Regulation of Protein Phosphatase Inhibitor-1 by Cyclin-dependent Kinase 5

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Chan Nguyen, Akinori Nishi, Janice W. Kansy, Joseph Fernandez, Kanehiro Hayashi, Frank Gillardon, Hugh C. Hemmings, Jr., Angus C. Nairn, and James A. Bibb

From the Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, Texas 75390; Department of Pharmacology, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan; Protein/DNA Technology Center and Laboratory of Cellular and Molecular Neuroscience, Rockefeller University, New York, New York 10021; Central Nervous System Research, Boehringer Ingelheim Pharma KG, 88397 Biberach an der Riss, Germany; Departments of Anesthesiology and Pharmacology, Weill Medical College of Cornell University, New York, New York 10021, and Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06508

Inhibitor-1, the first identified endogenous inhibitor of protein phosphatase 1 (PP-1), was previously reported to be a substrate for cyclin-dependent kinase 5 (Cdk5) at Ser67. Further investigation has revealed the presence of an additional Cdk5 site identified by mass spectrometry and confirmed by site-directed mutagenesis as Ser5. Basal levels of phospho-Ser6 inhibitor-1, as detected by a phosphorylation state-specific antibody against the site, existed in specific regions of the brain and varied with age. In the striatum, basal in vivo phosphorylation and dephosphorylation of Ser6 were mediated by Cdk5, PP-2A, and PP-1, respectively. Additionally, calcineurin contributed to dephosphorylation under conditions of high Ca2+. In biochemical assays the function of Cdk5-dependent phosphorylation of inhibitor-1 at Ser6 and Ser67 was demonstrated to be an intramolecular impairment of the ability of inhibitor-1 to be dephosphorylated at Thr35; this effect was recapitulated in two systems in vivo. Dephosphorylation of inhibitor-1 at Thr35 is equivalent to inactivation of the protein, as inhibitor-1 only serves as an inhibitor of PP-1 when phosphorylated by cAMP-dependent kinase (PKA) at Thr35. Thus, inhibitor-1 serves as a critical junction between kinase- and phosphatase-signaling pathways, linking PP-1 to not only PKA and calcineurin but also Cdk5.

Phosphatases are now recognized as important players in processes ranging from muscle contraction to synaptic plasticity. Protein phosphatase inhibitor-1, or simply inhibitor-1 (I-1), was identified from rabbit skeletal muscle in 1976 as an inhibitor of protein phosphatase 1 (PP-1) and a regulator of glycogen metabolism (1). In recent years, interest in the protein has focused on its role in heart failure and neuronal plasticity. In the brain, inhibitor-1 can be found in many areas including the olfactory bulb, neostriatum, cerebral cortex, and the dentate gyrus of the hippocampus. The level of inhibitor-1 in the rest of the hippocampus is a matter of some debate; reports range from little to none to moderate amounts (2–6).

Inhibitor-1 is a heat-stable 19-kDa protein that possesses little ordered structure and a preponderance of glutamic acid and proline in its amino acid sequence. As a regulator of one of the three major serine/threonine phosphatases in mammalian cells that can itself be regulated by a major kinase, inhibitor-1 occupies an important position in neuronal signal transduction cascades. Only when phosphorylated by cAMP-dependent protein kinase (PKA) at Thr35 does inhibitor-1 become a potent inhibitor of PP-1 and had only a mild effect on the phosphorylation of sites it shares with PP-1. That calcineurin (PP-2B, or Ca2+/calmodulin-dependent protein phosphatase) can inactivate I-1 by dephosphorylating the PKA site (10–12) provides an added layer of complexity, as well as a point of integration for cAMP- and Ca2+-dependent second messenger systems. The ability of inhibitor-1 to link the actions of PKA and calcineurin to PP-1 has allowed it to occupy a central position in molecular models of synaptic plasticity.

