Phosphorylation of Protein Phosphatase Inhibitor-1 by Cdk5*

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Protein phosphatase inhibitor-1 is a prototypical mediator of cross-talk between protein kinases and protein phosphatases. Activation of cAMP-dependent protein kinase results in phosphorylation of inhibitor-1 at Thr-35, converting it into a potent inhibitor of protein phosphatase-1. Here we report that inhibitor-1 is phosphorylated in vitro at Ser-67 by the proline-directed kinases, Cdk1, Cdk5, and mitogen-activated protein kinase. By using phosphorylation state-specific antibodies and selective protein kinase inhibitors, Cdk5 was found to be the only kinase that phosphorylates inhibitor-1 at Ser-67 in intact striatal brain tissue. In vitro and in vivo studies indicated that phoso-Ser-67 inhibitor-1 was dephosphorylated by protein phosphatases-2A and -2B. The state of phosphorylation of inhibitor-1 at Ser-67 was dynamically regulated in striatal tissue by glutamate-dependent regulation of N-methyl-D-aspartic acid-type channels. Phosphorylation of Ser-67 did not convert inhibitor-1 into an inhibitor of protein phosphatase-1. However, inhibitor-1 phosphorylated at Ser-67 was a less efficient substrate for cAMP-dependent protein kinase. These results demonstrate regulation of a Cdk5-dependent phosphorylation site in inhibitor-1 and suggest a role for this site in modulating the amplitude of signal transduction events that involve cAMP-dependent protein kinase activation.

Control of protein phosphorylation/depolyphorylation occurs through regulation of protein kinase and protein phosphatase activities and is an integral component of intracellular signal transduction. Inhibitor-1 was the first endogenous molecule found to regulate protein phosphatase activity (1). Inhibitor-1 purified from rabbit skeletal muscle is an 18,700-kDa acid- and heat-stable protein composed of 166 amino acids that are highly conserved throughout phylogeny (2, 3). When phosphorylated at Thr-35 by cAMP-dependent protein kinase (PKA), inhibitor-1 selectively and potently inhibits type 1 protein phosphatase (protein phosphatase-1, PP-1) with an IC_{50} value of ~1 nM (4–7). Phospho-Thr-35 inhibitor-1 is dephosphorylated by Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP-2B, calcineurin) and protein phosphatase 2A (PP-2A), with PP-2B activity predominating in the presence of Ca^{2+} (8–11). First messengers such as neurotransmitters (e.g. dopamine and acetylcholine) and hormones (e.g. adrenaline) that elevate intracellular cAMP levels promote PKA-dependent phosphorylation of inhibitor-1 at Thr-35 in various tissues. PP-1 inhibition by phospho-Thr-35 inhibitor-1 provides substantial amplification of PKA-dependent signaling cascades and modulates the intensity and duration of a number of physiological responses including regulatory aspects of the cell cycle, gene expression, carbohydrate and lipid metabolism, and synaptic plasticity (12–17).

Inhibitor-1 is widely expressed in mammalian tissue with highest levels occurring in the brain, skeletal muscle, adipose, and kidney tissues (18–26). Within the brain, the highest levels of inhibitor-1 immunoreactivity are associated with the dentate gyrus of the hippocampus and the neostriatum and substantia nigra of the basal ganglia (21). Control of PP-1 by inhibitor-1 in the hippocampus is thought to be an important component of the mechanisms underlying learning and memory, including long term potentiation and long term depression (15, 27). Mice lacking inhibitor-1 display deficits in long term potentiation induction (25).

In the dopaminergic medium spiny neurons of the striatum, inhibitor-1 is co-expressed with a homologous PP-1 inhibitor, DARPP-32 (28). Inhibitor-1 is ~10 times less abundant than DARPP-32 in the striatum (21, 29, 30), which constitutes about 0.25% of total striatal protein and is estimated to occur at a concentration of 50 μM. The NH_{2}-terminal residues 9–50 of inhibitor-1 and DARPP-32 display 60% identity (31) and phosphorylation of the homologous residue on DARPP-32 (Thr-34) converts it into an inhibitor of PP-1 with an IC_{50} value identical to that of inhibitor-1 (7). The activity of DARPP-32 is regulated through phosphorylation at other sites. Phosphorylation of Ser-102 by casein kinase 2 and Ser-137 by casein kinase 1 potentiates PKA phosphorylation and attenuates PP-2B dephosphorylation of Thr-34, respectively (32–34). Phosphorylation of DARPP-32 by cyclin-dependent kinase 5 (Cdk5) at Thr-75 prevents DARPP-32 from serving as a PKA substrate and converts...
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DARPP-32 into a competitive inhibitor of PKA (35). Following residue 50, very little primary sequence homology exists between inhibitor-1 and DARPP-32, and inhibitor-1 does not contain phosphorylation sites homologous to those found in DARPP-32 other than Thr-37. Therefore, it seemed possible that the activity of inhibitor-1 is differentially regulated by phosphorylation of other putative sites.

