Cyclin-dependent Kinase-5 Is Involved in Neuregulin-dependent Activation of Phosphatidylinositol 3-Kinase and Akt Activity Mediating Neuronal Survival*

Bing-Sheng Li†, Wu Ma§, Howard Jaffe‡, Yali Zheng‡, Satoru Takahashi‡, Lei Zhang‡, Ashok B. Kulkarni¶, and Harish C. Pant**

From the Laboratory of Neurochemistry, NINDS, National Institutes of Health, Bethesda, Maryland 20892-4130, the §Center for Biomolecular Science and Engineering, Naval Research Laboratory, Washington, D. C. 20375, ¶Functional Genomics Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892, and |Behavioral and Endocrinology Branch, NIMH, National Institutes of Health, Bethesda, Maryland 20892

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an important role in mediating survival signals in a wide variety of neurons and cells. Recent studies show that Akt also regulates metabolic pathways to regulate cell survival. In this study, we reported that cyclin-dependent kinase-5 (Cdk5) regulates Akt activity and cell survival through the neuregulin-mediated PI 3-kinase signaling pathway. We found that brain extracts of Cdk5−/− mice display a lower PI 3-kinase activity and phosphorylation of Akt compared with that in wild type mice. Moreover, we demonstrated that Cdk5 phosphorylated Ser-1176 in the neuregulin receptor ErbB2 and phosphorylated Thr-871 and Ser-1120 in the ErbB3 receptor. We identified the Ser-1120 sequence RSRSPR in ErbB3 as a novel phosphorylation consensus sequence of Cdk5. Finally, we found that Cdk5 activity is involved in neuregulin-induced Akt activity and neuregulin-mediated neuronal survival. These findings suggest that Cdk5 may exert a key role in promoting neuronal survival by regulating Akt activity through the neuregulin/PI 3-kinase signaling pathway.

Cyclin-dependent kinase 5 (Cdk5)1 is a serine/threonine kinase, which is predominantly expressed in postmitotic neurons (1). Cdk5 kinase activity requires association with its neuron-specific activators, p35 and p39. Cdk5 kinase activity is essential for neuronal migration, neurite outgrowth, and laminar configuration of the cerebral cortex (2–4). Recently, we reported that cyclin-dependent kinase-5 (Cdk5) regulates Akt activity and cell survival through the neuregulin-mediated PI 3-kinase signaling pathway. We found that brain extracts of Cdk5−/− mice display a lower PI 3-kinase activity and phosphorylation of Akt compared with that in wild type mice. Moreover, we demonstrated that Cdk5 phosphorylated Ser-1176 in the neuregulin receptor ErbB2 and phosphorylated Thr-871 and Ser-1120 in the ErbB3 receptor. We identified the Ser-1120 sequence RSRSPR in ErbB3 as a novel phosphorylation consensus sequence of Cdk5. Finally, we found that Cdk5 activity is involved in neuregulin-induced Akt activity and neuregulin-mediated neuronal survival. These findings suggest that Cdk5 may exert a key role in promoting neuronal survival by regulating Akt activity through the neuregulin/PI 3-kinase signaling pathway.

1 The abbreviations used are: Cdk5, cyclin-dependent kinase-5; NRG, neuregulin; PEG, polyethylene glycol; MG, methylglyoxal; PBS, phosphate-buffered saline; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PtdIns(4,5)P2, phosphatidylinositol 4,5-diphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent kinase-1; IP, immunoprecipitation; E18, embryonic day 18; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated DUTP nick end labeling.

This is an Open Access article under the CC BY license.
neuregulin family includes heregulin, acetylcholine receptor-
inducing activity, neu differentiation factor, and glial growth
factor (36, 37). Neuregulin proteins mediate their action
through the ErbB family of receptor tyrosine kinases, including
ErbB2, ErbB3, and ErbB4 (36, 38). NRG receptors differ in
kinase activity and substrate selectivity. Each ErbB protein
has an extracellular ligand binding domain, a single trans-
membrane domain, a short intracellular juxtamembrane re-
domain, tyrosine kinase domain, and a protein-rich carboxy-
terminal tail (36). NRG-1 binds to ErbB3 and induces the
formation of heterodimers between ErbB2 and ErbB3 and thus
activates the receptor (36). Targeted disruptions of the NRG-1
gene as well as the neuregulin receptors demonstrate that
neuregulins are essential for the formation of the heart and
nervous system (39, 40). Neuregulins have been implicated in
a number of events in cell survival, mitosis, migration, and dif-
ferentiation (41–44). The survival of Schwann cells, for exam-
ple, is mediated by neuregulin signaling through the phospho-
inositide 3-kinase (PI3K)/Akt pathway, a critical survival
pathway in neurons and in most cell types (45). Accordingly, it
seemed possible that an alternative site for Cdk5 regulation of
neuronal cell survival might be the neuregulin-mediated PI3K/
Akt signaling pathway. To explore this possibility, we took
advantage of the Cdk5 null mouse, which exhibited no Cdk5
activity (46), and examined the activities of PI3K and Akt.