In 2001, inhibitor-1 was found to be phosphorylated at another residue (Ser285) by cyclin-dependent kinase 5 (Cdk5) (13), a proline-directed serine/threonine kinase. This phosphorylation did not have an effect on the ability of phospho-Thr35 inhibitor-1 to inhibit PP-1 and had only a mild effect on the ability of PKA to phosphorylate inhibitor-1 at Thr35. Despite its name, Cdk5 is not cyclin-dependent. Furthermore, unlike all other cyclin-dependent kinases, Cdk5 is most active in postmitotic neurons because of its requirement for the relatively neuron-specific cofactor, p35 (14, 15), or its homologue, p39 (16, 17). Although early studies focused on its ability to hyperphosphorylate tau and its relationship to Alzheimer disease.
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Cdk5 now boasts an extensive list of substrates, including N-methyl-D-aspartate (NMDA) receptor (19), postsynaptic density, M, 95,000 (PSD-95) (20), meiosis-specific kinase 1 (MEK1) (21), c-Jun N-terminal kinase 3 (JNK3) (22), P/Q-type voltage-gated Ca$^{2+}$ channel (23), synapsin I (24), mammalian unc-18 (Munc18) (25, 26), amphiphysin I (27–30), dynamin I (28–30), microtubule-associated protein 1B (MAP1B) (31, 32), β-catenin (33, 34), focal adhesion kinase (FAK) (35), stathmin (36), tyrosine hydroxylase (37, 38), and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, $M_\text{r}$ 32,000) (39), an inhibitor-1 homologue. In the past decade, Cdk5 has been implicated in many aspects of normal and abnormal neuronal function, including neurodevelopment, synaptic plasticity, and neurodegeneration.

In this report, we show that inhibitor-1 is actually phosphorylated at two sites by Cdk5 in vitro and in vivo. Following discovery of the second site by phosphopeptide and phosphoamino acid analyses, mass spectrometric identification of the novel site as Ser$^6$ was confirmed by in vitro phosphorylation of site-directed mutants. Basal levels of phospho-Ser$^6$ inhibitor-1 as detected by a phosphorylation-state specific antibody generated against the site allowed demonstration of its relevance to in vivo systems. Pharmacological manipulation of both striatal lysates and striatal slices suggested that basal levels of phosphorylation at Ser$^6$ are controlled by the opposing actions of Cdk5, PP-2A, and PP-1, whereas biochemical analyses revealed a novel intramolecular regulatory function for Cdk5-dependent phosphorylation of inhibitor-1 that was recapitulated in two in vivo systems.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—All chemicals were from Sigma, except where indicated. Trypsin, shrimp alkaline phosphatase, and endoproteinase Lys-C were from Promega. Phosphorylase $b$ was purchased from Calzyme. Protease inhibitors, dithiothreitol, isopropyl-β-D-thiogalactopyranoside, and ATP were from Roche. [γ-$^32$P]ATP was from PerkinElmer Life Sciences. Cyclosporin A, calyculin A, okadaic acid, and forskolin were from LC Laboratories, U0126 was from Tocris, and butyrolactone 1 was from Biomol. Roscovitine and indolinone A and B were generously provided by Laurent Meijer (CNRS, Roscoff, France) and Frank Gillardon (Boehringer Ingelheim), respectively. The catalytic subunits of PKA and PP-1 were purified as described previously (45). Cell culture reagents were from Invitrogen. Oligonucleotides were ordered from Integrated DNA Technologies, and peptides and phosphopeptides were synthesized at Rockefeller University. Horseradish peroxidase-conjugated anti-rabbit secondary antibody was from Chemicon, and the enhanced chemiluminescence immunoblotting detection system from Amersham Biosciences.

Site-directed Mutagenesis—The pET-15b expression vector incorporating the cDNA for rat inhibitor-1-His$_6$ (13) served as a template for site-directed mutagenesis (43) using Stratagene’s QuickChange kit. The manufacturer’s recommendations for mutagenic primer design were followed, and mutations were confirmed by DNA sequencing.

Purification of Inhibitor-1—Recombinant His-tagged-inhibitor-1 was generated as described previously (13). Proteins were stored at −80 °C following analysis for purity by 15% SDS-PAGE and Coomassie Brilliant Blue staining.

