Phosphorylation of Protein Phosphatase 1 (PP1) by Cyclin-dependent Protein Kinase 5 During Nerve Growth Factor-Induced PC12 Cell Differentiation

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Running title: PP1 phosphorylation by Cdk5

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The transcription factor Egr-1 activates cyclin-dependent protein kinase 5 (Cdk5) during nerve growth factor (NGF)-induced differentiation of PC12 cells into neurons (Harada, T., Morooka, T., Ogawa, S. and Nishida, E. (2001) Nature Cell Biol. 3, 453-459). The downstream target of Cdk5 in the Egr-1/Cdk5 pathway is not clear. In this study, we observed that phosphorylation of protein phosphatase 1 (PP1) on T320 is reduced in brain extracts from Egr-1 -/- mice indicating that a kinase downstream of Egr-1 phosphorylates PP1. In HEK-293 cells co-transfected with PP1 and Cdk5, Cdk5 phosphorylates PP1. In vitro, Cdk5 purified from bovine brain phosphorylates bacterially expressed recombinant PP1. In NGF-treated PC12 cells, inhibition of Cdk5 by olomoucine or silencing Cdk5 expression by siRNA strategy, suppresses PP1 phosphorylation. Silencing Cdk5 expression by siRNA also blocks NGF-induced neurite outgrowth. Overexpression of PP1 (WT) promotes whereas that of PP1 (T320A) has no effect on NGF-induced differentiation of PC12 cells. Our data indicate that PP1 is a downstream target of the NGF/Egr-1/Cdk5 pathway during NGF-induced differentiation of PC12 cells and suggest that PP1 phosphorylation promotes neuronal differentiation.

Protein phosphatase 1 (PP1) is a major Ser/Thr phosphatase in eukaryotic cells participating in a wide variety of cell functions including cell cycle regulation, muscle contraction, glycogen metabolism, cell differentiation, neural function and signal transduction (for reviews see Refs 1-3). PP1 activity is regulated by separate inhibitory and targeting subunits. Inhibitory subunits suppress PP1 activity whereas targeting subunits specify substrate specificity and subcellular localization (2).

PP1 activity is also regulated by phosphorylation (3-9). In dividing mammalian cells, PP1 is phosphorylated by cyclin-dependent protein kinases Cdk1 and Cdk2 on T320 (3-6). When T320 is phosphorylated, PP1 activity is inhibited (4-6). In yeast PP1 homologue dis2 is phosphorylated on T316 (corresponding to T320 of mammalian PP1) and the overexpression of a dis2 (T316A) mutant causes cell cycle arrest (7). Introduction of a PP1-T320A mutant into synchronized mammalian cells at late G1 prevented cells from entering S phase (8). It was concluded that PP1 T320 phosphorylation is required for S-phase initiation (3, 8). The role of PP1 phosphorylation in other cell activities is unclear.

Cyclin-dependent protein kinase 5 (Cdk5) is a heterodimer of a catalytic Cdk5 and a regulatory p25 subunit (10-13). The p25 subunit is a proteolytic fragment of p35 protein and both p35 and p25 activate the catalytic activity of Cdk5 subunit (13). Cdk5 is inactive in dividing cells, but becomes progressively more active in differentiating cells (14, 15) and is predominantly expressed in terminally differentiated neurons (10-13). Cdk5 is involved in brain development, neuronal differentiation, cell signaling and regulates microtubule dynamics. When PC12 cells are treated with nerve growth factor (NGF), they differentiate into neurons. The transcription factor early growth factor-1 (Egr-1) is induced by NGF.
and is essential for the differentiation (16, 17). NGF activates Cdk5 via Egr-1 (15, 18) and inhibition of Cdk5 activity, blocks NGF-induced neurite outgrowth (15). These observations indicate that Cdk5 is a component of the Egr-1-dependent signaling pathway and that an Egr-1-driven increase in Cdk5 activity is required for differentiation of PC12 cells to neurons.

In this study, we observed that PP1 phosphorylation is reduced in the brain extracts of Egr-1-/- mice suggesting that a kinase which acts downstream of Egr-1 phosphorylates PP1 in the brain. This prompted us to investigate if Cdk5 phosphorylates PP1. Herein, we show that PP1 is phosphorylated by Cdk5 in vitro, in transfected mammalian cells and in differentiating PC12 cells. We also demonstrate that in NGF-exposed PC12 cells, blocking PP1 phosphorylation by inhibiting Cdk5 activity, blocks neurite outgrowth. Overexpression of PP1 (WT) but not PP1 (T320A) promotes differentiation. Our data indicate that Cdk5 phosphorylates PP1 in vivo and suggest that PP1 phosphorylation has a role in neuronal differentiation.

**EXPERIMENTAL PROCEDURES**

**Animals** – Experimental mouse studies were conducted in adult male wild type (+/-) C57Bl/6 or Egr-1 deficient (-/-) mice, n=3. Egr-1-/- mice have a C57Bl/6 genetic background. The originating parental mice lacking Egr-1 (19) were a gift from Dr. Jeffrey Milbrandt (University of St. Louis, St Louis, MO). All experiments were performed according to the regulations of the Canadian Council of Animal Care and the Animal Care Committee of the Lady Davis Institute for Medical Research. Mice were genotyped by polymerase chain reaction (PCR) using DNA obtained by tail docking and amplification using gene-specific primers and Taq DNA polymerase. Primer pairs were neo-929 5’-CTCGTGCTTTACGGTATGC-3’, Egr-1-1263 5’-AACCGGCCCCAGCAAGACACC- 3’ and Egr-1-1677 5’-GGGCACAGGGGATGGGAATG-3’. A 414 bp DNA was amplified from C57Bl/6 mice using the 1263+1677 primer combination and no DNA amplified using the neo929+1677 primers. A 520 bp DNA fragment was amplified from Egr-1-/- mice neo 929+1677 primers and no DNA was amplified using the 1263+1677 primers. Mice were killed at 3 months of age by cervical dislocation and their brains were removed immediately. Each brain was quickly homogenized in an equal amount (W/V) of cold extraction buffer (50 mM Hepes (pH 7.2), 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 50 mM β-glycerol phosphate, 0.2 mM okadaic acid, 10 mM NaF and 1 μg/ml each of leupeptin, pepstatin and aprotinin) using a Down’s glass homogenizer. Samples were centrifuged at 14,000 x g for 20 min using a bench top centrifuge. The resulting supernatants were used to generate Fig. 1.