In this study, we report that Cdk5 regulates Akt activity
through phosphorylation of the neuregulin receptors (ErbB2/
ErbB3) and regulation of PI3K/Akt kinase signaling pathways.
We found that brain extracts and cortical neurons from Cdk5
knockout mice exhibited reduced PI3K/Akt activities and in-
creased apoptosis. The Ser-1120 residue in the RSRSPR se-
quence of the receptor itself.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Cortical neurons from embryonic day 16.5 and 18
Cdk5 wild type and knockout mice (Cdk5+/−) were prepared as de-
scribed by Li et al. (30). In brief, embryos were dissected and minced
well with scissors. The dissociated cells were collected by centrifugation
and resuspended in a serum-free neurobasal medium supplemented
with B27 and 0.5 mM β-glutamine. Cells (25–100,000 cells/ml) were plated
on 35-mm plastic dishes precoated with polylysine (10 μg/ml) (Gibco) for
7 days. HEK-293T and COS-7 cells were cultured in Dulbecco’s modified
Eagle’s medium with 5% fetal calf serum.

**Preparation of Brain Extracts—**Brain of E18.5 from Cdk5−/− and
wild type mice were rinsed with cold phosphate-buffered saline (PBS)
and homogenized in a buffer containing 0.32 M sucrose, 5 mM HEPES,
and protease inhibitor mixture using poltron. The homogenate was
centrifuged at 10,000 × g for 60 min. The supernatant was ana-
lized for **in vitro** phosphorylation and PI3K activity.

**Phospholipid Extraction—**Equal aliquots of 50 μl (1 μg/μl) of super-
natant from Cdk5−/− and wild type mice brain (E18) were phos-
phorylated as described by Li et al. (30). In brief, the samples were incu-
bated in 15 μl of [γ-32P]ATP in a buffer containing Tris-HCl (pH 7.5),
50 mM NaCl, 5 mM MgCl2, 2 mM dithiothreitol for 2 h at 30 °C with
constant mixing. The reactions were stopped by the addition of chloro-
form/methanol (2:1, v/v). Following vigorous overtaxing, tubes were
precipitates were resuspended in 50 μl of kinase assay buffer (10 mM
Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, and 100 μM Na2VO4). The
activity of PI3K was assayed essentially as described by Hui et al. (73).
Brieﬂy, 10 μl of MgCl2 (stock concentration of 100 mM), 10 μl of PtdIns
(stock concentration of 1 μg), and 5 μl of 400 mM Tris-HCl, pH 7.6, were
added to the 50-μl precipitates, and the reaction was started by the
addition of 25 μl of [γ-32P]ATP. The reaction was incubated at room
temperature for 10 min with constant mixing. The reaction was termi-
nated by the addition of 40 μl of 8 × HCl and 320 μl of chloroform/
methanol (1:1). After vigorous mixing and centrifugation, the lipid
phase was collected and washed with 500 ml of CH3OH/H2O (1:1). The
isotol 3,4,5-trisphosphate and phosphatidylinositols were separated
by TLC using CHCl3/CH3OH/H2O (30/30/30) NH4OH (90:70:14.6:5.4, v/v/v/v)
developing mixture. Phosphorylated phospholipids were detected by
autoradiography.

**Phosphorylation Studies—**PTT rat brain extracts were prepared, and
Cdk5 was immunoprecipitated using anti-Cdk5 antibody as described
by Li et al. (30). The peptides were synthesized commercially (Peptide
Technologies Inc.). For in vitro phosphorylation studies, we incubated
peptides or histone H1 with the Cdk5 immunoprecipitated (IP). An Akt
IP was used to phosphorylate H2B to determine Akt activity. Sub-
strates (peptides or H2B) were incubated with [γ-32P]ATP (0.1 mM) in a
buffer containing 50 mM Tris-HCl (pH 7.4) with 1 mM EGTA, 1 mM
dithiothreitol, 5 mM MgCl2, 0.5 μM microcystin LR, and immunoprecipi-
ted Cdk5 or Akt for 30 min at room temperature as described by Li et
al. (30). To study the phosphorylation of ErbB-specific sites by Cdk5,
the mutant and wild type C-terminal domain (residues 663–1339) of rat
ErbB3 plasmids were transfected into COS-7 cells using Lipo-
fectAMINE. HA-tagged ErbB3 wild type and mutant (T871A and
S1120A) C-terminal domains (residues 663–1339) of the receptor were
generated by standard cloning methods. The putative phosphorylation
sites in ErbB3 were mutated using the QuikChange™ site-directed
mutagenesis kit (Stratagene) according to the manufacturer’s instruc-
tions. The mutations were verified by DNA sequencing.