In Vitro Protein Phosphorylation and Dephosphorylation Reactions—Protein phosphorylation reactions and PP-1 inhibition assays were performed as described (13, 44, 45). Calcineurin-mediated dephosphorylation assays were conducted similar to PP-1 inhibition assays, except in 33 mM Tris, pH 7.0, 0.01% Brij, 0.1% β-mercaptoethanol, 0.3 mg/ml bovine serum albumin, 100 μM CaCl$_2$, and 1 μM calmodulin with 1 μM $^32$P-labeled phospho-inhibitor-1. Substrates for calcineurin assays were phosphorylated first by PKA and then by Cdk5.

In some studies, preparative phosphorylation of inhibitor-1 by a protein kinase was followed by repurification of phospho-inhibitor-1 prior to use in a subsequent assay. For these experiments, phospho-inhibitor-1 was repurified from in vitro protein phosphorylation reaction mixtures by trichloroacetic acid precipitation and dialysis as described previously (13).

Phosphatase assays employing tissue lysates were performed as described (13). [γ-$^32$P]Ser$^6$/S67D was generated by preparative phosphorylation of S67D inhibitor-1 with Cdk5 in the presence of [γ-$^32$P]ATP. 15 μg of striatal lysate were used for each reaction.

Phosphopeptide Maps and Phosphoamino Acid Analysis—Phosphopeptide maps and phosphoamino acid analysis were conducted as described (46).

Phosphorylation Site Identification by Mass Spectrometry—$^32$P-labeled phospho-Ser$^6$/S67A inhibitor-1, resulting from phosphorylation by Cdk5 in the presence of [γ-$^32$P]ATP, was analyzed by SDS-PAGE and digested with Asp-N. Some of the digest mixture was subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (47). The remainder was fractionated by reversed phase high-performance liquid chromatography (HPLC) on a C$_{18}$ column (Vydac, 1.0 mm inner diameter × 150 mm), and collected fractions were screened for radioactivity (13). The fraction containing the radiolabel was analyzed by MALDI-TOF MS. A small aliquot of the fraction was also treated with shrimp alkaline phosphatase in 50 mM NH$_4$HCO$_3$ at 37 °C for 30 min before MALDI-TOF MS to confirm phosphorylation. The identity of the peptide was verified by Edman degradation.

Generation of Phosphorylation State-specific Antibodies—Polyclonal phosphorylation state-specific antibodies for phospho-Ser$^6$ inhibitor-1 were generated and affinity-purified as described previously (48) using synthetic phosphopeptides encompassing the local amino acid sequence around Ser$^6$ of inhibitor-1. Purified antibodies were evaluated for specificity by immunoblot analysis of dephospho- and phospho-inhibitor-1 standards (50 ng).

Preparation and Incubation of Acute Dorsal Striatal Slices—Slices from male C57BL/6 mice (6–10 weeks old) were prepared in Krebs buffer as described (45). Each 400-μm slice was transferred to a net-well (Costar) resting in one well of a 12-well plate containing 3 ml of Krebs buffer and allowed to recover at
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Discovery, Identification, and Confirmation of Ser6 as a Novel Cdk5 Site of Inhibitor-1—Inhibitor-1 was reported previously to be phosphorylated by Cdk5 at Ser67 (13). Phosphoprotein maps of wild-type and S67A inhibitor-1 phosphorylated by the proline-directed serine/threonine kinases Cdk1, MAPK, and Cdk5 were generated during the characterization of this phosphorylation site (Fig. 1A). Surprisingly, unlike that for Cdk1 and MAPK, the phosphopeptide map for wild-type inhibitor-1 phosphorylated by Cdk5 revealed two major phosphopeptide species. Mutation of Ser67 to an alanine eliminated one of these phosphopeptide species, suggesting the presence of an additional site of Cdk5-dependent phosphorylation. Phosphoamino acid analysis of wild-type and S67A inhibitor-1 revealed the novel site to be a serine (Fig. 1B).

S67A inhibitor-1 preparatively phosphorylated by Cdk5 in the presence of [γ-32P]ATP was next digested with Asp-N to facilitate identification of the novel site. MALDI-TOF MS of the digest revealed peaks at 1707.86 and 1613.20, representing phosphopeptide maps of tryptic digests of wild-type and S67A I-1 phosphorylated by Cdk5/p25 in vitro. B, phosphoamino acid analysis of acid-hydrolyzed tryptic digests of wild-type and S67A I-1 phosphorylated by Cdk5/p25 in vitro.

