Multiple Structural Elements Define the Specificity of Recombinant Human Inhibitor-1 as a Protein Phosphatase-1 Inhibitor[†,‡]

Shogo Endo,[§,||] Xiaozhen Zhou,[§] John Connor,[§] Bo Wang,[⊥,#] and Shirish Shenolikar*[,§]

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27708, and Department of Pharmacology, University of Texas Medical School at Houston, Houston, Texas 77030

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ABSTRACT: The cDNA encoding human brain protein phosphatase inhibitor-1 (I-1) was expressed in *Escherichia coli*. Following PKA phosphorylation at a threonine, recombinant human I-1 was indistinguishable from rabbit skeletal muscle I-1 as a potent and specific inhibitor of the type-1 protein serine/threonine phosphatase (PP1). N-Terminal phosphopeptides of I-1 that retained the selectivity of intact human I-1 highlighted a functional domain that mediates PP1 inhibition. Substituting alanine in place of threonine-35 eliminated I-1 phosphorylation by PKA and its phosphatase inhibitor activity. An acidic residue was substituted in place of the phosphoacceptor to produce I-1(T35D), a constitutive phosphatase inhibitor. I-1(T35D) was an equally effective inhibitor of PP1 and the type-2 phosphatase, PP2A. However, CNBr digestion of I-1(T35D) yielded an N-terminal peptide that showed 100-fold increased specificity as a PP1 inhibitor. This provided new insight into a unique conformation of the phosphorylated I-1 that accounts for selective inhibition of PP1 activity. Truncation of an active I-1 phosphopeptide identified an N-terminal sequence that was required in addition to threonine-35 phosphorylation to inhibit PP1 activity. Biosensor studies demonstrated that PP1 bound to both phosphorylated and dephosphorylated I-1 and suggested that distinct elements of I-1 structure accounted for PP1 binding and inhibition. Our data point to multiple interactions between the I-1 functional domain and the PP1 catalytic subunit that define this phosphoprotein as a physiological regulator of the type-1 protein phosphatase.

Reversible protein phosphorylation controls many processes in plant and animal cells (Cohen, 1988). As physiological responses of cells to hormones and other extracellular stimuli are defined by the phosphorylation state of key proteins, coordinating the opposing actions of protein kinases and phosphatases becomes important for cells to evoke an appropriate hormonal response. Huang and Glinsmann (1976) identified a protein from rabbit skeletal muscle extracts that, when phosphorylated by PKA, inhibited protein phosphatase activity. The phosphoprotein, inhibitor-1 (I-1), provided a potential mechanism for cross-talk between a protein kinase and phosphatase that amplified hormonal signals mediated by the second messenger, cAMP. Nimmo and Cohen (1978a) purified I-1 from rabbit skeletal muscle and showed that following PKA phosphorylation nanomolar concentrations of I-1 inhibited protein phosphatase-1 (PP1). By comparison, concentrations of dephosphorylated I-1 approaching 1 μM did not inhibit PP1 activity. Hormones that elevate intracellular cAMP increased I-1 activity in many tissues (Khatra et al., 1980; Foulkes & Cohen, 1981; Foulkes et al., 1982; Nemenoff et al., 1983; Neumann et al., 1991; MacDougall et al., 1989; Snyder et al., 1992). As PP1 dephosphorylates numerous phosphoproteins, I-1 activation may impose cAMP control over proteins that are not directly phosphorylated by PKA. Following a rise in intracellular calcium, I-1 was inactivated by a calcium/calmodulin-activated protein phosphatase, known as calcineurin or PP2B, resulting in increased PP1 activity. Such a cascade of phosphatases controlled synaptic plasticity in hippocampal neurons (Mulkey et al., 1994).

Inhibition by phosphorylated I-1 and structurally unrelated inhibitor-2 (I-2) became a cornerstone of the classification scheme (Ingebritsen & Cohen, 1983) that characterized protein serine/threonine phosphatases in mammalian tissues (Ingebritsen et al., 1983) and many organisms (Cohen, 1989; Shenolikar & Nairn, 1991). Type-1 phosphatases (PP1) were inhibited by I-1 and I-2. In contrast, "several hundred fold" higher concentrations of these proteins had no effect on type-2 phosphatases (Nimmo & Cohen, 1978b). However, I-1 was an excellent substrate for the type-2 phosphatases, PP2A and PP2B, whose catalytic subunits share some structural homology with PP1. The molecular basis underlying I-1's function as a PP1 inhibitor and a substrate for PP2A and PP2B remains poorly understood.

To investigate the mode of action of I-1 as a PP1 inhibitor, we have cloned and expressed a cDNA encoding human I-1. Recombinant I-1 demonstrated many of the properties previously ascribed to I-1 isolated from rabbit skeletal

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* Correspondence should be addressed to this author at the Department of Pharmacology, Duke University Medical Center, Box 3813, Durham, NC 27708. Phone: 919-681-6178. FAX: 919-684-8922. E-mail: sheno001@mc.duke.edu.

[§] Duke University Medical Center.

[||] Present address: Department of Biochemistry, University of Houston, Houston, TX 77204-5934.

[⊥] University of Texas Medical School at Houston.

[#] Present address: Department of Internal Medicine, University of Texas Health Science Center−Medical School, Houston, TX 77225.

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muscle. Analysis of N-terminal peptides defined the functional properties of a functional domain of human I-1. Mutagenesis of I-1 protein and peptide showed that multiple elements of the I-1 structure contribute to potent and selective inhibition of PP1. Our data provide new insight into the molecular basis underlying I-1's physiological function as a regulator of type-1 protein serine/threonine phosphatases.

MATERIALS AND METHODS

Restriction enzymes, isopropyl 1-thio-β-D-galactopyranoside (IPTG), human thrombin, and *Staphylococcus aureus* V8 protease (endoproteinase Glu-C) were purchased from Boehringer Mannheim. Thrombin and factor Xa were obtained from Sigma. Glutathione−Sepharose was purchased from Pharmacia. Phosphorylase *b* and phosphorylase kinase were obtained from GIBCO-BRL. [γ-^{32}P]ATP (>4000 Ci/mmol) and [α-^{32}P]dCTP (>3000 Ci/mmol) were purchased from ICN. Sequenase II was purchased from U.S. Biochemicals. Catalytic subunits of PP1 and PP2A (DeGuzman & Lee, 1988), I-1 (Cohen et al., 1988), and the PKA catalytic subunit (Beavo et al., 1974) were purified from rabbit skeletal muscle. The polyclonal antibody against rabbit I-1 (G185) was provided by Drs. P. Greengard and A. C. Nairn, Rockefeller University. Bovine anhydrotrypsin−Sepharose was provided by Drs. T. Kumazaki and H. Yokosawa of Hokkaido University, Sapporo, Japan. Anti-GST antibody was prepared by injecting rabbits with recombinant *Schistosomal* GST prepared in *E. coli.*

Protein concentration was determined by the method of Bradford (1976) using BSA as standard ($E^{1\%}_{280} = 6.54$).

