Neuronal Cdc2-like Protein Kinase (Cdk5/p25) Is Associated with Protein Phosphatase 1 and Phosphorylates Inhibitor-2* 

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Protein phosphatase 1 (PP1) is complexed with inhibitor 2 (I-2) in the cytosol. In rabbit muscle extract PP1-I-2 is activated upon preincubation with ATP/Mg. This activation is caused by phosphorylation of I-2 on Thr$^{72}$ by glycogen synthase kinase 3 (GSK3). We have found that PP1-I-2 in bovine brain extract is also activated upon preincubation with ATP/Mg. However, blocking GSK3 action by LiCl inhibited only ~29% of PP1 activity and indicated that GSK3 is not the sole PP1-I-2 activator in the brain. When bovine brain extract was analyzed by gel filtration PP1-I-2 and neuronal Cdc2-like protein kinase (NCLK), a heterodimer of Cdk5 and the regulatory p25 subunit, co-eluted as a ~450-kDa size species. The NCLK from the eluted column fractions bound to PP1-specific microcystin-Sepharose and glutathione S-transferase (GST)-I-2-coated glutathione-agarose beads. Similarly, PP1 from the eluted column fractions was pulled down with GST-Cdk5-coated glutathione-agarose beads. In vitro, NCLK phosphorylated I-2 on Thr$^{72}$ and activated PP1-I-2 in an ATP/Mg-dependent manner. NCLK bound to PP1 through its Cdk5 subunit and the PP1 binding region was localized to Cdk5 residues 28–41. Our data demonstrate that in brain extract PP1-I-2 and NCLK are associated within a complex of ~450 kDa and suggest that NCLK is one of the PP1-I-2-activating kinases in the mammalian brain.

Protein phosphatase 1 (PP1)$^1$ is a major Ser/Thr phosphatase involved in the regulation of metabolism, cell cycle, cell signaling, muscle contraction, and gene expression (for reviews see Refs. 1, 2). PP1 is a 37-kDa catalytic subunit bound to two types of regulatory subunits: a targeting subunit and an inhibitory subunit. Targeting subunits confer substrate specificity and localize PP1 to various subcellular compartments. Inhibitory subunits suppress PP1 activity. There are three PP1 inhibitory subunits: inhibitor 1 (I-1), DARPP-32, and inhibitor 2 (I-2) (1–3). I-1 and DARPP-32 require phosphorylation for PP1 inhibitory activity, whereas nonphosphorylated I-2 inhibits PP1. These inhibitors are phosphorylated in response to many extracellular stimuli and allow PP1 to respond to various growth factors and hormones (3).

In rabbit skeletal muscle extract PP1 is found in both particulate and cytosolic fractions. PP1 in the particulate fraction is active, whereas in the cytosolic fraction it is inactive (1, 2). The inactive cytosolic enzyme, a PP1-I-2 complex, is activated upon incubation with ATP/Mg and is hence called ATP/Mg-dependent PP1 (1). An activating factor named Fa is necessary for ATP/Mg-dependent activation of PP1-I-2. Fa has been identified to be glycogen synthase kinase 3 (GSK3) (4–6). The ATP/Mg-dependent activation is due to the phosphorylation of I-2 within the PP1-I-2 complex by GSK3. Nonphosphorylated I-2 suppresses PP1 activity within the PP1-I-2 complex. GSK3 phosphorylates I-2 on Thr$^{72}$ and relieves PP1 from I-2 inhibition (4–9). Even though GSK3 is a well-characterized PP1-I-2-activating kinase, several reports suggest that other kinases also phosphorylate I-2 and activate PP1-I-2 (10–12).

PP1 is highly expressed in brain (1). An earlier study found that most of the PP1 in brain extract is inactive and requires incubation with ATP/Mg to become active (13). The purified enzyme is a PP1-I-2 complex, which is activated upon incubation with ATP/Mg in the presence of muscle GSK3. It was suggested that brain ATP/Mg-dependent PP1 is regulated in a manner similar to its muscle counterpart, via phosphorylation of I-2 (13). A type of Fa activity was partially purified from porcine brain extract (13), but the identity of this activity has remained unknown. Thus, until now it has not been clear as to which kinase activates PP1-I-2 in the brain.