**Western Blot Analysis—**Brain tissues or cells were homogenized in
a buffer (5 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM
glycerol, 1 mM EDTA, 2 mM Na3VO4, 5 mM phenylmethylsulfonyl
fluoride, 5 μg/ml aprotinin, leupeptin, and pepstatin). Proteins were re-
solved by 10–20% SDS-PAGE, blotted onto a polyvinylidene difluoride
membrane (Roche Applied Science); blocked in 5% skim milk, 1% PBS,
0.05% Tween 20; and probed with primary antibodies. Anti-Cdk5 poly-
clonal antibody, anti-ErbB3 polyclonal antibody (C-17) or anti-ErbB3
monoclonal antibody (G-4) (C-8, Santa Cruz Biotechnology, Inc., Santa
Cruz, CA), anti-phospho-Ser-473 and Thr-308 Akt, or anti-total Akt was
used for Western blot analysis using the ECL kit (Amersham Bio-
sciences) following the manufacturer’s procedures.

**Apoptosis Assays—**DNA fragmentation associated with apoptosis
was detected by TUNEL histochemistry staining. Cortical neurons with
or without inhibitor treatment were directly mounted on cover slides
and fixed with 4% paraformaldehyde in PBS and permeabilized with
0.2% Triton X-100 (20 min at room temperature and then incubated for
nick end-labeling for 2 h at 37 °C with TdT according to standard
procedures (Roche Applied Science).

**Immunofluorescence—**Cortical neurons or brain sections were fixed
in 4% paraformaldehyde in PBS for 30 min, washed in several changes
of PBS for 30 min, and permeabilized in 0.2% Triton X-100 in PBS
for 15 min. Monoclonal or polyclonal anti-Cdk5 antibody (1:200; Santa
Cruz Biotechnology), polyclonal antibody ErbB3 (C-17; Santa Cruz),
and phospho-Akt (Thr206) (1:200; New England Biolabs) was incubated
overnight at 4 °C. After a wash in PBS (three times for 15 min each).
Cells or sections were incubated with fluorescein isothiocyanate-conju-
gated anti-mouse IgG and rhodamine-labeled goat anti-rabbit IgG
together reacted with anti-mouse IgG secondary antibody for 1 h at
room temperature. Fluorescent images were obtained using a Zeiss
LSM-410 laser-scanning confocal microscope. Images were processed
and merged using Adobe Photoshop software.

**Mass Spectrometry—**Peptides were phosphorylated using nonradio-
active ATP as described above. Phosphorylation sites of the peptides
was determined by MALDI-TOF mass spectra on a Voyager-DE STR Bio-
spectrometry Work station (Applied Biosystems) operating in the neg-
ative linear mode. α-Cyano-4-hydroxycinnamic acid matrix was used.

**RESULTS**

**Reduced Brain Lipid Phosphorylation and PI3K Activity in Cdk5−/− Mouse Brain—**Previous studies have shown that
neuronal Cdk5 activity is necessary for survival and nervous
system development (29, 30, 46). Phospholipid kinases, the
Phosphoinositide kinases are responsible for the phospholipid phosphorylation. Among three general families, PI3K selectively phosphorylates phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)P2) and produces phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) upon stimulation by a variety of ligands in vivo as well as in vitro (47). This data presented in Fig. 1A show a 6-fold decrease in \( \gamma^{32P} \) incorporation into the phospholipids in brain extracts of Cdk5-/- mice compared with wild-type mice. In the absence of Cdk5, the synthesis of phospholipids is compromised. In order to explore the possibility of Cdk5-mediated interaction with the phospholipid-dependent PI3K signaling pathway, we compared PI3K activity in the cortical neurons derived from wild type and Cdk5-/- mice. The cortical neurons of Cdk5-/- mice showed a significantly decreased PI3K activity (Fig. 1B).

Akt Phosphorylation and Activity Is Decreased in Cdk5-/- Mouse Brain—Since Akt phosphorylation on both Thr-308 and Ser-473 by Cdk5-/- mice brain homogenates were analyzed by in vitro phosphorylation in the presence of \( \gamma^{32P} \)ATP as described under “Experimental Procedures.” Lipid phosphorylation was reduced in Cdk5-/- mouse brain. Phospholipids were extracted from brain extract of wild type and Cdk5-/- mice after phosphorylating the brain extract. The level of lipid phosphorylation was greatly reduced in the Cdk5-/- mice brain. Values represent means ± S.D. from three independent experiments. B. Cortical tissue lysates from Cdk5-/- mice and wild type mice (200 µg of total protein) were immunoprecipitated with the anti-phosho Akt antibody. The PI3K activity was measured as described under “Experimental Procedures.” Values represent means ± S.D. from three independent experiments.