Molecular Cloning of Human I-1 cDNA. The human brain cDNA library constructed in λZAP II (Stratagene) was provided by Dr. R. Joho, University of Texas Southwestern Medical School. The cDNA library was transformed into *E. coli* BB4 grown in 1.0% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), and 0.5% (w/v) NaCl at pH 7.5 at 42 °C. Nitrocellulose filter lifts with denatured phage DNA were prehybridized in 6× SSC, 5× Denhardt's solution, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.3 M sodium phosphate, pH 6.8, and 100 μg of denatured salmon sperm DNA/mL. The filters were hybridized overnight at 42 °C with ^{32}P-labeled 168 bp *Eco*RI−*Bam*HI restriction fragment (0.5×10^6 cpm/mL) from rat I-1 cDNA (Elbrecht et al., 1990) random-primed with [α-^{32}P]dCTP. The filters were washed 4 times with 2× SSC and 0.1% (w/v) SDS at room temperature for 15 min each, followed by four washes with 1X SSC and 0.1% (w/v) SDS at 50 °C for 15 min each. Positive plaques were identified by aligning duplicate filters subjected to autoradiography. Individual clones were purified through secondary and tertiary screens, excised *in vivo* using helper phage, transferred to *E. coli* XL1-blue, and characterized by double-stranded DNA sequencing using Sequenase II.

Bacterial Expression of Human I-1. The *Nco*I−*Bst*XI fragment of human I-1 was excised from pBluescript SK-, blunt-ended with the Klenow fragment, and subcloned into the *Sma*I site in pGEX-2T (Pharmacia). The plasmids were transformed into competent *E. coli* BL21 (Novagen), JM109 (Promega), or DH5α (GIBCO-BRL) to express I-1 as a fusion protein with glutathione−*S*-transferase (GST).

A seed culture (5 mL) of *E. coli* was grown overnight at 37 °C in Terrific Broth (Tartof & Hobbs, 1987) containing ampicillin (50 μg/mL). The seed culture was added to 250 mL of medium maintained at 37 °C, and bacterial growth was continued until an A_{600} of 0.6 was reached. The culture was cooled to 25 °C, and bacterial growth was allowed to proceed until A_{600} reached 0.8. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 1 mM, and GST−I-1 was induced by incubation at 25 °C for 3 h. The bacteria were centrifuged at 3000*g* for 15 min and lysed by sonication in 20 mL of 50 mM Tris-HCl, pH 7.5, containing 1% (v/v) Nonidet P-40 (NP-40), 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, and 1 mM PMSF at 4 °C. The bacterial lysate was gently shaken with glutathione−Sepharose (10 mL) equilibrated in 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS) for 5 min at 4 °C. The affinity matrix was washed with lysis buffer (2 × 20 mL), followed by TBS containing 1% (v/v) NP-40 (3 × 30 mL). GST−I-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.

GST−I-1 (3−4 mg of total protein) was applied to a 9% preparative SDS−PAGE (gel volume 60 mL) using Prep-Cell 490 (Bio-Rad) and subjected to electrophoresis at 50 mA constant current at room temperature for 17 h. Fractions were analyzed by SDS−PAGE, and GST−I-1 (apparent molecular mass 47 kDa) was pooled and dialyzed against 0.5 mM Tris-HCl, pH 7.5, containing 0.005% (w/v) Brij 35 at 4 °C. Purified GST−I-1 was lyophilized and stored at −80 °C.

GST−I-1 (5 mg) was digested with thrombin (0.5−1.0 NIH unit/mL) in 10 mL of 50 mM Tris-HCl, pH 8.5, containing 5 mM $CaCl_2$ at 30 °C for 30 min. The reaction was terminated by adding PMSF (1 mM) followed by heating in a boiling water bath for 5 min. The reaction mixture was centrifuged at 40000*g* for 20 min, and the supernatant was subjected to preparative SDS−PAGE as described above. Fractions containing a single 28 kDa polypeptide representing human I-1 were pooled, dialyzed, and freeze-dried.

Bacterial Expression of I-1 Peptides. A synthetic gene (154 bp) was constructed using overlapping synthetic oligonucleotides that encoded I-1(9−54); the construction of the synthetic gene will be described elsewhere (S. Shenolikar, M. Planas-Silva, X. Zhou, C. D. Rasmussen, and A. R. Means, unpublished data). The *Eco*RI−*Bam*HI fragment representing the I-1(9−54) synthetic gene and an N-terminal cleavage site for factor Xa was inserted into pRIT-2T (Pharmacia) vector. *E. coli* N4830 (Pharmacia) were transformed using the expression plasmid and grown overnight in LB broth (250 mL) containing ampicillin (50 μg/mL) at 30 °C. An equal volume of LB maintained at 54 °C was added and bacterial growth continued at 42 °C for 2 h. Bacteria were harvested by centrifugation at 3000*g* for 15 min and lysed by sonication in 20 mL of 50 mM Tris-HCl, pH 7.5, containing 1% (v/v) NP-40, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, and 1 mM PMSF at 4 °C.

The bacterial lysate was shaken with 10 mL of IgG−Sepharose (Pharmacia) for 10 min at 4 °C. The gel was washed with PBS (3 × 30 mL) and protein A−I-1(9−54) fusion protein was eluted with 0.2 M glycine hydrochloride, pH 2.3 (2 × 10 mL). The fusion protein was dialyzed overnight against 50 mM Tris-HCl, pH 7.5, containing 0.005% (w/v) Brij 35 and digested with factor Xa (2 units/mg of fusion protein) at 37 °C for 90 min. The digest was heated in a boiling water bath for 5 min, and denatured

proteins were removed by centrifugation at 30000*g* for 30 min. The supernatant containing the I-1(9−54) peptide was stored at −20 °C.

Site-Directed Mutagenesis. The *Nco*I−*Eco*RI insert from pGEX-2T was subcloned into pGEM-3Zf(-) (Promega). Site-directed mutagenesis was performed by PCR using Taq polymerase (USB), with a "forward" primer and the SP6 primer in one reaction and a "backward" primer and the T7 primer in another reaction. 5′-GCCGCCCCGCCCCTGCC-3′ and 5′-GGCAGGGGCGGGGCGGC-3′ were used as "forward" and "backward" primers to substitute alanine in place of the phosphoacceptor, threonine-35, and 5′-GCCGC-CCCGACCCTGCC-3′ and 5′-GGCAGGGTCGGGGCGGC-3′ substituted aspartic acid in this position. PCR reactions (35 cycles) were undertaken at 94 °C for 30 s, for 1 min at 55 °C, and for 1 min at 72 °C. PCR products were purified by electrophoresis in 1% (w/v) agarose. For each mutation, equimolar amounts of products from "forward" and "backward" reactions were mixed and further amplified using only the external T7 and SP6 primers. The second PCR product was digested with *Nco*I and *Eco*RI and subcloned into pGEM-3Zf(-). Mutations were verified by double-stranded sequencing using Sequenase II.

Phosphorylation of I-1 by PKA. I-1 (0.25 mg/mL) or GST−I-1 (0.5 mg/mL) was phosphorylated with purified PKA catalytic subunit (0.6 unit/mL, specific activity 3000 units/mg) in 50 mM Tris-HCl, pH 7.5, containing 100 μM ATP and 1 mM $MgCl_2$ at 30 °C for 1−4 h. The extent of I-1 phosphorylation was monitored by including trace amounts of [γ-^{32}P]ATP in the reaction. Incorporation of [^{32}P]-phosphate into I-1 was followed by SDS−PAGE and autoradiography or by trichloroacetic acid (15%, w/v) precipitation. The concentration of I-1 was established by the incorporation of 1 mol of [^{32}P]phosphate into 1 mol of protein.

Protein Phosphatase Assay. [^{32}P]Phosphorylase *b* was phosphorylated with phosphorylase kinase (Shenolikar & Ingebritsen, 1984). The concentration of phosphorylase was determined using an $E^{1\%}_{280}$ of 13.1 (Cohen et al., 1971). PP1 and PP2A were 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. Reaction was terminated by adding 0.1 mL of 20% (w/v) trichloroacetic acid and 0.1 mL of BSA (6 mg/mL), cooled on ice for 5 min, and centrifuged at 15000*g* for 5 min. [^{32}P]Phosphate release was monitored by mixing 200 μL of supernatant with 2 mL of Safety-Solve scintillation fluid (RPI) and counting in a liquid scintillation counter. One unit of phosphatase was defined as the release of 1 nmol of phosphate in 1 min under the assay conditions. One unit of I-1 activity was defined as the amount required to inhibit 0.02 unit of PP1 by 50% in this assay.