In this report we show that inhibitor-1 is phosphorylated on Ser-67 by MAP kinase and by two members of the cyclin-dependent protein kinase family in vitro but serves as a substrate only for Cdk5 in striatal neurons. We demonstrate that this site of phosphorylation is predominantly dephosphorylated by PP-2A and PP-2B. Kinetic analyses indicate that phosphorylation at Ser-67 reduces the ability of inhibitor-1 to serve as a substrate for PKA but has no effect on PP-1 inhibition.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Operon Technologies, Inc. Restriction and DNA-modifying enzymes and electrophoresis media were from Life Technologies, Inc. Protease inhibitors, proteolytic enzymes, dithiothreitol, and ATP were from Roche Molecular Biochemicals. [γ-32P]ATP was from PerkinElmer Life Sciences. Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. Antibodies to cyclin-dependent protein kinases were from Santa Cruz Biotechnology, Inc. Phospho-p44/42 MAP kinase antibodies were from New England Biolabs, Inc. NMDA was from Research Biochemicals. Anti-phospho-p44/42 MAP kinase antibodies were from Santa Cruz Biotechnology. Phospho-p44/42 MAP kinase antibodies were from New England Biolabs, Inc. NMDA was from Research Biochemicals. Okadaic acid and cyclosporin A were from Alexis Biochemicals. Calmodulin was purified as described previously (36). Peptides and phosphopeptides were synthesized at the Rockefeller University Protein/DNA Technology Center.

Preparation of Inhibitor-1—Inhibitor-1 was purified from rabbit skeletal muscle as reported previously (3) or purified as a recombinant protein from Escherichia coli. For recombinant inhibitor-1, a cDNA fragment encompassing the nucleotide sequence for rat inhibitor-1 (2) was cloned into pC120 subclone and expressed as a protein encoded by a gene constructed by a gene sequencing procedure. The inhibitor-1 sequence was amplified by PCR. The NheI-terminus oligonucleotide primer, 5'-GGGCCCATGATGGCCGCAAC-3', included an NcoI restriction enzyme cleavage site containing the ATG start codon (inhibitor-1 sequence underlined). The COOH-terminal primer, 5'-GGCTGCTGATGCTGATGAGTGGATGGTTGATCTCCATGGATGATGATGGCAAGTTGCTGCTAATGATGATGATGGACCAAGCTGTCGACTTCGTTGCTGGG-3', encoded 6 histidine residues, a stop codon, and an XhoI restriction enzyme site following the COOH-terminal valine codon. A PCR product of the correct size was ligated into the NcoI/XhoI restriction enzyme sites of the bacterial expression vector pET 15B (Novagen). Conservation of the inhibitor-1 ORF sequence was confirmed using recombinant rat inhibitor-1/His6. Expression of recombinant rat inhibitor-1/His6 was observed by agarose gel electrophoresis of total protein microsequencing and matrix-assisted laser desorption ionization mass spectrometry (46). For identification of the Cdk1 site, a 250-μg sample of inhibitor-1 was phosphorylated in the presence of 250-μg sample of PKA in the presence of [γ-32P]ATP in the presence of actinomycin D (1 μg/ml). Phosphorylation reactions were conducted under linear conditions as described previously (7) using recombinant rat inhibitor-1. The reaction was then subjected to proteolytic digestion with AP-2A and MAP kinase and subjected to SDS-PAGE and autoradiography. The identity of the phosphorylated serine residue was confirmed by phosphopeptide mapping and phosphoamino acid analysis.