The above observations suggest that Cdk5 activity is involved in the PI3K signaling pathway. To determine the target substrate for Cdk5, we based our analysis on the observation that the Cdk5-p35 complex is implicated in neuregulin-induced acetylcholine receptor expression at the neuromuscular junction (13). This suggested that ErbB receptors might be a substrate for Cdk5 phosphorylation. Indeed, we found that the putative Cdk5 phosphorylation consensus sequence motifs are present in the ErbB2 and ErbB3 receptor molecules. Ser-1176 in the sequence SPGK of ErbB2, Thr-871 in the TPIK sequence, and Ser-1204 in the SPPR sequence of ErbB3 are putative phosphorylation sites in Cdk5 consensus motifs. To test whether these and other proline-directed serine residues in the peptides derived from ErbB2 and ErbB3 (see Fig. 3A) are the sites phosphorylated by Cdk5, we incubated these peptides and their mutant forms with Cdk5 IP from brain extracts and determined their phosphorylation by in vitro kinase assays. We found that Cdk5 significantly phosphorylated the Ser-1176 in the ErbB2 peptide RPKTLSPGKN and Thr-871 in the ErbB3 peptide AKTPIKWAL but weakly phosphorylated Ser-1204 in the ErbB3 proline-rich peptide RRGSPPRRPR (Fig. 3C). Interestingly, we found that the Ser-1120 in the basic amino acid-rich sequence RRSRSRPRPR of ErbB3 was the most favorable substrate for Cdk5 (Fig. 3C). The peptide RRSRSRPRPR does not have the conventional consensus sequence ([K/R](S/T)P[X](K/R), where X represents a basic residue) of cyclin-de-
dependent kinase including Cdk5, but it is rich in basic amino acids and appears to be a novel Cdk5 phosphorylation consensus sequence. To confirm whether Cdk5 phosphorylates the Ser-1120 residue, we conducted in vitro kinase assays using various mutant peptides derived from RSRSRSPRPR in which alanines were substituted for serines (Fig. 3, B and D). Ser-1120 appeared to be the site for Cdk5 phosphorylation. The phosphorylation of Thr-871 of RPKTLSPGKN in ErbB2 and Ser-1120 of RSRSRSPRPR in ErbB3 were further investigated and confirmed by mass spectrometry (Fig. 3, E and F).

To investigate the Cdk5 phosphorylation of ErbB3 in vivo, the Thr-871 and Ser-1120 in the C-terminal domain of rat ErbB3 were mutated to Ala (T871A and S1120A). COS-7 cells were cotransfected with wild type HA-ErbB3 or mutant HA-ErbB3 (T871A and S1120A) C-terminal domains with Cdk5-p35. The levels of phosphorylated wild type HA-ErbB3 C terminal and HA-ErbB3 (T871A and S1120A) were measured by immunoprecipitation using anti-HA-tagged antibody, and their phosphorylation state was detected by Western blot using anti-phosphoserine and anti-phosphothreonine antibody. We found that mutation of Thr-871 or Ser-1120 to Ala in ErbB3 showed no detectable Cdk5-dependent serine and threonine phosphorylation in HA-ErbB3 (T871A and S1120A) compared with the wild type HA-ErbB3 (Fig. 4, A and B). Similar results are obtained using HA-ErbB2 mutant (S1176A) and wild type (data not shown).

Cdk5 Associates with and Phosphorylates ErbB3 in Vivo—If Cdk5 phosphorylated the ErbB3 receptor, we would expect the kinase to co-localize with the receptor in cells. To examine this possibility in cortical neurons, we performed double-labeled immunofluorescence staining in rat cortical neurons and found that Cdk5 and ErbB3 are uniformly expressed throughout the cell (Fig. 5, A–C). To investigate whether Cdk5 is associated with ErbB3, we immunoprecipitated cell lysates from the rat cortical neurons with Cdk5 antibody by Western blot analysis and assayed for the co-elution of ErbB3. We found that ErbB3 is present in Cdk5 immunoprecipitate (Fig. 5 D).

To examine whether Cdk5 specifically phosphorylated ErbB3, cell extracts from the wild type and Cdk5+/H11002 brain were immunoprecipitated with anti-ErbB3 antibody and detected by Western blot using phospho-Thr or phospho-Ser antibody. Data in the histogram represent means ± S.D. from four independent experiments.