Purification of I-1 Peptides. I-1 peptides were purified by preparative SDS−PAGE on a 14% (w/v) polyacrylamide gel followed by reversed-phase HPLC on C_{18}-μBondapak (300 × 7.5 mm, Waters) eluted with 0.1% (v/v) trifluoroacetic acid in water using a linear gradient of 1−65% (v/v) acetonitrile at a flow rate of 1.0 mL/min for 30 min. Fractions were pooled, dried, and redissolved in 1.0 mM Tris-HCl, pH 7.5, containing 0.005% (w/v) Brij 35.

For mass spectrometry, I-1 peptides were further separated by reversed-phase HPLC using a C_8 (5 μm)-Deltabond-LC column (100 × 1 mm, Keystone Scientific) equilibrated with 0.08% (v/v) trifluoroacetic acid in ultrapure water (solvent A). Peptides were eluted with a linear gradient of 10−70% solvent B [90% (v/v) acetonitrile in solvent A] at a flow rate of 50 μL/min for 45 min. The purified peptides were vacuum-dried and resuspended in 50% aqueous acetonitrile containing 1% (v/v) formic acid for electrospray mass spectrometry.

Sequence Analysis of I-1 Protein and Peptides. I-1 proteins and peptides were separated by SDS−PAGE and electrophoretically transferred to a Trans-Blot membrane (Bio-Rad). The membranes were stained with Coomassie Blue, and protein bands were excised for N-terminal sequence determination using the Applied Biosystems Model 477A peptide sequencer (Duke University Sequencing Center).

C-Terminal sequences were determined by digestion of selected I-1 peptides with *Staphylococcus* V8 protease (1%, w/w) in 50 mM ammonium bicarbonate, pH 8.6 at 37 °C. The digest was adjusted to pH 5.0 using 1 mM HCl to terminate the reaction. An equal volume of 50 mM sodium acetate, pH 5.0, containing 20 mM $CaCl_2$ was added, and the sample was applied to anhydrotrypsin−Sepharose. The column was washed with 50 mM sodium acetate, pH 5.0, containing 20 mM $CaCl_2$, and bound peptides were eluted with 5 mM HCl (Yokosawa & Ishii, 1976; Ishii et al., 1983). The peptides were lyophilized, redissolved in 0.1% trifluoroacetic acid, and subjected to reversed-phase HPLC on a Vydac C_{18} column prior to sequence determination.

Electrospray Ionization Mass Spectrometry. I-1 proteins and peptides were analyzed using a Fisons-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure (Duke University Biomolecular Mass Spectrometry Laboratory). Samples were introduced by loop injection into a stream of 50% aqueous acetonitrile containing 1% (v/v) formic acid at 6 μL/min, and spectra were acquired in the multichannel analyzer mode from *m/e* 700 to 1400 with a scan time of 10 s. Mass spectra were transformed to a molecular mass scale calibrated with equine cardiac myoglobin (M_r 16 951.48) with resolution corresponding to a peak width at half-height of 1.4 Da for *m/e* 893.

Biosensor Analysis. Phosphatase/inhibitor interactions were analyzed using the Fisons IAsys optical biosensor system. Affinity-purified polyclonal antibody (16 μg of protein) against *Schistosomal* glutathione−*S*-transferase (GST) was covalently linked to an IAsys cuvette activated with EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride)−NHS (*N*-hydroxysuccinimide) in 10 mM acetate buffer, pH 4.5 (Fisons application note 3.1). Residual reactive groups were blocked with 1 M ethanolamine, pH 8.5, and unbound protein was removed by washing with 10 mM HCl. The cuvette was equilibrated with phosphate-buffered saline (10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) containing 0.05% (v/v) Tween 20 (PBS−Tween) prior to loading GST−I-1 (10 μg). Time-dependent binding of GST−I-1 to the immobilized anti-GST antibody was monitored as an increase in the evanescent response (measured in arc seconds). Excess GST−I-1 was removed with PBS−Tween. Purified PP1 (or PP2A) catalytic subunits were added, and binding was observed as a time-dependent increase in the evanescent field response. The cuvette was then washed with PBS−Tween, and dissociation of the phosphatase from GST−I-1 was analyzed. The IAsys

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                10         20         30         40         50
HUMAN     MEQDNSPRKI QFTVPLLEPH LDPEAAEQIR RRRPTPATLV LTSDQSSPEI
RAT       **P******* ********** ********** ********** *********V
RABBIT    ********** ********** ********** ********** *********V

                60         70         80         90        100
HUMAN     DEDRIPNPHL KSTLAMSPRQ RKKMTRITPT MKELQMMVEH HLGQQQQGEE
RAT       ********L* ****S***** ******T*** *****T**** *****K****
RABBIT    ********L* *PS******* ******T*** ********** *****E****

               110        120        130        140        150
HUMAN     PEGAAESTGT QESRPPGIPD TEVESRLGTS GTAKKTAECI PKTHERGSKE
RAT       ****T****N ***C****** *GSA**PD** ***Q*P***K ***Q*QRGV*
RABBIT    ******G**A ***Q***T*G *GA*****P* A**Q*P*QPS *RAQ**RGE*

               160        170
HUMAN     PSTKEPSTHI PPLDSKGANS V
RAT       ***EDL*A*M L****Q**SL *
RABBIT    ***AKT*Q-- ----**Q**SA *
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FIGURE 1: Primary sequence alignment of rat, rabbit, and human inhibitor-1. The amino acid sequence predicted by the human brain I-1 cDNA was aligned with the primary sequences for rat (Elbrecht et al., 1990) and rabbit I-1 (Aitken et al., 1982). Asterisks represent identical amino acids, and dashes represent gaps inserted in the sequence to optimize the alignment.

cuvette was regenerated with 10 mM HCl prior to binding phosphorylated GST−I-1 to the immobilized anti-GST antibody. PP1 (or PP2A) binding to phosphorylated GST−I-1 was analyzed as described.

Circular Dichroism Analyses. Purified I-1 proteins and peptides (1 mg of protein/mL of PBS) were analyzed for their secondary structure using an Aviv Model 62DS circular dichroism spectrophotometer (Lakewood, NJ). Phosphorylated and unphosphorylated I-1 proteins and peptides were scanned at wavelengths of 180−320 nm with a path length of 1 cm and a slit width between 0.25 and 0.3 mm. CD spectra at a range of temperatures between 10 and 50 °C were undertaken with the I-1 proteins and peptides to examine the stability of their secondary structure.

Western Immunoblot Analysis. SDS−PAGE was carried out by the method of Laemmli (1970). Proteins were transferred to a Trans-Blot membrane (Bio-Rad) at 100 V for 1 h in 25 mM Tris−192 mM glycine, pH 8.3, containing 20% (v/v) methanol (Towbin & Gordon, 1984). The membranes were blocked with 1% (w/v) dried milk in 20 mM Tris-HCl, pH 7.5, containing 0.1% (v/v) Tween 20 and 150 mM NaCl (TTBS) for 1 h at room temperature prior to incubation with a polyclonal antibody generated against rabbit I-1, diluted 1/500 or 1/1000 in TTBS, for 1 h at room temperature or overnight at 4 °C. Immunoblots were washed with TTBS and incubated with anti-rabbit IgG linked to horseradish peroxidase in TTBS for 1 h at room temperature. Immunodetection of I-1 was carried out using the ECL system (Amersham) or the color reaction developed with 4-chloro-1-naphthol and H_2O_2.

