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Conversion of Protein Phosphatase 1 Catalytic Subunit to a Mn^{2+}-Dependent Enzyme Impairs Its Regulation by Inhibitor 1†

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ABSTRACT: The phosphorylase phosphatase activity of protein phosphatase 1 (PP1) catalytic subunit from freshly purified rabbit skeletal muscle was inhibited by $MnCl_2$. Prolonged storage or inhibition by nonspecific phosphatase inhibitors ATP, sodium pyrophosphate, and NaF converted the muscle PP1 to a form that required Mn^{2+} for enzyme activity. Recombinant PP1 catalytic subunit expressed in *Escherichia coli* was also a Mn^{2+}-dependent enzyme. While native PP1 was inhibited by the phosphoprotein inhibitor 1 (I-1), with an IC_{50} of 1 nM, 40−50-fold higher concentrations of I-1 were required to inhibit the Mn^{2+}-dependent PP1 enzymes. Conversion to the Mn^{2+}-dependent state was accompanied by a 20-fold increase in PP1's ability to dephosphorylate and inactivate I-1. Inhibition by thiophosphorylated I-1 established that dephosphorylation does not play a significant role in I-1's reduced potency as an inhibitor of Mn^{2+}-dependent PP1. The Mn^{2+}-dependent PP1 enzymes were poorly inhibited by N-terminal phosphopeptides of I-1, indicating their impaired interaction with the I-1 functional domain. Mutation of a residue conserved in I-1 and DARPP-32, a structurally related PP1 inhibitor, preferentially attenuated I-1's activity as an inhibitor of Mn^{2+}-dependent PP1. These data showed that, in addition to changes in its catalytic properties, Mn^{2+}-dependent PP1 was modified in its interaction with I-1 at a site that was distinct from its catalytic domain. Our studies suggest that conversion to a Mn^{2+}-dependent state alters multiple structural elements in PP1 catalytic subunit that together define its regulation by I-1.

Protein phosphatase 1 (PP1) is a major eukaryotic protein serine/threonine phosphatase (Cohen, 1989; Shenolikar & Nairn, 1991: Bollen & Stalmans, 1992; Shenolikar, 1994) that is characterized by preferential dephosphorylation of the β-subunit of phosphorylase kinase and inhibition by two endogenous proteins, inhibitor 1 (I-1) and inhibitor 2 (I-2). Molecular cloning has identified four distinct PP1 cDNAs (Sasaki et al., 1990). The recombinant PP1 isoenzymes share similar substrate specificity toward several phosphoprotein substrates, association with regulatory subunits, and inhibition by toxins (Zhang et al., 1992, 1993a,b, 1994a,b; Alessi et al., 1993; Zhao et al., 1994). The three-dimensional structures of two recombinant PP1 isoenzymes have been solved (Goldberg et al., 1995; Egloff et al., 1995), opening the way for a better understanding of the structure, function, and regulation of PP1.

Unlike PP1 purified from mammalian tissues, recombinant PP1 catalytic subunits are inactive in the absence of divalent cations. Following reactivation by Mn^{2+}, the recombinant PP1 enzymes show higher activity toward some substrates than native PP1. These substrates include *p*-nitrophenyl phosphate and histone H1 phosphorylated by a growth-associated protein kinase (Alessi et al., 1993) or PKA (Zhao et al., 1994). Interestingly, the native PP1 also requires Mn^{2+} for efficient dephosphorylation of these substrates (Silberman et al., 1984). Treatments that convert native PP1 into a Mn^{2+}-dependent enzymes elevate its activity toward histone H1 and PNPP (Cohen, 1989; Shenolikar & Nairn, 1991; Bollen & Stalmans, 1992). It has been postulated that loss of metals from the substrate-binding site converts PP1 into a Mn^{2+}-dependent enzyme and alters the recognition of some substrates (Egloff et al., 1995).

Native and recombinant PP1 show similar sensitivity to many toxins and I-2, which inhibit their activity. In contrast, the recombinant PP1 is 100−600-fold less sensitive to inhibition by I-1. Conversion of native PP1 to a Mn^{2+}-dependent enzyme is also accompanied by a 500-fold reduction in its sensitivity to I-1 (Alessi et al., 1993). The molecular basis for this defect in regulation by I-1 remains unknown. Our recent studies suggest that multiple regions of I-1 interact with the PP1 catalytic subunit to mediate enzyme inhibition (Endo et al., 1996). Alterations in one or more of these PP1/I-1 interactions may contribute to the impaired regulation of the Mn^{2+}-dependent PP1.

PP1 purified from tissues is commonly stored in Mn^{2+}-containing buffers to stabilize its activity (Nimmo & Cohen, 1978). Mn^{2+} is also included in the growth medium to increase the expression of recombinant PP1 in *Escherichia coli* (Zhang et al., 1992; Alessi et al., 1993). These procedures may promote PP1 conversion to the Mn^{2+}-dependent enzyme which is compromised in its regulation by I-1. To determine the mechanism(s) underlying this

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defect in regulation, we analyzed Mn^{2+}-dependent and -independent forms of rabbit skeletal muscle PP1 catalytic subunits and recombinant PP1α for inhibition by I-1 proteins and peptides under well-defined conditions. Mn^{2+}-dependent PP1 enzymes were uniformly less sensitive than native PP1 to I-1 proteins and peptides. Moreover, the Mn^{2+}-dependent PP1 was also more effective in dephosphorylating and inactivating I-1 than native PP1. Using thiophosphorylated I-1, we established that the decreased sensitivity of the Mn^{2+}-dependent PP1 did not result from extensive dephosphorylation of I-1. Mutation of human I-1 that uniquely impaired its ability to inhibit the Mn^{2+}-dependent PP1 provided further evidence for its altered structure. Our studies suggest that changes in PP1 structure associated with its conversion to a Mn^{2+}-dependent state abrogate its regulation by I-1. Delineating these changes may provide new insight into the mechanism of PP1 inhibition by I-1.

