Identification and Characterization of the Human HCG V Gene Product as a Novel Inhibitor of Protein Phosphatase-1†

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ABSTRACT: The catalytic subunit of mammalian protein phosphatase-1 (PP1) is known to bind to a number of regulatory subunits, whose functions include the targeting of the catalytic subunit to the molecular proximity of its substrate proteins. In addition, PP1 is potently inhibited by several inhibitory polypeptides that include inhibitor-1 and inhibitor-2. In this study the yeast two-hybrid system was used to screen a human cDNA library for putative PP1-binding proteins. Ten putative positive clones were identified, one of which was found to be a partial cDNA of the hemochromatosis candidate gene V (HCG V) whose function was previously unknown. The full-length protein of 126 amino acid residues was expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein and also as a nonfusion protein. The recombinant protein inhibited recombinant and rabbit muscle protein phosphatase-1 with IC_{50}s of ca. 1 nM, but did not inhibit PP2A. The term inhibitor-3 is proposed for this novel inhibitor. It is extremely hydrophilic, is heat stable, and behaves anomalously on SDS−PAGE with an apparent molecular mass of 23 kDa and on gel filtration with a relative molecular weight of 55 000, in contrast to its calculated molecular mass of 14 kDa. These characteristics are shared by the previously described protein phosphatase-1 inhibitor-2 and inhibitor-1 proteins.

Protein phosphatase-1, also known as phosphorylase phosphatase, was first studied because of its role in the regulation of glycogen metabolism, but is now known to be important in a variety of cellular functions (for reviews, see *1*−*4*). The catalytic subunit of protein phosphatase-1 (PP1) is a protein of 37 kDa, with a primary structure that is highly conserved in evolution. PP1 has been isolated in several heterodimeric forms as a complex with different regulatory subunits from muscle; these include the ATP/Mg-dependent PP1, the glycogen-bound PP1, and the myosin-bound PP1. The study of the latter two forms led to the proposal that these subunits serve to target PP1 to glycogen and myosin, respectively (*5*). A number of genes that encode PP1-binding proteins have been identified in yeast (*GAC1, GIP2, REG1, GIP1, RED1, SDS22, SCD5, GLC8*). These have been suggested to be involved in functions that include glycogen metabolism, glucose repression, mitosis, meiosis, and protein translation (*6*−*8*). Several mammalian PP1-binding proteins have been identified by the use of the yeast two-hybrid system. These include Rb (*9*), p53BP2 (*10*), ribosomal protein L5 (*11*), and the splicing factor PSF (*12*). Thus, the multiple cellular functions in which PP1 is implicated can be regarded as being mediated by the existence of multiple targeting proteins which serve to localize PP1 to specific microenvironments in the cell. PP1 is also inhibited by several heat-stable inhibitor proteins, which include inhibitor-1, its neuronal analogue, DARPP-32, and inhibitor-2 (*1*−*3*). Inhibitor-2 is a subunit of the ATP/Mg-dependent form of PP1, and this inactive enzyme form is transiently activated when inhibitor-2 in the complex is phosphorylated by GSK-3 (*13*). Inhibitor-1 and DARPP-32 are both inhibitory only when phosphorylated by cAMP-dependent protein kinase (*1*−*4*). In addition, PP1 is potently inhibited by a nuclear protein, NIPP, as well as a ribosomal inhibitor protein (*14*, *15*). As well as the various polypeptide inhibitors of PP1 that have been described, there also exist a number of xenobiotic toxins that potently inhibit PP1 and PP2A. These include okadaic acid, microcystin, calyculin A, and tautomycin (*16*). In this study we report the identification of a novel heat-stable protein inhibitor of PP1 from a human cDNA library by use of the yeast two-hybrid system.

EXPERIMENTAL PROCEDURES

Materials. The yeast strain Y190 and vectors for the two-hybrid system (pAS2 and pACT2) were kindly provided by Dr. S. Elledge, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX (*13*). *E. coli* DH5α was used as the recipient cell for all plasmid constructions. The PP1 cDNA used in this study was the rabbit muscle PP1α isoform (*17*). Recombinant PP1α was expressed in *E. coli* as described by Zhang et al. (*18*) and purified to homogeneity (*19*). The inhibitor-2 cDNA was that isolated by Zhang et al. (*20*).