**Protein and antibodies** – Cdk5 was purified from an extract of fresh bovine brain as described previously (20, 21). PP1 (unless otherwise indicated PP1 means PP1α) was purified from *E. coli* overexpressing human PP1α (22). Monoclonal antibodies against Cdk1, Cdk2 and Cdk5 were purchased from Upstate Biotechnology Inc (Lake Placid, NY). A polyclonal anti-p35 antibody that recognizes both p35 and p25 and an anti-PP1 monoclonal antibody were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Monoclonal anti-Flag, anti-Myc, anti-Xpress and anti-HA antibodies were described previously (23, 24). Polyclonal anti-pT320 antibody specific for PP1 phosphorylated on T320 was from Cell Signaling Technology (Beverly, MA). NGF (2.5 S) was obtained from Upstate Signaling Solution (Temecula, CA).

**cDNA cloning** – PP1 was subcloned into the mammalian expression vector Myc-pcDNA3.1/Zeo by PCR. *pfu* DNA polymerase-catalyzed PCR was performed using forward primer 5’-CGG ATA TCA ATG TCC GAC AGC GAG AAG CTC-3’ and the reverse primer, 5’-CGG GAT CCC TAT TTC TTG GCT TTG GCG GT-3’ and human PP1α in pDR540 vector as the template. Site-specific mutant PP1 (T320A) was generated by PCR using Myc-PP1α–pcDNA3.1 as the template as described previously (23). The forward and reverse primers were: 5’-GGA ATT GCG GGG TGG GGC GAT GGG TCG GCC TCC-3’ and the reverse primer, 5’-GGG GAT CCC CAC TTC TTG GCT TTG GCG GT-3’ and human PP1α in pDR540 vector as the template. Site-specific mutant PP1 (T320A) was generated by PCR using Myc-PP1α–pcDNA3.1 as the template as described previously (23). The forward and reverse primers were: 5’-GGA ATT GCG GGG TGG GGC GAT GGG TCG GCC TCC-3’ and 5’-GGG GAT CCC CAC TTC TTG GCT TTG GCG GT-3’. All cDNA clones were confirmed by DNA sequencing. Dominant negative HA-Cdk5 (DN) in pcDNA 3.1 vector was a gift from Dr. L.-H. Tsai (Harvard University, Boston MA). Mammalian vectors that express Xpress-Cdk5, Flag-p25 and Flag-p35 were described previously (24).

**Cell culture and cDNA transfection** – PC12...
cells were grown in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, penicillin-streptomycin and glutamine. HEK-293 cells were cultured using standard techniques (23, 24). All cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Unless otherwise indicated, cells were harvested 48 hr after transfection.

PC12 cells were plated on polylysine coated dishes. After 24 hr NGF (0.1 μg/ml) was added and cell differentiation monitored microscopically as described (25). At the indicated time points, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM β-glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM MgCl₂, 0.2% Nonidet-P40, 0.4 μM okadaic acid, 2 nM cypermethrin and aprotinin, pepstatin and leupeptin (0.2 μg/ml of each)).

Transfection of siRNA – siRNA-Cdk5 (SMARTpool containing four pooled SMARTselected siRNA duplex) and a nonspecific control were purchased from Upstate Cell Signaling Solution (Temecula, CA). PC12 cells were transfected with siRNA using siMPORTER™ (Upstate Cell Signaling solution) reagent following the manufacturer's instructions. NGF was added 6 hr after transfection. Cells were harvested at the indicated time points.

Neurite outgrowth assay and immunocytochemical staining – NGF-treated cells grown on cover slips were fixed at room temperature with 4% paraformaldehyde in PBS for 30 min, washed with PBS containing 0.1% Triton X-100 and then permeabilized by incubation with PBS containing 1% BSA and 0.1% Triton X-100 for 60 min. Cells were then incubated with monoclonal anti-Myc antibody (1:500 in PBS containing 1% BSA and 0.1% Triton X-100) for ~12 hr at 4 °C. The washed cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) for 1 hr at room temperature, washed and finally visualized and photographed using a Nikon fluorescent microscope. Neurite length was measured on the photographs using a micrometer (26, 27). Only those neurites which were equal or longer than the cell diameter were considered to be neurites and cells which contained one or more neurites were regarded as having neurite outgrowth. Cells that had one or more neurites with the length twice or more than twice the diameter of the cell body were considered differentiated according to previously described criteria (28). More than 100 cells were quantitated in each experimental group.

In vitro phosphorylation – Phosphorylation of PP1 by Cdk5 was carried out at 30°C in a reaction mixture containing 0.5 mg/ml of PP1, 1 mM EDTA, 1 mM DTT, 10 mM MgCl₂, 10 mM NaF, 50 mM β-glycerol phosphate, 0.2 μM okadaic acid, 1 mM ATP and 400 units/ ml of Cdk5. The reaction was initiated by adding an aliquot of Cdk5 into the mixture containing the assay components. At the indicated time points, aliquots were withdrawn and mixed with an equal volume of SDS/PAGE sample buffer, boiled and analyzed by Western blot analysis using anti-PP1 or anti-pT320 antibody.

Cdk5 activity in mouse brain extract was assayed as described previously (20) using a synthetic peptide substrate of Cdk5 (KTPKKAKPKTPKKAKKL) (29). The protein concentration of each brain extract was adjusted to 2 mg/ml by dilution with extraction buffer. The final concentrations of the assay components were: 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 10 mM NaF, 50 mM β-glycerol phosphate, 20 nM okadaic acid, 10 mM MgCl₂, 0.5 mM [γ³²P]ATP and 50 μM peptide substrate. Reaction was initiated by the addition of 10 μl of brain extract to 40 μl of the assay mixture. After 30 min at 30 °C, the reaction was stopped by adding 10 μl of 50% TCA. Vials were incubated at 4 °C for 10 min and then centrifuged for 5 min using a bench top centrifuge at 10,000 rpm. The supernatant (20 μl each) was withdrawn and analyzed for the amount of radioactivity incorporated into the substrate using a phosphocellulose filter paper assay (20). Cdk5 specific activity is expressed as the nanomoles of phosphate transferred to the peptide substrate/min/mg protein in the brain extract.