MATERIALS AND METHODS

Restriction enzymes, isopropyl 1-thio-β-D-galactopyranoside (IPTG), and human thrombin were purchased from Boehringer Mannheim. Glutathione-Sepharose was purchased from Pharmacia. Phosphorylase *b* and phosphorylase kinase were obtained from Gibco-BRL. [γ-^{32}P]ATP (>4000 Ci/mmol) was purchased from ICN. The PP1 catalytic subunit (DeGuzman & Lee, 1988), I-1 (Connor et al., 1997), and the PKA catalytic subunit (Beavo et al., 1974) were purified from rabbit skeletal muscle. Recombinant PP1α was expressed in *E. coli* and purified to homogeneity as described by Zhang et al. (1992). Protein concentration was determined by the method of Bradford (1976) using BSA as standard ($E^{1\%}_{280} = 6.54$).

Site-Directed Mutagenesis of Human I-1. Site-directed mutagenesis to substitute an asparagine in place of isoleucine$_{10}$ in human I-1 was undertaken by PCR using human I-1 cDNA subcloned into pGEM-3Zf(−) as template (Promega). The forward primer, 5′-CCGAAAGAACCAGTTCACG-3′, and the SP6 primer were used in one amplification reaction, while the backward primer, 5′-CGTGAACTGGT-TCTTTCGG-3′, and the T7 primer were used in another. The amplifications were carried out for 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 35 cycles. The PCR products were purified by electrophoresis in 1% (w/v) agarose. Equimolar amounts of the two PCR products were mixed and further amplified using the T7 and SP6 primers. The product of the second PCR amplification was digested with *Nco*I and *Eco*RI and subcloned into pGEM-3Zf(−). The mutation, $I_{10}N$, was verified by double-stranded sequencing with Sequenase II (U.S. Biochemical Corp.).

Expression of Recombinant Human I-1. The hI-1 cDNA was subcloned into pGEX-2T (Pharmacia) and expressed as a fusion protein with glutathione S-transferase (GST) in *E. coli* BL21 (Novagen) as described by Endo et al. (1996).

Briefly described, the transformed bacteria were grown at 37 °C in 250 mL of Terrific Broth (Tartof & Hobbs, 1987) containing ampicillin (50 μg/mL) unit A_{600} of the culture was 0.6. The culture was cooled and incubated at 25 °C until A_{600} reached 0.8. GST-hI-1 was induced for 3 h at 25 °C by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The bacteria were sonicated in 20 mL of 50 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, and 1 mM PMSF (lysis buffer) at 4 °C and the extract was gently shaken with glutathione−Sepharose equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS) at 4 °C. The affinity matrix was washed with lysis buffer followed by TBS containing 1% Nonidet P-40. The GST-hI-1 was eluted with 50 mM Tris-HCl, pH 8.5, containing 10 mM glutathione and dialyzed against 0.5 mM Tris-HCl, pH 7.5, containing 0.005% (w/v) Brij 35. The GST-hI-1 was further purified to homogeneity by preparative SDS−9% (w/v) PAGE at 50 mA for 17 h using Prep-Cell 490 (Bio-Rad). Fractions containing GST-hI-1 were pooled and dialyzed against 0.5 mM Tris-HCl, pH 7.5, containing 0.005% Brij 35 at 4 °C.

Purified GST-hI-1 (apparent molecular mass 47 kDa) was digested with thrombin (0.5−1.0 NIH unit/mL) in 50 mM Tris-HCl, pH 8.5, containing 5 mM $CaCl_2$ at 30 °C and the digest was subjected to preparative SDS−PAGE to obtain a single 28 kDa polypeptide representing purified hI-1.

Isolation of Recombinant hI-1 Peptides. When GST-h1-1 was expressed in *E. coli* DH5α, a 37 kDa fusion protein was obtained. Thrombin cleavage of this fusion protein yielded two I-1 peptides with apparent molecular masses 12 and 14 kDa. Each peptide was purified by preparative SDS−14% (w/v) PAGE (described above) and reversed-phase HPLC on a C_{18}-μBondapak column (300 × 7.5 mm, Waters) in 0.1% trifluoroacetic acid using a gradient of 1−65% acetonitrile. The HPLC fractions were dried and redissolved in 1.0 mM Tris-HCl, pH 7.5, containing 0.005% Brij 35. N- and C-terminal sequence determination and mass spectrometry defined the two peptides as I-1(3−61) and I-1(9−61) (Endo et al., 1996).

Phosphorylation of I-1 by PKA. GST-hI-1 as well as the I-1 proteins and peptides were phosphorylated with the catalytic subunit of PKA in 50 mM Tris-HCl, pH 7.5, containing 100 μM ATP and 1 mM $MgCl_2$ at 30 °C for 1−4 h (Shenolikar & Ingebritsen, 1984). Phosphorylation was monitored using trace [γ-^{32}P]ATP in the reaction. Incorporation of ^{32}P-phosphate into I-1 was followed by SDS−PAGE/autoradiography or 15% (w/v) trichloroacetic acid precipitation. Concentration of activated I-1 was determined by incorporation of 1 mol of ^{32}P-phosphate into 1 mol of I-1.