Screening for PP1-Binding Proteins with the Yeast Two-Hybrid System. The rabbit muscle PP1α cDNA was cloned into the pAS2 vector by *Nde*I/*Bam*HI digestion and expressed as a fusion protein with the GAL4 DNA-binding domain in yeast. The rabbit muscle cDNA of inhibitor-2 (*21*) was cloned into pACT2 vector by *Nde*I/*Bam*HI digestion. The

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plasmid DNAs were purified by alkaline lysis and CsCl centrifugation. A GAL4 activation domain-tagged human brain cDNA expression library was obtained from Clontech. Preliminary experiments were performed to confirm that the two-hybrid system could be used for the screening of PP1-binding proteins. The PP1 sequence was cloned into the pAS2 vector containing the GAL4 DNA-binding domain and tested against pACT2 and pACT2 fused to the inhibitor-2 sequence. The plasmids were transformed in yeast alone, or with either pACT2 or the pACT2−inhibitor-2 construct. Cotransformants in yeast were tested for expression for β-galactosidase activity. The results (not shown) confirmed that PP1 itself could not activate the LacZ gene while the cotransformation of PP1 with inhibitor-2 restored the β-galactosidase activity. Yeast strains were grown in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose for liquid culture, plus 2% agar for plates) or synthetic dropout medium ("SD", 0.67% yeast nitrogen base, 2% glucose) with omitted amino acids as indicated.

The yeast strain Y190 was cotransformed with plasmids of the GAL4 DNA-binding domain-tagged PP1 and the GAL4 activation domain-tagged human brain cDNA expression library (*9*). The transformation mixtures were plated on 15 cm Petri dishes containing synthetic dropout medium (SD/−Trp/−Leu/−His) with 30 mM 3-aminotriazole. The plates were incubated at 28 °C for 1 week and then screened for β-galactoside activity with a filter lift assay using X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) as the substrate. Colonies were transferred onto nitrocellulose filters, permeabilized by freezing at −80 °C, and then placed cell side up in a Petri dish over 3 MM chromatography paper soaked with Z buffer (0.1 M sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0) containing 1 mg/mL X-Gal. The filters were then incubated at 30 °C for 30 min or overnight for development of color. Blue colonies were subjected to a second round of screening. Loss of the pAS2-PP1 plasmid was selected for on SD/Leu, containing 2.5 mg/mL cycloheximide medium. Forty-five putative positive clones were obtained. These were maintained by growth on SD/−Leu containing 2.5 mg/mL cycloheximide. The false positives were eliminated by examining transformants with the candidate genes alone, and cotransformation of candidate DNAs with the pAS1 plasmid containing the sequence of an unrelated protein (pAS1-SNF1, kindly provided by Dr. S. Elledge), and the candidate genes alone. Only those clones that were negative in both cotransformations were considered as positives. This reduced the number of positive clones to 10. The plasmids were isolated from the yeast and then used to transform *E. coli* DH5α. The positive clones were then sequenced. The sequence analyses were carried out with the Genetic Computer Group, Inc., sequence analysis package (Madison, WI). One of these, clone 45, was found to contain the coding sequence for the HCG V cDNA (*21*).

Construction of the Full-Length Inhibitor-3/HCG V Sequence and Its Expression as a GST-Fusion Protein. Clone 45 contained the coding sequence of HCG V (*21*) but was truncated by the first 13 codons. The full-length coding sequence of HCG V was constructed from clone 45 by PCR amplification. The primers were 5′-CCG**GAATTC**ATGGCCGAGGCAGGGGCTGGGCTGAGCGAGACCGTCACTGAGACAACGGTTACCGTGACAACC (the *Eco*RI site is in boldface, while the 13 missing codons that were reconstructed are underlined) and 5′-AATCCG**CTCGAG**TTAGTGCTGCATTGGCCCTGGAGG (the *Xho*I site is in boldface). The conditions for PCR amplification were the following: 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min for 30 cycles, then 72 °C for 10 min. The PCR product was digested with *Eco*RI/*Xho*I and cloned into the pGEX-4T-1 vector (Pharmacia-LKB Biotech) for expression as a fusion protein with glutathione-*S*-transferase (GST). The correctness of the insertion was confirmed by DNA sequence analysis. The construct was used to transform *E. coli* BL21-(DE_3) cells (Novagen). An overnight culture was diluted 1:100 into 2× YTA medium (16 g of peptone, 10 g of yeast extract, 5 g of NaCl per liter) containing 0.1 mg/mL ampicillin and grown at 37 °C with shaking until the OD_{600} reached 1−2. Isopropyl β-thiogalactoside (IPTG) was then added to a final concentration of 0.1 mM, and the culture was allowed to grow for an additional 2 h. Cells were harvested and resuspended in 50 mM TrisHCl, 2 mM dithiothreitol, pH 7.4, with 0.1 mM phenylmethylsulfonyl fluoride and then disrupted by passage through a French Press at 1000 psi. The cell lysate was centrifuged at 10 000 rpm to remove cell debris. The supernatant was then subjected to purification using GSH-beads (glutathione-Sepharose 4B, Pharmacia-LKB Biotech) preequilibrated in the same buffer. The crude lysate was incubated with GSH-beads for 1 h at 4 °C with rotation. The suspension was centrifuged to remove the supernatant, and the beads were washed 3 times with the same buffer. The beads were eluted with reduced glutathione (10 mM reduced glutathione in 50 mM Tris, pH 8.0). The eluted fusion protein was dialyzed against 10 mM TrisHCl, 2 mM dithiothreitol, pH 7.4, and stored at −20 °C. The yield of the fusion protein was about 10 mg/L of culture.

