Identification and Characterization of a Novel Protein Inhibitor of Type 1 Protein Phosphatase[†,‡]

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ABSTRACT: We have isolated human cDNA for a novel type 1 protein phosphatase (PP1) inhibitory protein, named inhibitor-4 (I-4), from a cDNA library of germ cell tumors. I-4, composed of 202 amino acids, is 44% identical to a PP1 inhibitor, inhibitor-2 (I-2). I-4 conserves functionally important structure of I-2 and exhibited similar biochemical properties. I-4 inhibited activity of the catalytic subunit of PP1 (PP1C), specifically with an IC_{50} of 0.2 nM, more potently than I-2 with an IC_{50} of 2 nM. I-4 weakly inhibited the activity of myosin-associated phosphates (PP1M). However, the level of inhibition of PP1M was increased during preincubation of PP1M with I-4, suggesting that the inhibition is caused by interaction of I-4 with PP1C in such a manner that it competes with the M subunit of PP1M. Gel overlay experiments showed that I-4 binds PP1C directly. Three I-4 peptides containing the N-terminal residues 1−123, 1−131, and 1−142 all showed strong binding ability to PP1C but did not show PP1 inhibitory activity, whereas an I-2 peptide (residues 1−134), lacking the corresponding C-terminal residues, potently inhibited PP1C activity as previously reported. Removal of the 18 N-terminal amino acid residues from I-4 dramatically reduced the PP1 binding activity with a correlated loss of inhibitory activity, whereas removal of the 10 N-terminal residues had only a little effect. The two peptides GST−I-4(19−131) and GST−I-4(132−202) showed ability to bind to PP1C, albeit very weakly. These results strongly suggest a multiple-point interaction between I-4 and PP1C, which is thought to cause the inhibition of I-4 which is stronger than the inhibition of I-2.

The reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a major mechanism for regulation of almost all cellular functions (*1*, *2*). Type 1 protein phosphatase (PP1)[1] is a major eukaryotic protein serine/threonine phosphatase that regulates diverse cellular processes such as cell cycle progression, protein synthesis, muscle contraction, glycogen synthesis, transcription, and neuronal signaling (*3*−*5*). PP1 exists in vivo as holoenzymes composed of the catalytic subunit (PP1C) and a wide variety of targeting and/or regulatory subunits (*4*, *5*). So far, four PP1C isoforms (α, γ1, γ2, and δ) were identified and found to be widely expressed in mammalian tissues (*6*−*8*). Biochemical analysis using bacterially expressed PP1C isoforms showed that the four isoforms have similar properties (*9*). PP1C is regulated by its interaction with a variety of subunits which appear to target PP1C to specific subcellular locations and define substrate specificity. PP1C is also regulated by various protein inhibitors, including inhibitor-1 (I-1), its homologue dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32), nuclear inhibitor of protein phosphatase-1 (NIPP-1), and inhibitor-2 (I-2). As a mechanism by which PP1C recognizes its regulatory subunits, it was reported that several PP1 regulatory subunits bind PP1C through the (R/K)(V/I)XF motif, although it is still possible that other motifs are also involved in an interaction between PP1C and regulatory subunits.

I-2 was isolated, together with I-1, as a heat-stable protein from skeletal muscle extracts, which specifically inhibit PP1 activity with a K_i of 3.1 nM. I-2 binds to PP1C to make an inactive PP1 complex, PP1I (*10*). PP1I can be activated by phosphorylation of I-2 at Thr73 by glycogen synthase kinase-3 (GSK-3) (*11*−*13*). I-2 is also phosphorylated at

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[‡] The inhibitor-4 sequence reported in this paper has been deposited at the DNA Data Bank of Japan under accession number AB044137.

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[1] Abbreviations: PP1, type 1 Ser/Thr protein phosphatase; PP1C, catalytic subunit of PP1; PP1M, myosin-associated phosphatase; PP2A, type 2A Ser/Thr protein phosphatase; PP5, Ser/Thr protein phosphatase-5; I-2, inhibitor-2; I-4, inhibitor-4; DARPP-32, dopamine- and cAMP-regulated phosphoprotein-32; NIPP-1, nuclear inhibitor of protein phosphatase-1; GSK-3, glycogen synthase kinase-3; CKII, casein kinase II; GST, glutathione *S*-transferase; NLS, nuclear localization signal; MLC, myosin light chain; MLCK, MLC kinase.

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Ser87, Ser121, and Ser122 by casein kinase II (CKII) (*14*, *15*). The phosphorylation by CKII does not alter the inhibitory activity of I-2 but greatly accelerates the subsequent phosphorylation at Thr73 by GSK-3 (*16*, *17*).

Recent in vitro studies suggest that I-2 interacts with misfolded PP1 to promote a rapid and effective refolding of this protein and yield an active enzyme. In support of this, recombinant PP1, which has characteristics different from those of native PP1, behaves much more like the native enzyme after incubation with I-2. This gave us a hint that I-2 is a chaperone for PP1. This hypothesis may also be consistent with previous reports that cotransfection of I-2 in mammalian cells enables the overexpression of PP1C protein (*18*−*20*). Studies with Glc8, the yeast homologue of I-2, came to a more complex conclusion that under different circumstances, Glc8 can work either as an inhibitor or as an "activator" of the yeast PP1 (*21*). So, despite extensive studies, the physiological functions of I-2 remain unclear.