Cdk5 Is Involved in Neuregulin-induced Akt Phosphorylation and Activity in Cortical Neurons—If Cdk5 acts as a modulator of the PI3K/Akt signaling pathway via its phosphorylation of
the ErbB3 receptor, then stimulation of the receptor by the addition of neuregulin should promote Cdk5 activity and ErbB3 phosphorylation. To test the prediction of this hypothesis, cortical neurons derived from rat E18 embryos were stimulated by neuregulin \( \beta_1 \) (100 ng/ml) for 10 min, and a PI3K inhibitor, LY294002 (50 \( \mu \)M), and Cdk5 inhibitor, roscovitine (25 \( \mu \)M), were added 1 h before treatment with NRG. The cell lysates were analyzed by Western blot using phospho-Akt (Thr-308 and Ser-473). B, the Akt kinase activity was assayed by in vitro kinase assay using histone H2B as a substrate. Phosphohistone H2B was quantified by liquid scintillation counting. Data in the histogram represent means ± S.D. from four independent experiments.

The roscovitine effect on Akt phosphorylation and activity were less inhibitory, reaching control levels in the absence of neuregulin (Fig. 6, lanes 2 and 5). Roscovitine reduced neuregulin-stimulated Akt phosphorylation and activity but had no significant effect on basal phosphorylation and activity. Akt activity and phosphorylation, however, were also reduced by half in the presence of roscovitine and neuregulin, suggesting that Cdk5 is involved in the neuregulin-PI3K/Akt signaling pathway (Fig. 6, lanes 2 and 4).

**Cdk5-mediated Neuroprotection Is Involved in the Neuregulin/PI3K Signaling Pathway**—One of the key signaling pathways involved in the regulation of neuronal survival is the PI3K/Akt kinase intracellular signaling transduction pathway (50, 51). The above results show that the phosphorylation of Akt induced by neuregulin was inhibited by a PI3K and a Cdk5 inhibitor, suggesting that Cdk5 may play an important role in neuronal survival through the regulation of Akt activity. Therefore, we investigated whether Cdk5 is involved in neuregulin-induced survival of cortical neurons after serum deprivation. Recent studies have indicated that the PI3K pathway is the primary signal transduction system that mediates the protective effect of neuregulin against serum deprivation in Schwann cells (52). We used cortical neurons derived from the rat embryonic brain (E18) as a model to measure cell apoptosis using TUNEL staining. The number of TUNEL-positive cells were quantified and compared. We found that the inhibitors, LY294002, a PI3K inhibitor, and roscovitine, a specific Cdk5 inhibitor, both increased the proportion of TUNEL-positive cells and reduced the extent of cell survival induced by neuregulin stimulation (Fig. 7). Roscovitine alone induced higher apoptosis compared in the presence of neuregulin and roscovitine. This suggests that Cdk5 activity is involved in neuron protection induced by neuregulin.

**DISCUSSION**

It has been demonstrated that a cell survival ligand, neuregulin, binds to ErbB receptors and recruits PI3K to the...
PRPR is a novel Cdk5 phosphorylation site suggesting that RSRSR is a preferred Cdk5 phosphorylation site, suggesting that Cdk5 may regulate PI3K/Akt signaling pathway by neuregulin.

Fig. 7. Cdk5 is involved in promoting neuronal survival by neuregulin. Rat cortical neurons were cultured for 5 days and switched to Dulbecco's modified Eagle's medium without any growth factors and serum-deprived for 20 h to induce apoptosis. A, control; B, with 100 ng/ml neuregulin β; C, with a 30-min pretreatment with LY294002 (50 μM); D, with a 30-min pretreatment with roscovitine (25 μM); E, roscovitine (25 μM) alone. F, apoptotic cells were detected by TUNEL staining. In each experiment, the number of TUNEL-positive cells was determined for each condition. The number of TUNEL-positive cells in control culture was defined as 100%. The number of TUNEL-positive cortical neurons in each experimental condition was then expressed as a percentage of control values. Bars, means ± S.D. from three independent experiments.

Fig. 8. Schematic representation of the phosphorylation of ErbB2 and ErbB3 by Cdk5. The diagram shows that neuregulin receptors ErbB2 and ErbB3 were phosphorylated by Cdk5–p35 at Cdk5 consensus motifs containing Ser-1176 of ErbB2 and Thr-871 and Ser-1120 of ErbB3. In turn, this phosphorylation affects activation of the PI3K/Akt signaling pathway by neuregulin.

Phosphorylation of ErbB receptors is probably essential to their function. ErbB3 has been shown to be well adapted to mediate PI3K signaling, because it contains in its C-terminal phosphorylation domain six such consensus p85 binding motifs (57). ErbB2 has also been found to associate with PI3K (58, 59). Thus, the ErbB2/ErbB3 neuregulin co-receptor has been shown to couple to PI3K in a neuregulin-dependent manner.