Neuronal Cdc2-like protein kinase (NCLK) is a heterodimer of cyclin-dependent protein kinase 5 (Cdk5) and a neuronal-specific p25 regulatory subunit (reviewed in Ref. 14). Cdk5, a member of the cyclin-dependent protein kinase family, is widely expressed in various tissues and cell lines (15). However, its kinase activity is detected only in terminally differentiated neurons where it is associated with a p25 subunit (16). p25 is a proteolytic fragment of a 35-kDa protein and is expressed only in neurons (17). NCLK is involved in brain development, neurite outgrowth, cell migration, cell signaling, microtubule dynamics regulation, and Alzheimer’s disease pathogenesis (18–24). Herein we show that NCLK is complexed with PP1-I-2 in brain extract, phosphorylates I-2 on Thr$^{72}$, and activates PP1-I-2. Our data suggest that NCLK is one of the kinases that activate PP1-I-2 in the central nervous system.

MATERIALS AND METHODS
cDNA Cloning—Human I-2 cDNA plasmid in pTT73D-pac vector (American Type Culture Collection, Manassas, VA) was subcloned into two different bacterial expression vectors: pET-9a (Promega, Madison, WI) and pGEX-6P-1 (Amersham Pharmacia Biotech, Baie’d’Urfe, Quebec, Canada). To subclone into the pET-9a vector Ffu DNA polymerase catalyzed 30 cycles polymerase chain reaction (PCR) was carried out.
using I-2 cDNA as the template and forward (5′-AAA AAA CAT ATG GCC GGC TCC AGG C-G-3′) and reverse (5′-AAA AAA GGG TCA TTA ACT TCG TAA TTT GGT TTG-3′) primers. The PCR condition was 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. After a 7-min extension at 72 °C was followed by a final incubation with Taq polymerase at 95 °C for 1 min. After the PCR product was purified from the gel, the PCR product was subcloned into pGEM-T Easy vector (Promega) and amplified. The I-2 cDNA insert from the pGEM-T Easy vector was excised by Ndel/BamHI and ligated into a Ndel/BamHI cloning site of the pET-9a vector. To subclone into pGEX-6P-1 vector, PCR was carried out using forward primer 5′-AAA AAA GGA TCC ATG CAG AAA TAC GAG A-3′, P2 (5′-AAA AAA GGA TCC ATG GGT CGG ACC TCC GCC-3′), and P3 (5′-AAA AAA GGA TCC ATC GTC GCT CAG AAA C-3′), each containing a BamHI site (underlined) and reverse primer 5′-AAA AAA GAA GAT TGA TTT TGG AAC TTG TCG TAA TTT GGT TTG-3′ containing an EcoRI site (underlined), except that the Taq polymerase and pGEM-T Easy vector steps were excluded. The PCR product was excised with BamHI/EcoRI and ligated into a BamHI/EcoRI cloning site of the pGEX-6P-1 vector.

Three forward primers, F1 (5′-AAA AAA GGA TCC ATG CAG AAA TAC GAG A-3′), F2 (5′-AAA AAA GGA TCC ATG GGT CGG ACC TCC GCC-3′), and F3 (5′-AAA AAA GGA TCC ATC GTC GCT CAG AAA C-3′), each containing a BamHI site (underlined) and two reverse primers R1 (5′-AAA AAA GTC GAC CTA GGG GAG ACT GCC CAG-3′), and R2 (5′-AAA AAA GGA TCC ATG CAA GGG ACA AAA GTT-3′), each containing an SalI site (underlined) were used in the following combination to generate three cdk5 deletion mutants: cdk5-(1–48) (F1 and R1), cdk5-(1–208) (F3 and R2), and cdk5-(1–257) (F2 and R2). All PCR conditions were the same as described above except for using human cdk5 cDNA (15) as the template and omitting the Taq polymerase and pGEM-T Easy vector steps. Each PCR product was excised with BamHI/SalI and ligated into the BamHI/SalI cloning site of the pGEX-6P-1 vector. All recombinant plasmids were transfected into DH5α first followed by BL21(DE3) Escherichia coli cells. All cDNA clones were confirmed by nucleotide sequencing.

