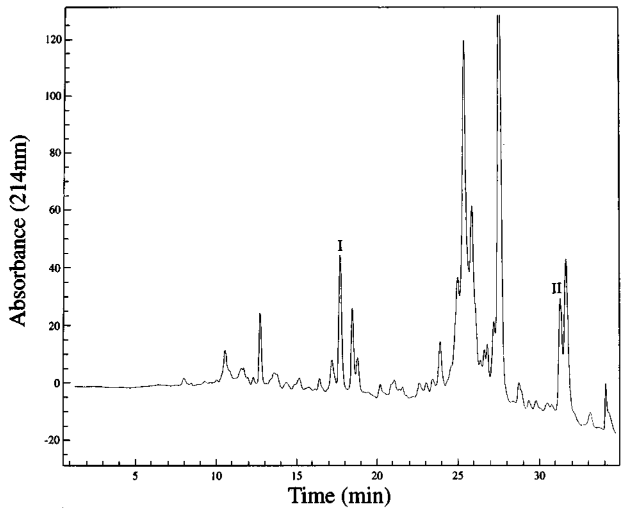

FIGURE 1: Purification of I_1^{PP2A}-derived tryptic peptides. Aliquots (2 μg) of purified bovine kidney I_1^{PP2A} were subjected to SDS−PAGE, followed by electrophoretic transfer onto Immobolin P membranes. After they were stained with Ponseau S, bands corresponding to I_1^{PP2A} were cut out and incubated overnight at 37 °C with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (0.5 mg) as described (Fernandez et al., 1994). The mixture was applied onto an HPLC Aquapore RP-300 column (250 mm × 1 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid. After being washed with this solution, the column was developed with a linear gradient from 0.1% trifluoroacetic acid to 0.08% trifluoroacetic acid containing 38.5% (v/v) acetonitrile in 30 min, followed by a linear gradient from 0.08% trifluoroacetic acid containing 38.5% acetonitrile (v/v) to 0.08% trifluoroacetic acid containing 59.5% (v/v) acetonitrile in 10 min. The flow rate was 0.15 mL/min, and 0.045 mL fractions were collected. The positions of peptides I and II are indicated.

RESULTS AND DISCUSSION

Amino Acid Sequencing. To establish the identity of I_1^{PP2A}, we set out to determine the amino acid sequence of this protein. Initially, the amino acid sequences of two tryptic peptides (peptide I, IHLELR; peptide II, SNEGKIEGLTDEFEEL) derived from the highly purified I_1^{PP2A} preparations (Figure 1) were determined. Comparison to amino acid sequences available at the PIR, SwissProt, and GenBank data bases indicated that peptides I and II were 100% and 94% identical (difference underlined) to residues 7−12 and 29−44 predicted for human PHAP-I, respectively (Vaesen et al., 1994). These results suggested that I_1^{PP2A} may be equivalent to PHAP-I and that the single amino acid difference noted may be species- and/or tissue-related. In this regard, it is pertinent that the purified preparations of bovine kidney I_1^{PP2A} (Li et al., 1995) and human PHAP-I (Vaesen et al., 1994) both exhibit an apparent M_r of approximately 30 000 ± 1000 as estimated by SDS−PAGE. However, because PHAP-I is blocked at its N-terminus (Vaesen et al., 1994), the results also suggested that the previously determined N-terminal amino acid sequence of I_1^{PP2A} (Li et al., 1995) may have been in error perhaps because of the presence of a contaminant in the highly purified preparations. To test this possibility, the purified preparations of bovine kidney I_1^{PP2A} were subjected to further purification by additional gel permeation chromatography on Sephacryl S-200 followed by chromatography on a small Q-Sepharose column (1 × 2 cm) as described (Li et al., 1995). N-terminal amino acid sequencing of these preparations showed that they were blocked at the N-terminus as expected for PHAP-I. In

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                                                          NcoI
                                                  97   GCGAGCC   103

  1   M  E  M  G  R  R  I  H  L  E  L  R  N  R  T  P  S  D  V  K    20
104   ATGGAGATGGGCAGACGGATTCATTTAGAGCTGCGGAACAGGACGCCCTCTGATGTGAAA   163