RESULTS

PP1 phosphorylation is reduced in Egr-1−/− mouse brain – To determine if Egr-1 affects PP1 expression in vivo, we homogenized fresh brains from three Egr-1+/+ and three Egr-1−/− mice under identical conditions. Each homogenate was analyzed by Western blot analysis. As shown in Fig. 1A, PP1 is highly expressed in Egr-1+/+ (lanes 1-3) and Egr-1−/− brains (lanes 4-6). Quantitation of
blot band intensities showed similar relative amount of PP1 protein in Egr-1+/+ brains (~0.32, ~0.33 and ~0.35) and Egr-1−/− brains (~0.39, ~0.36 and ~0.35) (Fig. 1B). These data indicate that Egr-1 may not regulate expression and/or stability of PP1 in adult mouse brain.

PP1 is phosphorylated on T320 in vivo (4-6). We observed that PP1 in fresh mouse brain extract is highly immunoreactive to an anti-pT320 antibody that specifically cross-reacts with PP1 phosphorylated on T320 (see below). This observation indicates that PP1 is phosphorylated in the adult mouse brain. To determine if Egr-1 regulates PP1 phosphorylation, we analyzed brain homogenates of Egr-1+/+ and Egr-1−/− mice by Western blot analysis using anti-pT320 antibody. We observed that PP1 is phosphorylated on T320 in brains of Egr-1+/+ as well as Egr-1−/− mice (Fig. 1A). Quantitation determined that the relative amount of T320-phosphorylated PP1 in Egr-1+/+ brains is twice (0.62, ~0.59 and ~0.57) that found in Egr-1−/− brains (~0.32, ~0.33 and ~0.29) (Fig. 1B). These observations indicate that PP1 phosphorylation is significantly suppressed in Egr-1−/− mouse brains.

Egr-1 is a transcription factor (16, 17) and so may regulate PP1 phosphorylation by controlling the expression of a kinase that phosphorylates PP1. T320 of PP1 is a proline-directed phosphorylation site recognized by cyclin-dependent kinases: Cdk1 and Cdk2 (3-6). Cdk1 and Cdk2 are not expressed significantly in adult brain whereas Cdk5 shares a very similar substrate specificity with Cdk1 and Cdk2 (30), and is the major cyclin-dependent kinase in the neurons of mammalian brain (10-12). Previous studies showed that Egr-1 activates Cdk5 in neurons by increasing the expression of p35 subunit of Cdk5 (15, 18). If Egr-1 activates brain Cdk5 then Egr-1+/+ brains would be expected to have reduced p35 level and Cdk5 activity. To test this possibility, we analyzed the levels of Cdk5, and p35 and p25 by Western blotting and measured Cdk5 activity in Egr-1+/+ and Egr-1−/− brain extracts. Since p35 is proteolyzed to p25, we determined the level of p35 based on the levels of p25 and p35. The Cdk5 level was similar but the sum of the levels of p35 and p25 was ~2.7 fold more in Egr-1+/+ than in Egr-1−/− brain extracts (Fig. 1A and B). The Cdk5 specific activity in Egr-1−/− brain extracts was ~38% less than that of Egr-1+/+ (Fig. 1C). Thus as expected, both p35/p25 level and Cdk5 activity are reduced in Egr-1+/+ brains. The observation of reduced PP1 phosphorylation coupled to reduced Cdk5 activity in Egr-1−/− brain suggests that Cdk5 may be a kinase that phosphorylates PP1 in the brain.

Cdk5 phosphorylates PP1 – To evaluate if Cdk5 phosphorylates PP1, we first tested the specificity of pT320 antibody. Since PP1 is phosphorylated on T320 in proliferating cells by endogenous Cdk1 and Cdk2, we transfected Myc-PP1 (WT) or site-specific mutant Myc-PP1 (T320A) in HEK-293 cells and analyzed cell lysates by Western blot analysis. Anti-Myc antibody detected both Myc-PP1 (WT) and Myc-PP1 (T320A) (Fig. 2), but anti-pT320 antibody cross-reacted with Myc-PP1 (WT) but completely failed to detect Myc-PP1 (T320A). This data demonstrated that anti-pT320 antibody is specific for PP1 phosphorylated on T320.

We next co-transfected Myc-PP1 (WT) with Xpress-Cdk5 and Flag-p25 (Xpress-Cdk5/Flag-p25) in HEK-293 cells. Controls included co-transfection of Myc-PP1 (T320A) with Xpress-Cdk5/Flag-p25, and Myc-PP1(WT) with Cdk5 mutant, HA-Cdk5 (DN). HA-Cdk5 (DN) with D144 mutated to N, although reported to contain no detectable kinase activity when co-transfected with p35 in C33A cervical carcinoma cells (13), displays kinase activity ~10% of Cdk5 (WT) when co-transfected with Flag-p25 in HEK-293 cells (data not shown). Transfected cells were lysed and analyzed by immunoblot analysis for the expression of the various transfected genes and PP1 phosphorylation. The relative amount of phosphorylated PP1 was calculated based on the band intensities of PP1 and phosphorylated PP1.