Expression of Inhibitor-3 in the pET3a Vector. The full-length inhibitor-3/HCG V cDNA was also inserted into the pET3a vector for expression as an intact protein. PCR amplification was used to introduce *Nde*I and *Bam*HI sites at the 5′- and 3′-ends of the coding sequence by the use of the following primers: GGGAATTCCATATGGCCGAGGCAGGGG, and CGCGGATCCTTAGTGCTGCATTGGCCC (*Nde*I and *Bam*HI sites are underlined). The template was the full-length cDNA constructed as described above. The conditions for amplification were the same as described above. The PCR product was digested with *Nde*I/*Bam*HI and inserted into the pET3a vector that had been digested with *Nde*I/*Bam*HI. The construct was used to transform *E. coli* BL21(DE_3), and the sequence was confirmed by DNA sequencing.

An overnight culture was inoculated into 1 L of Terrific media (24 g of yeast, 12 g of peptone, 10 g of NaCl, 4 mL of glycerol per liter, adjusted to pH 7.4 with Tris base) containing 100 μg/mL ampicillin. The culture was grown until the OD_{600} reached a value of 0.6. IPTG was then added to a final concentration of 0.5 mM. The culture was grown for an additional 6 h and harvested. The breakage of cells was described as above. The cell lysate was then heated at 100 °C for 20 min and then centrifuged at 10 000 rpm for 30 min. The supernatant was chromatographed on a DEAE-Sepharose column (1.5 × 25 cm) equilibrated with 50 mM imidazole, 1 mM EDTA, 2 mM dithiothreitol, pH 7.4, and eluted with a gradient of 0.1−0.4 M NaCl in a total volume

of 150 mL. The peak fractions of the expressed protein were identified by SDS−PAGE, pooled, and applied to a heparin−Sepharose column (1.5 × 15 cm) equilibrated with the same buffer as for the DEAE-Sepharose chromatography, and eluted with a gradient of 0.1−0.4 M NaCl in a total volume of 75 mL. The peak fractions were identified by SDS−PAGE, pooled, and stored at −20 °C.

Preparation of Recombinant PP1α. The Mn^{2+}-dependent recombinant rabbit muscle PP1α was expressed and purified as previously described (*19*). A novel metal-independent form of PP1 [Co(II)-PP1] was prepared by addition of 1 mM $CoCl_2$ to the growth medium instead of 1 mM $MnCl_2$ (Zhang and Lee, unpublished results). Under these conditions, the recombinant PP1 is active in the absence of added metal ions, and behaves in a manner similar to purified recombinant PP1 which has been treated with cobalt ion (*22*) and exhibits comparable specific activities to the Mn^{2+}-dependent recombinant PP1.

Affinity Chromatography of Inhibitor-3. Recombinant Co(II)-PP1 was immobilized on Sepharose as described by Zhao et al. (*23*). The column (5 mL) was equilibrated with 50 mM Tris, 2 mM DTT, 20% glycerol, pH 7.4. The crude lysate from 1 L of bacterial culture expressing the fusion protein was applied to the column, washed with 50 mM NaCl, and eluted with 1 M NaCl. The yield was 0.5 mg of protein.

Assay of PP1 Activity. PP1 was assayed using ^{32}P-labeled phosphorylase *a* (*18*).

Purification of Rabbit Skeletal Muscle Protein Phosphatases 1 and 2A. The catalytic subunits of PP1 and PP2A were purified from rabbit skeletal muscle as described by Silberman et al. (*24*).

RESULTS

Screening for PP1-Binding Proteins with the Yeast Two-Hybrid System. A human brain cDNA expression library expressing the proteins fused with the GAL4 activation domain was used for the screening of PP1-binding proteins by the yeast two-hybrid system (Experimental Procedures). Forty-five putative positive clones were isolated after 2 rounds of screening, and this number was further reduced to 10 clones after elimination of false positives (Experimental Procedures). The 10 clones were partially sequenced and the sequences searched against the Genbank database. One of these (clone 45) exhibited identity to a known sequence, that of the HCG V gene (*21*). HCG V is one of the several candidate genes for the hemochromatosis gene (Genbank X81003; ref *21*). Clone 45 was completely sequenced and found to consist of 768 base pairs that were identical to the cDNA for HCG V, except for a 138 bp deletion at the 5′ end, such that it starts at amino acid 14 of the encoded sequence, and a 614 bp deletion in the 3′-noncoding region (662−1275) (Figure 1). The HCG V cDNA encodes a polypeptide of 14 kDa. The C-terminal region is rich in proline, glutamic acid, serine, and threonine, and it has been suggested that these may represent a PEST sequence (*21*), which often characterizes proteins with short intracellular half-lives (*25*). The function of the HCG V gene is unknown, and a recent report indicates that it can be eliminated as a candidate for the hemochromatosis gene (*21*). As shown below, the protein encoded by the clone 45/HCG V gene is a potent inhibitor of PP1, and it will be referred to as inhibitor-3.