Identification of the Phosphorylation Sites on Inhibitor-1 and Generation of Phosphorylation State-Specific Antibodies—Two-dimensional phosphopeptide map and phosphoamino acid analyses were performed as described (45). Two methods were employed for identification of the sites of phosphorylation by Cdk1 and MAP kinase. For identification of the Cdk1 site, a 250-μg sample of inhibitor-1 was phosphorylated in the presence of 250-μg sample of PKA in the presence of [γ-32P]ATP. The reaction was then subjected to SDS-PAGE and autoradiography. The identity of the phosphorylated serine residue was confirmed by phosphopeptide mapping and phosphoamino acid analysis.

To determine the site of MAP kinase phosphorylation, a smaller scale sequencing procedure was used. A 10-μg aliquot of inhibitor-1 was phosphorylated by MAP kinase in the presence of the plasma membrane fraction was then subjected to SDS-PAGE using an 15% acrylamide gel, and protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The 32P-labeled phospho-inhibitor-1 was localized by autoradiography, and the membrane was excised and subjected to proteolytic digestion with trypsin (47, 48) or trypsin (29). Peptides were excised and subjected to reversed phase HPLC using a linear gradient elution in the buffer containing 0.1% trifluoroacetic acid and increasing concentrations of acetonitrile with monitoring for absorbance at 214 nm. Eluted fractions were subjected to mass spectrometry analysis, phosphopeptide mapping, or mass spectrometry.

In Vitro Phosphorylation and Dephosphorylation Reactions—The MAP kinase reaction solution included 1 μM inhibitor-1 in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 20 mM EGTA, 200 μM ATP, and 0.6 mM dithiothreitol, and estimated to be greater than 95% pure by Coomassie Blue staining. Protein concentrations were determined by quantitative amino acid analysis.

In vitro phosphorylation reactions were conducted using recombinant GST-Cdk5 with [γ-32P]ATP. Activated MAP kinase, 1 μM, was isolated from sea star oocytes (39–41) or recombinant p34cdc2/cyclin B (New England Biolabs). Cdk5 assays were conducted using either partially purified recombinant GST-Cdk5 with [γ-32P]ATP. Phospho-inhibitor-1 was precipitated by addition of trichloroacetic acid to 20% in the presence of 1 mg/ml bovine serum albumin. Inhibitor-1 was resuspended in 1 mM Tris-HCl, pH 8, and samples were dialyzed overnight in 10 mM Hepes, pH 7.4, with two changes of buffer. Dephospho-inhibitor-1 was treated identically except for omission of protein kinase from the in vitro phosphorylation reactions. Dephospho- and phospho-Ser-67 inhibitor-1 was phosphorylated by PKA in the presence of [γ-32P]ATP, as previously described (34).

Phosphatase assays, in which soluble extracts from mouse striatal homogenates were used as the source of phosphatase, were conducted as described previously (43, 44). Strial homogenates to which 5 mM EDTA and 1 mM EGTA were added were used to observe basal levels of [γ-32P]ATP. Phospho-Ser-67 inhibitor-1 was precipitated by addition of trichloroacetic acid to 20% in the presence of 1 mg/ml bovine serum albumin. Inhibitor-1 was resuspended in 1 mM Tris-HCl, pH 8, and samples were dialyzed overnight in 10 mM Hepes, pH 7.4, with two changes of buffer. Dephospho-inhibitor-1 was treated identically except for omission of protein kinase from the in vitro phosphorylation reactions. Dephospho- and phospho-Ser-67 inhibitor-1 was phosphorylated by PKA in the presence of [γ-32P]ATP, as previously described (34).

To determine the site of MAP kinase phosphorylation, a smaller scale sequencing procedure was used. A 10-μg aliquot of inhibitor-1 was phosphorylated by MAP kinase in the presence of the plasma membrane fraction was then subjected to SDS-PAGE using an 15% acrylamide gel, and protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The 32P-labeled phospho-inhibitor-1 was localized by autoradiography, and the membrane was excised and subjected to proteolytic digestion with trypsin (47, 48) or trypsin (29). Peptides were excised and subjected to reversed phase HPLC using a linear gradient elution in the buffer containing 0.1% trifluoroacetic acid and increasing concentrations of acetonitrile with monitoring for absorbance at 214 nm. Eluted fractions were subjected to mass spectrometry analysis, phosphopeptide mapping, or mass spectrometry.
were determined to be 1.5 and 0.9 for the two kinase families.

contains consensus phosphorylation sites for proline-directed sites. The amino acid sequence of inhibitor-1 phosphorylated by Cdk1 and MAP kinase was identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown).