Thiophosphorylation of rabbit skeletal muscle I-1 was undertaken essentially as described above except that 0.2 mM ATP-γ-S and 2 mM $MgCl_2$ were used in place of ATP-Mg and the reaction was incubated at 30 °C for 24 h. The incubation mixture was desalted on Sephadex G25 or through Centricon-30 to remove ATP-γ-S. A small aliquot of the incubation mixture was analyzed by SDS−PAGE for possible I-1 degradation. Another aliquot of thiophosphorylated I-1 was "back-phosphorylated" in the presence of ^{32}P-ATP as described above. A parallel reaction containing unphosphorylated I-1 was used as control. Maximal radiolabeling or back-phosphorylation of the thiophosphorylated I-1 did not exceed 5% of that incorporated into control I-1. This demonstrated that the thiophosphorylated I-1 was >95% covalently modified.

Protein Phosphatase Assay. PP1 was routinely assayed using ^{32}P-phosphorylase *a* phosphorylated *in vitro* with phosphorylase kinase (Shenolikar & Ingebritsen, 1984). PP1 was incubated with 10 μM ^{32}P-phosphorylase *a* in 50 mM Tris-HCl, pH 7.0, 1 mg/mL BSA, 1 mM EDTA, and 0.3% (v/v) 2-mercaptoethanol (total volume 60 μL) at 30 °C. The reaction was terminated after 10 min by the addition of 0.1

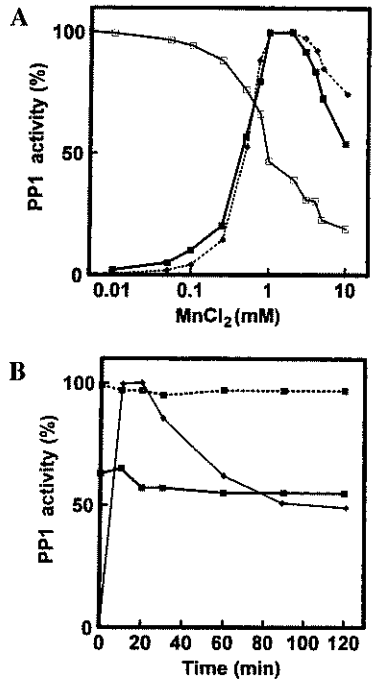

FIGURE 1: Effects of $MnCl_2$ on PP1 activity. (Panel A) Native PP1a purified from rabbit muscle (□, solid line), Mn^{2+}-dependent muscle PP1b (■, bold line) and recombinant PP1α (◆, dotted line) were preincubated in assay buffer containing varying concentrations of $MnCl_2$ for 10 min at 37 °C and assayed for phosphorylase phosphatase activity. (Panel B) Native PP1a was incubated in the presence (■, bold line) and absence (■, dotted line) of 1 mM $MnCl_2$ for increasing periods of time prior to assaying for phosphorylase phosphatase. Time course for activation of PP1b by 1 mM $MnCl_2$ is also shown (◆, solid line).

mL of 20% (w/v) trichloroacetic acid and 0.1 mL of BSA (6 mg/mL). Following centrifugation at 15000*g* for 5 min, the supernatant (200 μL) was analyzed for ^{32}P-phosphate release by liquid scintillation counting. Phosphatase assays were also undertaken using the recombinant hI-1 (9 μM) phosphorylated with PKA as substrate.

RESULTS

Effect of Mn^{2+} on Phosphorylase Phosphatase Activity of Native and Recombinant PP1 Catalytic Subunits. The PP1 catalytic subunit freshly purified from rabbit skeletal muscle, termed PP1a, showed maximal phosphorylase phosphatase activity in the absence of divalent cations. PP1a was inhibited by Mn^{2+} concentrations above 0.1 mM (Figure 1A). An approximately 50% reduction in PP1 activity was observed with 1 mM $MnCl_2$. Prolonged storage of PP1a at −20 °C or incubation with nonspecific phosphatase inhibitors ATP (1 mM), NaF (50 mM), and sodium pyrophosphate (10 mM) for 30 min at 37 °C resulted in the complete loss of phosphorylase phosphatase activity. This enzyme, termed PP1b, was not reactivated by dialysis or desalting on Sepadex G50 to remove the low molecular weight inhibitors (data not shown). Instead, incubation with $MnCl_2$ was used to restore PP1b activity. Maximal PP1b activity was observed when the enzyme was incubated with 1.0−3.0 mM $MnCl_2$ for 10 min at 37 °C (Figure 1A). $MnCl_2$ concentrations above 3 mM progressively decreased PP1b activity. The dose−response curves for reactivation by $MnCl_2$ were essentially the same whether PP1b was produced by prolonged storage or inactivation with NaF, ATP, or pyrophosphate (data not shown). As reported in earlier studies (Zhang et al., 1992, 1993a,b, 1994a,b; Alessi et al., 1993; Zhao et al., 1994), recombinant PP1α catalytic subunit expressed in *E. coli* was inactive in the absence of metal ions. The concentration curve for reactivation of recombinant PP1α by $MnCl_2$ was essentially identical to that for PP1b (Figure 1A).

The time course for the activation of PP1b (Figure 1B) or PP1α (data not shown) by 1 mM $MnCl_2$ at 4 °C was complex. $MnCl_2$ initially increased PP1b (and PP1α) activity to the level of the native PP1a assayed in the absence of divalent cations. Continued incubation with $MnCl_2$ resulted in a slow loss of PP1b activity, which stabilized after 60−90 min to a level equivalent to that of PP1a assayed in 1 mM $MnCl_2$ (Figure 1B). There was no further change in the phosphorylase phosphatase activity of PP1b and PP1α on continued incubation for 3 h. Thus, we defined the preincubation with 1 mM $MnCl_2$ at 4 °C for 60 min as the most stable conditions for studying the regulation of Mn^{2+}-dependent PP1 by I-1.