RESULTS

Molecular Cloning of Human Brain I-1 cDNA. A total of 900 000 recombinants were screened from a human brain cDNA library, and 14 positive clones were obtained. Eight of these were further purified through secondary and tertiary screens, and all contained an *Eco*RI insert of approximately 700 bp. Three independent clones were sequenced in both directions and yielded the identical nucleotide sequence that terminated with the 3′-poly(A)$^+$ tail. The cDNA predicted a polypeptide of 171 amino acids with 85% overall sequence identity to rabbit (Aitken et al., 1982) and rat I-1 (Elbrecht et al., 1990) (Figure 1). C-Terminal sequences of the 3 I-1 proteins showed considerable differences (37% identity in the last 51 residues). Indeed, rat and human I-1 contained a five amino acid insert near the C-terminus not found in

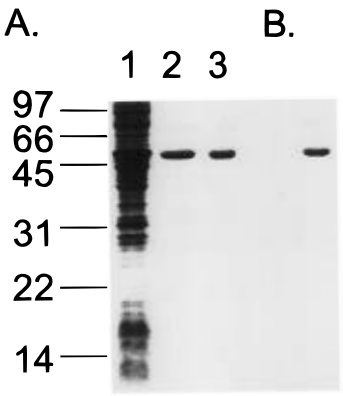

FIGURE 2: Purification of recombinant human inhibitor-1. *E. coli* (BL21) were transformed with plasmid pGEX-2T-hI-1, and GST−I-1 expression was induced with IPTG. Panel A shows SDS−PAGE of total bacterial extract (40 μg of total protein) in lane 1; lane 2, GST−I-1 (1 μg) after affinity chromatography on glutathione−Sepharose; lane 3, GST−I-1 (1 μg) after preparative SDS−PAGE. Phosphorylase *b* (M_r 97 400), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) were used as molecular weight markers, and the proteins were stained with Coomassie Blue. Panel B shows an autoradiogram of GST−I-1 phosphorylated with PKA and [γ-^{32}P]ATP−Mg.

rabbit I-1. This further highlighted the remarkable conservation of the N-terminal region (97% identity in the first 58 residues) containing the threonine phosphorylated by PKA (Cohen et al., 1977).

Characterization of Recombinant Human I-1. Maximum expression of GST−I-1 (apparent molecular mass 47 kDa on SDS−PAGE) in several strains of *E. coli* was obtained by 2−3 h growth in media containing IPTG. The fusion protein was rapidly purified from bacterial extracts by affinity chromatography on glutathione−Sepharose (Figure 2A, lane 2). This was followed by preparative SDS−PAGE which purified GST−I-1 to homogeneity (Figure 2A, lane 3). The fusion protein was phosphorylated by PKA (Figure 2B) to a stoichiometry of 1 mol of phosphate/mol of protein, and phosphoamino acid analysis established that the modification occurred exclusively on threonine (data not shown). For most studies, we expressed GST−I-1 in the *E. coli* BL21 strain that showed significantly reduced degradation of the fusion protein. A typical 250 mL culture of BL21 bacteria yielded 7−9 mg of GST−I-1.

GST−I-1 was digested with thrombin, and I-1 was purified by preparative SDS−PAGE. Human I-1 migrated on SDS−PAGE with an apparent molecular mass of 28 kDa, slightly larger than the rabbit skeletal muscle I-1 (Figure 3A), and readily cross-reacted with a polyclonal antibody generated against the rabbit I-1 (Figure 3B). Mass spectrometry of the recombinant human I-1 confirmed its molecular size at 19 179 Da, precisely as predicted by the cDNA. Thus, the anomalous electrophoretic mobility of rabbit, rat, and human I-1 on SDS−PAGE most likely reflects low detergent binding (Nimmo & Cohen, 1978a).

Following stoichiometric phosphorylation (1 mol of phosphate/mol of protein) by PKA, GST−I-1 inhibited PP1 activity with an IC_{50} of approximately 30 nM (Table 1). More than 1000-fold higher concentration of phosphorylated GST−I-1 was required to inhibit PP2A. Thrombin cleavage yielded free human I-1 and increased its potency as a PP1 inhibitor to that of I-1 purified from rabbit skeletal muscle (IC_{50} 1 nM). Phosphorylated rabbit and human I-1 also inhibited

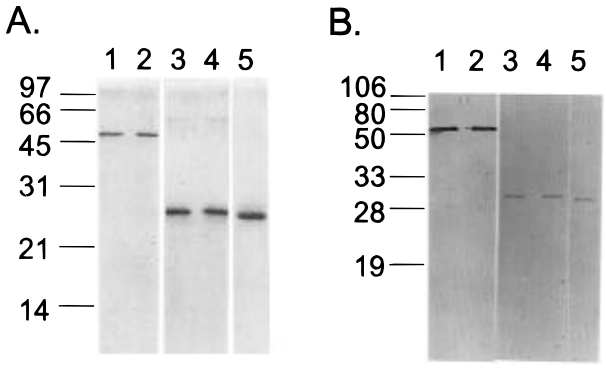

FIGURE 3: Purification of wild-type and mutant inhibitor-1. Wild-type GST−I-1 and GST−I-1(T35A) were purified from bacterial extracts by affinity chromatography on glutathione−Sepharose. The fusion proteins were digested with thrombin, and wild-type and mutant I-1 were purified on preparative SDS−PAGE. Panel A shows SDS−PAGE of the fusion proteins [wild-type GST−I-1 (lane 1), GST−I-1(T35A) (lane 2), I-1 wild-type (lane 3), and mutant (lane 4)] obtained from thrombin digestion of the fusion proteins. Lane 5 shows rabbit skeletal muscle I-1. Proteins were stained with Coomassie Blue. Panel B: Immunoblot of recombinant human I-1 proteins with a polyclonal antibody generated against rabbit I-1. Lane 1, wild-type GST−I-1; lane 2, GST−I-1(T35A); lane 3, wild-type I-1; lane 4, I-1(T35A); lane 5, rabbit skeletal muscle I-1. Prestained molecular weight markers [phosphorylase *b* (M_r 106 000), bovine serum albumin (M_r 80 000), ovalbumin (M_r 49 500), carbonic anhydrase (M_r 32 500), soybean trypsin inhibitor (M_r 27 500), and lysozyme (M_r 18 500)] were obtained from BioRad.

Table 1: Inhibition of Type-1 and Type-2 Phosphatases[a]

	IC_{50} (nM)	
inhibitor	PP1	PP2A
rabbit skeletal muscle I-1	1.1 ± 0.21	21000 ± 2200
human (recombinant)		
GST−I-1	32 ± 2.4	32000 ± 1500
I-1(−3−171)	0.98 ± 0.15	25000 ± 1300
I-1(−3−61)	3.7 ± 0.14	>10000
I-1(9−61)	4.5 ± 0.51	>10000

[a] Rabbit skeletal muscle I-1 was purified according to Cohen et al. (1988). Human I-1 fused to GST was expressed in *E. coli*. The fusion protein was digested with thrombin, and the I-1 proteins and peptides were purified to homogeneity as described under Materials and Methods. I-1 was phosphorylated with PKA prior to analyzing the inhibition of purified PP1 and PP2A catalytic subunits using [^{32}P]phosphorylase *a* as substrate. IC_{50} values were calculated from an average of five independent experiments. I-1 peptide a (−3−61) and peptide b (9−61) were analyzed in three and two separate experiments, respectively. All values are presented with standard errors.