A unique feature of our analysis is the utilization of the Cdk5 knockout mouse that lacks Cdk5 kinase activity. We demonstrated that brain extracts and/or cortical neurons of Cdk5−/− mice exhibit reduced PI3K and Akt kinase activities. The Akt activity was reduced significantly in the Cdk5−/− animals; however, PI3K activity was only reduced to 40% of wild type (Figs. 1B and 2D). These observations suggest that Cdk5 may regulate Akt activity independent of PI3K activation. In fact, there are several reports that the Akt activity can be regulated by PI3K-independent pathways; for example, Ca²⁺/calmodulin-dependent protein kinase kinase (68) and reagents that increase cAMP levels (69) as well as stress factors (70) can activate Akt. We suggest that some of these pathways may be compromised to reduce Akt activity in addition to PI3K activity in Cdk5−/− mice. In addition, the possibility exists that Cdk5 may regulate PDK1 activity by phosphorylating some of its proline-directed Ser/Thr residues, of which there are six. Two of them, QQTPPKL and VHTPNRT, are Cdk5 consensus motifs, whereas the Ser-244 residue in the KVLSPESK motif is essential for PDK1 activity. PDK1 exists in an active phosphorylated configuration, which can be membrane-localized due to its pleckstrin homology domain by PtdIns(4,5)P₂. Accordingly, PDK1 does not require receptor activation; the presence of PtdIns(4,5)P₂ in unstimulated cells is sufficient to activate PDK1 and Akt phosphorylation (49). ErbB3 immunoprecipitates from E18 cortical neurons of
Cdk5-deficient mice exhibited a decreased serine and threonine phosphorylation but no significant reduction in tyrosine phosphorylation (Fig. 5G) compared with wild type. A conventional read-out for ErbB activity is usually associated with the levels of tyrosine phosphorylation. The data presented in Fig. 5G suggest that ErbB activity is modulated without affecting the tyrosine phosphorylation of some of the individual receptor subunits. Active ErbB receptors are heterodimers. It is important to note that ErbB3 is a unique in the sense that it has a kinase domain but shows no activity (71, 72). ErbB2, which has both kinase domain and activity, is essential for receptor activity. In the present experiments, we used ErbB3 antibody to analyze the phosphorylation state of the receptor. The possibility exists that the ErbB3 tyrosine phosphorylation may have less effect on activation, and ErbB2 tyrosine phosphorylation may play a major role in ErbB2/ErbB3 activation. From these studies, we propose that proline-directed Ser/Thr phosphorylation of receptor in the cytoplasmic domain is required to fully activate the ErbBs. This study also suggests that neuregulin-dependent activation of Akt-enhanced survival of rat cortical neurons deprived of serum could be inhibited by roscovitine, a specific Cdk5 inhibitor. These results implicate that Cdk5 is involved in neuregulin-dependent activation of the PI3K/Akt neuronal survival pathway by regulating the phosphorylation of ErbB2/ErbB3.

Our results are consistent with studies showing that Cdk5 is involved in the neuregulin-mediated regulation of neuromuscular junction development (13). Cdk5 and its activator, p35, are highly concentrated at the neuromuscular junction, colocalize with acetylcholine receptors on the postsynaptic muscle membrane, and are physically associated with ErbBs, suggesting that the Cdk5-p35 complex is close to ErbB receptors in the signaling pathway. Moreover, neuregulin-induced Cdk5 activation in myotubes that correlated with the expression of acetylcholine receptor. Cdk5 has been implicated in synaptic function in other systems, phosphorylating components of the presynaptic complex or the postsynaptic receptor (7, 11, 12). Our results suggest that the neuregulin/ErbB receptor signaling cascade that regulates neuronal survival is another important site for Cdk5 modulation. Our data showed that specific inhibition of Cdk5 activity results in significantly reduced protection from induced apoptosis in cortical neurons by inactivating the neuregulin/ErbB survival pathway (see Fig. 5D).

Apoptosis is an active process occurring during both normal maturation of the nervous system and under stress-induced situations such as neurodegenerative diseases and stroke (60–62). Activation of the PI3K/Akt pathway has been observed in promoting survival downstream of extracellular stimuli (63–65). What is most relevant to our observations is the recent evidence of neuregulin-induced Schwann cell survival via the PI3K/Akt pathway. In this system, both the Akt and mitogen-activated protein kinase pathways were activated in parallel downstream from PI3K, resulting in the phosphorylation and inactivation of BAD, a principal inducer of cell apoptosis. However, inactivation of the mitogen-activated protein kinase pathway by selective inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase did not affect neuregulin-mediated survival, leaving the Akt pathway as primarily involved. Our data on cortical neurons are in agreement with these observations but introduce Cdk5 as an important modulator of the pathway by virtue of its regulation of ErbB2/3 receptor phosphorylation.