RESULTS

Phosphorylation of Inhibitor-1 by Proline-directed Protein Kinases—In initial experiments, phosphoamino acid analysis of radiolabeled inhibitor-1 immunoprecipitated from 32P-prelabeled striatal slices indicated that basal phosphorylation was due to the presence of phosphoserine. Furthermore, it has been reported previously that inhibitor-1 purified from rabbit skeletal muscle was phosphorylated at Ser-67 to a stoichiometry of 0.5–0.7 mol/mol (50). The amino acid sequence of inhibitor-1 contained consensus phosphorylation sites for proline-directed kinases including members of the cyclin-dependent and MAP kinase families. In vitro phosphorylation reactions indicated that inhibitor-1 was an efficient substrate for Cdk1, Cdk5, and MAP kinase. In typical experiments, inhibitor-1 isolated from rabbit skeletal muscle was phosphorylated by Cdk1 and MAP kinase to a stoichiometry of 1.15 and 0.82 mol/mol, respectively (Fig. 1, A, C, and D). Recombinant rat inhibitor-1 was phosphorylated by Cdk5 to a stoichiometry of 0.86 (Fig. 1, B and D). In kinetic studies conducted under initial rate conditions, the apparent $K_m$ values for recombinant inhibitor-1 phosphorylation by sea star Cdk1, baculovirus-derived Cdk5, and sea star MAP kinase were determined to be 11.4, 5.5, and 14.1 $\mu$M, respectively. The apparent $V_{max}$ values for Cdk1 and Cdk5 were determined to be 1.5 and 0.9 $\mu$mol/min/mg, respectively. Velocity parameters for MAP kinase were not defined due to the unknown proportion of the kinase preparation in the active state.

Identification of Phosphorylation Sites and Generation of Phosphorylation State-specific Antibodies—To determine if Cdk1 and Cdk5 phosphorylated the same site, a sample of 32P-labeled phospho-inhibitor-1 from each kinase reaction was subjected to phosphoamino acid analysis and phosphopeptide mapping (Fig. 2A). For both of these kinases, the predominant phosphorylated residue was serine. Tryptic phosphopeptide maps of rabbit inhibitor-1 phosphorylated by Cdk1 and Cdk5 generated were very similar, with two major and three minor phosphopeptides migrating to the same positions. By using conventional methodology (51), phosphopeptides corresponding to spots 1 and 3 were isolated, and microsequencing analysis indicated a single site of phosphorylation, Ser-67. These results were supported by matrix-assisted laser desorption ionization/time of flight mass spectrometry analysis of the same purified phosphopeptides (data not shown).

Phosphoamino acid analysis indicated that MAP kinase phosphorylated inhibitor-1 on serine, and phosphopeptide maps of recombinant rat inhibitor-1 phosphorylated with Cdk1 and MAP kinase were identical (data not shown). A novel methodology was used to identify the site of rat recombinant inhibitor-1 phosphorylation by MAP kinase (47) (Fig. 2B). 32P-Labeled phospho-inhibitor-1 from in vitro MAP kinase phosphorylation reaction mixtures was purified by SDS-PAGE chromatography and transferred to PVDF membrane. This material was proteolytically digested, eluted from the membrane, and subjected to small scale capillary reversed phase HPLC. Eluted peptides were directly spotted onto PVDF strips that were used to generate autoradiograms. Small pieces of PVDF containing pure radiolabeled peptides, based on A$_210$ absorbance profiles (Fig. 2B, left), were excised and subjected to mass spectrometry analysis and microsequencing (Fig. 2B, right). Analysis of the major radiolabeled peptide yielded the amino acid sequence, IPNPLLKSTSMMSPR. The predicted mass of this peptide (1654 daltons plus 16 daltons for an oxidized methionine and 80 additional Da for the PO$_4$ group) is 1750. The observed mass was determined to be 1750 by matrix-assisted laser desorption ionization/time of flight analysis. Analysis of a minor phosphopeptide yielded the same sequence minus the first isoleucine. These results indicated that Ser-67 was the single site of inhibitor-1 phosphorylated by MAP kinase.