PP2A, albeit at concentrations exceeding 20 μM. Neither PP1 nor PP2A was inhibited by dephosphorylated rabbit and human I-1 (data not shown). Thus, the overall functional properties of recombinant human I-1 were similar to I-1 purified from tissues.

When pGEX-2T-hI-1 was transformed in *E. coli* JM109 or DH5α and the bacteria were grown at 37 °C in the presence of IPTG, a significantly smaller fusion protein was expressed. Chromatography of JM109 or DH5α extracts on glutathione−agarose yielded a polypeptide of apparent molecular mass 37 kDa by SDS−PAGE. Following thrombin digestion, two peptides of apparent mass 14 and 12 kDa were obtained (Figure 4A). Both were readily phosphorylated by PKA, and the mixture of phosphopeptides inhibited PP1. Thus, the individual I-1 peptides (a and b) were purified by preparative SDS−PAGE and reversed-phase HPLC. Peptide a yielded the N-terminal sequence GSPMEQDNSRKIQF and corresponded to the N-terminus of human I-1 with the additional three amino acids derived from the pGEX-2T linker (Figure 4C). Peptide b had the sequence KIQFTVPLLEPHLDP and represented cleavage between residues 8 and 9 in human I-1. The molecular mass for peptides *a* and *b* was estimated by mass spectrometry as 7265.82 and 6066.38 Da, respectively (Figure 4C). This suggested that both peptides terminated at lysine-61, which was confirmed by digesting peptides a and b with *Staphylococcus* V8 protease and chromatography on anhydrotrypsin−Sepharose. The predicted C-terminal lysine-containing peptides were eluted from the affinity matrix and further purified by HPLC. In both cases, a major peptide (Figure 4B) with the sequence DRIPNPHLK was obtained. This established that peptides a and b terminated at lysine-61 and molecular weights calculated from their amino acid sequence precisely matched those determined by mass spectrometry (Figure 4C).

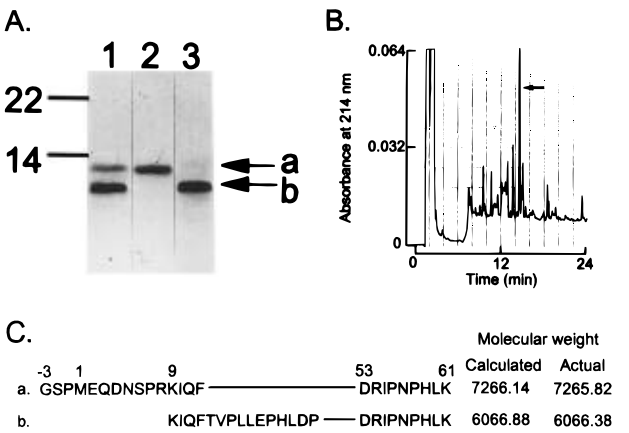

FIGURE 4: Characterization of inhibitor-1 peptides. GST−I-1 was expressed in *E. coli* JM109 at 37 °C. Panel A shows an SDS−PAGE of two I-1 peptides obtained by thrombin cleavage of the 37 kDa fusion protein (lane 1). Peptides a (lane 2) and b (lane 3) were individually purified by preparative SDS−PAGE and reversed-phase HPLC. To determine their C-termini, each peptide was digested with endoproteinase Glu-C and subjected to affinity chromatography on anhydrotrypsin−Sepharose. Peptides eluted from this affinity matrix were further purified by reversed-phase HPLC (panel B). A single major peptide (marked by arrow) was obtained from peptide a and peptide b. Panel C shows N- and C-terminal sequences (in capital letters) obtained from each I-1 peptide. Molecular weights of the peptides calculated from their amino acid sequence matched their mass determined by mass spectrometry.

Following PKA phosphorylation, peptide a or I-1(−3−61) inhibited PP1 with an IC_{50} of 3.7 nM, and peptide b or I-1(9−61) inhibited with an IC_{50} of 4.5 nM. Thus, the two N-terminal peptides of human I-1 were nearly as potent as full-length I-1 in inhibiting PP1 activity. Neither phosphopeptide inhibited PP2A activity at the highest concentration examined (10 μM).

Mutagenesis of the Phosphoacceptor Site at Threonine-35. Site-directed mutagenesis was used to substitute a nonphosphorylated residue, alanine, in place of the phosphoacceptor at threonine-35 and establish the role of PKA phosphorylation for I-1 function. GST−I-1(T35A) expressed in *E. coli* BL21 was purified to homogeneity (Figure 3A), using immunoblotting with the polyclonal antibody against rabbit I-1 to monitor the purification. As anticipated, GST−I-1(T35A) was not phosphorylated by PKA and at 50 μM, the highest concentration of the fusion protein that did not

Table 2: Mutations of the Phosphoacceptor Site in Human Inhibitor-1[a]

inhibitor-1	IC_{50} (nM) PP1	PP2A
GST−I-1(T35A)	inactive[b]	inactive[b]
I-1(−3−171, T35A)	inactive[b]	inactive[b]
GST−I-1(T35D)	30000 ± 2300	44000 ± 3500
I-1(−3−171, T35D)	24000 ± 2100	25000 ± 2400
CNBr−GST−I-1(T35D)	1700 ± 1100	20000 ± 1900
I-1(2−65, T35D)	235 ± 72[c]	>10000[c]

[a] Site-directed mutagenesis was used to substitute alanine or aspartic acid in place of threonine-35. Mutants GST−I-1(T35A) and GST−I-1(T35D) were expressed in *E. coli* and purified as described under Materials and Methods. I-1 proteins and peptides were analyzed for inhibition of PP1 and PP2A catalytic subunits. Each result represents an average of three independent experiments and is shown with standard errors. [b] Inactive is defined as the inability of the mutant I-1 to inhibit the phosphatases at 50 μM. [c] The HPLC-purified I-1(2−65, T35D) was assayed in triplicate.

aggregate during the assay, GST−I-1(T35A) had no effect on PP1 or PP2A activity (Table 2). Thrombin digestion of the fusion protein and subsequent purification of I-1(T35A) further confirmed that the mutant protein was inactive as a phosphatase inhibitor. These data established that PKA phosphorylation of threonine-35 was essential for I-1 to inhibit PP1 at nanomolar concentrations and PP2A at micromolar concentrations.

To mimic the functional effects of phosphorylation, we substituted an aspartic acid in place of the phosphoacceptor. In contrast to GST−I-1(T35A) that at 50 μM did not inhibit PP1 or PP2A, GST−I-1(T35D) was more effective and a constitutive inhibitor of these phosphatases (Table 2). However, the efficacy of GST−I-1(T35D) as a PP1 inhibitor (IC_{50} 30 μM) was very similar to PP2A (IC_{50} 44 μM). Following thrombin cleavage, the purified I-1(T35D) no longer distinguished between PP1 and PP2A (IC_{50} 24−25 μM). Earlier studies (Shenolikar et al., 1978) used CNBr cleavage to isolate an active phosphopeptide of rabbit I-1. CNBr cleavage of GST−I-1(T35D) increased its potency as a PP1 inhibitor. HPLC-purified I-1(2−65, T35D) inhibited PP1 with an IC_{50} of 235 nM, an 100-fold increase over the activity of the parent I-1(T35D) protein. Moreover, the I-1(2−65, T35D) peptide at 10 μM completely inhibited PP1 but only resulted in a 10% decrease in PP2A activity. These data provided new insight into the conformation of intact I-1 induced by phosphorylation that yields a potent and selective PP1 inhibitor. Moreover, functions associated with this phosphorylation-induced conformation were not changed by truncation to the N-terminal functional domain. By comparison, substitution of an acidic residue in place of the phosphoacceptor had different functional effects in the context of the full-length I-1 and the N-terminal peptide.