Inhibition of Mn^{2+}-Dependent and -Independent PP1 by I-1 Proteins and Peptides. PP1a from rabbit skeletal muscle was inhibited by I-1 only after it had been phosphorylated on a threonine by PKA. The IC_{50} for PP1a inhibition by skeletal muscle I-1 was approximately 1 nM, in the absence of divalent cations (Table 1). Although 1 mM $MnCl_2$ reduced PP1a activity by nearly 50%, it had little effect on the apparent IC_{50} for I-1 (Table 1 and Figure 2). However, $MnCl_2$ induced a significant change in the shape of the dose−response curve for I-1. The sigmoidal dose−response curve for I-1 seen in the absence of divalent cations was noticeably biphasic when 1 mM $MnCl_2$ was included in the assay (Figure 2). By comparison, PP1b following its activation with 1 mM $MnCl_2$ (60 min at 4 °C) was inhibited by I-1 with an IC_{50} of 46 nM (Table 1). Mn^{2+}-activated recombinant PP1α also required 40 nM phospho-I-1 for half-maximal inhibition.

Recombinant human I-1 (hI-1) was a potent PP1a inhibitor. N-Terminal fusion of hI-1 to GST, however, reduced its inhibitory potency by nearly 30-fold (Table 1). The presence of 1 mM $MnCl_2$ in these assays had little effect on the IC_{50} for GST-hI-1. Surprisingly, GST-hI-1 was only 3−4-fold less effective in inhibiting the Mn^{2+}-dependent enzymes, PP1b and PP1α. Thrombin cleavage of GST-hI-1 yielded free hI-1, increasing its efficacy as a PP1 inhibitor. Recombinant hI-1, like the rabbit muscle I-1, inhibited PP1a with an IC_{50} of 1 nM in the presence or absence of Mn^{2+}. In contrast, hI-1 inhibited Mn^{2+}-dependent PP1b with an IC_{50} of 35 nM. Thus, I-1 discriminated between the Mn^{2+}-dependent and Mn^{2+}-independent PP1 much better when it was not fused to GST.

The primary structural determinants for PP1 inhibition are localized to a N-terminal domain in hI-1 that contains the PKA phosphorylation site (Endo et al., 1996). N-Terminal peptides of hI-1, −3 to 61 and 9 to 61, were potent inhibitors of native PP1a (Table 1). By contrast, 50-fold higher concentrations of the phosphopeptides were required to inhibit PP1b. IC_{50}'s for PP1b inhibition by hI-1 peptides exceeded 200 nM. These data suggest that the altered regulation of Mn^{2+}-dependent PP1b results from its defective interaction with the N-terminal domain of I-1.

I-1 Dephosphorylation and Inactivation by Native and Recombinant PP1. Nimmo and Cohen (1978) showed that PP1 purified from rabbit muscle required Mn^{2+} to dephos-

Table 1: PP1 Inhibition by I-1 Proteins and Peptides[a]

	IC_{50} (nM)			
	PP1a			
	−Mn	+Mn	PP1b + Mn	PP1α + Mn
rabbit skeletal muscle inhibitor 1				
phosphorylated	1.1 ± 0.21[b]	0.9 ± 0.20	46 ± 3.8	40 ± 2.3
thiophosphorylated	1.3 ± 0.23	1.1 ± 0.31	35 ± 2.6	33 ± 2.6
recombinant human inhibitor 1				
GST-hI-1 (phosphorylated)	32 ± 2.4[b]	26 ± 4.3	85 ± 16	82 ± 13
hI-1 (-3-171, phosphorylated)	0.98 ± 0.15[b]	1.0 ± 0.3	ND	35 ± 2.7
hI-1 (-3-61, phosphorylated)	3.7 ± 0.14[b]	35 ± 2.7	200 ± 18	ND
hI-1 (9-61, phosphorylated)	4.5 ± 0.51[b]	ND	230 ± 21	ND

[a] I-1 purified from rabbit skeletal muscle was phosphorylated by PKA catalytic subunit using ATP or ATP-γ-S as described in Materials and Methods. Recombinant hI-1 was expressed as a GST fusion in *E. coli*. Following thrombin cleavage, hI-1 and two N-terminal peptides were purified by preparative SDS−PAGE and HPLC. The hI-1 proteins and peptides, phosphorylated by PKA, were analyzed for PP1 inhibition. Native Mn^{2+}-independent PP1a was purified from rabbit skeletal muscle and assayed in the presence or absence of 1 mM $MnCl_2$. Mn^{2+}-dependent muscle PP1b was generated by long-term storage at −20 °C or by inhibition with 1 mM ATP or 50 mM NaF. PP1b and recombinant PP1α expressed in *E. coli* were incubated in buffer containing 1 mM $MnCl_2$ for 60 min at 4 °C prior to assaying for phosphorylase phosphatase activity in the presence of 1 mM $MnCl_2$. IC_{50} values for I-1 proteins and peptides were obtained in 3−5 independent experiments (shown with standard errors). Values not determined are indicated as ND. [b] Taken from Endo et al. (1996).