The precise role of Cdk5 in induction and protection from apoptosis remains controversial. For example, in the developing mouse limb, regions of programmed cell death during digit formation correlate with elevated levels of Cdk5-p35, suggesting that Cdk5 activity is proapoptotic (14). Similar correlations between Cdk5 activity and apoptosis were reported in tissue remodeling of mammalian reproductive organs (15). Likewise, heat-shocked astrocytoma cells exhibit changes in morphology and increased apoptosis that correlate with Cdk5-p35 activity (16). Finally, in some neurodegenerative disorders, it has been suggested that deregulation of Cdk5 activity by cleavage of p35 to p25 correlates with neuronal pathology in Alzheimer’s disease brains (18). On the other hand, a number of studies in addition to the results reported here show that Cdk5 is involved in cell survival. The absence of Cdk5 activity in the Cdk5 knockout mouse causes embryonic-lethal and induces cortical and cerebellar abnormalities and increased cortical apoptosis (24, 30). The phenotype is rescued when Cdk5 (driven by a neuron-specific promoter, p35) is overexpressed in a transgenic Cdk5 knockout mouse host, suggesting a key role of neuronal Cdk5 activity in sustaining neuronal survival (29). Furthermore, we have also shown that Cdk5 can modulate neuronal survival by phosphorylating and inhibiting e-Jun N-terminal kinase 3 kinase activity, thereby blocking this apoptotic pathway (30). In this case, we see that Cdk5 supports neuronal survival by inhibiting a key site in an apoptotic pathway, whereas its phosphorylation of the ErbB receptors promotes neuronal survival by activating the PI3K/Akt survival pathway. The data presented in this study are summarized in Fig. 8. The ErbB receptors are activated by Cdk5 phosphorylation in their proline-directed Ser/Thr residues in the C-terminal tail domain; this in turn activates PI3K kinase, which induces activation of PDK1. PDK1 phosphorylates and activates Akt. Active Akt phosphorylates and down-regulates the molecules involved in cell death. The versatility of Cdk5 in development, function, and survival of the nervous system is probably due to a number of different factors, in addition to the wide range of target substrates, from cytoskeletal to synaptic proteins (66, 67); its activity is also dependent upon the specific cell type, its regulatory proteins (p35, p39, p25), its localization within different cellular compartments, and the developmental and physiological state of the neuron.

Acknowledgments—We thank Dr. Philip Grant for critically reading the manuscript and Dr. Niranjana D. Amin for experimental help. We also thank Dr. Carolyn Smith in the NINDS Light Microscopy Facility for assistance in confocal microscopy.

REFERENCES

1. Hellmich, M. R., Pant, H. C., Wada, E., and Battey, J. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10867–10871
2. Lew, J., Huang, Q. Q., Q. Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) Nature 371, 423–426
3. Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) Nature 371, 419–423
4. Humbert, S., Dhavan, R., and Tsai, L-H. (2000) J. Cell Sci. 113, 975–983
5. Oshima, T., and Mikoshiba, K. (2002) Mol. Neurobiol. 26, 153–166
6. Sasaki, Y., Cheng, C., Uchida, Y., Nakajima, O., Ohoshima, T., Yagi, T., Tani-guchi, M., Nakarama, T., Kishida, R., Kudo, Y., Ohno, S., Nakamura, F., and Goshima, Y. (2002) Neuron 35, 907–920
7. Shuang, R., Zhang, L., Fletcher, A., Grohowski, G. E., Pesnev, J., and Stuenkel, E. L. (1999) J. Biol. Chem. 274, 4957–4966
8. Fisher, A. I., Shuang, R., Giovannucci, D. B., Zhang, L., Bittner, M. A., and Stuenkel, E. L. (1999) J. Biol. Chem. 274, 4027–4035
9. Rosales, J. L., Nedwell, M. J., Johnston, R. N., and Lee, K. Y. (2000) J. Cell. Biochem. 76, 151–159
10. Floyd, S. R., Porro, E. B., Sllepnev, V. I. Ochoa, G. C., Tsai, L. H., and De Camilli, P. (2001) J. Biol. Chem. 276, 8104–8110
11. Li, B. S., Sun, K. R., Zhang, L., Takahashi, S. M., Wu, Kukarni, A. B., and Pant, H. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12742–12747
12. Bibb, J. A., Snyder, G. L., Nishi, A., Yan, Z., Meijer, L., Fienberg, A. B., Tsai, L. H., Kwon, Y. T., Girault, J. A., Czernik, A. J., Huganir, R. L., Hemmings, H. C., Jr., Nairn, A. C., and Greengard, P. (1999) Nature 402, 669–671
13. Fu, A. K., Fu, W. Y., Cheung, J., Tsam, K. W., Ip, F. C., Wang, J. H., and Ip, N. Y. (2001) Nat. Neurosci. 4, 374–381
14. Ahuja, H. S., Zhu, Y., and Zakeri, Z. (1997) Dev. Genet. 21, 258–267
15. Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J., and Godowski, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9562–9567
16. Gao, C., Negash, S., Wang, H. S., Lodee, D., Gue, H., Russell, P., and Zelenka, P. (2001) J. Cell Sci. 114, 1145–1153
Cyclin-dependent Kinase-5 Regulates Akt Activity