 21   E  L  V  L  D  N  S  R  S  N  E  G  K  L  E  G  L  T  D  E    40
164   GAACTTGTCCTGGACAACAGTCGGTCGAATGAAGGCAAACTCGAAGGCCTCACAGATGAA   223

 41   F  E  E  L  E  F  L  S  T  I  N  V  G  L  T  S  I  A  N  L    60
224   TTTGAAGAACTGGAATTCTTAAGTACAATCAACGTAGGCCTCACCTCAATCGCAAACTTA   283

 61   P  K  L  N  K  L  K  K  L  E  L  S  D  N  R  V  S  G  G  L    80
284   CCAAAGTTAAACAAACTTAAGAAGCTTGAACTAAGCGATAACAGAGTCTCAGGGGGCCTG   343

 81   E  V  L  A  E  K  C  P  N  L  T  H  L  N  L  S  G  N  K  I   100
344   GAAGTATTGGCAGAAAAGTGTCCGAACCTCACGCATCTAAATTTAAGTGGCAACAAAATT   403

101   K  D  L  S  T  I  E  P  L  K  K  L  E  N  L  K  S  L  D  L   120
404   AAAGACCTCAGCACAATAGAGCCACTGAAAAAGTTAGAAAACCTCAAGAGCTTAGACCTT   463

121   F  N  C  E  V  T  N  L  N  D  Y  R  E  N  V  F  K  L  L  P   140
464   TTCAATTGCGAGGTAACCAACCTGAACGACTACCGAGAAAATGTGTTCAAGCTCCTCCCG   523

141   Q  L  T  Y  L  D  G  Y  D  R  D  D  K  E  A  P  D  S  D  A   160
524   CAACTCACATATCTCGACGGCTATGACCGGGACGACAAGGAGGCCCCTGACTCGGATGCT   583

161   E  G  Y  V  E  G  L  D  D  E  E  E  D  E  D  E  E  E  Y  D   180
584   GAGGGCTACGTGGAGGGCCTGGATGATGAGGAGGAGGATGAGGATGAGGAGGAGTATGAT   643

181   E  D  A  Q  V  V  E  D  E  E  D  E  D  E  E  E  E  G  E  E   200
644   GAAGATGCTCAGGTAGTGGAAGACGAGGAGGACGAGGATGAGGAGGAGGAAGGTGAAGAG   703

201   E  D  V  S  G  E  E  E  E  D  E  E  G  Y  N  D  G  E  V  D   220
704   GAGGACGTGAGTGGAGAGGAGGAGGAGGATGAAGAAGGTTATAACGATGGAGAGGTAGAT   763

221   D  E  E  D  E  E  E  L  G  E  E  E  R  G  Q  K  R  K  R  E   240
764   GACGAGGAAGATGAAGAAGAGCTTGGTGAAGAAGAAAGGGGTCAGAAGCGAAAACGAGAA   823

241   P  E  D  E  G  E  D  D  D  *                                  249
824   CCTGAAGATGAGGGAGAAGATGATGACTAAGTGGAATCC                        862
                                         BamH I
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FIGURE 2: Nucleotide and deduced amino acid sequence of PHAP-I cDNA. Bold lines indicate the oligonucleotides used to generate the cDNA and the location of the introduced *Bam*HI and *Nco*I sites. Double-underlined amino acid sequences indicate the directly determined I_1^{PP2A} internal tryptic peptide amino acid sequences.

contrast, prior to the second gel permeation chromatography step, the I_1^{PP2A} preparations exhibited an N-terminal amino acid sequence identical to that reported previously (Li et al., 1995). These results confirm that the previously reported N-terminal amino acid sequence (Li et al., 1995) was that of a contaminant in the I_1^{PP2A} preparations.