Of great interest is the fact that levels of protein and mRNA of I-2 fluctuate during the cell cycle, peaking twice, at S phase and mitosis (*22*). The experiments using I-2 fused to the green fluorescent protein (GFP) showed that I-2 is cytosolic during G1 phase but translocates into the nucleus in S phase (*23*). Moreover, phosphorylation at the four sites was necessary for the nuclear translocation of I-2 since mutations at any of the residues prevented the nuclear entry. These studies also identified a putative nuclear localization sequence of I-2 and showed that mutations of two Lys residues in this sequence abolished the translocation of I-2 into the nucleus. Changes in the phosphorylation of I-2 during G1 and S phases also supported a role of I-2 phosphorylation in its subcellular translocation.

We report here an identification of a novel I-2-related PP-1 inhibitor protein, named inhibitor-4 (I-4), with an inhibitory activity that is about 10 times stronger than that of I-2. The mode of action of the inhibitory activity was compared between I-4 and I-2.

MATERIALS AND METHODS

cDNA Cloning and Nucleotide Sequencing. We searched the expressed sequence tag library (EST library) of the GenBank database at the National Center for Biotechnology Information (NCBI) using human I-2 as a probe. The EST cDNA clone (IMAGE entry 1470424) was identified to encode a protein which is very homologous to human I-2. This clone was obtained from the Research Genetics Inc., and both strands of the cDNA were sequenced by the chain termination method using a dye termination cycle sequencing kit (Amersham Pharmacia Biotech).

Expression of I-2 and I-4 as GST Fusion Proteins in E. coli. The primers were designed on the basis of the human I-4 or I-2 sequences, as follows: 4F1, 5′GGGAATTCGATGTCAGCCTCCACCTCCTCG3′; 4F2, 5′GGGAATTCAATCAAGGGGATCCTGAAAAAC3′; 4F3, 5′GGGAATTCAAGCTCGTCGGGTTCCTCGGTG3′; 4F4, 5′GGGAATTCGAGAAAAATCCTCCTCCACAAA3′; 4F5, 5′GGGAATTCGGAGAAAAAGCGGCAGTTCGAA3′; 4F6, 5′GGGAATTCGCTTCACTACAACGAAGAATTG3′; 4R1, 5′GGGAATTCCTAAGGGTCGCATGACTGGGT3′; 4R2, 5′GGGAATTCCTACCTTCTTCTCATTTCGAA3′; 4R3, 5′GGGAATTCCTACTGTTTGTGGAGGAGGAT3′; 4R4, 5′GGGAATTCCTACATGTAGGCCTCACTGCT3′; 4R5, 5′TTGAATTCCTATTTGTTTTTCAGGATCCC3′; 2F, 5′GTG**GGATCC**CCATGGCGGCCTCGACGGCCTCG3′; 2R1, 5′GTG**GGATCC**CTATGAACTTCGTAATTTGTT3′; and 2R2, 5′GGGAATTCCTATCGTTCTTCAGGTGAGAG3′. *Eco*RI sites are underlined. *Bam*HI sites are boldface. PCR was carried out using 4F1/4R1, 4F1/4R5, 4F1/4R4, 4F1/4R3, 4F1/4R2, 4F2/4R1, 4F3/4R1, 4F3/4R3, 4F3/4R2, 4F4/4R1, 4F5/4R1, 4F6/4R1, 2F/2R1, and 2F/2R2 primer pairs for GST−I-4, GST−I-4(1−18), GST−I-4(1−123), GST−I-4(1−131), GST−I-4(1−142), GST−I-4(11−202), GST−I-4(19−202), GST−I-4(19−131), GST−I-4(19−142), GST−I-4(124−202), GST−I-4(132−202), GST−I-4(143−202), GST−I-2, and GST−I-2(1−134), respectively. The PCR products were digested with *Bam*HI for I-2 or *Eco*RI for I-4 and ligated into the respective enzyme sites of the pGEX-3X plasmid vector (Amersham Pharmacia Biotech). No misincorporation during the PCR was verified by nucleotide sequencing. The induction and purification of the fusion protein were performed according to the manufacturer's protocol (Amersham Pharmacia Biotech). The size and purity of the recombinant protein were determined by 12% SDS−PAGE, and the amount of protein was determined with the Bradford protein assay kit (Bio-Rad) using BSA as the standard protein.

Cleavage of Fusion Protein and Heat Treatment. The purified GST−I-4 fusion protein was incubated with factor Xa (Roche) as described in ref *24* to cleave the N-terminal GST protein from GST−I-4. To separate the cleaved I-4 protein from the uncleaved GST−I-4 bound to glutathione-Sepharose 4B (Amersham Pharmacia Biotech), the incubation mixture was boiled for 15 min, cooled on ice, and then centrifuged at 15 000 rpm for 20 min. The supernatant was used as the heat-treated I-4 fraction.

Assay of Inhibitory Activity against Protein Phosphatases. The inhibitory activities of I-4 protein were examined against PP1, PP2A, or PP5 using phosphorylase *a* as a substrate by the method of MacKintosh (*25*). Assay mixtures (final volume of 30 μL) contained 50 mM Tris-HCl, 0.15 mM EDTA, 15 mM 2-mercaptoethanol, 0.01% (w/w) Brij 35, 0.3 mg/mL bovine serum albumin, 5 mM caffeine, 10 μM [^{32}P]phosphorylase *a*, and 100 ng/mL protein phosphatases with various concentrations of inhibitors. For enzyme assays, protein phosphatases and inhibitors were preincubated at 30 °C for 10 min, and then the dephosphorylation reaction was initiated by the addition of substrate and carried out for 10 min at 30 °C. The PP1 catalytic subunit was purified from rabbit skeletal muscle as described by Cohen et al. (*26*). The PP2A holoenzyme composed of catalytic subunit C and regulatory subunits A and B was obtained from Calbiochem. GST−PP5 was expressed and purified as previously described (*27*). Four recombinant PP1C isoforms (α, γ1, γ2, and δ) were prepared as previously described (*28*). Smooth muscle myosin-associated phosphatase (PP1M) was purified from chicken gizzard by the method of Okubo et al. (*29*). Smooth muscle myosin and myosin light chain kinase (MLCK) were purified as described previously (*30*). PP1M activity was measured using 1 μM [^{32}P]myosin light chain (MLC) as a substrate at 30 °C with various concentrations of inhibitors as described previously (*31*).