RESULTS

Immunoblot Analysis of Cell and Tissue Homogenates—Lysis and immunoblot analysis of striatal slices, as well as gross dissections of brain and body parts, were performed essentially as described (45). However, samples for the peripheral distribution of inhibitor-1 had to be lysed in a different buffer (5 mM Tris, pH 8.4, 0.1 mM EGTA), boiled for 40 min, and centrifuged at 15,000 × g for 15 min to eliminate a cross-reactive band at the same molecular weight. An equal amount of total protein (80–100 μg of brain homogenate, 25 μg of cleared peripheral tissue homogenate, or 25 μg of PC12 cell lysate) from each sample was determinated by the bicinchoninic acid protein assay (BCA, Pierce) was analyzed. The membranes were immunoblotted using antibodies for phospho-Ser67 inhibitor-1 (1:750) (see “Results”), phospho-Ser67 inhibitor-1 (1:4000) (13), total inhibitor-1 (1:2000) (4), or phospho-Thr34 DARPP-32/phospho-Thr35 inhibitor-1 (1:750) (49) following published protocols. All antibody incubations were in 5% milk plus Tris-buffered saline-Tween 20 except that for phospho-Thr35 inhibitor-1, in which bovine serum albumin replaced milk.

Data Analysis—NIH Image J was used to quantitate immunoblots. All results are stated as percentage decrease or fold increase of the mean ± error. Error of change was calculated from standard errors of the mean using error propagation formulas.

Discovery, Identification, and Confirmation of Ser6 as a Novel Cdk5 Site of Inhibitor-1—Inhibitor-1 was reported previously to be phosphorylated by Cdk5 at Ser67 (13). Phosphoprotein maps of wild-type and S67A inhibitor-1 phosphorylated by the proline-directed serine/threonine kinases Cdk1, MAPK, and Cdk5 were generated during the characterization of this phosphorylation site (Fig. 1A). Surprisingly, unlike that for Cdk1 and MAPK, the phosphopeptide map for wild-type inhibitor-1 phosphorylated by Cdk5 revealed two major phosphopeptide species. Mutation of Ser67 to an alanine eliminated one of these phosphopeptide species, suggesting the presence of an additional site of Cdk5-dependent phosphorylation. Phosphoamino acid analysis of wild-type and S67A inhibitor-1 revealed the novel site to be a serine (Fig. 1B).

S67A inhibitor-1 preparatively phosphorylated by Cdk5 in the presence of [γ-32P]ATP was next digested with Asp-N to facilitate identification of the novel site. MALDI-TOF MS of the digest revealed peaks at 1707.86 and 1613.20, representing the phosphorylated peptide DNSPRKIQFTVPLL17 and its daughter ion, respectively (Fig. 2A). The daughter ion, which resulted from a loss of H3PO4, formed a characteristic broad peak because of its inability to be focused on the MALDI-TOF detector (47). Because Ser6 is the only serine or threonine directly followed by a proline in this fragment, it was concluded that it was the novel site of phosphorylation. As further confirmation, the proteolytic fragments were fractionated by capillary HPLC into a 96-well plate, and collected fractions were screened for radioactivity (Fig. 2B, top panel). Counts were found primarily in a single fraction, which produced a predominant M, of 1708.26 by MALDI-TOF MS analysis (Fig. 2B, middle panel). Upon treatment of a small portion of the fraction with alkaline phosphatase, a mass of 1708.26–80 Da (M, PO3) = 1628.67 was observed (Fig. 2B, bottom panel),
confirming that the peptide was phosphorylated. The identity of this peptide as "DNSPRKIQFTVPLL" was verified by Edman degradation microsequencing.