As shown in Fig. 3A, in cells co-transfected with Myc-PP1 (WT) and Xpress-Cdk5/Flag-p25, Myc-PP1 was phosphorylated ~3.5-fold more than basal level (compare lanes 2 and 3 and see panel F). In cells co-transfected with Myc-PP1 (WT) and HA-Cdk5 (DN)/p25-Flag, Myc-PP1 phosphorylation was slightly above basal level (compare lanes 2 and 6 and see panel F). These data indicate that PP1 is highly phosphorylated by Cdk5 and very modestly by Cdk5 (DN). To extend and confirm these results, we treated HEK-293 cells, co-transfected with Myc-PP1 and Xpress-Cdk5/Flag-p25, with the Cdk5 inhibitor olomoucine (21, 31) or vehicle.
Treated cells were then analyzed for PP1 phosphorylation by Western blot analysis as described above. PP1 was highly phosphorylated in vehicle treated cells, however, PP1 phosphorylation progressively diminished with increasing olomoucine concentration. When the concentration of olomoucine reached 200 μM, PP1 phosphorylation was almost at basal level (Fig. S1 of supplemental data). Based on these results, we conclude that Cdk5 phosphorylates PP1 on T320 in HEK-293 cells transfected with Myc-PP1 and Xpress-Cdk5/Flag-p25.

The p25 regulatory subunit of Cdk5 is formed by the proteolytic cleavage of p35 protein (13). Although p25 activates Cdk5, p35 is the natural activator of Cdk5 (11, 12). To evaluate if Cdk5/p35 phosphorylates PP1, we transfected Myc-PP1 and Xpress-Cdk5/Flag-p35 in HEK-293 cells. Transfected cells were lysed, analyzed for expression of the transfected genes and Myc-PP1 phosphorylation as described above. The relative amount of phosphorylated Myc-PP1 in cells transfected with Myc-PP1 and Xpress-Cdk5/Flag-p35 was ~3 fold more than in those cells transfected with Myc-PP1 alone (data not included). These results determined that Cdk5/p35, like Cdk5/p25, phosphorylates PP1.

In vitro phosphorylation – To test if Cdk5 phosphorylates PP1 directly, we performed in vitro kinase assays. Bacterially expressed recombinant PP1 was incubated with purified Cdk5 in the presence of ATP/Mg2+ for various time points. Incubated samples were analyzed for PP1 phosphorylation by immunoblot analyses using anti-pT320 antibody (Fig. 4A). No immunoreactive band against anti-pT320 was observed in samples containing Cdk5 alone incubated with ATP/Mg2+ (lane 1) and PP1 did not become phosphorylated when incubated alone with ATP/Mg2+ (lane 2). In samples containing PP1, Cdk5 and ATP/Mg2+, PP1 phosphorylation increased with increasing incubation time (lanes 3-7). These observations demonstrate that Cdk5 phosphorylates PP1 in vitro. The Cdk5 used in this experiment was purified from bovine brain extract. To rule out the possibility that a contaminant kinase in our Cdk5 preparation may have phosphorylated PP1, we performed in vitro kinase assay as described above for 30 min in the presence of increasing concentrations of the Cdk5 inhibitor olomoucine (Fig. 4C). Incubation with 50 μM olomoucine suppressed ~60% of PP1 phosphorylation (lane 5). When the concentration of olomoucine was increased to 100 μM, PP1 phosphorylation was almost completely suppressed (lane 6). Taken together, these observations indicate that Cdk5 phosphorylates PP1 in vitro.

PP1 associates with Cdk5 – To characterize the interaction between Cdk5 and PP1, we co-transfected Xpress-Cdk5 with Myc-PP1 (WT) or Myc-PP1 (T320A) in HEK-293 cells. Transfected cells were lysed and each lysate was subjected to immunoprecipitation using either anti-Myc antibody or control IgG. Each immunocomplex was then immunoblotted against anti-Xpress or anti-Myc antibody. As shown in Fig. 5A, Xpress-Cdk5 specifically co-immunoprecipitated with Myc-PP1 (WT) (lane 3). This data is consistent with our previous report (21) and indicates that PP1 binds with Cdk5. Xpress-Cdk5 also co-immunoprecipitated with Myc-PP1 (T320A) (lane 4) indicating that mutation of T320 to A does not significantly interfere with the binding of PP1 to Cdk5. Since PP1 is phosphorylated on T320 in HEK 293 cells (Fig. 3, lane 2), this data also indicates that blocking T320 phosphorylation does not prevent PP1 from binding to Cdk5.

PP1 is phosphorylated during neuronal differentiation – PC12 cells treated with NGF differentiate into neurons (32). In these cells, NGF activates Egr-1 which in turn activates Cdk5 by inducing the expression of p35 (15). Cdk5 activity is required for PC12 cell differentiation (15). It is possible that NGF-activated Cdk5 phosphorylates PP1 and this phosphorylation may be an important event for neuronal differentiation. To test this idea, we treated PC12 cells with NGF for various time points and then analyzed the treated cells.

Exposure with NGF for 1 day caused ~20-30% of PC12 cells to extend neurites. On day 2, ~40-50% PC12 cells displayed long neurites with multiple branches, varicosities and growth cones. Almost 90% of NGF-treated PC12 cells exposed for 6 days displayed the fully differentiated phenotype (data not shown). As shown in Fig. 6, PP1 is highly expressed (panel A) and is phosphorylated (panel B) in PC12 cells exposed to NGF. Blot band quantitation determined that the relative amounts of PP1 (1, ~0.8, ~1.1, ~1, ~0.9 and 1) and phosphorylated PP1 (1, ~1.4, ~1.3, ~1.6) increased in PC12 cells during neuronal differentiation.
compared (Fig. 7D-G). This data indicates that the level of total PP1 and phosphorylated PP1 are not affected during NGF-induced PC12 cell differentiation.

**Cdk5 is involved in PP1 phosphorylation in NGF-induced differentiating PC12 cells** – Consistent with a previous report (14), Western blot analysis determined that Cdk1 and Cdk2 proteins were highly expressed in PC12 cells exposed to NGF for 0 day but became undetectable on day 3 of NGF exposure (data not shown). These data suggest that PP1 may have been phosphorylated by Cdk1 and/or Cdk2 at day 0 and day 1, but that these kinases are less likely to be responsible for significant PP1 phosphorylation with increasing time of differentiation.