Proteins and Peptides—I-2 was purified from bacterial culture medium as described previously (25) with some modifications. Overnight bacterial culture (10 ml) was diluted 100-fold in fresh medium and incubated with vigorous shaking at 37 °C. When the A_600 of the medium reached ~0.6, isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM. Shaking then continued for another 3 h at 37°C. The medium was centrifuged, and the bacterial pellet was suspended in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 M NaCl, and 0.05% Tween 20. The washed beads were mixed with 50 μl of microcystin-Sepharose (Upstate Biotechnology) beads pre-equilibrated in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM MnCl2, 15 mM β-mercaptoethanol, 0.05% Nonidet P-40 with 0.2 ml of combined gel filtration fractions from Fig. 1A. The mixture was incubated at 4 °C overnight with end-over-end shaking. The incubated mixture was centrifuged, and the recovered beads were washed three times with 0.5 ml of 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.2 mM NaCl, and 0.05% of Tween 20. The washed beads were mixed with 50 μl of SDS-PAGE sample buffer, boiled, and centrifuged, and 10 μl of the supernatant was analyzed by immunoblot analysis using the indicated antibody. To perform the GST pull-down assay 50 μl of glutathione-agarose beads (Sigma-Aldrich Ltd.) coated with the indicated GST fusion protein were mixed with 150 μl of the indicated protein solution and incubated overnight with end-over-end shaking at 4 °C. Incubated beads were washed four times with 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM NaCl, and 0.05% Tween 20. The washed beads were mixed with 50 μl of SDS-PAGE sample buffer, boiled, and centrifuged, and 10 μl of the supernatant was analyzed by immunoblot analysis using the indicated antibody.

Activity Assays—ATP/Mg-dependent PP1 activity was assayed as described previously (9) except that the assay mixture also contained okadaic acid (Sigma-Aldrich Ltd.) (to inhibit PP2A and PP2B inhibitor c-mercaptoethanol (Calbiochem). Samples were preincubated at 30 °C for 15 min in a mixture containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM MgCl2, 0.5 mM dithiothreitol, and 0.5% BSA. The assay was initiated by the addition of 10 μl of the preincubated sample to a vial containing 30 μl of the rest of the assay components. The final concentrations of the various components in the assay were 50 mM Tris-HCl (pH 7.4), 0.2% β-mercaptoethanol, 5 mM caffeine, 0.5 mM MnCl2, 10 μM (2′)-Polyphosphorylase, 5 mM okadaic acid, 40 μM cAMP-dependent protein kinase, 1 mM EGTA, and carboxyxy-cysteinubination mixture components. After 20 min at 30 °C, 10 μl of 50% trichloroacetic acid was added to the assay mixture. The assay mixture was cooled on ice for 10 min and centrifuged for 5 min using a bench top centrifuge. The supernatant (20 μl) was withdrawn, spotted onto a filter paper, and counted in a liquid scintillation counter to determine the amount of 32P released. NCLK, GSK3, and PKA activities were assayed by using their respective peptide substrates (27). 1-2 phosphorylation by NCLK was carried out in a reaction mixture containing 25 μm Hepes (pH 7.2), 0.1 mM EDTA, 1 mM DTT, 0.5 mM (2′)-32P-ATP, 10 mM MnCl2, 0.5 mM MgCl2/l, and 400 units/ml NCLK. Phosphorylation was initiated by the addition of NCLK to the mixture containing the rest of the assay components. After indicated time points at 30 °C aliquots were withdrawn, mixed with an equal volume of SDS-PAGE sample buffer, and electrophoresed on a 12% SDS-PAGE gel. The gel was sliced out and counted in a liquid scintillation counter to determine the amount of radioactivity that was incorporated. 1-2 phosphorylation by CK2 was carried out as above by using 10 μg/ml CK2 in the phosphorylation mixture. Over 4 h, CK2 incorporated 2.8 mol of phosphate/mol of 1-2. CK2-phosphorylated 1-2 was desalted by a Sephadex G-25 column and used to generate the data shown in Fig. 4.
Partial Purification of PP1 from Brain Extract—All procedures were performed at 4 °C. Fresh bovine brain (0.5 kg) was homogenized for 1 min in 1 liter of buffer C (20 mM MOPS (pH 7.4), 50 mM β-glycerophosphate, 1 mM EDTA, 1 mM DTT, and 15 mM MgCl₂), which contained protease inhibitor mixture. The homogenate was centrifuged at 104,000 × g for 30 min, and the supernatant was centrifuged at 10^6 × g for 45 min. The resulting clear supernatant was loaded onto a DEAE-Sephacel column (2.5 × 45 cm) previously equilibrated with buffer C. The flow-through fraction containing PP1 activity was loaded onto an SP-Sepharose (Amersham Pharmacia Biotech) column (1 × 60 cm) pre-equilibrated with buffer C. The column was washed with buffer C and was eluted with a linear NaCl gradient (0–0.5 M) in buffer C. Fractions containing PP1 activity were pooled, dialyzed against buffer C, concentrated by dialysis against Aquacide III to ~8 ml, and chromatographed through an FPLC Superose 12 gel filtration column (Amersham Pharmacia Biotech) column (2 × 70 cm) equilibrated and eluted with 20 mM MOPS (pH 7.4), 1 mM EDTA, 1 mM DTT, and 0.15 M NaCl. Fractions 41–43, containing PP1 activity, were combined. A portion of the combined fractions was used to generate Figs. 1B and 2, and the rest was loaded onto a hydroxylapatite column (1 × 15 cm) pre-equilibrated with buffer C. The column was washed with ~40 ml of buffer C and eluted with a 50-ml linear gradient (0–0.5 M) of K₂HPO₄ (pH 7.4) in buffer C.