Assay of Binding of PP1C. Far-Western analysis was performed by the method of Campos with minor modifica-

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    cgtcatacgcagcgccctttgtgacaccagggccctggtgctttaactagggcgttggga   60
    cctgttgcccacacagaccgccctgcagtttcagactggagggcggtggacggctactca  120
    gcggcccaactctctcgcagcccttctctccgcaaaatgtcagcctccacctcctcgcac  180
  1                                     M  S  A  S  T  S  S  H
    cggcccatcaaggggatcctgaaaaacaaaagctcgtcgggttcctcggtggcgacttcc  240
  9 R  P  I  K  G  I  L  K  N  K  S  S  S  G  S  S  V  A  T  S
    ggtcagcagtctggagggactattcaagatgtgaagagaaagaaatcccaaaagtgggac  300
 29 G  Q  Q  S  G  G  T  I  Q  D  V  K  R  K  K  S  Q  K  W  D
    gaatcaagcatccttgcggcacaccgcgcaacgtacagagattacgatttaatgaaggca  360
 49 E  S  S  I  L  A  A  H  R  A  T  Y  R  D  Y  D  L  M  K  A
    aatgagcccggcacttcctacatgagtgtgcaagataatggggaagattcagtgcgcgat  420
 69 N  E  P  G  T  S  Y  M  S  V  Q  D  N  G  E  D  S  V  R  D
    gtcgaaggagaagattcagtgcgtggtgtcgaaggaaaggaagccaccgatgcttccgac  480
 89 V  E  G  E  D  S  V  R  G  V  E  G  K  E  A  T  D  A  S  D
    cacagctgtgaggtggacgagcaagagagcagtgaggcctacatgagaaaaatcctcctc  540
109 H  S  C  E  V  D  E  Q  E  S  S  E  A  Y  M  R  K  I  L  L
    cacaaacaggagaaaaagcggcagttcgaaatgagaagaaggcttcactacaacgaagaa  600
129 H  K  Q  E  K  K  R  Q  F  E  M  R  R  R  L  H  Y  N  E  E
    ttgaacatcaaattagctagacaattaatgtggaaagagctacaaagtgaagataatgaa  660
149 L  N  I  K  L  A  R  Q  L  M  W  K  E  L  Q  S  E  D  N  E
    aacgaagaaacgccacaaggcacgaacgaagagaagactgctgcggaagaatcagaggaa  720
169 N  E  E  T  P  Q  G  T  N  E  E  K  T  A  A  E  E  S  E  E
    gctcctctgaccggtggactgcaaacccagtcatgcgaccctagaagatgcctgcttca  780
189 A  P  L  T  G  G  L  Q  T  Q  S  C  D  P  *
    cccttgcaattgtttgtgaatatgtgacgcttagaagatatctgcttcacccttgcaatt  840
    gtttgtgaaataca aaccttgttactgtaaaaaaaaaaaaaaa                  894
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FIGURE 1: Nucleotide and predicted amino acid sequences of human I-4. The predicted amino acid sequence is shown in one-letter code below the nucleotide sequence. The initiation codon and termination codon are boxed, and the in-frame termination codon upstream to the initiation codon is underlined. A putative poly(A) addition signal is double-underlined.

tions (*32*). Several GST fusion proteins were separated by 12% SDS−PAGE and then transferred to nitrocellulose membrane. The blot was incubated in TBST [10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween (pH 7.4)] for 1 h at room temperature, which was then followed by incubation for 4 h at 4 °C with 0.5 μg/mL purified recombinant PP1Cγ1 in TBST containing 0.5 mM $MnCl_2$. After the membrane had been washed three times with TBST, it was incubated for 2 h with either antibody against PP1Cγ1 (*33*) or antibody against GST which was raised by injecting bacterically expressed GST into rabbit, and then incubated with anti-rabbit antibody coupled to horseradish peroxidase. After the mixture had been washed three times with TBST, either PP1C binding proteins or GST fusion proteins were detected by ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

Nucleotide and Predicted Amino Acid Sequences of I-4. To search for a novel PP1 inhibitor, we screened a dbEST library with the nucleotide sequence of human I-2 as a probe. A human clone (IMAGE clone entry 1470424), obtained from the cDNA library synthesized from three pooled human germ cell tumors, was identified to encode a novel protein related to I-2. We named this protein as inhibitor-4 (I-4).

The entire 606-nucleotide open reading frame encoded 202 amino acid residues with a predicted molecular mass of 22.6 kDa (Figure 1). Comparison of the sequence of human I-4 with human I-2 revealed that they are 61.5% identical at the nucleotide level and 43.7% identical at the amino acid level (*34*). The human I-4 has a much higher degree of sequence similarity at the amino acid level with human I-2 than with human I-3 (22.9%) (*35*), *Drosophila* I-2Dm (24.8%) (*19*), or *Drosophila* I-t (22.0%) (*36*) (Figure 2). In the rat, it is known that there are three I-2 isotypes (I-2α1, I-2α2, and I-2β), the former two of which are derived from alternative splicing from a single gene and the latter of which is a very weak inhibitor and encoded by another gene (*37*). The alignment shows that human I-4 has a much higher degree of sequence similarity with I-2α1, a I-2 counterpart, among the three rat I-2 isotypes (Figure 2). The amino acid sequence of I-4 showed some characteristic feature in comparison with human I-2. Human I-4 conserves the Thr73 residue, whose phosphorylation is critical for relief of PP1 from the inhibition by I-2; however, I-4 lacks Pro74 which is essential for the phosphorylation of I-2 at Thr73 by the proline-directed protein kinases, such as MAPK or cdc2. The Ser87 residue of I-2, whose phosphorylation by CKII accelerates the phosphorylation of Thr73 by GSK-3, is conserved at Ser85 of I-4. The other CKII sites at Ser121 and Ser122 of I-2 are conserved in I-4 as well. The cluster of basic amino acids, which was shown to work as a nuclear localization signal (NLS) in I-2, is conserved at amino acid residues 133−142 of I-4.