PHAP-I cDNA Generation and Expression. To test whether PHAP-I inhibited PP2A in a manner similar to that of I_1^{PP2A}, a cDNA coding for the open reading frame of human PHAP-I was generated, placed into a pET-3d vector under the control of the T7 promotor, and then expressed in bacteria as described in Experimental Procedures. The nucleotide sequence of this cDNA (Figure 2) was identical to that reported previously for PHAP-I (Vaesen et al., 1994; GenBank accession number X75090). Consistent with the possibility that PHAP-I inhibits PP2A, IPTG-induced expression of this cDNA in bacteria resulted in about a 10-fold increase in PP2A inhibitory activity as determined following poly(L-lysine)agarose chromatography (Figure 3A,B). In addition, a protein with an apparent M_r of approximately 30 000 as estimated by SDS−PAGE was detected in the eluates from poly(L-lysine)agarose containing the IPTG-induced inhibitor (Figure 3C). Furthermore, the activity of this inhibitor was destroyed by incubation with trypsin (1:10 w/w) (not shown). Because of interference in the assays from unidentified endogenous inhibitor(s), differences in PP2A inhibitor activity in the extracts from control and IPTG-treated cells could not be distinguished prior to chromatography on poly(L-lysine)agarose. The unidentified endogenous inhibitor(s) was not a product(s) of the introduced cDNA because similar activity was detected in extracts of the bacterial cells that had been mock-transformed or transformed with the vector alone.

Purification of IPTG-Induced PP2A Inhibitor. To more directly test the possibility that the IPTG-induced PP2A

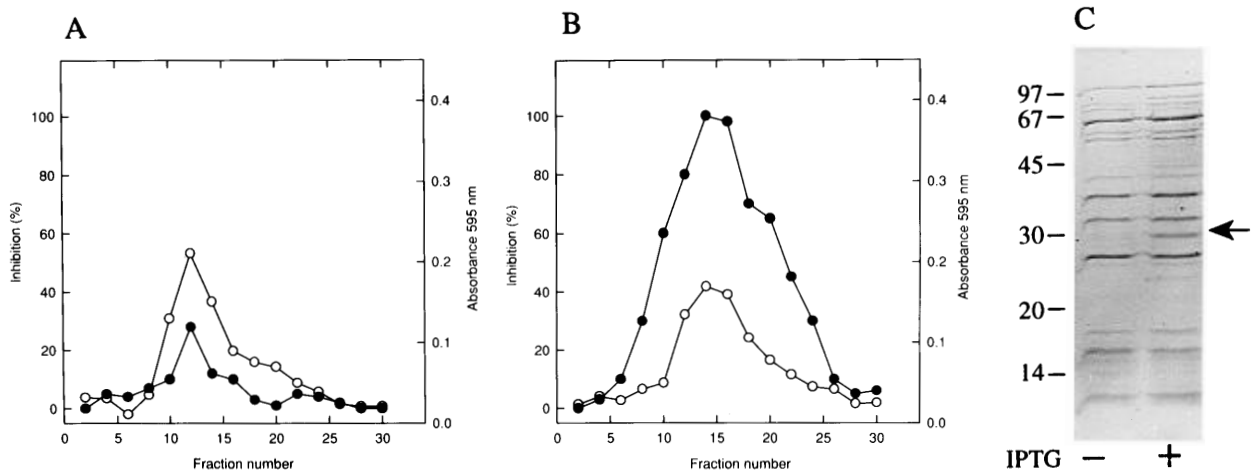

FIGURE 3: Induction of PP2A inhibitor. Extracts from 500 mL cultures of control (A) and IPTG-treated (B) bacteria containing PHAP-I cDNA were prepared as described in Experimental Procedures. Each extract was then applied onto a separate column (2.5 × 8.5 cm) of poly(L-lysine)agarose equilibrated with buffer B. Each column was washed with 500 mL of buffer B, followed by buffer B containing 0.3 M NaCl. Inhibitor activity was recovered with buffer B containing 0.8 M NaCl. Fractions (3 mL) were collected, and PP2A inhibitor activity (●) (Li et al., 1995) and absorbance at 595 nm (○) (Bradford, 1976) were determined as described. Panel C shows the SDS−PAGE pattern of the 0.8 M NaCl poly(L-lysine)agarose eluates of the extracts from control (lane 1) and IPTG-treated (lane 2) cells. PAGE was performed in slab gels (12% acrylamide) with 0.1% SDS and Tris-glycine buffer (pH 8.3) (Laemmli, 1970).