Phosphopeptide Purification and Peptide Sequencing—I-2 was phosphorylated for 16 h by NCLK in a 0.7-ml reaction mixture containing 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 10 mM NaF, 0.5 mM [γ-32P]ATP, 10 mM MgCl₂, 1 mg/ml I-2, and 400 units/ml NCLK. Phosphorylated I-2 was desalted through a Sephadex G-25 column, lyophilized, dissolved in 0.2 ml of 50 mM NH₄HCO₃ (pH 8.0) containing 50 μg/ml trypsin, and incubated at 37 °C for 16 h. The incubated tryptic digest was separated by a HPLC reverse phase column previously equilibrated with 0.1% trifluoroacetic acid as described (26) using 0–40% acetonitrile gradient in 50 min. Radioactive fractions were equilibrated with 0.1% trifluoroacetic acid as described (26) using 0–40% acetonitrile gradient in 50 min. Radioactive fractions were eluted with 400 ml of a linear NaCl gradient (0–0.5M) in buffer C. The column was washed, and bound peptide was eluted with 0.3 M NaCl in the equilibration buffer. Fractions containing radioactivity were lyophilized, dissolved in 50 mM NH₄HCO₃ containing 50 μg/ml of chymotrypsin, and incubated for 16 h at 37 °C. The chymotryptic digest was lyophilized, dissolved in 25 mM Hepes (pH 7.2), 1 mM EDTA, 1 mM EGTA, and loaded onto a DEAE-Sephacel column (~2 ml) previously equilibrated in 25 mM Hepes (pH 7.2). The column was washed, and bound peptide was eluted with 0.3 M NaCl in the equilibration buffer. Fractions containing radioactivity were lyophilized, dissolved in 25 mM Hepes (pH 7.2), 1 mM EDTA, 1 mM EGTA, and chromatographed through an HPLC column as described above. Only one radioactive peptide was recovered. This peptide was sequenced at the Department of Biochemistry and Microbiology, University of Victoria by using a gas-phase microsequencer.

RESULTS

PP1 in Bovine Brain Extract—In a previous study PP1-I-2 was found to be the predominant form of PP1 in brain extract (13). To learn more about brain PP1-I-2, we chromatographed a fresh bovine brain extract through a DEAE-Sephacel column then an SP-Sepharose column. The effluent containing ATP/Mg-dependent PP1 activity was then analyzed by an FPLC Superose 12 gel filtration column. PP1 activity eluted from the gel filtration column as a broad peak (Fig. 1A). Immunoblot analyses of various gel filtration fractions (by using anti-PP1 or anti-I-2 antibodies) indicated that both PP1 and I-2 were present in column fractions containing PP1 activity (data not shown). The gel filtration analysis indicated that the molecular size of PP1-I-2 in Fig. 1A (fraction 42) is ~450 kDa. Because the molecular size of PP1-I-2 is ~70 kDa, these observations corroborate previous reports (2) and indicate that PP1-I-2 is complexed with other protein(s) in the brain.