Effects of I-4 on Various Phosphatases. I-4 cDNA was expressed as a GST fusion protein and purified to a single band in SDS−PAGE (Figure 3A). The purified human GST−I-4 protein was examined for its ability to inhibit PP1, PP2A, and PP5 using phosphorylase *a* as a substrate (Figure 3B, panels a−c). PP1C purified from skeletal muscle, the PP2A holoenzyme composed of a catalytic subunit with A and B regulatory subunits, and GST−PP5 were used as PP1,

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human I-4 MSASTSSHRP IKGILKNKSS SGSSVATSGQ QSGGTIQDVK RKKSQKWDES 50
human I-2 MAASTASHRP IKGILKNKTS TTSSMVASAE QPRGNVDEEL SKKSQKWDEM 50
rat I-2α1 MAASTASHRP IKGILKNKTS TTSSVVASAE QPRRTVEEEL SKKSQKWDEM 50
rat I-2α2 MAASTASHRP IKGILKNKTS TTSSVVASAE QPRRTVEEEL SKKSQKWDEM 50
rat I-2β  MAS------- ----L----- ----AVASIE HPFGTFEEEL GKKSQKWDEM 30
dro.I-2Dm MQNNPSPQLP CKGILK---- --------TS RSFDKSGASF -RKSAKFDEL 37
                                  *              ▼
human I-4 SILAAHRATY RDYDLMKANE PGTSYMSVQD NGEDSVRDVE GEDSV-RGVE 99
human I-2 NILATYHPAD KDYGLMKIDE PSTPYHSMMG DDEDACSDTE ATEAMAPDIL 100
rat I-2α1 NILATYHPAD KDYGLMKIDE PDTPYHNMIG DDEDVCSDSE GNEVMTPEIL 100
rat I-2α2 NILATYHPAD KDYGLMKIDE PDTPYHNMIG DDEDVCSDSE GNEVMTPEIL 100
rat I-2β  NILETYHPAN KDYGLMKKSK PNTLYRNTVA DYDSDNSDSE VNEIMNPDFL 80
dro.I-2Dm NVMQTFHPAD KDYGHMKIDE PKTPYNYTEG FDEN--RDEL DTELL-VEKL 84
                                  ▼▼
human I-4 GK--EATDAS DHSCEVDEQE SSEAYMRKIL LHKQEKKRQF EMRRRLHYNE 147
human I-2 ARKLAAAEGL EPKYRIQEQE SSGEEDSDLS PEEREKKRQF EMKRKLHYNE 150
rat I-2α1 AKKLAAAEGS EPKFRTREQE SSGEEDNDLS PEEREKKRQF EMKRKLHYNE 150
rat I-2α2 AKKLAAAEGS EPKFRTREQE SSGEEDNDLS PEEREKKRQF EMKRKLHYNE 150
rat I-2β  TKKLAVDEAS EPTNVILEQE SSGEKDDE-- --ELEKKQQF DMKRKLHYNE 126
dro.I-2Dm -R--IAANTQ PSTESIEDDG SSGDDQP-LS EEERQRREF ERRRKAHYRE 130

human I-4 ELNIKLARQL MWKELQSEDN EN-------- ---------- --EETPQGTN 177
human I-2 GLNIKLARQL ISKDLHDDDE DE-------- ---------- --EMLETADG 180
rat I-2α1 GLNIKLARQL ISKDLHDDDE DE-------- ---------- --EMSETADA 180
rat I-2α2 GLNIKLARQL ISKDLHDDDE DE-------- ---------- --EMSETADA 180
rat I-2β  ---------- ---------- ---------- ---------- ---------- 126
dro.I-2Dm FEAVKLARKL IQEEDDDDDD EDKGADSRPS GSSQGASSSG RFASSSTKRS 180

human I-4 EEKTAAEESE EAPLTGGLQT QSCDP   202
human I-2 ESMNTEESNQ GSTPSDQQQN KLRSS   205
rat I-2α1 DSMNIEESNQ GSTAGDHLQH KSQSS   205
rat I-2α2 DSMNIEESNQ G--------- --QSS   194
rat I-2β  ---------- ---------- -----   126
dro.I-2Dm SSQADSTTSP STSAGQNMDL EPSNN   205
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FIGURE 2: Alignment of amino acid sequences of human I-4 and the related proteins. Alignment of amino acid sequences of human I-4 and human I-2 (*33*), rat I-2α1, I-2α2, and I-2*β* (*35*), and *Drosophila melanogaster* I-2Dm (*19*) is shown. Numbers indicate the amino acid position from the first methionine. Identical amino acid residues are shaded. A gap indicated by a dash was introduced to optimize the alignment. Thr73, a site of phosphorylation by GSK-3, is indicated by an asterisk. Arrowheads indicate sites of putative phosphorylation by CKII.

PP2A, and PP5, respectively. GST−I-4 inhibited PP1 activity potently, but had no effect on both activities of PP2A and PP5. The inhibitory activity of GST−I-4 toward PP1C was much stronger than that of GST−I-2, with IC_{50} values of 0.2 and 2 nM for GST−I-4 and GST−I-2, respectively (Figure 3B, panel a).