The identity of Ser-67 as the site of phosphorylation by Cdk1, MAP kinase, and Cdk5 was confirmed using site-directed mutagenesis to generate purified recombinant Ser-67→Ala inhibitor-1. Inhibitor-1 phosphorylation by Cdk1, MAP kinase, and Cdk5 was greatly attenuated by the mutation of Ser-67 to Ala in comparison to the wild type isoform in time course in

FIG. 1. Phosphorylation of inhibitor-1 by proline-directed protein kinases. Cdk1 (A), Cdk5 (B), and MAP kinase (C) were used to phosphorylate dephospho-inhibitor-1 in in vitro time course reactions. For Cdk1 and MAP kinase, rabbit inhibitor-1 was used. For Cdk5, recombinant rat inhibitor-1 was used. The higher intensity of signal for the Cdk5 reaction is due to higher specific activity of the [γ-32P]ATP used in the reaction. The radiographic images shown in A–C were used to derive the quantified and plotted values (D).
vitro phosphorylation reactions (Fig. 3A).

To directly monitor phosphorylation in vitro and in vivo, a phosphorylation state-specific antibody was generated that detected inhibitor-1 only when phosphorylated at Ser-67 (Fig. 3B). Immuno blot analyses of time course in vitro phosphorylation reaction mixtures with Cdk1, MAP kinase, and Cdk5 demonstrated that the antibody did not detect dephospho-inhibitor-1 (0-min time point). Signal intensity increased with the period of incubation of the phosphorylation reactions. In contrast, no signal was detected at any time point if the recombinant Ser-67 → Ala mutant was used as a substrate in the reactions. Equal amounts of protein could be detected at all time points when blots were reprobed using an antibody specific for total inhibitor-1. In addition to demonstrating the specificity of the phospho-Ser-67 inhibitor-1 phosphorylation state-specific antibody, these results confirm that Cdk1, Cdk5, and MAP kinase phosphorylate inhibitor-1 at Ser-67.

Expression of Cyclin-dependent Protein Kinases in the Striatum during Development—In order to determine which of the proline-directed protein kinase/inhibitor-1 phosphorylation reactions could be physiologically relevant, an analysis was conducted with antibodies to detect various kinases, cofactors, and inhibitor-1 in the striatum. Immunoblot analysis of striatal tissue taken at different stages of development indicated that inhibitor-1 is expressed at substantial levels in adult post-mitotic striatal neurons (Fig. 4) as reported previously (18). Cdk1 is involved in cell cycle regulation and is not expressed in undifferentiated neurons. Cdk5 is a neuronal cyclin-dependent kinase that is regulated by the neuron-specific activating cofactor, p35 (52, 53). Immunoblot analysis of striatal homogenates with antibodies to Cdk1, Cdk2, Cdk4, and Cdk5 indicated that, while all three species were expressed during early stages of development, only Cdk5 was expressed in detectable levels in adult mouse striatum, as was p35 (Fig. 4). Furthermore, both ERK1 and ERK2 isoforms of MAP kinase and the upstream MAP kinase-activating kinase, MEK-1, are expressed throughout development and in adult striatum (data not shown). These results indicated that the physiologically relevant kinases that might phosphorylate Ser-67 of inhibitor-1 in adult brain are Cdk5 and/or MAP kinase.

Regulation of Phospho-Ser-67 Inhibitor-1 by Selective Protein Kinase Inhibitors in Striatal Slices—In homogenates prepared from acutely dissected striatal slices, phospho-Ser-67 inhibitor-1 was detected with the phosphorylation state-specific antibody, and the basal stoichiometry of phosphorylation was determined to be 0.34 mol/mol, based on a comparison with standard curves constructed using in vitro phosphorylated material (data not shown). To determine if MAP kinase, Cdk5, or both could be responsible for catalyzing this phosphorylation in intact neurons, striatal slices were treated with various concentrations of the selective inhibitor of MEK/MAP kinase activation, PD 98059 or the selective Cdk5 inhibitor, roscovitine. Homogenates from these slices were analyzed by immunoblotting for phospho-Ser-67 inhibitor-1, total inhibitor-1, phospho-Thr-75 DARPP-32, and phospho-Thr-202/phospho-Tyr-204 MAP kinase (Fig. 5). PD 98059 did not affect phospho-Ser-67 inhibitor-1 levels. Similarly, there was no effect upon levels of phospho-Thr-75 DARPP-32, which has previously been shown to be a Cdk5-dependent phosphorylation site (35). Phosphorylation of MAP kinase was reduced to near undetectable levels by treatment of striatal slices with 100 μM PD 98059. Conversely, treatment of slices with roscovitine caused a reduction in the levels of phospho-Ser-67 inhibitor-1 and phospho-Thr-75 DARPP-32 without affecting phospho-MAP kinase levels. These data indicate that Cdk5, but not MAP kinase, is responsible for phosphorylation of inhibitor-1 on Ser-67 in intact neurons of the adult striatum.