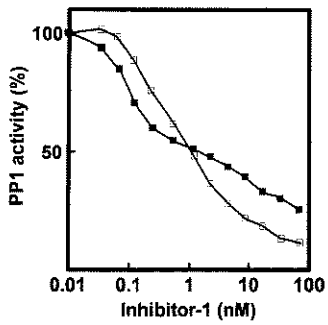

FIGURE 2: Inhibition of skeletal muscle PP1 catalytic subunit by I-1. Phosphorylase *a* phosphatase activity of native muscle PP1a was assayed in the presence of increasing concentrations of PKA-phosphorylated rabbit skeletal muscle I-1. The PP1 inhibitor assays were carried out in the absence (□) or presence (■) of 1 mM $MnCl_2$.

phorylate I-1. Thus, the dephosphorylation and inactivation of I-1 by the Mn^{2+}-dependent PP1 enzymes could represent a mechanism for their reduced sensitivity to I-1 (Alessi et al., 1993). Therefore, we compared PP1a, PP1b, and PP1α for their ability to dephosphorylate ^{32}P-labeled hI-1. Concentrations of the PP1 enzymes were set so that they dephosphorylated phosphorylase *a* at nearly equivalent rates (Figure 3A). In the absence of Mn^{2+}, PP1a was an ineffective I-1 phosphatase, removing less than 3% of the protein-bound phosphate in 40 min at 37 °C (Figure 3B). Addition of 1 mM $MnCl_2$ increased its I-1 phosphatase activity by approximately 2-fold. In contrast, PP1b and PP1α, which were active only in the presence of 1 mM $MnCl_2$, were 20-fold more efficient in dephosphorylating I-1. Nearly 70% of phospho-I-1 was hydrolyzed by PP1b and PP1α in 40 min at 37 °C. This rate was nearly twice that seen with phosphorylase *a* as substrate under the same conditions.

Inhibition of Native and Recombinant PP1 by Thiophosphorylated I-1. Earlier studies showed that I-1 dephosphorylation by PP1 was not inhibited by phosphorylase *a* (Nimmo & Cohen, 1978). Thus, extensive dephosphorylation and inactivation of I-1 could occur even in the presence of the competing substrate, phosphorylase *a*, and be seen as the reduced sensitivity of Mn^{2+}-dependent PP1 to I-1. To address this, we utilized thiophosphorylated I-1 that was not dephosphorylated by either Mn^{2+}-dependent PP1b, PP1α, or PP2A, an I-1-insensitive phosphatase, when incubated at 37

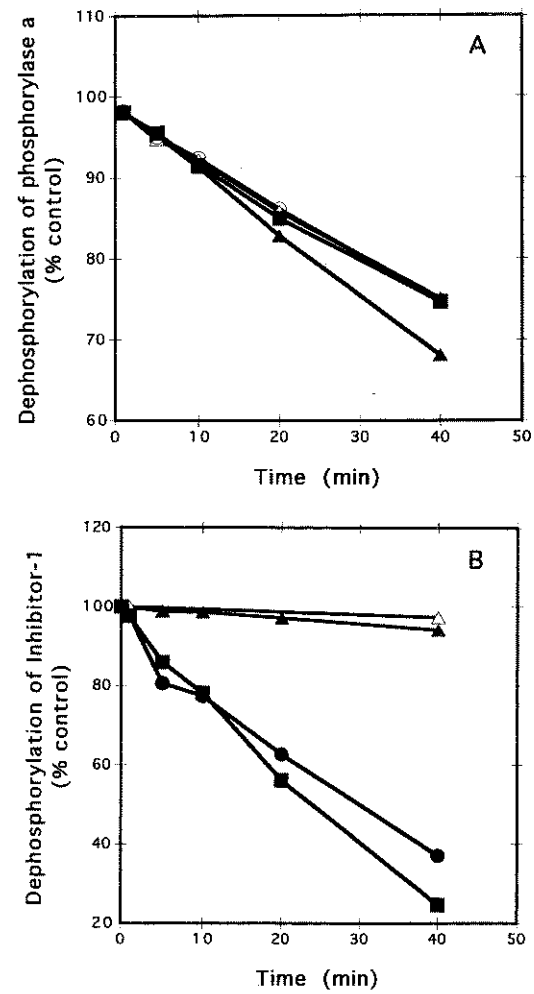

FIGURE 3: Substrate specificity of native and Mn^{2+}-dependent PP1. Native PP1a was assayed in the absence (△) and presence (▲) of 1 mM $MnCl_2$ using either phosphorylase *a* (panel A) or hI-1 (panel B) as substrate. Mn^{2+}-dependent PP1b (●) and recombinant PP1α (■) were also assayed for dephosphorylation of these substrates.

°C for 24 h (data not shown). The thiophosphorylated I-1 was functionally indistinguishable from phospho-I-1 and inhibited PP1a with an IC_{50} of 1 nM (Table 1). As with all the I-1 proteins and peptides examined, $MnCl_2$ induced a pronounced biphasic dose−response curve for PP1a inhibition by thiophosphorylated I-1 without changing its apparent

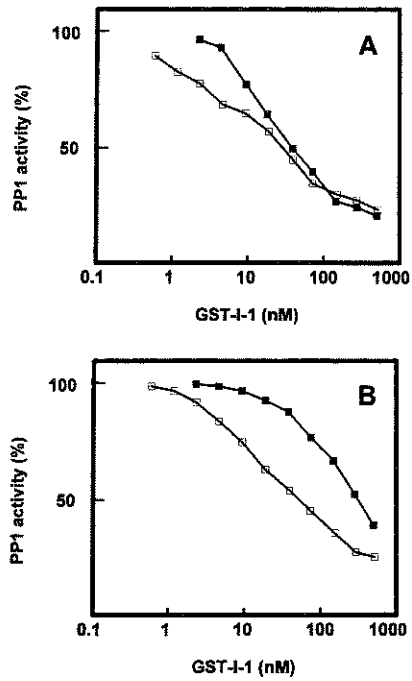

FIGURE 4: Wild-type and mutant I-1 as PP1 inhibitors. Wild-type hI-1 (□) and mutant hI-1(I_{10}N) (■) were expressed in *E. coli* as GST fusion proteins. The affinity-purified GST-hI-1 proteins were phosphorylated with PKA and analyzed for inhibition of native PP1a assayed in the absence of divalent cations (panel A) and recombinant PP1α assayed in the presence of 1 mM $MnCl_2$ (panel B) with phosphorylase *a* as substrate.