Since heat stability is a characteristic feature common for I-2 and I-1, the heat stability was examined with a bacterially expressed I-4 protein. I-4 boiled for 15 min was able to inhibit PP1C with an IC_{50} of 0.2 nM, which is equivalent to the IC_{50} of GST−I-4 without the heat treatment (lane 4 of Figure 3A and panel a of Figure 3B).

Effects of I-4 on a Holoenzyme PP1M. To analyze effects of I-4 on the PP1 holoenzyme, inhibition of PP1M by I-4 was examined. In contrast to the inhibitory effects on myosin phosphatase activity of PP1C by I-4 and I-2, where the IC_{50} values were approximately 0.2 and 1.0 nM for I-4 and I-2, respectively, either with or without the preincubation, the level of reduction of PP1M activity by I-4 was increased during preincubation of PP1M with these inhibitor proteins (Figure 4). The reduction in PP1M activity was not due to the inactivation but the inhibition, because there was no reduction in phosphatase activity of PP1M by the preincubation without the inhibitor for 12 h (data not shown). After preincubation of PP1M with I-4 or I-2 at 4 °C overnight, IC_{50} values for I-4 and I-2 were 2 and 200 nM, respectively, strongly suggesting that I-4 as well as I-2 replaces regulatory subunit M of the holoenzyme PP1M in binding of these inhibitor proteins to PP1C. The replacement was confirmed independently by pull-down analysis, since the extent of association between I-4 and PP1C was increased in the I-4/PP1M mixture by preincubation for 12 h (data not shown).

Effects of Human I-4 on Four Recombinant PP1C Isoforms. The specificity of I-4 inhibitory activity toward four PP1C isoforms was then examined using bacterially expressed PP1Cs, although the bacterially expressed ones were known to be less sensitive to I-2 than PP1C purified from tissues (*9*). As shown in Figure 5, the inhibitory activities of GST-I-4 toward four PP1C isoforms were about the same, suggesting that I-4 interacts with the region conserved among those isoforms.

Binding of GST−I-4 Deletion Mutant Proteins to PP1C. To elucidate the regions of I-4 involved in binding to or inhibition of PP1C, various GST−I-4 deletion mutants were designed as shown in Figure 6, expressed, and purified (data not shown). These GST−I-4 deletion mutant proteins were examined for their activity of binding to PP1C by far-Western blot analysis (Figure 7A). The same blot was incubated with anti-GST antibody to ascertain whether the same amounts of protein were loaded in each lane (Figure 7B). GST protein

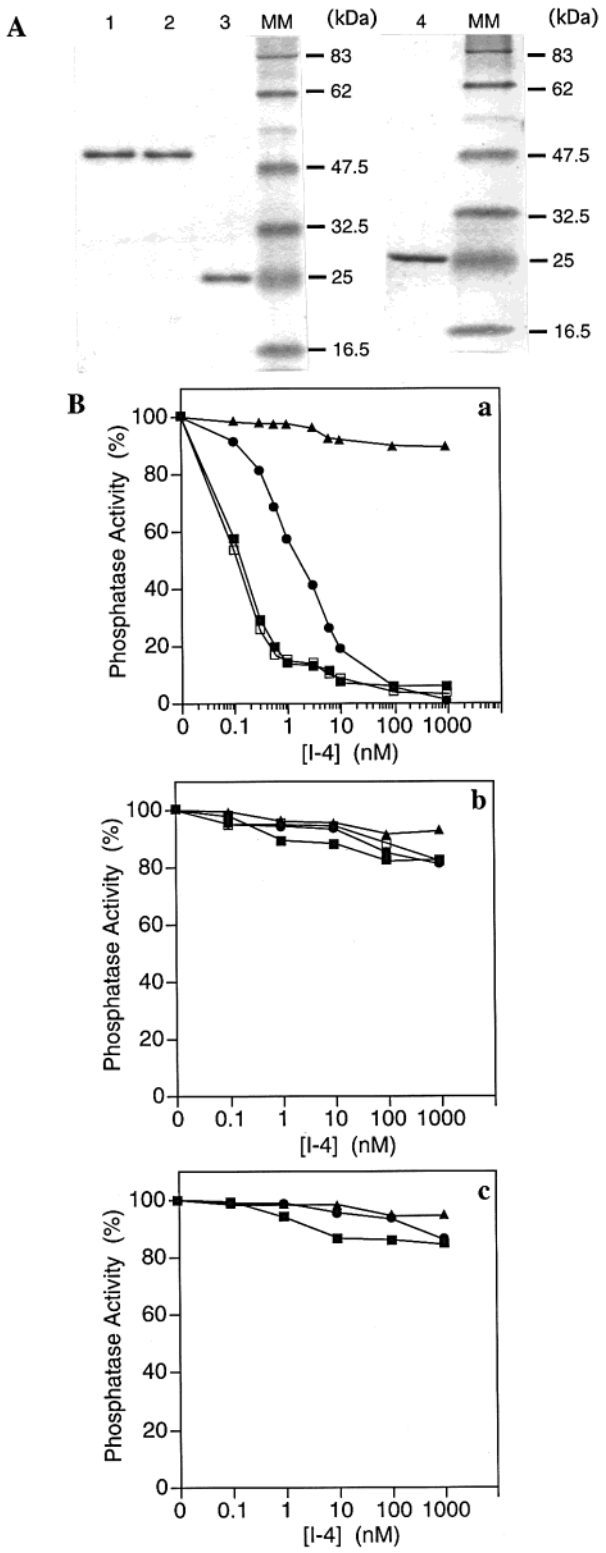

FIGURE 3: Effects of GST−I-4 on various protein phosphatases. (A) The GST fusion proteins were expressed in *E. coli* and purified on a glutathione-Sepharose column. Two micrograms of the fusion proteins was subjected to SDS−PAGE (12% acrylamide gel): lane 1, GST−I-4; lane 2, GST−I-2; lane 3, GST; lane 4, I-4; and lane MM, prestained protein marker (New England BioLabs). (B) Phosphatase activity was assayed using 10 μM [^{32}P]phosphorylase *a* as a substrate and expressed as a percentage of the activity in the absence of inhibitors. These phosphatase activities of PP1C (a), PP2A (b), and GST−PP5 (c) are plotted against various concentrations of GST−I-4 (■), heat-treated I-4 (□), GST−I-2 (●), and GST (▲). Heat treatment was carried out by boiling the I-4 protein cleaved by factor Xa for 15 min.