Dephosphorylation of Phospho-Ser-67 Inhibitor-1 in Vitro—To identify the endogenous protein phosphatase(s) responsible for dephosphorylation of phospho-Ser-67 inhibitor-1, reactions were conducted using striatal homogenate as the source of phosphatase activity and 32P-labeled phospho-Ser-67 inhibitor-1 prepared in vitro using Cdk1 (Fig. 6A). Thio-phospho-Thr-34 DARPP-32 (100 nM), a potent and selective inhibitor of PP-1 (54), had no effect on basal phosphatase activity. Selective inhibition of PP-2A activity by the addition of 1 μM okadaic acid had the same effect. Activation of PP-2B by the addition of Ca2+ and calmodulin resulted in a marked increase in

**FIG. 2. Identification of Ser-67 as the site on inhibitor-1 phosphorylated by Cdk1, Cdk5, and MAP kinase.** A, phosphoamino acid analysis and tryptic phosphopeptide maps of 32P-labeled rabbit phospho-inhibitor-1 from in vitro phosphorylation reactions using Cdk1 (left) and Cdk5 (right). For the phosphoamino acid analyses, the positions of co-migrating phosphoserine, phosphothreonine, and phosphotyrosine standards and the origin are indicated. For the phosphopeptide maps, the positions of comigrating phosphopeptides are indicated by numbers. The positions of the origins (circles, bottom center) and phenol red markers (ovals, upper left) are also indicated. B, determination of the site of inhibitor-1 phosphorylation by MAP kinase. Peaks corresponding to dye marker origins (left) are also indicated. D, site of the Ser-67 phosphorylation determined by MAP kinase are shown in alignment. Peaks corresponding to dye marker and radiolabeled peptides (filled circles) are indicated (left). Matrix-assisted laser desorption, mass spectrometry, and Edman amino acid microsequencing indicated the peptide mass and sequence shown (right), with the phosphorylated serine preceding a proline (underlined).
phosphatase activity. Activation of PP-2C by the addition of Mg\(^{2+}\) had no detectable effect upon basal phosphatase activity. These results indicate that both PP-2A and PP-2B may contribute to the dephosphorylation of phospho-Ser-67 inhibitor-1 in striatal homogenates.

**Regulation of Phospho-Ser-67 Inhibitor-1 by NMDA and Protein Phosphatase Inhibitors in Striatal Slices**—The state of phosphorylation of inhibitor-1 at Ser-67 was characterized in striatal slices prepared from wild type and genetically altered (PP-2B\(^{a2}\)/\(^2\)) mice. Phospho-Ser-67 levels were assessed under basal conditions and in response to activation of NMDA-type glutamate channels (Fig. 6B). In slices from wild type mice the basal level of phospho-Ser-67 inhibitor-1 was dramatically reduced by NMDA treatment for 5 min. The basal level was higher in the PP-2Bc\(^{-/-}\) mice. NMDA caused a reduction in the level of phosphorylation, possibly due to a Ca\(^{2+}\)-dependent activation of the \(\beta\)-isoform of PP-2B, which is expressed in these mice (56). However, detectable levels of phospho-Ser-67 inhibitor-1 remained after NMDA treatment. Total levels of inhibitor-1 were the same in control and treated slices from both wild type and PP-2Bc\(^{-/-}\) mice.