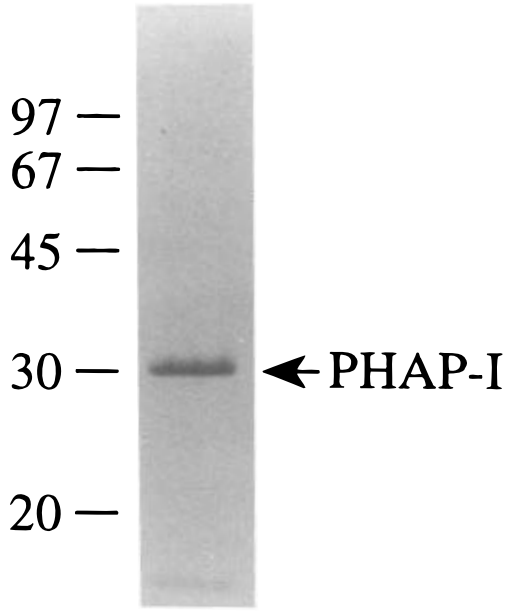

FIGURE 4: SDS−PAGE pattern of purified recombinant PHAP-I (1 μg). SDS−PAGE was performed as described in the legend to Figure 3. Purification of PHAP-I was as described in Experimental Procedures.

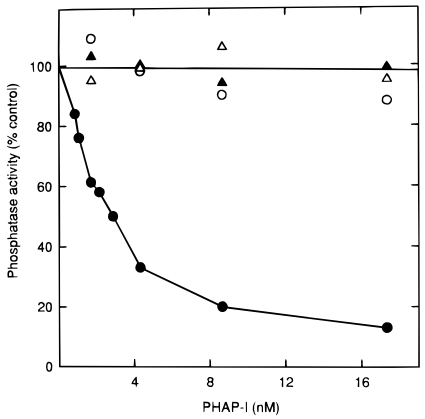

FIGURE 5: Effect of PHAP-I on protein phosphatases. The activities (~0.005 unit) of 0.1 ng of $PP2A_2$ (●), 0.7 ng of $PP1_C$ (○), 2 ng of PP2B (▲), and 0.8 ng of PP2C (△) were measured with ^{32}P-labeled MBP as substrate as described (Li et al., 1995) in the presence of the indicated concentration of purified recombinant PHAP-I. The calculated molecular weight of 28 400 was used to determine the concentration of PHAP-I in the assays.

inhibitor was indeed PHAP-I, a procedure was developed to purify it to apparent homogeneity from the bacterial extracts as described in Experimental Procedures. This procedure was based on the one employed previously to purify I_1^{PP2A} (Li et al., 1995) and included chromatography of the extracts on poly(L-lysine)agarose, precipitation with ethanol, and chromatography on Sephacryl S-200, followed by trichloroacetic acid and ethanol precipitation. The purified preparations consisted of a single Coomassie blue-staining polypeptide with an apparent M_r of approximately 30 000 ± 1000 as estimated by SDS−PAGE (Figure 4), similar to the apparent M_r values of PHAP-I (Vaesen et al., 1994) and I_1^{PP2A} (Li et al., 1995). Furthermore, the amino acid sequence of a tryptic peptide derived from these preparations, corresponding to the I_1^{PP2A}-derived peptide II (Figure 1), was identical to that of residues 29−44 of human PHAP-I, confirming the identity of the purified preparations. A typical 500 mL culture of transformed cells yielded about 4 mg of purified recombinant PHAP-I.