alone did not bind to PP1C, while GST−I-4 as well as GST−I-2 bound to PP1C, suggesting that wild-type I-4 is a PP1C-binding protein like I-2 (Figure 7A). To specify the region for PP1C binding, various deletion mutant proteins of GST−I-4 were prepared and analyzed for their binding activities. Removal of the 10 N-terminal residues did not affect its ability to bind PP1C, while removal of the 18 N-terminal residues definitely reduced the binding activity. The 18 N-terminal amino acid residues alone showed weak but significant binding to PP1C. Removal of C-terminal regions containing residues 124−202, 132−202, and 143−202 of I-4 definitely affected its binding (Figure 7A, lanes 3−5). GST−I-4(19−202) also reduced its binding activity by removal of residues 132−202 and 143−202 (Figure 7A, lanes 7−9). Under these conditions, the C-terminal GST−I-4 peptides containing residues 124−202, 132−202, and 143−202 did not exhibit the binding activity (Figure 7A, lanes 10−12). However, it should be noted that under prolonged exposure these C-terminal peptides (residues 124−202, 132−202, and 143−202) exhibited weak bands (data not shown). Furthermore, the middle portion containing residues 19−131 or 19−142 has certain binding activity that is more potent than the C-terminal region (Figure 7A, lanes 8 and 9). These results demonstrate that the 11−19 N-terminal amino acid residues of I-4 are critically important for binding of I-4 to PP1-C and also that other middle and C-terminal regions, even if weakly, interact with PP1C. The other regions except the 19 N-terminal residues might work to potentiate the binding between I-4 and PP1C.

Inhibitory Activities of GST−I-4 Deletion Mutant Proteins toward PP1C. Although removal of the 10 N-terminal amino acid residues of I-4 did not significantly affect its inhibitory activity, removal of the 18 N-terminal amino acid residues increased the IC_{50} by 200-fold (Figure 8), being well correlated to the level of reduction in the binding activity as shown in Figure 7. It has been previously reported that removal of C-terminal residues of I-2 affected its binding activity only slightly (*38*). Actually, GST−I-2(1−134) inhibits PP1C with an IC_{50} of 3 nM which is equivalent to that of GST−I-2(1−205) under our assay conditions (data not shown). In contrast to that, I-4(1−131) as well as GST−I-4(1−131) did not inhibit PP1C (unpublished data and Figure 8), which suggested that the C-terminal region of I-4 is involved in its inhibitory activity. It should be noted that the C-terminal I-4 peptides alone containing residues 124−202 or 132−202 showed very weak but significant inhibitory activity, being consistent with their weak binding ability shown in Figure 7A.

Then we performed a competition analysis against the I-4 inhibition by using the GST−I-4 deletion mutant proteins lacking the inhibitory activity (Figure 9). The GST−I-4 deletion mutant proteins containing residues 1−123 or 1−131 antagonized the inhibitory activity of I-4 with concentration dependence. The polypeptide of residues 1−131 reversed the inhibition more efficiently than that of residues 1−123. On the other hand, polypeptides of residues 1−18, 19−202, and 124−202 did not show any competitive activity. These results demonstrate that the regions of amino acid residues 1−123 or 1−131 of I-4 do not have any inhibitory activity to PP1C but possesses an ability to compete with I-4 in binding to PP1C.

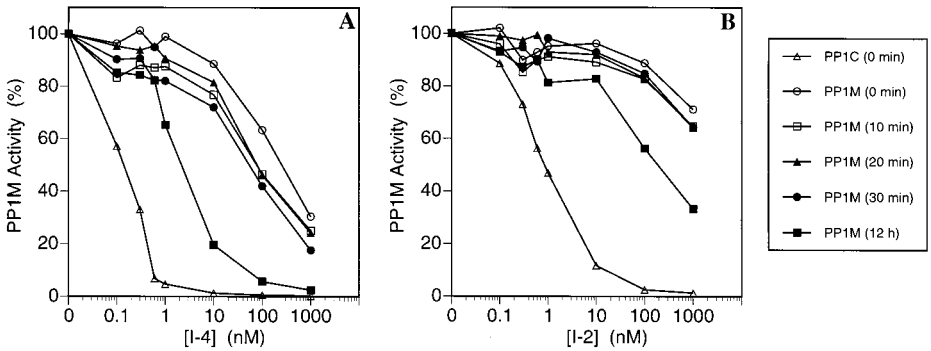

FIGURE 4: Effects of GST−I-4 on PP1M activity. Phosphatase activities of PP1M were measured using MLC as a substrate in the presence of the indicated concentrations of GST−I-4 (A) or GST−I-2 (B). Effects of GST−I-4 or GST−I-2 were measured after preincubation of PP1M at 4 °C for the indicated periods of time. Myosin phosphatase activities are shown as a percentage of the activity without the inhibitors.