To evaluate if Cdk5 is involved, PC12 cells were exposed to NGF for 3 days and then transfected with Cdk5 inhibitor olomoucine. Treated cells were lysed and analyzed for PP1 phosphorylation. As shown in Fig. 7, olomoucine inhibited PP1 phosphorylation in a dose-dependent manner (panel A and C) without affecting the level of PP1 (panel A and C). This data indicates that a kinase sensitive to olomoucine phosphorylates PP1 in NGF-exposed PC12 cells. Olomoucine, in addition to Cdk5 also inhibits Cdk1 and Cdk2 (31). Therefore to confirm if Cdk5 phosphorylates PP1, we suppressed Cdk5 expression by siRNA strategy. We transfected PC12 cells with siRNA-Cdk5 or siRNA-control. Transfected cells were then exposed to NGF for various time points and analyzed by immunoblot analysis. Levels of Cdk5, PP1 and phosphorylated PP1 were quantitated and compared (Fig. 7D-G).

Cdk5 expression was not affected by siRNA-Cdk5 in day 0 and day 1 cells as the relative amount of Cdk5 was similar in cells transfected with siRNA-Cdk5 and siRNA-control (Fig. 7G). By day 2, siRNA-Cdk5 suppressed Cdk5 expression by ~80% (Fig. 7D, lane 6 and G). PP1 expression was similar at all time points (Fig. 7E and G) indicating that suppression of Cdk5 expression does not affect PP1 level. PP1 phosphorylation on the other hand was similar in siRNA-Cdk5 and siRNA-control transfected day 0 cells (compare lanes 1 and 2 in Fig. 7F). However, on day 1, PP1 phosphorylation was suppressed by ~30% and by day 2, the relative amount of phosphorylated PP1 was ~1/10 that of day 0 (Fig. 7G). Thus, silencing ~80% Cdk5 expression, suppressed ~90% PP1 phosphorylation in PC12 cells treated with NGF for two days. Based on these results, we concluded that Cdk5 phosphorylates PP1 during NGF-induced differentiation of PC12 cells.

**Effect of silencing Cdk5 expression on PC12 cell differentiation** – To determine the role of PP1 phosphorylation, we suppressed PP1 phosphorylation by suppressing Cdk5 expression and quantitated neurite outgrowth. We transfected PC12 cells with siRNA-control and SiRNA-Cdk5 and exposed the cells to NGF. The relative amount of phosphorylated PP1 in cells transfected with siRNA-control remained unchanged (~0.78 versus ~0.79) after 1 and 2 days of NGF treatment (data not shown). The relative number of cells displaying neurite outgrowth in these cells is 18% at day 1 and increased to ~36% at day 2 (Fig. 8A and B). However, in cells transfected with siRNA-Cdk5 the relative amount of phosphorylated PP1 is reduced (~0.72 versus ~0.30) (data not shown) and the number of cells showing neurite outgrowth did not change significantly (~20% and ~15%) (Fig. 8A and B) at day 1 and 2 day, respectively. Thus, from day 1 to day 2, siRNA-control transfected cells maintained a constant level of phosphorylated PP1 and doubled the number of cells displaying neurite outgrowth. Cells transfected with siRNA-Cdk5 maintained PP1 phosphorylation at day 1, and contained a level of neurite out growth similar to that observed in cell transfected with siRNA-control. By day 2, cells transfected with siRNA-Cdk5 had significantly suppressed amounts of phosphorylated PP1 and did not show the expected increase in the number of cells displaying neurite outgrowth. These data demonstrate that suppression of Cdk5 expression by siRNA-Cdk5, blocks PP1 phosphorylation and neurite outgrowth.

**Effect of overexpression of PP1 (WT) and PP1 (T320A) on NGF-induced differentiating PC12 cells** – Cdk5 in addition to PP1 also phosphorylates a number of cellular proteins (11). Suppressing Cdk5 expression by siRNA will also suppress phosphorylation of these other targets. Hence, the failure of cells transfected with siRNA-Cdk5 to advance to differentiation, could be due to suppressed phosphorylation of Cdk5 targets other than PP1. Therefore to more specifically evaluate the role of PP1 phosphorylation, we transfected
PC12 cells with Myc-PP1 (WT), Myc-PP1 (T320A) and Myc vector control. Transfected cells were treated with NGF to activate cellular Cdk5. In Myc-PP1 (WT) transfected cells, activated Cdk5 would be expected to phosphorylate Myc-PP1 (WT) and increase the cellular level of phosphorylated PP1. In Myc-PP1 (T320A) and Myc-vector transfected control cells, the cellular level of phosphorylated PP1 would be expected to remain at similar but basal level. We then determined the number of cells committed to differentiation by measuring neurite outgrowth. Cells were considered fully differentiated when they displayed one or more neurites equal or longer than twice the diameter of the cell body (28). Since multipolar neurons that predominate mammalian brain contain multiple neurites (a single axon and one or more dendrites that emerge from all parts of the cell body), we also analyzed the number of neurites per cell.

As shown in Fig. 9A and B, ~64 and ~52% Myc-vector and Myc-PP1 (T320A) transfected cells displayed neurites, respectively. In Myc-PP1 (WT) transfected cells, this number rose to ~79%. Only ~15% each of Myc-vector and Myc-PP1 (T320A) transfected cells displayed fully differentiated phenotype (Fig. 9A and C). For Myc-PP1 (WT) transfected cells ~27% cells were fully differentiated. Furthermore, among neurite containing Myc-vector transfected cells, ~52 and ~33% contained one and two neurites, respectively and ~15% contained three or more neurites (Fig. 9D). Among neurite containing Myc-PP1 (T320A) transfected cells, ~47% and 32% contained one or two neurites, respectively and ~21% advanced to three or more neuritic stage. In contrast, ~35% and 27% of Myc-PP1 (WT) transfected cells had one and two neurite, respectively and ~38% advanced to higher differentiation stage containing three or more neurites. Thus, overexpression of Myc-PP1 (T320A) did not affect PC12 differentiation. Overexpression of Myc-PP1 (WT) however significantly promoted neurite outgrowth, neurite extension and number of neurites per cell. These observations indicate that overexpression of PP1 (WT) promotes neuronal differentiation.