Demonstration That the Clone 45/HCG V Protein Binds to PP1. Attempts were made to express the 113 residue truncated protein encoded by clone 45 as a GST-fusion protein in the pGEX-4T-1 plasmid. However, only poor expression of the fusion protein was observed, because of rapid proteolysis of the expressed protein. When the fusion protein was expressed and purified with glutathione−Sepharose 4B and analyzed on SDS−PAGE, GST (29 kDa) was the major band, and a minor band near the expected size (43 kDa) was observed (not shown). The degradation could not be prevented even when multiple protease inhibitors were added to the lysis buffer.

Clone 45 was reconstructed to restore the missing N-terminal 13 amino acids present in the HCG V cDNA by PCR amplification (Experimental Procedures) and expressed as the GST-fusion protein. The latter was isolated on GSH−Sepharose (Figure 2). The major GST-binding protein was 43 kDa, with several smaller proteins, the most prominent of which was 38 kDa. These may be degraded forms of the GST-fusion protein. The size of the fusion protein is consistent with the estimated molecular mass of HCG V (14 kDa) plus that of the GST protein (29 kDa).

To confirm that the clone 45/HCG V protein binds to PP1, the crude bacterial lysate expressing the GST−HCG V fusion protein was chromatographed on a PP1−Sepharose column. The results are shown in Figure 3. The 43 kDa protein was bound to PP1−Sepharose while the GST protein (29 kDa) did not bind to the column.

Characterization of the Recombinant Inhibitor-3 Protein. The full-length coding sequence for inhibitor-3/HCG V was then cloned into the pET3a vector and expressed as the free protein. The purification of the protein could be achieved by affinity chromatography on PP1−Sepharose. However, it was observed that the yields of inhibitor-3 were very low (less than 0.1 mg from the crude extract from 1 L of culture) because the binding efficiency was very poor as compared to the isolation of inhibitor-2 on the same column support. An alternative method was developed for the preparation of milligram amounts of the protein. The SDS−PAGE analysis of the preparation at different stages is shown in Figure 4. The bacterial lysate was first heated at 100 °C, as it was observed that the inhibitory activity of the protein was heat-stable. After heat treatment, the expressed protein was a significant component in the preparation (Figure 4, lane 2), a situation similar to that we have observed in the isolation of recombinant inhibitor-2 (*26*). This was followed by chromatography on DEAE-Sepharose and heparin−Sepharose (Experimental Procedures). The amounts of protein recovered in the crude extract, heated extract, DEAE-Sepharose, and heparin−Sepharose steps for a typical preparation from a 2 L culture were 190, 40, 4, and 2 mg of protein, respectively. This indicates that the expression of the protein was at least 1% of the soluble protein.

The recombinant protein behaved anomalously on SDS−PAGE with a relative molecular mass of 23 kDa, much higher than its estimated molecular mass of 14 kDa (Figure 4). This may be due to the high content of hydrophilic amino acids in the polypeptide, which could result in a lower binding of SDS and a lower motility on SDS−PAGE. The protein also behaved anomalously on HPLC gel filtra-