35709

17. Zhu, X., Lai, C., Thomas, S., and Burden, S. J. (1995) EMBO J. 14, 5842–5848
18. Patrick, G. N., Zukerberg, L., Nicolici, M. de la Monte, S., Dikkes, P., and Tsai, L. H. (2001) Nature 411, 764–765
19. Lee, M. S., Kwon, Y. T., Jin, L. W., and Vincent, I. (2002) Neurobiol. Dis. 11, 285–297
20. Ohshima, T., Ogawa, M., Veeranna, Hirasawa, M., Longenecker, G., Ishiguro, K., Pant, H. C., Brady, R. O., Kulkarni, A. B., and Mikoshiba, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2764–2769
21. Tanaka, T., Veeranna, Pant, H. C., Brady, R. O., and Kulkarni, A. B. (2001) J. Neurosci. 21, 550–558
22. Holmes, W. R., Sliekowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N, Raab, H., Lewis, G. D., Shepard, H. M., Kiang, W. J., Wood, W. I., Goeddel, D. V., and Vanden, R. L. (1992) Science 255, 1205–1210
23. Peles, E., Lamprecht, R., Ben-Levy, R., Tzahar, E. Y., and Arden, Y. (1992) J. Biol. Chem. 267, 12266–12274
24. Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993) Cell 72, 801–815
25. Chang, H., Riese, D. J. 2 rd., Gilbert, W., Stern, D. F., and Mcmahon, U. J. (1997) Nature 387, 509–512
26. Meyer, D., and Birchmeier, C. (1997) Nature 386, 380–389
27. Gassmann, M., and Lemke, G. (1997) Curr. Opin. Cell Biol. 9, 87–92
28. Lin, W., Sanchez, H. B., Deertnick, T., Morris, J. K., Ellisman, M., and Lee, K. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1299–1304
29. Gassmann, M., Casagrande, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) Nature 376, 390–394
30. Dey, Z., Brennan, A., Lai, C., Yarden, Y., Leikowitz, G., Mirsky, R., and Jessen, K. R. (1995) Neuron 15, 585–596
31. Anton, E. S., Marchionni, M. A., Pane, K. F., and Rakic, P. (1997) Development 124, 3501–3510
32. Rio, C., Rieff, H. I., Qi, P., Khurana, T. S., and Corfas, G. (1997) Neuron 19, 39–50
33. Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994) Cell 77, 349–360
34. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotob, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
35. Ko, J., Human, S., Brown, B., Takahashi, S., Kulkarni, A. B., Li, E., and Tsai, L. H. (2001) J. Neurosci. 21, 6758–6771
36. Stephens, L., Gruenauer, A., Corey, S., Jackson, T., and Hawkins, P. T. (1993) EMBO J. 12, 2265–2273
37. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brokman, G. M., Mirtzos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998) Cell 95, 29–39
38. Oppenheim, R. W. (1991) Annu. Rev. Neurosci. 14, 453–501
39. Pettmann, B., and Henderson, C. E. (1998) Neuron 20, 633–647
40. Yao, R., and Cooper, G. M. (1995) Science 267, 2003–2006
41. Segal, R. A., and Greenberg, M. E. (1996) Neuron 19, 463–489
42. Weiner, J. A., and Chun, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5233–5238
43. Grant, P., and Pant, H. C. (2001) J. Neurosci. 29, 843–872
44. Smith, D. S., and Tsai, L. H. (2002) Trends Cell Biol. 12, 28–36
45. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–587
46. Filippa, N., Sable, C. L., Filleux, C., Hemmings, B., and van Obberghen, E. (1999) Mol. Cell. Biol. 19, 4898–5000
47. Konishi, H., Matsuhashi, A., Tanaka, M., Ono, Y., Tokunaga, C., Kuroda, S., and Ikeda, U. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7639–7643
48. Mickey, P. M., Plakto, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8132–8136
49. Sierke, S. L., Cheng, K., Kim, H-H., and Yen, M. (1997) Biochem. J. 322, 757–763
50. Hii, C. S., Moghadammi, N., Dunbar, A., and Ferrante, A. (2001) J. Biol. Chem. 276, 27344–27355