Dephosphorylation of phospho-Ser-67 inhibitor-1 was further characterized by treatment of striatal slices from wild type mice with selective protein phosphatase inhibitors (Fig. 6C). Treatment with cyclosporin A, an inhibitor of PP-2B, caused a 1.8-fold increase. We have previously reported that pretreatment of striatal slices with 1 \(\mu\)M okadaic acid inhibited PP-2A activity completely (IC\(_{50}\); 100 nM) and PP-1 activity by about 30% (43). Okadaic acid (1 \(\mu\)M) caused an increase in the level of phospho-Ser-67 inhibitor-1 (2.1-fold) similar to that seen using cyclosporin A. Together with the results from the in vitro studies, these results indicate that both PP-2A and PP-2B can dephosphorylate phospho-Ser-67 in the striatum.

**Analysis of the Effect of Phospho-Ser-67 upon Inhibitor-1 Function**—To determine the effect of phosphorylation at Ser-67 upon the ability of inhibitor-1 to function as an inhibitor of PP-1...
activity, protein phosphatase inhibition assays were performed in vitro in the presence of various concentrations of either dephospho-, phospho-Thr-35, phospho-Ser-67, or phospho-Thr-35/phospho-Ser-67 inhibitor-1 (Fig. 7). Neither dephospho- nor phospho-Ser-67 inhibitor-1 inhibited PP-1 activity at any of the concentrations tested (0–1 μM). Phospho-Thr-35 inhibitor-1 potently inhibited the activity of PP-1 with an IC₅₀ value of 3.0 nM. Phosphorylation of Ser-67 had no significant effect on the inhibitory activity of phospho-Thr-35 inhibitor-1.

To determine the effect of this proline-directed phosphorylation upon the ability of PKA to phosphorylate Thr-35, inhibitor-1 was phosphorylated to a stoichiometry of greater than 0.9 by Cdk1 and repurified to homogeneity. Phosphorylation of Ser-67 altered the apparent Kₘ and Vₘₐₓ for phosphorylation of inhibitor-1 by PKA from 2.1 ± 0.3 (n = 6) and 0.9 ± 0.04 (n = 4) to 9.5 ± 2.3 (n = 6) and 1.4 ± 0.1 (n = 4) (Fig. 8), respectfully. Thus, phosphorylation at Ser-67 caused a significant reduction in catalytic efficiency (Vₘₐₓ/Kₘ) from 0.42 ± 0.08 to 0.23 ± 0.03 (n = 4, p = 0.0076, Student’s paired t test).
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FIG. 7. Comparison of PP-1 inhibition by dephospho-, phospho-Thr-35, and phospho-Ser-67 inhibitor-1. Protein phosphatase-1 inhibition was analyzed using dephospho- (open circles), phospho-Thr-35 (open squares), phospho-Ser-67 (filled squares), or phospho-Thr-35/phospho-Ser-67 (open triangles) inhibitor-1. Quantified values represent the average of 4–6 different experiments performed in duplicate.

FIG. 8. Effect of phosphorylation of inhibitor-1 at Ser-67 on the ability of PKA to phosphorylate inhibitor-1. Lineweaver-Burk analysis of PKA phosphorylation of dephospho- (open squares) and phospho-Ser-67 (filled circles) inhibitor-1. Values represent the average of four experiments using duplicate samples.

DISCUSSION

We report here that protein phosphatase inhibitor-1 is efficiently phosphorylated at Ser-67 by three different proline-directed kinases in vitro, Cdk1, Cdk5, and MAP kinase. The apparent $K_{m}$ values for these phosphorylation reactions were similar to the concentration of inhibitor-1 estimated to occur in striatal medium spiny neurons (29, 30, 57, 58). Moreover, immunoblot analysis indicated that Cdk5 and MAP kinase, but not Cdk1, are present in adult striatum. Treatment of striatal slices with selective protein kinase inhibitors indicated that Cdk5 phosphorylates Ser-67 inhibitor-1 in the striatum but that MAP kinase does not. These studies using striatal slices were consistent with our biochemical results and indicate that Cdk5 is the predominant kinase responsible for phosphorylation of inhibitor-1 at Ser-67 in intact striatal tissue. Cdk5 functions in neurite outgrowth (59), development of the nervous system (60), and regulation of dopamine signaling through phosphorylation of DARPP-32 in the striatum (35). Cdk5 may also play a role in muscle development (61). A substantial level of basal phosphorylation of inhibitor-1 at Ser-67 was also detected in adult rat hippocampus, and in skeletal muscle and kidney tissue. These observations suggest that Cdk1 and/or MAP kinase could also be responsible for the phosphorylation of Ser-67 in peripheral tissue.