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   1  cggcgagccggaagtggggttagccaggttatccccaggggtggagaagcggaggcccag
  61  gaggaggggaataaagaaggtggaggatcctggctaccactctgaatccgataccgctt
 121  ctcttagacctcagcgacagaaaaagggaagggtgtctcatccccctcctcctctcctc
 181  cctgtcctgagccttagccatggccgaggcaggggctgggctgagcgagaccgtcactGA
                          M  A  E  A  G  A  G  L  S  E  T  V  T  E      14
 241  GACAACGGTTACCGTGACAACCGAGCCCGAGAACCGGAGCCTTACCATCAAACTTCGGAA
       T  T  V  T  V  T  T  E  P  E  N  R  S  L  T  I  K  L  R  K      34
 301  ACGGAAGCCAGAGAAAAAGGTAGAATGGACAAGTGACACTGTGGACAATGAACACATGGG
       R  K  P  E  K  K  V  E  W  T  S  D  T  V  D  N  E  H  M  G      54
 361  CCGCCGCTCATCCAAATGCTGCTGTATTTATGAGAAACCTCGGGCCTTTGGCGAGAGCTC
       R  R  S  S  K  C  C  C  I  Y  E  K  P  R  A  F  G  E  S  S      74
 421  CACGGAAAGTGATGAGGAGGAAGAAGAGGGCTGTGGTCATACACACTGTGTACGTGGCCA
       T  E  S  D  E  E  E  E  E  G  C  G  H  T  H  C  V  R  G  H      94
 481  CCGCAAAGGACGGCGTCGTGCAACCCTAGGACCGACCCCCACCACCCCTCCCCAGCCTCC
       R  K  G  R  R  R  A  T  L  G  P  T  P  T  T  P  P  Q  P  P     114
 541  TGACCCTTCCCAGCCCCCTCCAGGGCCAATGCAGCACTAAATCCCTCTCTCCTCCAGCAT
       D  P  S  Q  P  P  P  G  P  M  Q  H  *                          126
 601  TCCTGTGTCTGTCTGGCCCTAAATGTATCCATGTGGCTACTTCTCCAGCCCCCTCCTTCC   660
 661  Ctctcttctgcctgatagagggaagaggaagaggaggacgaacagagatcctgaaattct   720
 721  gacttgctgctattccagaacccagcctcctgggtttccccagtcctcatttttcctccc   780
 781  aatacccacccttctctctcgagggatctaggcaccttggtcccagtgtcttccttttgt   840
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1261  acttagagccgactgTCTCCTCTGCCTTTTGTTTTTCAGCTTCAGAGACAGATCCAATAT  1320
1321  AGTCCCAGGGACCTGGGTCTCTGGGAGAGGAAGGAAGAGGGAGGGAGCAAAGAGATTGGG  1380
1381  GTATGTCCCCTGTAGTACACTCTTACCTCTTACTTCCTAGACTTTGATTTCTCCGGCAGC  1440
1441  CCAGATGTTCAGTTCTCTTGGCCCCTCTCTACCCCTTACTGGGATCTGGTTTTCATTTTC  1500
1501  CGGTCCTTTTGCCATACACAGTTACAGAGATCAGTCAAATCCATACCACCACTGAGATCT  1560
1561  CATTTATTGCCACAGATGCACAAAATAAATAACCCAAAATCACAAAAAAAAAAAAAAAAA  1620
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FIGURE 1: Nucleotide sequence of clone 45. The diagram shows the complete (1620 nt) cDNA of human HCG V (Genbank X81003). The cDNA contains an open reading frame for a protein of 126 amino acid residues. The sequence of clone 45 (768 bp) is identical to the HCG V cDNA with deletions between 1 and 238 and 662−1275 (shown in lower case). The start codon and polyadenylation signal are underlined, and the starting sequences of clone 45 are in boldface type.

tion, with an apparent molecular weight of 55 000 (Figure 5). Because of the anomalous behavior of the protein, its identity was also confirmed by N-terminal amino acid sequencing where the first eight N-terminal residues were found to correspond to those expected from the cDNA sequence.

The HCG V Gene Product (Inhibitor-3) Is a Specific Inhibitor of PP1. The ability of the recombinant protein to inhibit the phosphorylase phosphatase activity of PP1 was investigated. For these experiments, we tested both the Mn^{2+}-dependent form of recombinant PP1 and also a recombinant metal-independent form of PP1 [Co(II)-PP1]. The latter form is a presumptive cobalt form of PP1 which is produced by including $CoCl_2$ in the growth media instead of $MnCl_2$ (Experimental Procedures). It had previously been shown that incubation of recombinant PP1 with divalent cobalt ion leads to the stable incorporation of cobalt in a 1:1 molar ratio (*22*). This enzyme form ["Co(II)-PP1"] has similar properties to native PP1 isolated from skeletal muscle in terms of metal dependency (unpublished results) in that it is active in the presence of EDTA. Both forms of PP1 are potently inhibited by inhibitor-3 in the nanomolar range. However the recombinant Co(II)-PP1 is inhibited ca. 20-fold more strongly than the Mn^{2+}-dependent PP1 (Figure 6). The IC_{50}s for Co(II)-PP1 and Mn^{2+} PP1 were found to be 0.3 and 6 nM, respectively (mean of two determinations with different preparations of I-3 and PP1). We also tested native rabbit muscle PP1 catalytic subunit. This was inhibited in a comparable manner to Co(II)-PP1. No inhibition of PP2A was observed. These results show that inhibitor-3 is a potent inhibitor of PP1 and, moreover, exhibits a differential sensitivity toward the metal-independent and metal-dependent forms of PP1.