Circular Dichroism Analysis of Recombinant I-1 Protein and Peptides. Earlier studies (Cohen et al., 1979) used circular dichroism to show that I-1 isolated from boiled skeletal muscle extracts was largely disordered in structure. PKA phosphorylation activated the rabbit I-1 as a PP1 inhibitor but had no effect on its limited secondary structure that could be discerned by denaturation with 6 M guanidine hydrochloride. Circular dichroism showed that recombinant human I-1 was also largely disordered in solution. Stoichiometric phosphorylation did not result in a significant change in its CD spectrum (data not shown). Comparison of spectra

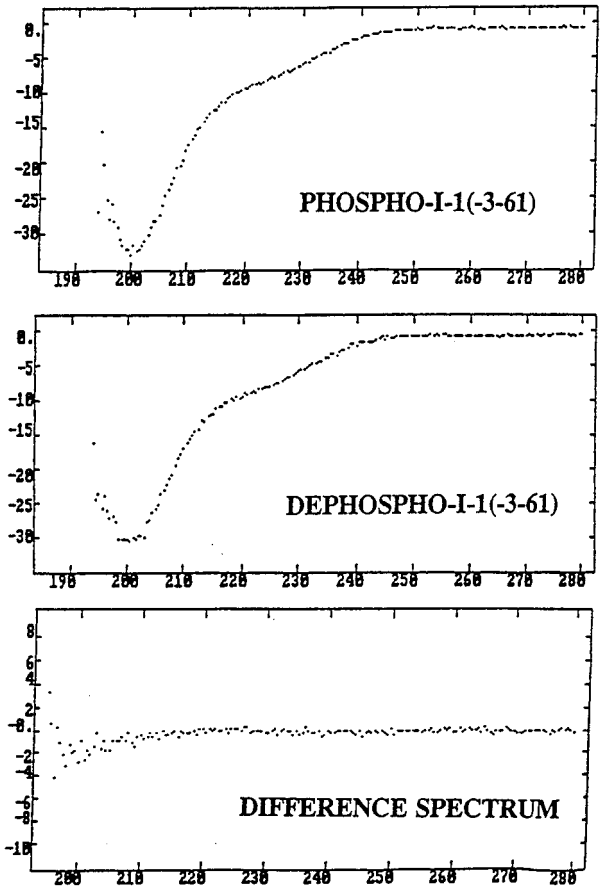

FIGURE 5: Circular dichroism of an active inhibitor-1 peptide. The spectra obtained for the I-1(−3−61) peptide in its active or phosphorylated state (top panel) and in its inactive or unphosphorylated state (middle panel) at 25 °C in PBS are shown. The absorbance due to buffer alone was subtracted in each case. The difference spectrum that indicates the change in secondary structure resulting from PKA phosphorylation of I-1(−3−61) is also shown (bottom panel).

at temperatures from 10 to 50 °C showed that the higher temperatures increased in the extent of random coil and established that both phosphorylated and unphosphorylated I-1 contained limited secondary structure. However, no difference in structure could be attributed to covalent modification (data not shown). Surprisingly, I-1(−3−61) that eliminated 110 residues from the C-terminus of I-1 protein was also largely disordered (Figure 5). Moreover, like full-length I-1, the overall secondary structure of the functional domain represented in I-1(−3−61) was unchanged by phosphorylation.

Association of I-1 with PP1 Catalytic Subunit. To investigate the binding of phosphorylated and unphosphorylated I-1 to purified PP1 and PP2A catalytic subunits, we used the Fisons IAsys optical biosensor system. A polyclonal anti-GST antibody was covalently coupled to the biosensor surface and used to subsequently immobilize either unphosphorylated or phosphorylated GST−I-1. Excess fusion protein was removed with PBS−Tween. The time-dependent increase in the evanescent field response following the binding of purified PP1 catalytic subunit (10 μg of protein) to the immobilized GST−I-1 saturated at a value 8−10-fold higher than the maximum response evoked by a similar amount of PP2A catalytic subunit (Figure 6). This suggested that, compared to PP1, less than 10% of PP2A bound to the immobilized GST−I-1. Neither PP1 nor PP2A bound to the

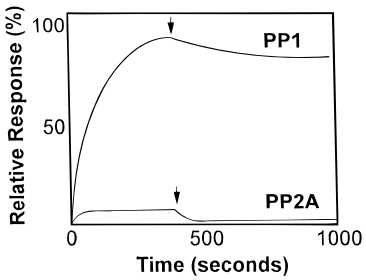

FIGURE 6: Inhibitor-1 binding to protein phosphatases. Polyclonal antibody against *Schistosome* GST was covalently immobilized to the biosensor surface in a IAsys cuvette. GST−I-1 was bound to the immobilized antibody, and PP1 or PP2A catalytic subunits (10 μg of protein) were applied. The time-dependent change in the evanescent field was monitored by the Fisons IAsys optical biosensor system. The figure shows a representative profile of the relative change in response elicited by PP1 and PP2A binding to the same surface containing dephosphorylated GST−I-1. The arrow marks the point at which the cuvette was washed with PBS−Tween. The dissociation constants were calculated using an average of 260 data points and were corrected for the very slow dissociation of GST−I-1 from the anti-GST antibody. k_{off} (s^{-1}) for PP1: dephosphorylated GST−I-1, $3.70 \times 10^{-3} \pm 1.30 \times 10^{-4}$; phosphorylated GST−I-1, $2.08 \times 10^{-3} \pm 6.61 \times 10^{-5}$; k_{off} (s^{-1}) for PP2A: dephosphorylated GST−I-1, $1.51 \times 10^{-1} \pm 5.94 \times 10^{-2}$; phosphorylated GST−I-1, $1.90 \times 10^{-1} \pm 1.88 \times 10^{-2}$.

biosensor surface when GST alone was immobilized, indicating that the association of the phosphatases was mediated through I-1. PP1 dissociated slowly from GST−I-1. While phosphorylation of GST−I-1 increased its phosphatase inhibitor activity by 1000-fold, it slowed the dissociation of the PP1 catalytic subunit by only 2-fold. The K_{off} values for the PP2A catalytic subunits from phosphorylated and unphosphorylated GST−I-1 were approximately 90- and 40-fold faster than those for PP1. The slow off-rate of the PP1 catalytic subunit may be an important determinant of I-1's specificity as a phosphatase inhibitor. However, the biosensor studies showed that PP1 binding was largely independent of I-1's phosphorylation state and by itself may not account for inhibition of this phosphatase.