**DISCUSSION**

Cdk1 and Cdk2 phosphorylate and inactivate PP1 in dividing cells and this phosphorylation plays an important role in cell cycle regulation (3-9). To examine PP1 phosphorylation by Cdk5 in the brain and in NGF-treated PC12 cells, we used an antibody that specifically cross-reacts with PP1 phosphorylated on T\textsuperscript{20}. We first confirmed the specificity of the antibody by using PP1 (T320A) mutant. Our subsequent results show that PP1 is highly phosphorylated when co-transfected with Cdk5 in HEK-293 cells (Fig. 3). In NGF-treated PC12 cells, silencing Cdk5 expression or suppressing Cdk5 activity significantly reduced PP1 phosphorylation (Fig. 7). Finally, in vitro purified Cdk5 phosphorylated bacterially expressed recombinant PP1 (Fig. 4). Taken together, our data indicate that Cdk5 phosphorylates PP1 in vitro and in vivo. It is interesting that in HEK-293 cells, the basal phosphorylation of PP1 is insensitive to Cdk inhibitor olomoucine (Fig. S1). This data suggests that PP1 is also phosphorylated by a kinase (s) other than Cdk1, Cdk2 and Cdk5 in vivo.

Morfini et al (33) demonstrated that in rat cortical neurons PP1 was activated when Cdk5 was inactivated indicating that Cdk5 suppresses PP1 activity in vivo. However, the authors also reported that Cdk5 did not phosphorylate PP1 in vitro and hence that Cdk5 inactivated PP1 by a mechanism that did not require PP1 phosphorylation. This study contradicts our in vitro phosphorylation data presented here.

Morfini et al (33) did not fully describe their in vitro phosphorylation procedure. Therefore, we are unable to determine the cause of the disparity between their results and our data. There are unique problems associated with in vitro phosphorylation of PP1 (4, 6). PP1 autodephosphorylates (4) and hence, its phosphorylation can only be detected in the presence of high concentration of PP1 inhibitors. Furthermore, PP1 phosphorylation greatly depends upon the purity of the kinase used. For example, highly purified Cdk1/cyclinA phosphorylates PP1, but when less purified Cdk1/cyclinA is used, PP1 is not phosphorylated significantly (4). It is possible that Morfini et al (33) may not have included sufficiently high doses of PP1 inhibitors and/or used a Cdk5 which was not purified in their phosphorylation mixture.

PC12 cells are an excellent and widely used model for studying neuronal differentiation (32). In these cells, binding of NGF to its cell
surface receptor leads to the activation MAP-kinase. Activated MAP-kinase phosphorylates ternary complex factor, which together with serum response factor induces Egr-1 (16, 17). Indeed Egr-1 was first identified as a transcription factor increased within hours after NGF treatment of PC12 cells (17). The MAP-kinase/Egr-1/Cdk5 pathway is one of the major signaling events triggered by NGF and leads to neurite extension. Inhibition of Cdk5 activity blocks neurite outgrowth (15), but the targets of Cdk5 in this pathway are not completely known.

In the current study, we observed that PP1 is phosphorylated in PC12 cells exposed to NGF (Fig. 6). When Cdk5 expression is silenced, PP1 phosphorylation is significantly suppressed (Fig. 7G). In vitro and in transfected HEK-293 cells, Cdk5 phosphorylates PP1 (Figs. 3, 4). Our data demonstrate that PP1 is phosphorylated by Cdk5 in NGF-exposed differentiating PC12 cells and suggest that PP1 is downstream of Cdk5 in the NGF/MAP-kinase/Egr-1/Cdk5 pathway.

In dividing PC12 cells, Cdk5 is inactive and is only activated when cells are treated with NGF (14, 15). Therefore one would expect PP1 phosphorylation to surge concomitant with Cdk5 activation. However, as shown in Fig. 6, PP1 is phosphorylated in dividing cells (day 0) and remains phosphorylated at the same level after cells are exposed to NGF and throughout the differentiation period. This observation indicates that PP1 phosphorylation is sustained at a constant level during the transition of dividing PC12 cells to neuronal differentiated PC12 cells.

Withdrawal from the cell cycle is a prerequisite for differentiation and it has been suggested that terminal mitosis and differentiation must be coupled for successful differentiation (34). Cdk1 and Cdk2, which are active in dividing cells, are inactivated early in the neuronal differentiation period (14). Cdk5 on the other hand is inactive in dividing cells, but becomes progressively more active in differentiating cells (14, 15). It is possible that when PC12 cells are exposed to NGF, PP1 is phosphorylated initially by Cdk1 and/or Cdk2. With increasing time of NGF exposure, PP1 may be less and less phosphorylated by Cdk1 and Cdk2 and more and more by Cdk5. Thus, a constant level of phosphorylated PP1 is maintained and this may be important to couple terminal mitosis to neuronal differentiation.

NGF-exposed PC12 cells with suppressed Cdk5 expression and PP1 phosphorylation displayed significantly impaired differentiation as exemplified by a reduced number of cells with neurite outgrowth when compared to control cells with normal levels of Cdk5 and phosphorylated PP1 (Fig. 8). This observation suggests that PP1 phosphorylation may be required for neurite outgrowth. However, Cdk5 phosphorylates a number of cellular proteins. Therefore to more clearly understand the importance of PP1 phosphorylation, we transfected PP1 (WT) or PP1 (T320A) into differentiating PC12 cells. PP1 (WT) enhanced neurite outgrowth, increased the number of neurites per cell and promoted neurite extension to the fully differentiated phenotype (Fig. 9). PP1 (T320A) on the other hand, did not influence significantly any of the differentiation characteristics analyzed in this study. Since the main difference between the PP1 (WT) and PP1 (T320A) is that only former is phosphorylated, these data suggest that PP1 phosphorylation promotes neuronal differentiation. Many proteins involved in the regulation of cytoskeletal dynamics and membrane trafficking are phosphorylated in vivo; and some of these proteins are PP1 targets (35). It is possible that PP1 phosphorylation may keep PP1 inactive and allow proper cytoskeletal remodeling and/or membrane trafficking required for differentiation (36).