DISCUSSION

We have identified a new PP1-binding protein by the use of the yeast two-hybrid system. This protein is encoded by the human gene HCG V (*H*emochromatosis *C*andidate *G*ene *V*). HCG V is one of a group of seven genes that were identified as candidate genes for hereditary hemochromatosis (HH), an abnormal iron storage disease in which excessive deposition of iron occurs within the parenchymal cells of many organs (*27*). Northern blot analysis showed that the HCG V gene is expressed as a 1.8 kb transcript in a variety of tissues (spleen, thymus, prostate, testis, ovary, placenta, colon, leucocyte, heart, kidney, pancreas, brain, liver, and lung) (*21*). HCG V is localized on the short arm of chromosome 6, within the major histocompatibility complex (MHC) class I region. Interestingly, one of several human inhibitor-2 genes also maps to the MHC region on chromo-

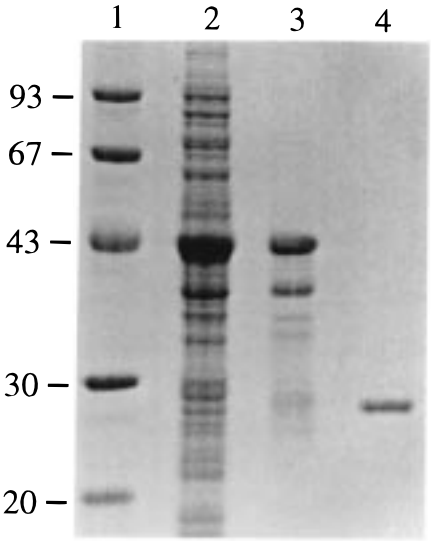

FIGURE 2: Purification of the GST−inhibitor-3 fusion protein on GST−Sepharose. The GST−inhibitor-3 fusion protein was expressed and purified on glutathione−Sepharose (Experimental Procedures). Samples of the crude bacterial lysate containing the fusion protein (lane 2), the affinity-purified fusion protein (lane 3), and the affinity-purified material from a control bacterial lysate containing recombinant glutathione-*S*-transferase (lane 4) were subjected to SDS−PAGE on 12% acrylamide gels and stained with Coomassie blue. Lane 1 contains the protein standards (phosphorylase, 93 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa).

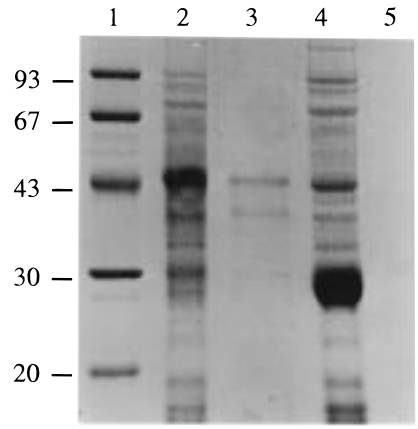

FIGURE 3: Purification of the GST−inhibitor-3 fusion protein on PP1−Sepharose. The GST−inhibitor-3 fusion protein was expressed and purified on PP1−Sepharose (Experimental Procedures). Samples of the crude bacterial lysate containing the GST−inhibitor-3 fusion protein (lane 2), the affinity-purified fusion protein after chromatography on PP1−Sepharose (lane 3), a control crude bacterial lysate expressing glutathione-*S*-transferase (lane 4), and the 1 M NaCl eluate when the control lysate was chromatographed on the same PP1−Sepharose column (lane 5) were subjected to SDS−PAGE on 12% acrylamide gels and stained with Coomassie blue. Lane 1 contains the protein standards (phosphorylase, 93 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa).

some 6 (*28*). Analysis of the gene in hemochromatosis patients revealed only a single polymorphism within the coding region of HCG V, and no disease-associated changes were found. Therefore, it was considered that HCG V is not involved in the pathogenesis of hemochromatosis (*21*). The gene responsible for hemochromatosis has now been positively identified as the HLA-H gene, which encodes a protein that binds transferrin and β2-microglobulin (*29*−*31*).

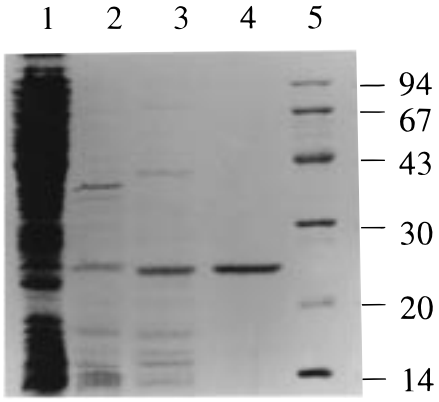

FIGURE 4: Purification of recombinant inhibitor-3. Inhibitor-3 was expressed in *E. coli* and purified as described under Experimental Procedures. A sample of the preparation at each step was subjected to SDS−PAGE on a 15% acrylamide gel and stained for protein with Coomassie blue. Lane 1, crude bacterial lysate; lane 2, heat-treated lysate; lane 3, DEAE-Sepharose chromatography; lane 4, heparin−Sepharose chromatography. Lane 5 contains the protein standards (phosphorylase, 93 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa).