An N-Terminal Truncation Inactivates I-1. Earlier studies (Aitken & Cohen, 1984) used proteolysis of rabbit I-1 to isolate the "minimal" active fragment, I-1(9−54). This sequence was entirely conserved in human I-1 and also shared homology with DARPP-32, a neuronal cAMP-regulated phosphoprotein that also inhibited PP1 (Figure 7). I-1(9−54) was expressed in *E. coli* N4830 as a fusion protein with the IgG-binding domain of *Staphylococcus aureus* protein A (described under Materials and Methods). The fusion protein was purified on IgG−Sepharose but was not phosphorylated *in vitro* by PKA. Thus, the ability of the fusion protein to inhibit PP1 activity could not be analyzed. Digestion with factor Xa at a unique site constructed in the linker yielded the I-1(9−54) peptide with two additional N-terminal residues. This peptide was readily phosphorylated by PKA and following phosphorylation inhibited PP1 activity in a dose-dependent manner (Figure 7). As expected, the unphosphorylated peptide was an ineffective PP1 inhibitor. *Eco*RI−*Kpn*I cleavage deleted the nucleotide sequence encoding the tetrapeptide, KIQF, conserved in I-1 and DARPP-32. Following cleavage of the altered fusion protein with factor Xa, I-1(13−54) with four additional N-terminal residues was obtained. I-1(13−54) was phosphorylated by PKA with similar efficiency to I-1(9−54), but the resulting

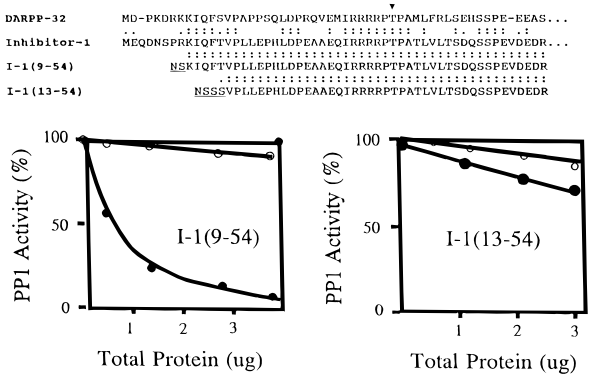

FIGURE 7: N-Terminal truncation inactivates an inhibitor-1 peptide. Synthetic gene encoding I-1(9−54) was expressed in *E. coli* N4830 as a fusion protein with *Staphylococcus aureus* protein A. The fusion protein was purified on IgG−Sepharose, digested with factor Xa, and rechromatographed on IgG−Sepharose to yield I-1(9−54) peptide. Parallel incubations (60 min at 37 °C) containing the peptide (25 μg) and the catalytic subunit of PKA (5 μg) were carried out with (solid circles) and without 1 mM ATP and 10 mM $MgCl_2$ (open circles). Reactions were terminated by heating at 95 °C for 10 min, and unphosphorylated and phosphorylated I-1(9−54) was assayed for PP1 inhibition (left panel). *Eco*RI−*Kpn*I digestion excised a short 5′-sequence to generate a synthetic gene that expressed protein A fused I-1(13−54). The fusion protein was expressed, purified, and digested with factor Xa to yield I-1(13−54). The peptide was incubated with PKA in the presence (solid circles) and absence of ATP−Mg (open circles) and subsequently assayed for PP1 inhibition (right panel). The alignment of amino acid sequences for I-1(9−54) and I-1(13−54) with regions of homology between I-1 and DARPP-32 is shown. Threonine phosphorylated by PKA is indicated by the arrowhead. Additional amino acids derived from the linker are underlined.

phosphopeptide was a very poor inhibitor of PP1 activity (Figure 7). This suggested that the KIQF sequence was required in addition to phosphorylation of threonine-35 for PP1 inhibition.

We immobilized protein A-I-1(9−54) to the biosensor surface via the anti-GST antibody. However, no significant binding of PP1 or PP2A catalytic subunits was observed to this fusion protein. Hence, the fusion of I-1(9−54) to protein A not only impaired its phosphorylation by PKA but also prevented PP1 binding.

DISCUSSION

A cDNA encoding I-1 from a human brain library was isolated. The 171 amino acids predicted by the cDNA shared extensive homology with rat (Elbrecht et al., 1990) and rabbit I-1 (Aitken et al., 1982). Bacterially expressed human I-1 was readily phosphorylated by PKA and following phosphorylation inhibited PP1 activity (IC_{50} 1 nM) in a manner identical to I-1 purified from rabbit skeletal muscle. With the availability of recombinant protein, we established that high concentrations of phospho-I-1 also inhibited PP2A (IC_{50} 25 μM), the major type-2 phosphatase in mammalian tissues. However, as the highest level of I-1 reported in mammalian tissues was 1 μM (Nimmo & Cohen, 1978a), even if fully phosphorylated, I-1 is likely to function solely as a PP1 inhibitor in intact cells.

DARPP-32, a functional homolog of I-1, inhibited PP1 activity in both its phosphorylated (IC_{50} 2 nM) and its unphosphorylated form (IC_{50} 1 μM) (Desdouits et al., 1995). Indeed, it has been suggested that in neurons containing high levels of DARPP-32, the dephosphoprotein may also sup-

press PP1 function. We substituted a nonphosphorylated residue, alanine, in place of threonine-35 in human I-1. This not only abolished I-1 phosphorylation by PKA but the mutant I-1(T35A) at concentrations up to 50 μM failed to inhibit either PP1 or PP2A activity. These data established that I-1 has a strict requirement for PKA phosphorylation for phosphatase inhibitor activity.

Substituting a glutamic acid in place of the threonine phosphorylated in DARPP-32 produced a mutant that was identical to the unphosphorylated wild-type DARPP-32 as a PP1 inhibitor (Desdouits et al., 1995). By comparison, substitution of an aspartic acid in place of threonine-35 activated I-1. To our surprise, I-1(T35D) was equally effective in inhibiting PP1 and PP2A activity. However, cleavage of I-1(T35D) to yield the N-terminal peptide, I-1(2−65, T35D), increased its potency and specificity as a PP1 inhibitor. This suggested the presence of autoinhibitory interactions between the N-terminal functional domain and C-terminal sequences that masked the specificity of I-1(T35D) as a PP1 inhibitor. Disruption of these autoinhibitory interactions by CNBr cleavage produced a "constitutive" peptide inhibitor of PP1, that can be used to investigate the physiological functions of PP1 in the intact cell (Hagiwara et al., 1992; Alberts et al., 1994). The data also argued that phosphorylation induced a unique conformation of I-1 protein that selectively recognized PP1.

Circular dichroism studies showed that the functional domain encompassing the I-1(−3−61) peptide was largely disordered in structure. Moreover, no change of its structure was observed following its phosphorylation by PKA. We hypothesized that association with the PP1 catalytic subunit may induce the "active" conformation of I-1 and promote enzyme inhibition by the phosphorylated I-1. Using a biosensor, we demonstrated that PP1, but not PP2A, bound tightly to I-1. While these data indicated a potential mechanism underlying I-1's specificity as a PP1 inhibitor, PP1 "docking" occurred with both phosphorylated (active) and unphosphorylated (inactive) I-1 and did not account for the orders of magnitude difference in their efficacy as PP1 inhibitors. The mutant I-1(T35D) also preferentially associated with the PP1 catalytic subunit (data not shown). Thus, autoinhibition that decreased the potency and specificity of I-1(T35D) as a PP1 inhibitor was not mediated by altered PP1 binding. We speculated that PP1 binding at the putative "docking site" and a unique conformation induced by phosphorylation at threonine-35 facilitated other interactions between the phosphorylated I-1 and PP1. These may include the association of the I-1 phosphorylation sequence with the PP1 catalytic site and account for enzyme inhibition. The three-dimensional structure of the PP1α catalytic subunit has indicated numerous potential interactions between a dodecapeptide representing the I-1 phosphorylation sequence and the PP1 catalytic site (Goldberg et al., 1995). This model proposed highly selective interactions between the four arginines preceding the phosphothreonine in I-1 and residues in an "acidic groove" in the PP1 catalytic subunit. Interactions of hydrophobic residues that immediately follow the phosphothreonine were proposed with a "hydrophobic groove" in the PP1 catalytic subunit. The specificity of these numerous interactions may increase the stability of PP1 binding to phosphorylated I-1 sufficiently to explain earlier observations that PP1 selectively bound to an affinity matrix containing phosphorylated I-1 (Ingebritsen & Ingebritsen, 1989). Negligible dephosphorylation of synthetic phosphopeptides modeled on I-1 (Pinna & Donella-Deana, 1994) also argued that the inability to dephosphorylate I-1 may be an important factor in PP1 inhibition. However, I-1 phosphopeptides that retained structural features required for binding at the PP1 catalytic site were ineffective inhibitors (Nimmo & Cohen, 1978b). This suggested that other interactions between PP1 and I-1, such as those involving a "docking" site, were also critical for I-1 function. DARPP-32 peptides (Hemmings et al., 1990) and proteolytic fragments of rabbit I-1 (Aitken & Cohen, 1984) that inhibited PP1 had suggested a shared functional domain. This was confirmed by purified N-terminal peptides of human I-1 that potently inhibited PP1 activity (IC_{50} 4 nM). The functional domain encompassed by these peptides appeared to contain the key structural elements that define I-1's potency and specificity as a PP1 inhibitor. A synthetic gene was constructed to express I-1(9−54), the "minimal" active fragment identified by proteolysis of rabbit I-1 (Aitken & Cohen, 1984). The recombinant I-1(9−54) peptide showed a phosphorylation-dependent inhibition of PP1 activity. An N-terminal deletion that significantly decreased the inhibitory activity of this peptide identified the conserved sequence, KIQF, as necessary for PP1 inhibition. As the KIQF sequence is located some distance from the phosphothreonine, it is unlikely to bind within the PP1 catalytic site and may represent the proposed "docking" site. Alternately, removal of KIQF could result in structural changes in I-1 that abrogate its binding to PP1 via one or more sites and decrease inhibitor activity.