Cdk5 requires the p35 subunit for its activity (11). In PC12 cells, Egr-1 induces the expression of p35 and thereby activates Cdk5 (15). Expression of NAB2, a co-repressor of Egr-1 almost completely abolishes NGF-induced p35 expression (15). These data indicate that Egr-1 controls p35 expression in NGF-treated PC12 cells. In the current study, we find that the level of p35/p25 protein in the Egr-1−/− brain extract is ~2.7 fold less than in the Egr-1+/+ mice brains (Fig. 1). Our results indicate that in adult rat brain Egr-1 promotes p35 expression but that p35 expression is also regulated independent of Egr-1.

In NGF exposed PC12 cells, suppression of ~80% of Cdk5 expression, inhibits ~90% of PP1 phosphorylation (Fig. 7G) indicating that Cdk5 is the major PP1 kinase in these cells. Egr-1−/− brains which have ~38% less Cdk5 activity than Egr-1+/+ brains, also display ~50% less PP1
phosphorylation than Egr-1+/+ brains (Fig. 1). This correlation between Cdk5 activity and PP1 phosphorylation suggests that Cdk5 may be a major PP1 kinase in the adult mouse brain also. It would be interesting to validate this suggestion by analyzing PP1 phosphorylation in adult mouse brains deficient in Cdk5 activity. However, Cdk5 null mice suffer perinatal death (37). The p35 null mice survive to adulthood (38), but in these mice p39, an isoforms of p35 (39), compensates for the loss of p35 function and Cdk5 remains active (38). It should be noted that the level of p35/p25 in Egr-1-/- brains is ~36% of Egr-1+/+ brains, but Cdk5 activity in Egr-1-/- brains is ~62% of Egr-1+/+ brains (Fig. 1). This data indicates that Cdk5 activity does not correlate with the level of p35 in Egr-1-/- brains. It is possible that deletion of Egr-1-/- gene suppresses p35 expression and the brain in turn enhances the expression of p39 to compensate the loss of p35. Analysis of p39 expression in Egr-1-/- and Egr-1+/+ brains will substantiate this hypothesis.

Previous studies showed that inactivation of PP1 is involved in the induction and maintenance of long term potentiation (LTP), a form of synaptic plasticity that contributes to learning and memory (35, 40-42). In vitro, PP1 is inactivated by T320 phosphorylation (3-6). In the brain, Egr-1 concentration increases with neuronal activity and Egr-1 is required for the induction of LTP (43-45). PP1 phosphorylation is reduced in Egr-1-/- mouse brains (this study) and Cdk5 which phosphorylates PP1 in response to Egr-1 activation is involved in the regulation of neuronal plasticity (46-48). These observations together suggest that PP1 phosphorylation may play a role in the induction and maintenance of LTP.

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FOOT NOTES

*Dr. H. K. Paudel would like to dedicate this study to Dr. Gerald M. Carlson, professor and Chairman of the Department of Biochemistry and Molecular Biology, the University of Kansas Medical Center, Kansas City in recognition and appreciation of his mentorship.

1 The abbreviation used in this paper are: Cdk5, cyclin-dependent protein kinase 5, NGF, nerve growth factor, PP1, protein phosphatase 1
FIGURE LEGENDS

FIG. 1. PP1 phosphorylation is reduced in Egr-1-/- mouse brain. Three Egr-1+/- and three Egr-1-/- mouse brains were homogenized under identical conditions. Each homogenate was analyzed by Western Blot analysis using the indicated antibodies or subjected to Cdk5 activity assay. Based on the blot band intensity, the relative amount of indicated proteins and phosphorylated PP1 in each sample was determined. A, Western blots. B, relative amounts. C, Cdk5 specific activity. To determine the relative amount, the band intensity value of indicated protein in each sample of corresponding blot was normalizing against the corresponding actin band intensity value of that sample. To calculate the relative amount of phosphorylated PP1, the phosphorylated PP1 band intensity value of each sample was normalized against the band intensity value of PP1 in that sample. Cdk5 specific activity was determined as described in the “Experimental Procedure” section. Values are average of three determinations.

FIG. 2. Anti-pT320 is specific for T320-phosphorylated PP1. Lysates of HEK-293 cells transfected with indicated genes were analyzed by Western Blot using indicated antibodies.

FIG. 3. Cdk5 phosphorylates PP1 in HEK 293 cells – Lysates of HEK-293 cells transfected with indicated genes were analyzed by Western blot analysis using the indicated antibodies. Blots corresponding to anti-Myc, anti-Xpress, anti-Flag and anti-HA demonstrate the expression of the corresponding tagged proteins. Anti-pT320 monitors PP1 phosphorylation. A-E, Western blots; F, relative amount of phosphorylated PP1. Blots A and B representing phosphorylated PP1 and total Myc-PP1, respectively were scanned. Band intensity value of each sample in blot A was normalized against the band intensity value of that sample in blot B. The resulting value is expressed as the fraction of the resulting value of lane 2 control representing cells transfected with PP1 (WT) alone. Values are average of three independent determinations.

FIG. 4. In vitro phosphorylation of PP1 by Cdk5 is inhibited by olomoucine. A and B, phosphorylation. PP1 and Cdk5 were incubated together or separately as indicated in the phosphorylation mixture containing ATP/Mg2+. At the indicated time points, aliquots were withdrawn and analyzed by Western blot analysis using anti-pT320 and anti-PP1 antibodies. C and D, inhibition by olomoucine. PP1 was phosphorylated by Cdk5 as described above in the presence of the indicated amounts of olomoucine. After 30 min, samples were analyzed by Western blot analysis. The amount of DMSO used to dissolve olomoucine was the same in all samples.

FIG. 5. PP1 and Cdk5 Co-immunoprecipitate – Myc-PP1 (WT) and Myc-PP1 (T320A) were immunoprecipitated from lysates of HEK-293 cells transfected with indicated genes using anti-Myc antibody and IgG control as described previously (23). Each immune complex was analyzed by Western blot using the indicated antibodies. Lanes 1 and 2 represent lysates of cells to monitor expression of the transfected genes.