Phospho-Ser-67 inhibitor-1 was found to be a substrate for protein phosphatases PP-2A and PP-2B in vitro. In other in vitro protein phosphatase assays using purified protein phosphatase catalytic subunits and standard substrates as controls, PP-2B was found to be more efficient than PP-2A and PP-2C at dephosphorylating phospho-Ser-67 inhibitor-1. PP-1 could not dephosphorylate phospho-Ser-67 inhibitor-1 at all (data not shown). Both phospho-Thr-35 of inhibitor-1 and phospho-Thr-34 of DARPP-32 have been shown to be very efficient substrates for PP-2B (62), and PP-2B is highly concentrated in striatal neurons (63). Treatment of slices with NMDA, which activates PP-2B by increasing intracellular Ca$^{2+}$, caused an almost complete loss of phospho-Ser-67 levels. Conversely, cyclosporin A, a PP-2B inhibitor, increased phospho-Ser-67 levels. Basal phospho-Ser-67 inhibitor-1 levels were increased in striatal slices from PP-2B$^{-/-}$ mice. The residual effects of NMDA on Ser-67 in PP-2B$^{-/-}$ may be attributed to the activity of the $\beta$-isoform of PP-2B (56). Thus, a variety of data suggest that both PP-2A and PP-2B may dephosphorylate phospho-Ser-67 inhibitor-1 under basal conditions, but PP-2B may function as the predominant phosphatase in the presence of elevated Ca$^{2+}$ levels.

Phosphorylation of inhibitor-1 at Ser-67 by Cdk5 had no effect on PP-1 inhibitory activity. These results are also in complete agreement with numerous previous reports that used inhibitor-1 purified from rabbit muscle. It has been demonstrated that inhibitor-1, as well as its homolog, DARPP-32, only become potent inhibitors of PP-1 after phosphorylation by PKA and that the Thr-35 nonphosphorylated form of inhibitor-1 is devoid of PP-1 inhibitory activity (1, 50, 58, 64). In early studies, inhibitor-1 isolated from rabbit skeletal muscle was found, by direct amino acid sequence analysis, to be phosphorylated at Ser-67 with a stoichiometry of 0.5–0.7 mol/mol (50). That preparation was found to inhibit PP-1 with a $K_{i}$ of 1.6 nM only when phosphorylated at Thr-35 by PKA. Without phosphorylation by PKA, the endogenous protein could not inhibit PP-1 at detectable levels even at a concentration 1,000-fold above the $K_{i}$. Peptide fragments lacking the sequence surrounding phospho-Ser-67 were fully active when phosphorylated by PKA, and a phosphopeptide containing residues 61–71 was inactive (5, 6). All these findings are in contradiction to a recent report by Huang and Paudel (65) that suggested that phosphorylation of inhibitor-1 at Ser-67 converted it into a potent inhibitor of recombinant PP-1 purified from bacteria.

The present studies are also completely consistent with extensive studies that have established that PP-1 is inhibited by a common conserved region at the NH$_2$ terminus of inhibitor-1 and DARPP-32 (5, 66, 67). In addition to the region surrounding the phosphorylated Thr-35 residue (Thr-34 in DARPP-32), which interacts with the active site of PP-1, a short docking motif (RKLXF, residues 8–12) in the two proteins is required for potent inhibition and interacts with a defined region that is removed from the active site on PP-1 (5, 54, 66, 67). Phosphorylation of DARPP-32 at other sites does not directly affect PP-1 inhibitory activity (33, 34).

Our results indicated that phosphorylation of inhibitor-1 at Ser-67 slightly altered its efficiency as a substrate for PKA when phosphorylated on Ser-67. The stoichiometry of phosphorylation in striatal tissue under basal conditions was determined to be 0.34 mol/mol, allowing an estimate of the concentration of phospho-Ser-67 in the striatum of about 1.7 $\mu$M. By increasing the $K_{m}$ for PKA phosphorylation from 1.7 to 9.5 $\mu$M, phospho-Ser-67 may serve as a fine control mechanism for regulating the degree to which the effects of PKA activation are amplified. Recently, additional PP-1 inhibitor proteins have been identified, some of which demonstrate selective inhibitory activity only when PP-1 occurs in association with other regu-
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