As observed previously (13), we found that PP1 has low activity in brain extract, is complexed with I-2, and is activated by preincubation with ATP/Mg (data not shown). These observations are consistent with a previous report (13) and indicate that the PP1-I-2-activating factor is present in brain. An immunoblot analysis using anti-GSK3 antibody showed that GSK3 is present in Fig. 1A column fractions (data not shown). To examine if GSK3 is responsible for ATP/Mg-dependent PP1-I-2 activation, we combined gel filtration fractions 41–43 containing peak PP1 activity from Fig. 1A. We preincubated an aliquot from the combined fractions with ATP/Mg then in the presence of the GSK3 inhibitor LiCl (29) to block GSK3 action and assayed PP1 activity. Surprisingly, PP1 activity was only ~29% inhibited (Fig. 1B), although a kinase assay confirmed that LiCl had completely suppressed GSK3 activity in our samples (data not shown). These data suggested that the brain contains a kinase other than GSK3 that phosphorylates I-2 and activates PP1.

To identify the PP1-I-2-activating kinase we included 2 mM Ca²⁺-chelator EGTA, or 50 μM PKA inhibitory peptide (PKI) in the preincubation mixture and assayed the Fig. 1A combined column fractions. PP1 activity was approximately the same in samples preincubated in the presence of EGTA, PKI, or buffer control (data not shown). These data indicated that LiCl-insensitive ATP/Mg-dependent PP1 activation in Fig. 1B could not be due to the involvement of PKA or any Ca²⁺-dependent kinases (protein kinase C, calmodulin-dependent kinase 2, or phosphorylase kinase).
MAPK phosphorylates I-2 in vitro (11). To determine if MAPK is a PP1-I-2-activating kinase, we analyzed Fig. 1A column fractions by immunoblot analysis using an anti-MAPK antibody. Our antibody that detected noggin of MAPKs (p43erk1 and p42erk2) in brain extract failed to show any immunoreactivity (data not shown). By similar immunoblot analyses we determined that CK1 and CK2 were also absent in Fig. 1A column fractions (data not shown). These observations indicated that MAPK, CK1, and CK2 are not responsible for activating PP1-I-2 in Fig. 1B.

I-2 Thr72 is followed by a proline residue (9), and NCLK recognizes an (S/T)P motif (22). An immunoblot analysis using anti-NCLK antibody (data not shown) as well as an NCLK activity assay demonstrated that NCLK was present in almost all fractions containing PP1 and displayed a gel filtration profile similar to PP1 (Fig. 1A). We therefore included the NCLK inhibitor olomoucine (27, 30) in the preincubation mixture, and assayed PP1 and NCLK activities in the combined column fractions from Fig. 1A. Interestingly, not only did olomoucine completely inhibit NCLK activity (data not shown), but it also suppressed ATP/Mg-dependent PP1 activity by ~30% (Fig. 1B). Inclusion of both olomoucine and LiCl in the preincubation mixture inhibited ~60% of ATP/Mg-dependent PP1 activity (Fig. 1B). Thus, blocking NCLK action partially suppressed ATP/Mg-dependent activation of PP1 in our samples. These data suggested that ATP/Mg-dependent PP1 activity in the brain might also be regulated by NCLK.

NCLK Is Associated with PP1 in Fig. 1A Column Fractions—To substantiate the above suggestion, we incubated combined column fractions from Fig. 1A with PP1-specific microcystin-Sepharose beads (31). The beads were washed and immunoblotted with either anti-NCLK or anti-PP1 antibody. PP1 was pulled down with microcystin-Sepharose beads as expected (Fig. 2B). Importantly, NCLK also was pulled down with microcystin-Sepharose beads (Fig. 2A). We then performed two GST pull-down assays. In the first assay, glutathione-agarose beads coated with GST-I-2 were mixed with the combined column fractions, washed, and immunoblotted by using an anti-PP1 or anti-NCLK antibody. In the second assay, glutathione-agarose beads coated with GST-Cdk5 were mixed with the combined column fractions, washed, and immunoblotted with anti-PP1 antibody. As shown in Fig. 2, C and D, both PP1 and NCLK specifically were pulled down with the GST-I-2-coated beads from the column fractions. Likewise, PP1 was pulled down with the beads coated with GST-Cdk5 but not GST control (Fig. 2E). Finally, we chromatographed the combined column fractions from Fig. 1A through a hydroxylapatite column. An immunoblot analysis of effluent fractions, using anti-PP1 and anti-NCLK antibodies, indicated that PP1 and NCLK co-eluted from the column (data not shown). Thus, NCLK and PP1 from brain extract could not be separated from each other by DEAE-Sepahcel, SP-Sepharose, FPLC gel filtration, or hydroxylapatite chromatographies. Taken together, these data indicated that NCLK is complexed with PP1-I-2 in the brain extract and is likely to be a PP1-I-2-activating kinase.