FIG. 6. Phosphorylation of PP1 is sustained during PC12 differentiation – PC12 cells were treated with NGF for the indicated time points then lysed. Protein (100 μg) from each lysate was analyzed by Western blot using the indicated antibodies. Based on the blot band intensities, the relative amount of PP1 and phosphorylated PP1 for each sample was calculated. A and B, Western blots. C, relative amounts. To determine relative amount of PP1, the band intensity value of each sample in the panel A representing total PP1 is expressed as the fraction of the band intensity value of day 0 sample in panel A. Values are average of three independent determinations. The differences at various time points are not significant (p > 0.05). To determine relative amount of phosphorylated PP1, band intensity value of each sample in panel B representing phosphorylated PP1 was normalized against the band intensity value of total PP1 of that sample in panel A. The resulting value of each sample was then expressed as the fraction of the resulting value of sample corresponding to day 0. Values are average of three independent determinations. The differences at various time points are not significant (p > 0.05).
FIG. 7. **PP1 phosphorylation is inhibited by olomoucine and Cdk5 targeted siRNA** – *A-C*, inhibition by olomoucine. PC12 cells exposed to NGF for 72 hr were treated with indicated concentration of olomoucine for 1 hr. Treated cells were lysed and then analyzed by Western blot analysis. Based on band intensities of the blots, relative amount of PP1 and phosphorylated PP1 in various samples was determined as in Fig. 6. *D-G*, inhibition by siRNA-Cdk5. PC12 cells transfected with siRNA-Cdk5 or siRNA-control and treated with NGF for the indicated time points were analyzed by Western blot. Based on blot band intensities, relative amounts of Cdk5, PP1, and phosphorylated PP1 were calculated. To calculate relative amount of Cdk5, band intensity value of each sample in panel *D* transfected with siRNA-Cdk5 (or siRNA control) is expressed as the fraction of the band intensity value of the sample in panel *D* transfected with siRNA-Cdk5 (or siRNA control) for 0 day. The relative amount of PP1 for indicated sample was also calculated as described above for Cdk5 except band intensity values of blot *E* representing total PP1 were used. To calculate relative amount of phosphorylated PP1, band intensity value of each sample in the blot *F* representing phosphorylated PP1 was normalized against the band intensity value of that sample in the blot *E* representing total PP1. Resulting value of each sample is then expressed as the fraction of the resulting value of corresponding sample representing cells treated with NGF for 0 day.

FIG. 8. **Silencing Cdk5 expression inhibits PP1 phosphorylation and blocks neurite outgrowth** – PC12 cells transfected with siRNA control or siRNA-Cdk5 were exposed to NGF for the indicated time and visualized under microscope. Nontransfected cells were included for comparison *A*, phase contrast micrographs of NGF exposed cells. *B*, % of cells with neurite outgrowth. Over 200 cells were counted. Values are the average of three determinations.

FIG. 9. **Effect of overexpression of Myc-PP1 (WT) or Myc-PP1 (T320A) on PC12 differentiation** – PC12 cells transfected with Myc-vector (control), Myc-PP1 (WT) or Myc-PP1 (T320A) were treated with NGF for 48 hr. Myc-tag was visualized in the transfected cells by immunofluorescent microscopy. *A*, immunofluorescent micrographs. *B*, percent of cells showing neurite outgrowth. *C*, percent of cells displaying differentiated neurites. *D*, percent of cells containing the indicated number of neurites. Values are the average of three independent experiments.
Figure 1

**A**

- Total PP1
- Phosphorylated PP1
- Actin
- Cdk5
- p35
- p25

IB: anti-PP1
IB: anti-pT320
IB: anti-β actin
IB: anti-Cdk5
IB: anti:p35

**B**

- PP1
- Phosphorylated PP1
- Relative amount of Cdk5
- p35/p25

**C**

Cdk5 specific activity
**Figure 2**

|               | Myc-PP1 (WT) | Myc-PP1 (T320A) | Mock  |
|---------------|--------------|-----------------|-------|
|               | -            | -               | +     |
| IB: anti-Myc  | -            | -               | -     |
| IB: anti-pT320| -            | +               | -     |
| kDa           | -33          | -33             |       |

- Mock - no expression
- Myc-PP1 WT - expression
- Myc-PP1 T320A - expression
- Myc-PP1 WT/(T320A) - expression
- T320-phosphorylated PP1 - expression
Figure 3
Figure 4
Figure 5

|                 | IP: anti-Myc | IP: anti-IgG | Cell lysate |
|-----------------|-------------|--------------|-------------|
| Myc-PP1 (WT)    | +           | -            | +           |
| Myc-PP1 (T320A)| -           | +            | +           |
| Xpress-Cdk5     | +           | +            | +           |

**A**

Xpress-Cdk5

IB: anti-Xpress

-45

**B**

Myc-PP1 (WT)/Myc-PP1 (T320A)

IB: anti-Myc

-45

1 2 3 4 5 6
Figure 7

A

Olomoucine (μM)

0 15 30 60 100

IB: anti-PP1

B

Phosphorylated PP1

IB: anti-pT320

C

Relative amount

Olomoucine (μM)

D

Cdk5

IB: anti-Cdk5

E

PP1

IB: anti-PP1

F

Phosphorylated PP1

IB: anti-pT320

G

Relative amount

Days

Figure 7

siRNA-Cdk5

siRNA-control

NGF exposure Days

0 0 1 1 2 2

Cdk5 Phosphorylated PP1

Relative amount

0.0 0.5 1.0 100 80 60 40 20 0

210

0.0 0.5 1.0 210

0.0 0.5 1.0 210

210
Figure 8
Figure 8

B

% of cells with neurite outgrowth

Day 1  Day 2  Day 1  Day 2  Day 1  Day 2

- Nontransfected
- siRNA-control
- siRNA-Cdk5
Figure 9

(A) Images of cells expressing Myc-vector, Myc-PP1 (WT), and Myc-PP1 (T320A).

(B) Graph showing the percentage of cells with neurite outgrowth.

(C) Graph showing the percentage of differentiated cells.

(D) Graph showing the neurite number per cell.

Legend:
- Black bars: Myc-vector
- Light gray bars: Myc-PP1
- White bars: Myc-PP1 (T320A)
Phosphorylation of protein phosphatase 1 (PP1) by cyclin-dependent protein kinase 5 during nerve growth factor-induced PC12 cell differentiation
Tong Li, Lorraine E. Chalifour and Hemant K. Paudel

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