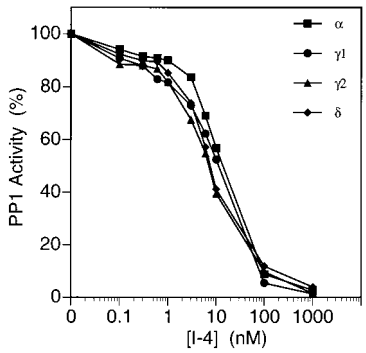

FIGURE 5: Effects of GST−I-4 on four recombinant PP1C isoforms. Phosphatase activities of four recombinant PP1C isoforms were assayed using 10 μM [^{32}P]phosphorylase *a* as a substrate. Phosphatase activity was expressed as a percentage of the activity in the absence of GST−I-4.

Then, we examined ability of the GST−I-4 deletion mutant proteins to compete with I-2 in the binding to PP1C. As shown in Figure 9, 1 μM peptide of residues 1−131 showed a release from the inhibition of PP1C activity from 90% to 20% by 0.5 nM I-4 or by 10 nM I-2, demonstrating that the affinity of amino acid residues 1−131 of I-4 for PP1C is much higher than that of the corresponding residues of I-2.

DISCUSSION

Here we identified and characterized a novel PP1 inhibitor protein, named I-4. I-4 conserves functionally important regions of I-2 and showed biochemical properties similar to those of I-2 except that the inhibition of PP1 by I-4 is much stronger than that by I-2.

The important phosphorylation sites of I-2 are almost totally conserved in I-4. However, Thr73 critical for the phosphorylation-induced release from the inhibition by I-2 is not adjoined with a Pro residue. Therefore, Thr73 in I-4 is thought to be phosphorylated by GSK-3, but not by Pro-targeted kinases such as cdc2 or MAPK. With respect to CKII sites of I-4, Ser85 fits the motif S/TXXE/D, but Ser118 and Ser119 do not. Thus, I-4 might be subject to a regulation in such a manner different from that of I-2.

The nuclear localization signal of I-2 proposed by Kakinoki et al. appears to be conserved at residues between amino acids 131 and 142 of I-4, although Lys is substituted with Arg at residues 140 and 142. Interestingly, the putative nuclear export signal is also present at amino acid residues 143−162 of I-4 (*39*). It is reported that I-2 has two high PEST score sequences (*20*, *37*). One of such sequences found between the CKII site and the NLS (residues 124−133) in I-2 is replaced with an unrelated sequence in I-4, suggesting

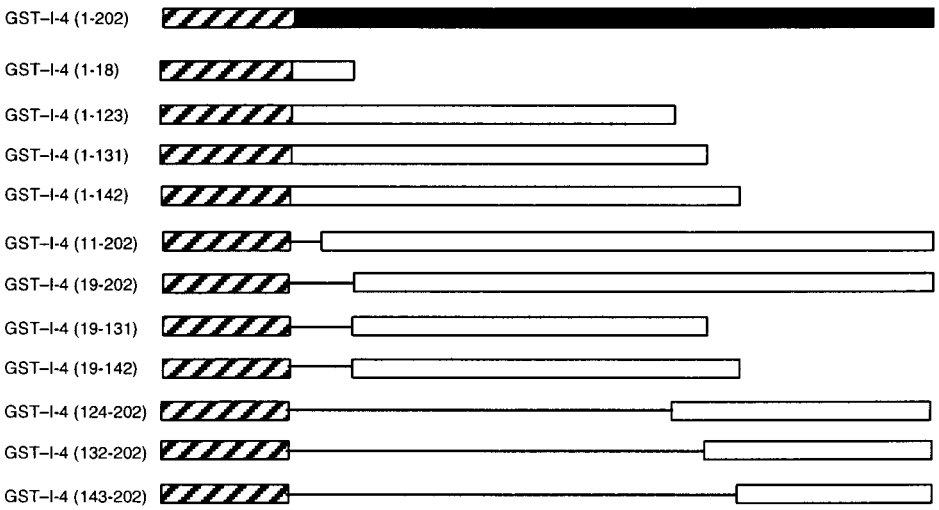

FIGURE 6: Construct of the GST−I-4 mutants and the GST−I-4 deletion mutants. The hatched boxes represent GST. Black and white boxes represent wild-type and deletion constructs of I-4, respectively.