NCLK Phosphorylates Thr72 of I-2—Because PP1-I-2 activation occurs via I-2 phosphorylation (5–9) we examined whether NCLK phosphorylates I-2. We incubated I-2 with NCLK in the presence of [γ-32P]ATP/Mg2+3 and the product analyzed by SDS-PAGE/autoradiography. I-2 was phosphorylated in a time-dependent manner (Fig. 3, A and B). Over 16 h, NCLK incorporated ~1.02 mol of phosphate/mol of I-2 because NCLK used in this study was purified from brain extract, we examined to see if our NCLK preparations were contaminated by any other kinase(s). Immunoblot analyses using antibodies directed to various kinases determined that our kinase preparations did not contain any detectable MAPK (p43erk1 and p42erk2), CK1, or CK2. Similarly, our kinase preparations contained neither PKA nor GSK3 activity (data not shown), and the phosphorylation of I-2 by our NCLK preparations was insensitive to 2 mM...
EGTA. Finally, we included NCLK inhibitor olomoucine in the assay mixture and monitored I-2 phosphorylation. Olomoucine inhibited I-2 phosphorylation by our NCLK preparation in a dose-dependent manner (Fig. 3, C and D). From these data we concluded that NCLK phosphorylates I-2.

PP1-I-2 activation requires phosphorylation of I-2 on Thr\textsuperscript{72} (5–9). To determine if NCLK phosphorylates I-2 Thr\textsuperscript{72}, NCLK-phosphorylated \textsuperscript{32}P-labeled I-2 was trypsinized and the product was fractionated over an HPLC reverse phase column. Only one radioactive peak eluted from the column (data not shown). Radioactive fractions were combined and digested with chymotrypsin. The chymotryptic digest was chromatographed sequentially through a DEAE-Sephaloc column and an HPLC reverse phase column. Only one radioactive peptide was recovered. Purified peptide was sequenced by using a gas-phase microsequencer. Ile, Asp, Glu, Pro, Ser, and Tyr were identified as the first, second, third, fourth, fifth, and seventh residues, respectively, of this peptide (Table I). The sixth residue (indicated by Xaa) could not be identified, indicating that this residue was phosphorylated. This notion was confirmed by the release of radioactivity during the sixth sequencing cycle. Based on these data and the amino acid sequence of human I-2 (32), we concluded that this phosphopeptide extends from I-2 residues 67–73 and that Thr\textsuperscript{72} is the phosphorylation site. Thus NCLK indeed phosphorylates Thr\textsuperscript{72} of I-2.

**Phosphorylation of I-2 by NCLK and GSK3—**

GSK3, on the other hand, phosphorylates CK2-phosphorylated I-2. However, GSK3 phospho-I-2 has identical substrate specificity for I-2 phosphorylation. NCLK directly binds to PP1—We found that NCLK is complexed with PP1 in brain extract (Fig. 2). To examine if NCLK binds to PP1, glutathione-agarose beads coated with GST, GST-Cdk5, or GST-p25 were mixed with recombinant PP1, washed, and immunoblotted with anti-PP1 antibody. PP1 was found to be associated with GST-Cdk5 but not with GST-p25 or GST (Fig. 5). These data indicate that NCLK binds to PP1 through its Cdk5 subunit.