Effect of PHAP-I on PP2A Activity. The effect of the purified recombinant PHAP-I preparations on PP2A activity was examined next. These preparations inhibited PP2A potently (Figure 5). Half-maximal inhibition of the phosphatase occurred at about 4 nM (Figure 5), similar to the potent inhibition observed with purified preparations of bovine kidney I_1^{PP2A} (Li et al., 1995). Previously, we showed that, by contrast to PP2A, purified preparations of $PP1_C$, PP2B, and PP2C were unaffected by I_1^{PP2A} (Li et al., 1995). Similar experiments revealed that recombinant human PHAP-I also exhibited little or no effect on the activities of $PP1_C$, PP2B, and PP2C (Figure 5). Together, these results indicate that PHAP-I is a potent and specific inhibitor of PP2A and corresponds to I_1^{PP2A}. Because the recombinant PHAP-I preparations inhibited $PP2A_1$ (not shown), $PP2A_2$ (Figure 5), and $PP2A_C$ (not shown), PHAP-I is also analogous to I_1^{PP2A} in that it appears to act by binding to the C subunit of

the phosphatase. On the basis of the results, we recommend that PHAP-I be renamed I_1^{PP2A} to indicate its function and to distinguish it from I_2^{PP2A} which is the product of a distinct gene (Li et al., 1996). In this connection, it is pertinent that, by contrast to I_2^{PP2A} which appears to be largely nuclear (Fornerod et al., 1995), I_1^{PP2A} is diffusely distributed largely throughout the cytosol, although significant amounts are also present in the nucleus (Vaesen et al., 1994). Whether I_1^{PP2A} occurs as a fusion protein in acute myeloid leukemia by analogy to I_2^{PP2A} (von Lindern et al., 1992) is an intriguing possibility.

The current work provides a firm basis for further characterization of the role of I_1^{PP2A} in the regulation of PP2A. Perhaps the most striking feature present in the deduced amino acid sequence of I_1^{PP2A} is a highly acidic C-terminal region, amino acid residues 168−249 (Figure 2). A similar acidic C-terminal tail is also present in I_2^{PP2A} (Li et al., 1996). These observations suggest that I_1^{PP2A} and I_2^{PP2A} may inhibit PP2A *via* their acidic tails. Interestingly, the N-terminal region of I_1^{PP2A} is rich in regularly spaced leucine/isoleucine residues, and this motif is repeated twice within its primary structure (Figure 2). Similar leucine/isoleucine motifs are not present in I_2^{PP2A} but have been found in proteins of different origin and function, including the sds22 proteins (Ohkura & Yanagida, 1991; Wilson et al., 1994; Hisamoto et al., 1995; Renouf et al., 1995) that are considered positive modulators of PP1. These sds22 proteins are characterized by 11 centrally located leucine-rich repeats that are essential for interaction with $PP1_C$. By contrast, leucine-rich domains at the N- and C-termini of sds22 are important for the stability and subcellular localization of these proteins, respectively. Studies on the leucine/isoleucine motifs of I_1^{PP2A} and the acidic tails of I_1^{PP2A} and I_2^{PP2A} are needed in order to determine their significance.

ACKNOWLEDGMENT

Tryptic peptide generation, purification, and sequencing were performed at the Worcester Foundation Microsequencing Facility, Worcester Foundation for Biomedical Research, Shrewsbury, MA. N-Terminal amino acid sequencing was performed at the Milton S. Hershey Medical Center Macromolecular Core Facility. We thank Dr. Samer Al-Murrani for helpful comments.