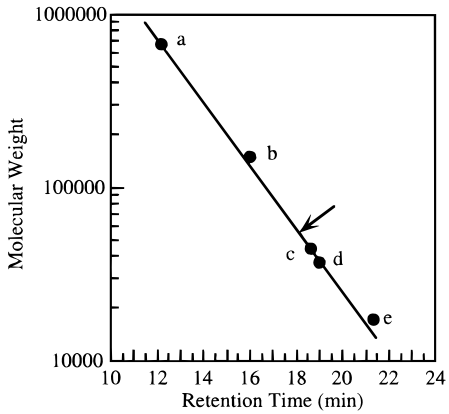

FIGURE 5: Apparent molecular weight of inhibitor-3 determined by gel permeation chromatography. Purified recombinant inhibitor-3 was chromatographed on a SEC-250 column on a Biologics HPLC system (BioRad). The protein standards used were: a, thyroglobulin (670 000); b, alcohol dehydrogenate (158 000); c, chicken ovalbumin (44 000); d, PP1 (37 000); and e, horse myoglobin (17 000). The retention times of the standards were determined by the absorbance at 280 nm. The elution position of inhibitor-3 was analyzed by SDS−PAGE. The estimated relative molecular weight of inhibitor-3 was 55 000.

Our studies now reveal that the protein encoded by the HCG V gene is a novel inhibitor of PP1, for which we propose the name inhibitor-3. Inhibitor-3 has some general similarities to the other two well-characterized heat-stable inhibitors of PP1, inhibitor-1 and inhibitor-2. All three are highly hydrophilic proteins that behave anomalously on SDS−PAGE, and are specific for the inhibition of PP1 (*1*−*4*). The percentage compositions of carboxylic amino acids (Asp + Glu) in inhibitor-3, inhibitor-2, and inhibitor-1 are 16, 22, and 14%, respectively, those of the hydroxyamino acids (Ser + Thr) are 20, 29, and 17%, respectively, and those of the basic amino acids (Lys + Arg) are 21, 23, and 16%, respectively. All three inhibitors also have high proline contents (11, 7.4, and 12.7% for inhibitor-3, inhibitor-2, and inhibitor-1, respectively). Our studies of inhibitor-3 suggest that it is extremely sensitive to proteolysis, as expression of

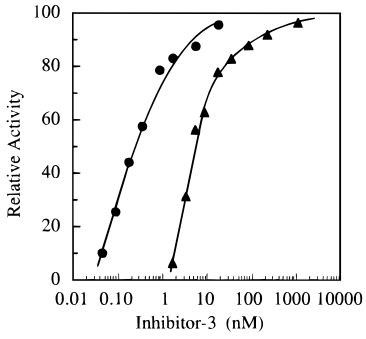

FIGURE 6: Inhibition of recombinant PP1 by inhibitor-3. PP1 activity was assayed by the dephosphorylation of ^{32}P-labeled phosphorylase *a*. Inhibitor-3 was purified to near-homogeneity and tested for inhibition of PP1 activity at the concentrations indicated. For these experiments, recombinant PP1 expressed in the presence of $MnCl_2$ (triangles) and purified as previously described (*18*) and recombinant PP1 expressed in the presence of $CoCl_2$ (circles), were both tested.

the partial cDNA as a GST-fusion protein resulted in the recovery of a mixture of partial degradation products.

Another heat-stable inhibitor of PP1 has been isolated from calf thymus, this being the nuclear inhibitor of PP1 (NIPP) as a low molecular weight heat-stable polypeptide of ca. 16−18 kDa (*14*). This was later found to be a fragment of a larger polypeptide by cloning of the cDNA (*15*). The inhibitory properties of NIPP, like inhibitor-2 and inhibitor-1, are modulated by protein phosphorylation by cAMP-dependent protein kinase and casein kinase 2 (*15, 32*). Examination of the sequence of inhibitor-3 reveals a number of potential phosphorylation sites: two for PKA at S58 and T102; two for protein kinase C at T29 and S57; five for casein kinase 2 at T11, T21, S73, T75, and S77. However, further investigation will be required to determine if phosphorylation of inhibitor-3 can modulate its inhibitory functions. A basic ribosomal protein (RIPP) that inhibits PP1 has been isolated (*33*), and the human homologue of the yeast sds22 has been shown to an inhibitory subunit of PP1 (*34*). Two other PP1-binding proteins that exhibit inhibitory activity toward PP1, p53BP2 (*10*) and the splicing factor PSF (*12*), have been identified by the use of the two-hybrid system.