These findings were supported by site-directed mutagenesis. Mutation of both Ser$^6$ and Ser$^{67}$ resulted in no detectable phosphorylation by Cdk5 (Fig. 2C). Thus, Ser$^6$ and Ser$^{67}$ are the only sites of phosphorylation by Cdk5. Curiously, in in vitro phosphorylation reactions mutation of Ser$^6$ to Ala decreased phosphorylation of inhibitor-1 by 44% after 1 h, whereas mutation of Ser$^{67}$ to Ala decreased phosphorylation by 85% instead of the expected 66%. The incongruence of these numbers suggests that phosphorylation of Ser$^6$ may have to follow that of Ser$^{67}$. Indeed, phosphomimetic mutation of Ser$^{67}$ to Asp more than doubled the ability of Cdk5 to phosphorylate Ser$^6$, whereas phosphomimetic mutation of Ser$^6$ did not affect the ability of Cdk5 to phosphorylate Ser$^{67}$.

**Demonstration of the in Vivo Phosphorylation of inhibitor-1 at Ser$^6$**—To confirm the in vivo relevance of this novel phosphorylation event, a phosphorylation state-specific antibody to phospho-Ser$^6$ inhibitor-1 was generated. Phosphospecificity of the purified antibody was demonstrated by immunoblot analysis of recombinant wild-type or S6A inhibitor-1 preparatively phosphorylated or mock phosphorylated by Cdk5 (Fig. 3A). A strong signal was detected from wild-type inhibitor-1 incubated with Cdk5 but not wild-type inhibitor-1 incubated without Cdk5 or S6A inhibitor-1 incubated with Cdk5. In a tissue distribution analysis, basal levels of phospho-Ser$^6$ inhibitor-1 were observed throughout the mouse brain in regions where total inhibitor-1 was detected, including the olfactory bulb, striatum, hippocampus, and cortex (Fig. 3B). In peripheral tissues, phospho-Ser$^{67}$ and total inhibitor-1 could be detected in kidney, fat, gastrocnemius, and abdominal skeletal muscle. However, phosphorylation of inhibitor-1 at Ser$^6$ was not detected in peripheral tissues, with the exception of fat (Fig. 3C). No signal was detected in the corresponding regions of I-1/+/H11002 mice (2), confirming the specificity of the observed band in tissue homogenates. The developmental profile of phospho-Ser$^6$ and phospho-Ser$^{67}$ with respect to total inhibitor-1 in mouse striatum showed high levels of phosphorylation of both sites at embryonic day 18, which steadily decreased to stable levels by about 4 weeks of age (Fig. 3D).

**In Vivo Phosphorylation and Dephosphorylation of Inhibitor-1 by Cdk5 and PP-2A, PP-1, and Calcineurin at Ser$^6$**—Acutely dissected striatal slices were treated with the commonly used Cdk5 inhibitor roscovitine to determine whether Cdk5 is actually the kinase responsible for phosphorylating inhibitor-1 at Ser$^6$ in vivo. Levels of phospho-Ser$^6$ inhibitor-1 were reduced by roscovitine in a dose-dependent manner, with 50 μM roscovitine causing a 65 ± 8% reduction (Fig. 4A). Another Cdk5 inhibitor, butyrolactone I, also caused a decrease (37 ± 7%), but the MAPK pathway inhibitor U0126 had no effect (Fig. 4B). As peptide "DNSPRKIQFTVPLL" and its daughter ion. Masses (Da) of peptides are indicated. B, autoradiogram of the 96-well plate into which fractions were robotically collected after capillary HPLC purification of the Asp-N digest (top) and MALDI-TOF MS of the radiolabeled fraction before and after alkaline phosphatase treatment (bottom). C, phosphorimages (top) and quantitation (bottom) of in vitro phosphorylation of wild-type (wt), S6A, S67A, S6A/S67A, S6E, and S67D I-1 by Cdk5/p25 in a time course experiment.
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Functional Significance of Cdk5-dependent Phosphorylation of Inhibitor-1 in Vitro—Functional analyses of phosphorylation are greatly facilitated by the use of phosphomimetic mutants, because complete phosphorylation can be difficult to achieve in vitro (43). Thus, we screened for potential functions using phosphomimetic mutants and confirmed positive results using preparatively phosphorylated material. Given the proximity of Ser6 to the inhibitor-1 PP-1 binding motif, RKIQF12, we conducted studies to assess the effect of Cdk5-dependent phosphorylation of inhibitor-1 on PP-1 inhibition. Phosphomimetic mutation of one or both Cdk5

Further evidence of the in vivo phosphorylation of inhibitor-1 by Cdk5 at Ser6, striatal slices were treated with the novel Cdk5 inhibitor indoline A (50–52). Indoline A reduced levels of phospho-Ser6 inhibitor-1 in a dose-dependent manner, with 5 μM indoline A causing a 36 ± 17% decrease (Fig. 4C). In contrast, indoline B, primarly an inhibitor of Cdk4 (52), had no effect (Fig. 4D).