REFERENCES

Amick, G. D., Reddy, S. A. G., & Damuni, Z. (1992) *Biochem. J. 287,* 1019−1022.
Arino, J., Woon, C. W., Brautigan, D. L., Miller, T. B., & Johnson, G. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 4252−4254.
Bradford, M. M. (1976) *Anal. Biochem. 72,* 248−254.
Cohen, P. (1989) *Annu. Rev. Biochem. 58,* 453−508.
Csortos, C., Zolnierowicz, S., Bako, E., Durbin, S. D., & DePaoli-Roach, A. A. (1996) *J. Biol. Chem. 271,* 2578−2588.
Da Cruz e Silva, O. B., & Cohen, P. T. W. (1987) *FEBS Lett. 226,* 176−178.
Da Cruz e Silva, O. B., Alemany, S., Campbell, D. G., & Cohen, P. T. W. (1987) *FEBS Lett. 221,* 415−422.
Deibler, G. E., Boyd, L. F., & Kies, M. W. (1984) *Prog. Clin. Biol. Res. 146,* 249−256.
Fernandez, J., Andrews, L., & Mische, S. M. (1994) *Anal. Biochem. 218,* 112−117.
Fornerod, M., Boer, J., van Baal, S., Jaegle, M., von Lindern, M., Murti, K. G., Davis, D., Bonten, J., Buijs, A., & Grosveld, G. (1995) *Oncogene 10,* 1739−1748.
Guo, H., Reddy, S. A. G., & Damuni, Z. (1993) *J. Biol. Chem. 268,* 11193−11198.
Hemmings, B. A., Adams-Pearson, C., Maurer, F., Muller, P., Goris, J., Merlevede, W., Hofsteenge, J., & Stone, S. R. (1990) *Biochemistry 29,* 3166−3173.
Hendrix, P., Mayer-Jackel, R. E., Cron, P., Goris, J., Hofsteenge, J., Merlevede, W., & Hemmings, B. A. (1993) *J. Biol. Chem. 268,* 15267−15276.
Hisamoto, N., Frederick, D. L., Sugimoto, K., Tatchell, K., & Matsumoto, K. (1995) *Mol. Cell. Biol. 15,* 3767−3776.
Laemmli, U. K. (1970) *Nature 227,* 680−685.
Li, M., Guo, H., & Damuni, Z. (1995) *Biochemistry 34,* 1988−1996.
Li, M., Makkinje, A., & Damuni, Z. (1996) *J. Biol. Chem. 271,* 11059−11062.
Makkinje, A., Xiong, H., Li, M., & Damuni, Z. (1995) *J. Biol. Chem. 270,* 14824−14828.
Mayer, R. E., Hendrix, P., Cron, P., Matthies, R., Stone, S. R., Goris, J., Merlevede, W., Hofsteenge, J., & Hemmings, B. A. (1991) *Biochemistry 30,* 3589−3597.
McCright, B., & Virshup, D. M. (1995) *J. Biol. Chem. 270,* 26123−26128.
Mumby, M. C., & Walter, G. (1993) *Physiol. Rev. 73,* 673−699.
Ohkura, H., & Yanagida, M. (1991) *Cell 64,* 149−157.
Reddy, S. A. G., Amick, G. D., Cooper, R. H., & Damuni, Z. (1990) *J. Biol. Chem. 265,* 7748−7752.
Reddy, S. A. G., Guo, H., Tarun, S. Z., Jr., & Damuni, Z. (1993) *J. Biol. Chem. 268,* 15298−15304.
Renouf, S., Beullens, M., Wera, S., van Eynde, A., Sikela, J., Stalmans, W., & Bollen, M. (1995) *FEBS Lett. 375,* 75−78.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular cloning: A laboratory manual, 2*nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
Shenolikar, S., & Nairn, A. C. (1991) *Adv. Second Messenger Phosphoprotein Res. 23,* 1−121.
Sneddon, M., Cohen, P. T. W., & Stark, M. J. R. (1990) *EMBO J. 9,* 4339−4345.
Stone, S. R., Hofsteenge, J., & Hemmings, B. A. (1987) *Biochemistry 26,* 7215−7220.
Tung, H. Y. L., Alemany, S., & Cohen, P. (1985) *Eur. J. Biochem. 138,* 635−641.
Vaesen, M., Barnikol-Watanabe, S., Gotz, H., Awni, L. A., Cole, T., Zimmerman, B., Kratzin, H. D., & Hilschmann, N. (1994) *Biol. Chem. Hoppe-Seyler 375,* 113−126.
von Lindern, M., van Baal, S., Wiegant, J., Raap, A., Hagemeijer, A., & Grosveld, G. (1992) *Mol. Cell. Biol. 12,* 3346−3355.
Wera, S., & Hemmings, B. A. (1994) *Biochem. J. 311,* 17−29.
Wilson, R., et al. (1994) *Nature 368,* 32−38.
Zolnierowicz, S., Csortos, C., Bondor, J., Verin, A., Mumby, M. C., & DePaoli-Roach, A. A. (1994) *Biochemistry 33,* 11858−11867.

BI960581Y