A BLAST search of the Genbank data against the inhibitor-3 sequence shows that there are related genes in *S. cerevisiae*, in *S. pombe*, and in *C. elegans*. The latter organism possesses two genes (Genbank Z83236, Z48544) encoding the hypothetical proteins K10H10.c (15 kDa) and ZK945.8 (12.3 kDa). K10H10.c and ZK945.8 exhibit 55% and 38% identity, respectively, with human inhibitor-3. The hypothetical *S. cerevisiae* protein YFR003c (18.2 kDa; Genbank D50617, D44605) shares a 21% identity with inhibitor-3, and has been shown to interact with yeast PP1 by the two-hybrid method (*6*). A hypothetical protein (SPAC6B12.13, Genbank Z98531) with a 27% identity to inhibitor-3 is also present in *S. pombe*. Inhibitor-3 is thus well conserved in evolution. The cellular functions of these genes are unknown, but it is reasonable to propose that these may all turn out to be inhibitory to PP1, and that their cellular functions are related to the targeting and/or regulation of PP1 activity. Further investigations are required to establish the potential cellular functions and the subcellular distribution of inhibitor-3. The identification of cognate genes in *C. elegans* and in yeast should also facilitate genetic studies of their roles in cellular function.

Inhibitor-3	33	RKRKPEKKVEWTSDTVDNEHMGRRSSKCCCIY	64
Ce K10H10.c	30	RAPPSPPHVTWAEGVVDNEHMGRLKSNCCCIY	61
Ce ZK945.8	24	RAPVERPRVTWGAGVIDNEHMGRLKSNCCCIY	55
Sc YFR003c	43	EAMPTRHNVRWEENVIDNENMNKKKTKICCIF	74
SPAC6B12.13	38	LQPEPVRRVRWTVSTVDNENMNKKKSKVCCIF	69

FIGURE 7: Sequence alignment of a conserved region in inhibitor-3. The diagram shows the alignment of residues 33−64 of human inhibitor-3 with those of the yeast and *C. elegans* genes. The alignment includes the *C. elegans* hypothetical proteins K10H10.c (Genbank Z83236) and ZK985.8 (Genbank Z48544), the hypothetical *S. cerevisiae* protein YFR003c (Genbank D50617, D44605), and the hypothetical *S. pombe* protein SPAC6B12.13 (Genbank Z98531). The boxed area shows a highly conserved region which starts with the VxW motif. Basic residues N-terminal to the VxW motif, and the acidic residues following, are underlined.

Inhibitor-3 joins a growing list of PP1-binding proteins that now number in the dozens. In the yeast system, a two-hybrid screen led to the identification of over a dozen PP1-binding proteins (*10*). As many as 40 polypeptides from muscle that bind to PP1 have been detected by the use of microcystin−Sepharose to which PP1 is bound (*35*). The interactions between some of these proteins and PP1 can now be rationalized by the structural identification of a hydrophobic binding site on PP1 for the binding of a motif that is present in a number of PP1-binding proteins (*36*). We have obtained independent evidence for a PP1-binding motif through the screening of a random peptide display library (*37*); this analysis resulted in the identification of the motif VxF/VxW, generally preceded by several basic residues and followed by an acidic residue. This motif is also present in inhibitor-3 (Figure 7). In the human, *C. elegans*, and yeast I-3 sequences, the PP1-binding motif is adjacent to a highly conserved region of sequence (Figure 7). While the peptide display analysis revealed that VHW was the most commonly observed sequence, inhibitor-3 is unusual in that x is an acidic residue whereas in the PP1-binding peptides the most common amino acid is a basic residue (*37*). Also, inhibitor-3 and a recently described nuclear protein, p99 (*38*), are the first two PP1-binding proteins found to exhibit the VxW motif.

It is likely that the PP1-binding ability of inhibitor-3 is due at least in part to the possession of the VxW motif. There is evidence that interaction at an additional site besides the one involving this motif is required for PP1 binding in the case of the glycogen-binding subunit (*39*, *40*), inhibitor-1 (*41*), and DARPP-32 (*42*). In addition, recent studies of inhibitor-2 and NIPP suggest that while these proteins are potent inhibitors of PP1, this does not exclude them from functioning as important targeting proteins. NIPP-1 has been shown to possess a C-terminal sequence that is almost identical to a human cDNA known as ARD-1 (*15*), and is able to bind to RNA, leading to the proposal that NIPP-1 may target PP1 to RNA where it may have a role in control of RNA processing (*43*). ARD-1 has been shown to possess endoribonuclease activity and is a human analogue of *E. coli* ribonuclease E (*44*). Inhibitor-2, which has previously been regarded as a regulator of cytosolic PP1, has been shown to be translocated to the nucleus during the S-phase in a phosphorylation-dependent manner, consistent with a role in the regulation of the cell cycle (*45*).

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