Phosphatase assays employing cell lysates were employed next to identify candidate phosphatases responsible for dephosphorylation of Ser6 (Fig. 4E). [γ-32P]Ser6/S67D, generated by preparative phosphorylation of S67D inhibitor-1 with Cdk5 in the presence of [γ-32P]ATP, was used as a substrate for dephosphorylation by endogenous phosphatases found in acutely prepared striatal homogenates. In the presence of the Ca2+ chelator EGTA, there was measurable dephosphorylation of [γ-32P]Ser6/S67D inhibitor-1 that was eliminated by inclusion of microcystin, an inhibitor of both PP-2A and PP-1. Inclusion of fostecrin, primarily an inhibitor of PP-2A, and neurabin (53), a protein inhibitor of PP-1, caused 66 ± 6% and 37 ± 4% decreases in dephosphorylation, respectively. In contrast, the calcineurin inhibitor cyclosporin A had no effect. When the assay was repeated without EGTA in the lysis buffer, the rate of dephosphorylation increased by 2.7 ± 0.2-fold, suggesting that calcineurin was activated under this condition. This conclusion was supported by the fact that the addition of cyclosporin A, a highly specific calcineurin inhibitor, resulted in a significant decrease in inhibitor-1 dephosphorylation (36 ± 16%). Thus, PP-2A and PP-1 dephosphorylate Ser6 under basal conditions, and calcineurin can contribute to dephosphorylation under conditions of high Ca2+.

Pharmacological treatment of acutely dissected striatal slices with various phosphatase inhibitors allowed further exploration in a more intact system (Fig. 4F). Calyculin A, an equally potent inhibitor of PP-1 and PP-2A, resulted in a 1.4 ± 0.2-fold increase in levels of phospho-Ser6 inhibitor-1. In striatal slices, 200 nM okadaic acid inhibits 80% of PP-2A activity and 5% of PP-1 activity, whereas 1 μM okadaic acid inhibits 95% of PP-2A activity and 35% of PP-1 activity (54). Both concentrations of okadaic acid raised levels of phospho-Ser6 inhibitor-1, 200 nM by 1.42 ± 0.2-fold and 1 μM by 2.1 ± 0.2-fold. In contrast, the calcineurin inhibitor cyclosporin A was ineffective.

Striatal slices were next treated with NMDA to see whether calcineurin could contribute to the dephosphorylation of Ser6 under conditions of increased intracellular Ca2+. NMDA caused levels of phospho-Ser6 inhibitor-1 to decrease by 80 ± 12%. Pairing the NMDA treatment with cyclosporin A resulted in a 3.1 ± 1.0-fold increase in the level of phosphorylation of Ser6 compared with NMDA treatment alone (Fig. 4G). Thus, by two different systems, PP-2A and PP-1 were found to dephosphorylate Ser6 under basal conditions, and calcineurin was found to act under conditions of elevated Ca2+.
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**FIGURE 4.** In vivo phosphorylation and dephosphorylation of inhibitor-1 by Cdk5 and PP-2A, PP-1, and calcineurin at Ser6. A–D, quantitative immunoblot analysis of acute striatal slices incubated with A, different concentrations of the Cdk5 inhibitor roscovitine (60 min), n = 4; B, the Cdk5 inhibitors roscovitine (rosco; 50 μM) and butyrolactone I (butoyo; 20 μM) or the MAPK pathway inhibitor U0126 (40 μM) for 60 min, n = 3; C, different concentrations of the novel Cdk5 inhibitor indoline A (60 min). **, p < 0.01, Kruskal-Wallis with Dunn’s multiple comparison test, n = 4–5; D, the Cdk4 inhibitor indolinone B (indo B, 5 μM, 1 h), n = 3–4. E, phosphatase reconstitution assays in which the dephosphorylation of [γ-32P]Thr35 inhibitor-1 was measured 15 min after the addition of ~15 μg striatal lysate with or without various phosphatase inhibitors (microcystin (μcyst, 1 μM), fostreicin (fostr, 5 μM), neurexin (0.1 μg/μl), cyclosporin A (cyA, 1 μM)) in the presence (+) or absence (−) of EGTA (2 mM), n = 3. F, quantitative immunoblot analysis of acute striatal slices incubated in the absence or presence of various phosphatase inhibitors (calyculin A (cala, 200 nM), okadaic acid (OA, 200 nM or 1 μM), cyclosporin A (cyA, 10 μM)) for 60 min, n = 4–G, quantitative immunoblot analysis of acute striatal slices treated with cyclosporin A (cyA, 10 μM, 60 min) and/or NMDA (50 μM, 5 min), n = 5–7. A, B, E–G, *p < 0.05; **, p < 0.01; †, p < 0.001, one-way analysis of variance with Newman-Keuls multiple comparison test versus control (cont).
versely, phosphomimetic mutation of one or both Cdk5 sites resulted in a 45% increase in levels of phospho-Thr35 inhibitor-1 as compared with controls.