IC_{50} (data not shown). The key finding, however, was that more than 30-fold higher concentrations of thiophosphorylated I-1 were required to inhibit PP1b and PP1α. This argued against the dephosphorylation of I-1 as the basis for decreased sensitivity of Mn^{2+}-dependent PP1 to I-1.

An N-Terminal Mutation of I-1 Attenuates Inhibition of Mn^{2+}-Dependent PP1. N-Terminal phosphopeptides of I-1, which failed to inhibit PP1 at nanomolar concentrations, identified a KIQF sequence that acts in concert with the phosphorylation of threonine$_{35}$ to inhibit PP1 activity (Aitken & Cohen, 1984; Endo et al., 1996). Serial deletions in a synthetic peptide of DARPP-32, a structurally related PP1 inhibitor, showed the most significant loss in PP1 inhibitor activity with the loss of the isoleucine in the KIQF sequence (Hemmings et al., 1990). To define the functional role of this conserved isoleuine, we substituted a neutral amino acid, asparagine, in place of isoleucine$_{10}$ in hI-1 and expressed the mutant GST-hI-1 protein in *E. coli*. GST-hI-1(I_{10}N) was phosphorylated by PKA at rates similar to wild-type GST-I-1 (data not shown). The wild-type GST-hI-1 inhibited native PP1a in the absence of Mn^{2+} with an IC_{50} of 32 nM (FIgure 4, panel A). In contrast, the mutant GST-hI-1(I_{10}N) was slightly less potent as a PP1a inhibitor with an apparent IC_{50} of 45 nM. The mutant hI-1 was most notably compromised in its ability to inhibit PP1a activity when analyzed at concentrations below 10 nM. The most striking finding was that GST-hI-1(I_{10}N) was severely impaired in its ability to inhibit the recombinant PP1α compared to wild-type GST-hI-1 (Figure 4, panel B). The IC_{50} values for PP1α inhibition by wild-type and mutant GST-hI-1 were 82 nM and 675 nM, respectively. This suggested that the defect in interaction of PP1α with the N-terminal functional domain of I-1 was further exacerbated by mutation of isoleucine$_{10}$.

DISCUSSION

Protein phosphatase 1 (PP1) is a ubiquitous eukaryotic protein serine/threonine phosphatase that dephosphorylates a broad range of phosphoprotein substrates. Long-term storage inactivates PP1, converting it to a form that requires divalent cations for activity. This conversion was accelerated by PP1 inhibition by the reversible inhibitors NaF (Burchell & Cohen, 1978), ATP, and sodium pyrophosphate (Hsiao et al., 1978; Brautigan et al., 1982; Yan & Graves, 1982). With the cloning of PP1 cDNAs, the recombinant isoenzymes were expressed in bacteria and found to be exclusively Mn^{2+}-dependent (Zhang et al., 1992, 1993a,b, 1994a,b; Alessi et al., 1993; Zhao et al., 1994). Indeed, poor yields of PP1 were reported when $MnCl_2$ was not added to the bacterial growth medium (Zhang et al., 1992; Alessi et al., 1993). Site-directed mutagenesis and analysis of mutant enzymes has begun to shed new light on the structure–function relationship for the PP1 catalytic subunit (Zhang et al., 1993b, 1994a,b, 1996a,b). Three-dimensional structures of two PP1 isoenzymes also offer new opportunities for understanding the function and regulation of PP1 (Goldberg et al., 1995; Egloff et al., 1995).

In these studies, we established that the phosphorylase phosphatase activity of PP1a catalytic subunit, freshly purified from rabbit skeletal muscle, was independent of metal ions and was unaffected by addition of chelator to the assay. Indeed, $MnCl_2$ above 0.1 mM inhibited the native enzyme. In contrast, the recombinant PP1α and the Mn^{2+}-dependent muscle enzyme, PP1b, were absolutely dependent on divalent cations for enzyme activity. In examining the time and concentration dependency of reactivation of recombinant PP1α and PP1b by Mn^{2+}, we saw that their activity was maximal after 10 min in 1 mM $MnCl_2$. On continued incubation, this activity slowly declined to reach a new steady-state level, equivalent to that of the native enzyme in the presence of Mn^{2+}. These data suggest that Mn^{2+} induces multiple conformations in the PP1 catalytic subunit that are reflected in these changes in enzyme activity.

Additional evidence suggesting structural alterations in the recombinant PP1 includes its enhanced activity against selected substrates, PNPP and histone H1, when compared with native PP1 (Alessi et al., 1993; Zhao et al., 1994). It has been speculated that the loss of one or more endogenous metals converts PP1 to a Mn^{2+}-dependent enzyme with a more relaxed catalytic site that allows access to otherwise poor substrates like the phosphotyrosine analog, PNPP (Egloff et al., 1995). Our data showed that the Mn^{2+}-dependent phosphatases were also modified in their ability to dephosphorylate I-1. Native PP1a was a poor I-1 phosphatase even in the presence of Mn^{2+}, a potentially important factor in determining I-1's potency as a PP1 inhibitor. In contrast, Mn^{2+}-dependent PP1b and recombinant PP1α were excellent I-1 phosphatases, preferring I-1 to phosphorylase *a* by nearly 2:1. So the proposed relaxation of catalytic site that occurs in PP1α and PP1b also increases access to a phosphothreonine-containing substrate, like I-1.