To locate the PP1 binding region within Cdk5 we generated three Cdk5 deletion mutants: Cdk5-(28–292) lacking residues 1–27, Cdk5-(42–292) lacking residues 1–41, and Cdk5-(1–48) lacking residues 49–292 (Fig. 6, A and B). From these data we concluded that the PP1 binding region is located within the N-terminal Cdk5 residues 28–41 (Fig. 6, C and D). Thus, strategies to remove Cdk5 residues 1–27 drastically reduced Cdk5-PP1 binding. These observations indicated that a major PP1 binding region is located within the N-terminal Cdk5 residues 28–41 (Fig. 6, A).

**Reconstitution of Brain ATP/Mg-dependent PP1—**

To directly demonstrate that NCLK activates PP1-I-2, we reconstituted the PP1-I-2 complex from purified PP1 and I-2. Reconstituted PP1-I-2 was preincubated with ATP/Mg in the presence of NCLK, and PP1 activity was assayed. As shown in Fig. 7, preincubation of PP1-I-2 with ATP/Mg did not affect PP1 activity, as expected. PP1-I-2 preincubation with ATP/Mg in the presence of NCLK under identical conditions, however, stimulated PP1 activity by \~6-fold.

**DISCUSSION**

PP1 activity is controlled by three PP1 inhibitory subunits: I-1, DARPP-32, and I-2 (1–3). I-1 is widely expressed in various tissues, whereas DARPP-32 is found in basal ganglion neurons where it is regulated by dopamine (1). PKA phosphorylates I-1 and DARPP-32, and both proteins inhibit PP1 upon PKA phos-

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**TABLE I**

**Sequence determination of \textsuperscript{32}P-labeled phosphopeptide**

| Cycle | Amino acid | Yield (pmol) | Amount released (cpm) |
|-------|------------|-------------|----------------------|
| 1     | Ile        | 154         | 39                   |
| 2     | Asp        | 24          | 27                   |
| 3     | Glu        | 20          | 30                   |
| 4     | Pro        | 21          | 26                   |
| 5     | Ser        | 4           | 26                   |
| 6     | Xaa        |             |                      |
| 7     | Tyr        | 8           | 50                   |

**Fig. 4.** Phosphorylation of CK2-phosphorylated I-2 by NCLK and GSK3. I-2 and CK2-phosphorylated I-2 were phosphorylated for 4 h by NCLK or GSK3 under identical conditions in a phosphorylation mixture containing 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 1 mM DTT, 0.5 mM [\textsuperscript{γ}-\textsuperscript{32}P]ATP, 10 mM MgCl\textsubscript{2}, 0.5 mg/ml of indicated I-2 species, and 400 units/ml NCLK or 2 μg/ml GSK3. I-2 phosphorylation was quantitated as in Fig. 3B. Values are mean ± S.D. of three independent experiments.

**Fig. 5.** GST pull-down assay. Glutathione-agarose beads coated with the indicated GST fusion protein were mixed with a PP1 solution (50 mM Tris-HCl (pH 7.5), 5 mM MnCl\textsubscript{2}, 0.1 mM EGTA, 15 mM β-mercaptoethanol, 0.05% Nonidet P-40, 1 mg/ml bovine serum albumin, and 0.5 μg/ml PP1), washed, and immunoblotted with anti-PP1 antibody as described under "Materials and Methods." Similar results were obtained in three independent experiments.
structure of various Cdk5 species that were used in the binding experiment. As discussed above, NCLK phosphor-
ylated I-1 and DARPP-32 (27, 33). In this study we showed that NCLK also phosphor-
ylates I-2. These observations suggest that NCLK plays a central role in neuronal signaling by phosphorylating PP1 inhibitory sub-
units I-1, DARPP-32, and I-2.

It is established that PP1 binds to I-2 (1–3, 8, 9). We found that PP1 also binds to NCLK (Fig. 5). These observations suggest that PP1 is the central molecule that holds I-2 and NCLK together within a PP1-I-2-NCLK complex. Our GST pull-
down assay demonstrated that, from the gel filtration column fractions containing PP1-I-2-NCLK complex, PP1 and NCLK are pulled down with GST-I-2 (Fig. 2, C and D) and PP1 is pulled down with GST-Cdk5 (Fig. 2E). These observations raise a question as to how the PP1-I-2-NCLK complex could bind to an exogenous GST-I-2 or GST-Cdk5.