In a second, more physiological approach, we used NMDA to manipulate levels of phospho-Ser6 and phospho-Ser67 inhibitor-1 in acutely dissected striatal slices and looked for a corresponding change in levels of phospho-Thr35 inhibitor-1 (Fig. 6B). Treatment of all slices with the PKA pathway activator forskolin was necessary, as endogenous levels of phospho-Thr35 inhibitor-1 in striatum are virtually undetectable. Indeed, the decrease in levels of phospho-Ser6 (87 ± 19%) and phospho-Ser67 (67 ± 19%) inhibitor-1 caused by treatment with NMDA correlated with a decrease in levels of phospho-Thr35 inhibitor-1 (88 ± 28%). Together these data serve as the first demonstration of the direct modulation of inhibitor-1 function by Cdk5 in living cells.

**DISCUSSION**

We report here the discovery, identification, and confirmation of a novel phosphorylation site on inhibitor-1. This phosphorylation event, as detected by a phosphorylation state-specific antibody, occurs in vivo and is mediated by Cdk5 in the striatum. Dependence upon Cdk5 was demonstrated through the use of the Cdk5 inhibitors roscovitine, butyrolactone I, and indolinone A. Though indolinone A (IC50 = 5 nM) (52) is a much more potent inhibitor of Cdk5 than roscovitine (IC50 = 0.2 μM) (56), it is a novel compound that has not been used extensively.

Higher expression of inhibitor-1 in the olfactory bulb, cortex, striatum, and hippocampus than in the brain stem and cerebellum agrees with results published at the protein (5) and mRNA levels (6). That phosphorylation of inhibitor-1 at Ser6 seems to occur wherever inhibitor-1 is present in the brain suggests its importance to the function of the protein. Phosphorylation of Ser67 in the striatum is attributed to Cdk5, as Cdk1 is not active in postmitotic neurons and pharmacological inhibition of MAPK does not alter levels of phospho-Ser67 inhibitor-1 (13). Although phosphorylation of Ser6 is relatively brain-specific, phosphorylation of Ser67 seems to be a general phenomenon occurring throughout the body.

Cdk5 activity is generally restricted to the brain (14, 15), so presumably other kinases such as Cdk1 and MAPK phosphorylate Ser67 in peripheral tissues. However, some evidence suggests the presence of Cdk5 in low levels in peripheral tissues (57), particularly the insulin-secreting cells of the pancreas, where it regulates insulin release (58, 59). Interestingly, PP-1 enhances the synthesis of insulin via the dephosphorylation of eIF2α (eukaryotic translation initiation factor 2α) (60), which has been shown to be regulated by inhibitor-1 in transfected HEK293T cells (61). Furthermore, inhibitor-1 and its homologue DARPP-32 have recently been shown to localize to pancreatic β cells (62). Thus, pancreatic β cells represent one non-neuronal tissue in which Cdk5-dependent phosphorylation of inhibitor-1 may be important.