In summary, we have shown that recombinant human I-1 possessed identical biochemical properties to the protein isolated from tissues. Our studies showed that I-1 phosphorylation increased PP1 inhibitor activity by several orders of magnitude but did not result in a stable conformational change detectable by circular dichroism. Elimination of autoinhibitory interactions between the functional domain and undefined C-terminal sequences following I-1 phosphorylation may also define its specificity for PP1. We have identified two independent structural elements in I-1, an N-terminal sequence represented by KIQF and the phosphorylation of threonine-35, that together determine its potency and selectivity as a PP1 inhibitor. More detailed structure−function studies with recombinant I-1 proteins and peptides should provide clearer understanding of the mode of action I-1 as a physiological regulator of the type-1 protein phosphatase.

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REFERENCES

Aitken, A. A., & Cohen, P. (1984) *FEBS Lett. 147,* 54−58.

Aitken, A. A., Bilham, T., & Cohen, P. (1982) *Eur. J. Biochem. 126,* 235−246.

Alberts, A. S., Montminy, M., Shenolikar, S., & Feramisco, J. R. (1994) *Mol. Cell. Biol. 14*, 4398−4407.
Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38*, 299−308.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Cohen, P. (1988) *Proc. R. Soc. London 234*, 115−144.
Cohen, P. (1989) *Annu. Rev. Biochem. 58*, 453−508.
Cohen, P., Duewer, T., & Fischer, E. H. (1971) *Biochemistry 10*, 2683−2694.
Cohen, P., Rylatt, D. B., & Nimmo, G. A (1977) *FEBS Lett. 76*, 182−186.
Cohen, P., Nimmo, G. A., Shenolikar, S., & Foulkes, J. G. (1979) *FEBS Symp. 54*, 161−169.
Cohen, P., Foulkes, J. G., Holmes, C. F. B., Nimmo, G. A., & Tonks, N. K. (1988) *Methods Enzymol. 159*, 427−437.
DeGuzman, A., & Lee, E. Y. C. (1988) *Methods Enzymol. 159*, 356−368.
Desdouits, F., Cheetham, J. J., Huang, H. B., Kwon, Y. G., da Cruz e Silva, E. F., Denefle, P., Ehrlich, M. E., Nairn, A. C., Greengard, P., & Girault, J. A. (1995) *Biochem. Biophys. Res. Commun. 206*, 652−658.
Elbrecht, A., DiRenzo, J., Smith, R. G., & Shenolikar, S. (1990) *J. Biol. Chem. 265*, 13415−13418.
Foulkes, J. G., & Cohen, P. (1981) *Eur. J. Biochem. 97*, 251−256.
Foulkes, J. G., Cohen, P., Strada, S. J., Everson, W. V., & Jefferson, L. S. (1982) *J. Biol. Chem. 257*, 12493−12496.
Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A. C., & Kuriyan, J. (1995) *Nature 376*, 745−753.
Hagiwara, M., Brindle, P., Alberts, A., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., & Montminy, M. (1992) *Cell 70*, 105−113.
Hemmings, H. C., Nairn, A. C., Elliot, J. I., & Greengard, P. (1990)- *J. Biol. Chem. 265*, 20369−20376.
Huang, F. L., & Glinsmann, W. H. (1976) *Eur. J. Biochem. 70*, 419−426.
Ingebritsen, T. S., & Cohen, P. (1983) *Science 221*, 331−338.
Ingebritsen, T. S., Stewart, A. A., & Cohen, P. (1983) *Eur. J. Biochem. 132*, 297−307.
Ingebritsen, V. M., & Ingebritsen, T. S. (1989) *Biochim. Biophys. Acta 1012*, 1−4.
Ishii, S., Yokosawa, H., Kumazaki, T., & Nakamura, I. (1983) *Methods Enzymol. 91*, 378−383.
Khatra, B. S., Chiasson, J. L., Shikama, H., Exton, J. H., & Soderling, T. R. (1980) *FEBS Lett. 114*, 253−256.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
MacDougall, L. K., Campbell, D. G., Hubbard, M. J., & Cohen, P. (1989) *Biochim. Biophys. Acta 1010*, 218−226.
Mulkey, R. M., Endo, S., Shenolikar, S., & Malenka, R. C. (1994) *Nature 369*, 486−488.
Nemenoff, R. A., Blackshear, P. J., & Avruch, J. (1983) *J. Biol Chem. 258*, 9437−9443.
Neumann, J., Gupta, R. C., Schmitz, W., Scholz, H., Nairn, A. C., & Watanabe, A. M. (1991) *Circ. Res. 69*, 1450−1457.
Nimmo, G. A., & Cohen, P. (1978a) *Eur. J. Biochem. 87*, 341−351.
Nimmo, G. A., & Cohen, P. (1978b) *Eur. J. Biochem. 87*, 353−365.
Pinna, L. A., & Donella-Deana, A. (1994) *Biochim. Biophys. Acta 1222*, 415−431.
Shenolikar, S. (1994) *Annu. Rev. Cell Biol. 10*, 55−86.
Shenolikar, S., & Ingebritsen, T. S. (1984) *Methods Enzymo*l. *107*, 102−129.
Shenolikar, S., & Nairn, A. C. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 1−121.
Shenolikar, S., Foulkes, J. G., & Cohen, P. (1978) *Biochem. Soc. Trans. 6*, 935−937.
Snyder, G. L., Girault, J. A., Chen, J. Y., Czernik, A. J., Kebabian, J. W., Nathanson, J. A., & Greengard, P. (1992) *J. Neurosci. 12*, 3071−3083.
Tartof, K. P., & Hobbs, C. A. (1987) *BRL Focus 9*(2), 12−14.
Towbin, H., & Gordon, J. (1984) *J. Immunol. Methods 72*, 313−340.
Williams, K. R., Hemmings, H. C., LoPresti, M. B., Konigsberg, W. H., & Greengard, P. (1986) *J. Biol. Chem. 261*, 1890−1903.
Yokosawa, H., & Ishii, S. (1976) *Biochem. Biophys. Res. Commun. 72*, 1443−1449.

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