PP1a was characterized by its potent inhibition by rabbit and human I-1 in the presence or absence of $MnCl_2$. The Mn^{2+} ions did not change the apparent IC_{50} for I-1 proteins and peptides but clearly modified their dose–response curves. As $MnCl_2$ did not perturb the secondary structure of I-1 proteins and peptides, as judged by circular dichroism (data not shown), its primary interaction is most likely with

the PP1a catalytic site, where it also enhanced the dephosphorylation of I-1 and several other substrates. Alessi et al. (1993) reported that 100−600-fold higher concentrations of I-1 were required to inhibit recombinant PP1. Tissue-derived PP1 catalytic subunit was impaired in its regulation by I-1 only following its conversion to a Mn^{2+}-dependent enzyme. Determining the structural basis for the altered regulation of recombinant PP1 by I-1 was a key goal of our studies. When we saw that PP1α and PP1b preferred I-1 as a substrate over phosphorylase *a*, it raised the possibility that dephosphorylation and inactivation of I-1 in the assay accounted for the apparent reduced sensitivity of these enzymes to I-1. Thus, we used thiophosphorylated I-1, which was resistant to dephosphorylation by cellular phosphatases and inhibited PP1 in living cells (Mulkey et al., 1994; Endo et al., 1995). Our finding that thiophosphorylated I-1 was indistinguishable from phospho-I-1 as an inhibitor of PP1a, PP1b, and PP1α made a strong argument against the dephosphorylation of the I-1 proteins and peptides as the basis for their reduced potency as inhibitors of Mn^{2+}-dependent PP1. Nanomolar concentrations of I-1 were used the PP1 inhibitor assay, which should also preclude its significant dephosphorylation during the course of these assays. The 30−50-fold reduction of I-1 potency against PP1α as seen in our studies was much less than the 100−600-fold decrease reported by Alessi et al. (1993). We can only speculate about the contributions of different assay conditions and PP1 preparations, but one explanation may come from our observation that recombinant PP1α, stored at high protein concentrations (>0.5 mg/mL in 1.0 mM $MnCl_2$), shows further changes in its IC_{50} for I-1. In preliminary studies, we have seen a time-dependent increase in IC_{50} for I-1 from 46 ± 3.8 nM as reported in Table 1 to 580 ± 24 nM (H. Quan, J. H. Connor, and S. Shenolikar, unpublished observations), a value close to that reported by Alessi et al. (1993). Under these conditions, PP1α's sensitivity to other inhibitors, toxins and I-2, was not changed. This suggests that regulation by I-1 may be a very useful monitor of the structural integrity of the PP1 catalytic subunit.

The first and foremost among the structural determinants in I-1 that mediate PP1 inhibition is its phosphorylation on $threonine_{35}$. Modeling a dodecapeptide, representing the phosphorylation site conserved in I-1 and DARPP-32, into the three-dimensional structure of PP1α highlights its excellent fit in the catalytic site containing two metals (labeled site 1 in Figure 5; Goldberg et al., 1995). However, this dodecapeptide does not inhibit PP1 activity (Nimmo & Cohen, 1978), suggesting that additional interactions are necessary for PP1 inhibition. Biosensor studies showed that the PP1 catalytic subunit also associated with the dephosphorylated and inactive I-1 (Endo et al., 1996). This clearly pointed to interactions of PP1 with I-1 that may not be solely mediated by the catalytic site. Inability of several I-1 phosphopeptides to inhibit PP1 (Aitken & Cohen, 1984; Endo et al., 1996) identified a N-terminal tetrapeptide sequence, KIQF, in I-1 that was essential for its potency as a PP1 inhibitor. N-Terminal truncation of a DARPP-32 peptide that removed the isoleucine present in this sequence resulted in a 1000-fold reduction in its PP1 inhibitor activity (Hemmings et al., 1990). Our studies used site-directed mutagenesis to substitute this isoleucine in hI-1 with asparagine. The mutant hI-1(I_{10}N) was nearly equivalent to wild-type hI-1 as a PP1a inhibitor. Thus, PP1a association with

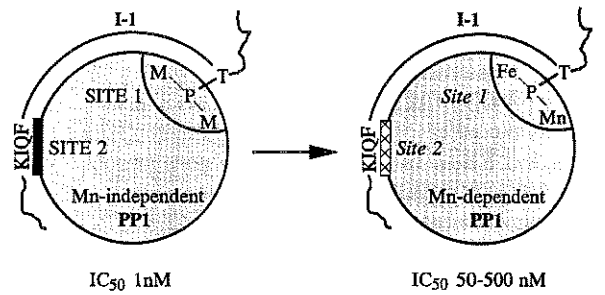

FIGURE 5: Model for PP1 association with I-1. PP1a, freshly purified from mammalian tissues, contains as yet unidentified endogenous metals (M) and does not require additional cations for enzyme activity. PP1a was inhibited by phospho-I-1 with IC_{50} 1 nM. Three-dimensional structure of PP1 catalytic subunit (Goldberg et al., 1995; Egloff et al., 1995) shows that the two metals in the catalytic site (site 1) coordinate phosphate binding (shown as T-P in I-1). Conversion to a Mn^{2+}-dependent enzyme may displace a metal from the M1 site (Egloff et al., 1995) and result in the loss of enzyme activity. This can be restored by incubation with Mn^{2+}. However, there is a resultant change in site 1 that enhances I-1 dephosphorylation. Requirement for higher concentrations of thiophosphorylated I-1 to inhibit the Mn^{2+}-dependent PP1 and mutation of $isoleucine_{10}$ in hI-1 that preferentially abrogates the inhibition of this enzyme argue for an additional PP1/I-1 interaction site (site 2) that recognizes the KIQF sequence in I-1.