It is unclear which protein kinase is responsible for the small amount of phospho-Ser6 inhibitor-1 detected in fat, as Ser6 was not shown to be a good substrate for Cdk1 or MAPK in vitro. Regardless, the major kinase responsible for phosphorylation of Ser67 in the striatum is Cdk5, because pharmacological inhibition of Cdk5 activity for 1 h with roscovitine decreased phospho-Ser67 inhibitor-1 levels by ~65%.

Expression of total inhibitor-1 in kidney, fat, and skeletal muscle, but not in lung, agrees well with previous results obtained at the protein (63) and mRNA levels (64). We could not detect inhibitor-1 in the liver, as rat and mouse liver possesses little or no inhibitor-1 protein (63) or mRNA (64), in contrast to rabbit, guinea pig, porcine, and sheep liver. Although inhibitor-1 is widely studied in relation to cardiac failure (65–68), we were also unable to detect a signal in heart.
This likely relates to the very low abundance of inhibitor-1 in this tissue (64, 69, 70).

That Cdk5-dependent phosphorylation of inhibitor-1 rendered it less susceptible to dephosphorylation at Thr35, rather than having a direct effect on its ability to inhibit PP-1, was somewhat surprising given the proximity of Ser6 to the inhibitor-1 PP-1 binding motif (RKRQF12). The consensus sequence of this degenerate motif is (K/R/H/N/S)(V/I/L)X(F/W/Y) (71), and phosphorylation of serine(s) close to or within this motif has been shown to impair PP-1 binding to several partner proteins, including nuclear protein phosphatase-1 (NIPP-1) (72), neurabin I (53), and muscle glycogen-binding subunit (GADD34) (73). One function of inhibitor-1 may be to facilitate interactions between PP-1 and various other proteins such as growth arrest and DNA damage-inducible protein (GADD34) (74). Although phosphorylation of Ser6 had no effect on PP-1 inhibition, it remains distinctly possible that it might impair these interactions.

Ironically, PP-1, the phosphatase that inhibitor-1 is charged with inhibiting, can dephosphorylate inhibitor-1 at Ser6. This circuit may function as a positive feedback loop in that activation of PP-1 will lead to phosphorylation at Thr35, with consequent inhibition of PP-1 activity and maintenance of inhibitor-1 activity by Cdk5 may provide an important point for the opportunity for NMDA receptor signaling to converge upon PKA-signaling pathways originating from Gs-coupled receptors. The indirect modulation of inhibitor-1 activity is glutamate acting via the ionotropic NMDA receptor. NMDA regulates Cdk5 activity (75, 76), and levels of phospho-Ser67 inhibitor-1 decrease upon treatment of acutely dissected striatal slices with NMDA (13). We have shown here that phosphorylation at Ser67 by Cdk5 facilitates phosphorylation of Ser6. Further adding to the complexity of multi-site phosphorylation is the fact that PP-1 does not exist in cells as a free catalytic subunit. Thus, the physiological impact of Cdk5-dependent phosphorylation of inhibitor-1 can only be understood in the context of multimeric PP-1 complexes. It is perhaps these types of complicated interactions that allow cells to fine-tune their responses to external stimuli that activate a whole host of intracellular signal transduction cascades.

Prominent among the external stimuli that control Cdk5 activity is glutamate acting via the ionotropic NMDA receptor. NMDA regulates Cdk5 activity (75, 76), and levels of phospho-Ser67 inhibitor-1 decrease upon treatment of acutely dissected striatal slices with NMDA (13). We have shown here that phospho-Ser6 is similarly affected but to a greater extent than phospho-Ser67 inhibitor-1. Together, Ser6 and Ser67 represent an opportunity for NMDA receptor signaling to converge upon PKA-signaling pathways originating from Gs-coupled receptors such as the D1 dopamine receptor, A2A adenosine receptor, or β-adrenergic receptor. The indirect modulation of inhibitor-1 activity by Cdk5 may provide an important point for the amplification of PKA signaling resulting from activation of these receptors. We have shown that inhibitor-1 serves as a critical junction between kinase- and phosphatase-signaling pathways, linking PP-1 not only to PKA and calcineurin but also to Cdk5.

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