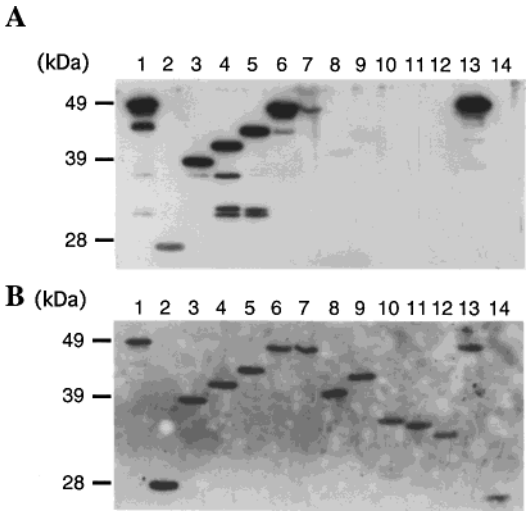

FIGURE 7: Binding of GST−I-4 and its mutant proteins to PP1C. GST−I-4 deletion mutant proteins (1 μg) were separated by SDS−PAGE (12% acrylamide gel) and transferred to nitrocellulose. Bound PP1Cγ to mutant proteins of I-4 or I-2 was detected by Western blotting using anti-PP1Cγ antibody. A: lane 1, GST−I-4(1−202); lane 2, GST−I-4(1−18); lane 3, GST−I-4(1−123); lane 4, GST−I-4(1−131); lane 5, GST−I-4(1−142); lane 6, GST−I-4(11−202); lane 7, GST−I-4(19−202); lane 8, GST−I-4(19−132); lane 9, GST−I-4(19−143); lane 10, GST−I-4(124−202); lane 11, GST−I-4(132−202); lane 12, GST−I-4(143−202); lane 13, GST−I-2(1−205); and lane 14, GST. (B) After the PP1Cγ immunocomplex had been stripped, the same filter was probed with anti-GST antibody to analyze protein amounts on the filter: lane 1, GST−I-4(1−202); lane 2, GST−I-4(1−18); lane 3, GST−I-4(1−123); lane 4, GST−I-4(1−131); lane 5, GST−I-4(1−142); lane 6, GST−I-4(11−202); lane 7, GST−I-4(19−202); lane 8, GST−I-4(19−132); lane 9, GST−I-4(19−143); lane 10, GST−I-4(124−202); lane 11, GST−I-4(132−202); lane 12, GST−I-4(143−202); lane 13, GST−I-2(1−205); and lane 14, GST.

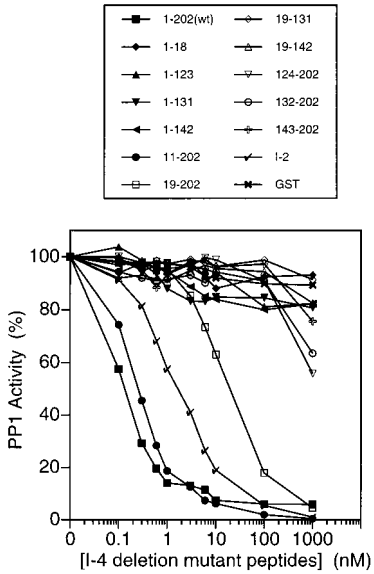

FIGURE 8: Effects of GST−I-4 and its mutant proteins on PP1C activity. Phosphatase activity was assayed using 10 μM [^{32}P]-phosphorylase *a* as a substrate and expressed as a percentage of the activity without the inhibitors.

that I-4 is more stable than I-2 in terms of PEST-mediated protein destabilization.

The present data demonstrating that I-4 binds PP1C directly and inhibits four PP1C isoforms similarly suggested that I-4 associates with PP1C via the common binding

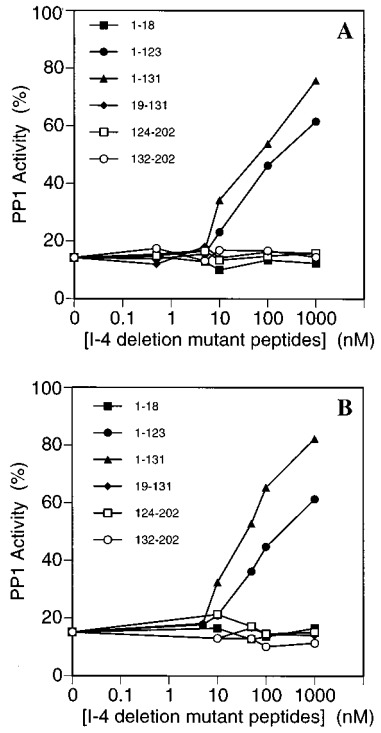

FIGURE 9: Competition of PP1 inhibition by GST−I-4 mutant proteins. Phosphatase activity was assayed using 10 μM [^{32}P]-phosphorylase *a* as a substrate and expressed as a percentage of the activity without the inhibitors. Several GST−I-4 mutant proteins were added at the indicated concentrations to the reaction mixtures with 0.5 nM I-4 (A) or 10 nM I-2 (B).

sequence. To elucidate further the interaction site on PP1C, the effects of I-4 on an activity of a PP1 holoenzyme, PP1M, were analyzed. As previously reported, the inhibition of PP1M by I-2 requires preincubation, suggesting that I-2 interacts with PP1C in such a manner to compete with the regulatory subunit M. Recently, it is reported by using several mutagenized PP1C that amino acid residues (E53, E55, D165, E166, and K167) are involved in the interaction with I-2 (*40*). Since I-4 showed much stronger binding activity to PP1C, similar experiments using I-4 instead of I-2 are required for further understanding a precise mechanism of interaction between PP1 and its regulatory proteins.

PP1 binding proteins are mostly thought to interact with PP1C via an R/KV/IXF or R/KXV/IF motif (*41*), but such motifs are not identified in I-4. Gel overlay experiments and the competition analysis against the inhibition suggested that the I-4 peptide of residues between 1 and 131 binds PP1 more potently than the corresponding residues of I-2. The region containing residues between amino acids 11 and 18 of I-4 was identified as a potent PP1 binding region, and the region containing residues 19−131 was required for potentiation of the binding activity. The binding activity of the sequence at residues 11−18 might be via the IKGI (residues 11−14) motif, which has already been identified as an important sequence for the binding of I-2 to PP1C (*38*). The I-4 peptides of residues 19−131 or 19−142 exhibited very weak binding to PP1 as shown in Figure 7. Therefore, residues 19−131 are thought to work to potentiate the binding through stabilization of formation of the PP1C−I-4 complex. Experiments using additional deletion mutants and chimeric constructs between I-4 and I-2 are required to

elucidate further a mechanism for the stronger binding of I-4. Taken together, these results strongly suggest that I-4 interacts with PP1C at multiple sites, which seems to cause the strong inhibitory activity of I-4 with respect to PP1C.

Furthermore, since I-4 is a much more potent inhibitor than I-2, I-4 could be used as a powerful tool to investigate physiological functions of PP1 by overexpressing it in the cells. Also, the I-4 peptide of residues 1−131, which is thought to work as a new dominant negative protein toward I-2, could be a useful tool for further understanding the precise function of I-2 in vivo.

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