It has been suggested that, in vitro, free PP1 and I-2 are in a dynamic equilibrium with PP1-I-2 and an excess of I-2 can replace PP1-bound I-2 (34, 35). As shown in Fig. 1A, PP1 in brain extract elutes form a gel filtration column as a component of various species with sizes of ~40 to ~450 kDa. Some of these species may represent PP1, PP1-I-2, PP1-NCLK, or PP1-I-2-NCLK. Thus, it is possible that PP1, I-2, and NCLK may also be in a dynamic equilibrium with the PP1-I-2-NCLK complex in the brain. Because I-2 within PP1-I-2 may be displaced by exogenous I-2 (34, 35), GST-I-2 could similarly displace I-2 from PP1-I-2-NCLK and form a PP1-GST-I-2-NCLK species. Likewise, GST-Cdk5 may compete with Cdk5 within the PP1-I-2-NCLK complex and displace NCLK to result in the formation of a PP1-I-2-GST-Cdk5 complex.

I-2 is bound to PP1 in tissue extracts and inhibits PP1 in its nonphosphorylated state. GSK3 phosphorylates I-2 on Thr\textsuperscript{72} within the PP1-I-2 complex. This phosphorylation causes a conformational change in I-2 and also within the PP1-I-2 complex, leading to PP1 activation without complex dissociation (3, 8, 9, 34). Activated PP1 rapidly dephosphorylates I-2. This dephosphorylation, however, does not cause immediate loss of PP1 activity and only after some time the complex returns to its inactive conformation (3, 8, 9, 34). GSK3 phosphorylation of I-2 within PP1-I-2 has been suggested to be short-lived (3, 8, 9).

To further investigate the phosphorylation of I-2 we incubated PP1-I-2 with NCLK in the presence of [γ\textsuperscript{32}P]ATP/Mg\textsuperscript{2+}. We found that the incubation robustly activated PP1 within
PP1-NCLK Interaction

PP1-I-2. However, when we analyzed the product of the incubation by SDS-PAGE/autoradiography, we could not detect any significant I-2 phosphorylation (data not shown). These observations suggest that phosphorylation of I-2 by NCLK within the PP1-I-2 complex is also a transient event.

I-2 is phosphorylated on Ser\textsuperscript{386}, Ser\textsuperscript{120}, and Ser\textsuperscript{121} in vitro (36), and these sites are phosphorylated by CK2 \textit{in vitro} (1, 37). CK2 phosphorylation does not activate PP1-I-2 but enhances Thr\textsuperscript{72} phosphorylation by GSK3 (8, 9). We found that NCLK also phosphorylates I-2 on Thr\textsuperscript{72}. However, NCLK phosphorylation of I-2 is insensitive to a prior phosphorylation by other proteins (45, 46). It should also be noted that deletion of the region there is a34RVRL37 sequence (Fig. 6), located within a 14-residue-long Cdk5 region (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6).

I-2 but enhanced Thr\textsuperscript{72} phosphorylation by GSK3 (8, 9). We found that NCLK also phosphorylates I-2 on Thr\textsuperscript{72}. However, NCLK phosphorylation of I-2 is insensitive to a prior phosphorylation by CK2 (Fig. 4). These data indicate that NCLK and GSK3 display different substrate specificity for I-2 phosphorylation. In neu-rons CK2/GSK3 and NCLK may activate PP1-I-2 in response to different cellular stimuli. They may also function in different regions of the brain or in different subcellular compartments.

In this study we showed that a major PP1-binding site is located within a 14-residue-long Cdk5 region (Fig. 6). Within this region there is a34RVRL37 sequence (Fig. 6) similar to an RVRF sequence motif found in many PP1-binding proteins (2, 3, 4, 5). This putative PP1-binding site is located in between the ATP binding region and the PSSLAIR helix of Cdk5. This site is identical in Cdk5 from human, bovine, mouse, rat, and Drosophila (15, 40–43) and has a conserved substitution D96N found in many PP1-binding proteins (2, 3, 4, 5). In crystal structure, this sequence is an exposed loop available for interaction with other proteins (44). In crystal structure, this sequence is an exposed loop available for interaction with other proteins (44). In crystal structure, this sequence is an exposed loop available for interaction with other proteins (44).

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