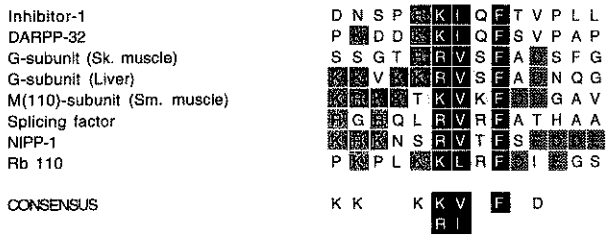

FIGURE 6: KIQF-like motifs in PP1-binding proteins. The KIQF sequence conserved in I-1 and DARPP-32 was aligned with the sequences of PP1-binding domains in G_M and G_L (residues 65−73), the skeletal muscle and liver glycogen-binding subunits; M_{110} (residues 1−38), the large subunit of the smooth muscle myosin-binding complex; the region of splicing factor (residues 349−430) that was encoded in a partial cDNA cloned by PP1 interaction in a yeast two-hybrid screen; NIPP-1 (residues 143−225), an inhibitory fragment of the nuclear PP1 inhibitor; and the C-terminal PP1-binding domain of RB_{110} (residues 773−928), the retinoblastoma gene product. This defined a consensus that was preceded by a preponderance of basic residues in many PP1-binding proteins. One or more acidic residues were also present C-terminal to the consensus in some proteins.

I-1 was not mediated exclusively by $isoleucine_{10}$, and in its absence, other residues in I-1 formed an effective PP1-binding domain. A small right shift in IC_{50} was observed for PP1a inhibition by GST-hI-1(I_{10}N), particularly at low concentrations. However, the most remarkable finding was that hI-1(I_{10}N) was much weaker than wild-type hI-1 as an inhibitor of a Mn^{2+}-dependent enzyme, PP1α (and PP1b; data not shown), providing support for $isoleucine_{10}$ and surrounding residues in I-1 as a potential site for association with the PP1 catalytic subunit.

Additional support for the KIQF sequence as a potential PP1-binding domain comes from comparing its primary sequence with that of proposed PP1-binding domains in other regulatory proteins (Figure 6). A 12-residue peptide (G_M-63−75) prevents PP1 regulation by G_M, the skeletal muscle glycogen-targeting subunit, and G_L, the liver glycogen-binding subunit (Johnson et al., 1996). This has identified a putative PP1-binding domain that is highly conserved in the two glycogen-binding subunits (Doherty et al., 1995).

G_M63−75 sequence shows homology with the I-1 functional domain, particularly the KIQF sequence. Interestingly, PP1-binding domains identified in M_{110}, the myosin-binding subunit (Gailly et al., 1996), NIPP-1, a nuclear PP1 inhibitor (Van Eynde et al., 1995), a splicing factor (Hirano et al., 1996), and RB_{110}, the retinoblastoma gene product (Durfee et al., 1993) also contain KIQF-like sequences. Thus, the KIQF sequence may represent a widely utilized PP1 recognition motif. The lack of KIQF-like sequences in other PP1-binding proteins also points to other modes of association with the PP1 catalytic subunit, such as the leucine-rich repeat found in *sds*22 (Stone et al., 1993). The presence of a common binding motif may have important implications for physiological regulation of cellular PP1 complexes via I-1. G_M, G_L, M_{110}, and RB_{110} also possess an additional PP1-binding site, which could also determine to the inhibition of an associated phosphatase by I-1. Wu et al. (1996) reported that rabbit muscle PP1 but not recombinant PP1α associated with a fragment of recombinant G_M in a solid-phase assay. This suggests that structural modification(s) in recombinant PP1 reduces its affinity not only for I-1 but also perhaps for other PP1 regulators. We have proposed a model for PP1 regulation by I-1 that indicates I-1's association with two sites on PP1 (Figure 5), both of which may be modified in the recombinant PP1 to account for its decreased sensitivity to I-1.

In summary, expression of PP1 catalytic subunits in bacteria modifies its structure. The requirement for divalent cations for enzyme activity, the enhanced dephosphorylation of I-1 (and other substrates), and impaired regulation by phospho-I-1 all argue for a change in the catalytic site of the recombinant PP1. New evidence has been presented for a second modification in the PP1 catalytic subunit that is uniquely sensitive to the mutation of I-1 at isoleucine$_{10}$. Identifying the structural defects in the recombinant PP1 catalytic subunit may provide new insight into the mechanism underlying its conversion to a divalent cation-dependent enzyme and also aid in our understanding of the mode of action of I-1, which regulates PP1 activty in response to hormones.

NOTE ADDED IN PROOF

Direct evidence in support of the proposed second binding site for I-1 and other PP1 regulators is provided by recent crystallization of the PP1 catalytic subunit with the G_M(63−75) peptide (Egloff et al., 1997). These data show that the two residues I/V and F conserved in all putative PP1-binding sites (shown in Figure 6) make direct contact with the PP1 catalytic subunit at a site distinct from the catalytic domain.

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