

Cdk5 Phosphorylates a Component of the HDAC Complex and Regulates Histone Acetylation during Neuronal Cell Death

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Key Words

Histone deacetylase · Cyclins · Neuronal apoptosis · Cyclin-dependent kinase · p35 · Transcription

Abstract

Cyclin-dependent kinase 5 (Cdk5), a member of the cyclin-dependent kinase family, is critical for regulating neural development and neuronal survival. Dysregulation of Cdk5 is associated with abnormal expression of cell cycle-related proteins during neuronal apoptosis. We have previously found that p35, a Cdk5 activator, interacts with mSds3, an integral component of the histone deacetylase complex in vitro, suggesting a functional role of Cdk5 in gene regulation through modulation of chromatin integrity. In this study, we further demonstrate that Cdk5-dependent phosphorylation of mSds3 at Ser228 occurs in mouse brain nuclei. The expression of mSds3 protein and its interaction with Cdk5 activators is developmentally regulated in the mouse brain. Importantly, our findings suggest that the ability of Cdk5 to regulate activity deprivation-induced apoptosis of cerebellar granule neurons is likely mediated by the regulation of histone acetylation. Suppression of Cdk5 not only attenuates the induction of histone H3 acetylation and the aberrant up-regulation of cyclin proteins in neurons after activity deprivation,

but also results in protection of neurons against apoptotic cell death. Taken together, our findings suggest that Cdk5 regulates neuronal survival by precise epigenetic control through modulation of histone acetylation.

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Introduction

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that plays important roles in various aspects of neuronal functions, including neuronal migration, synaptic functions, and neuronal survival and apoptosis [1–3]. Unlike classical Cdk activity, the activity of Cdk5 is tightly controlled by its association of two neuronal-specific activators, p35 and p39, which are believed to be enriched at the plasma membrane [3]. Interestingly, it was recently reported that p35 can undergo nuclear import via the importin pathway [4], raising the possibility that Cdk5 directly regulates gene transcription in the nucleus. Indeed, precise regulation of nuclear Cdk5 activity has been suggested to be critical for maintaining neuronal survival [5–8], but the underlying mechanisms remain largely unknown.

Neuronal apoptosis has been implicated in various neurodegenerative diseases such as Alzheimer's disease. Accumulating studies reveal that aberrant gene activation and neuronal cell cycle reentry also contribute to activation of the apoptotic pathway [9]. Several lines of evidence have demonstrated that Cdk5 is involved in aberrant transcriptional activation of cell cycle proteins in cancer cell lines or in neuronal cells undergoing apoptosis [10, 11]. Importantly, Cdk5 has been demonstrated to regulate transcription at multiple levels. For example, various transcription factors, such as STAT3 [12], MEF2 [13], p53 [14] and RB [15], have been identified as substrates of Cdk5, and phosphorylation of these transcription factors results in regulation of their transcriptional activity. In addition, Cdk5 also regulates histone acetylation modification [16, 17]. Acetylation of histones is coordinately controlled by two chromatin-modifying enzymes, histone acetyltransferases and histone deacetylases (HDACs) [18]. Our earlier studies revealed that Cdk5/p35 interacts with mSds3, an integral component of the mSin3-HDAC complex that plays a key role in the repression of gene expression through remodeling of the chromatin structure. We found that Cdk5 phosphorylates mSds3 at Ser228 *in vitro*, and this specific phosphorylation of mSds3 regulates the HDAC1-dependent histone acetylation modification *in vitro* [17]. Furthermore, HDAC1 exists as a signaling complex with p25, a cleavage product of p35 induced upon neuronal cell death [16]. Together, Cdk5 is suggested to regulate histone acetylation, thereby revealing a role of Cdk5 in regulating gene transcription during normal neuronal functions or upon neuronal apoptosis.

In this study, we further characterize the regulation of mSds3 by the active Cdk5 complex in the mouse brain, and study the potential roles of Cdk5 in histone acetylation during neuronal apoptosis. We confirm the endogenous phosphorylation of mSds3 at Ser228 by Cdk5 in the mouse brain and show that the interaction of mSds3 with the Cdk5 activators p35 and p39 is differentially regulated in the mouse brain during development. These findings suggest that mSds3/mSin3-associated HDAC1 activity is regulated by Cdk5 in the mouse brain during development. On the other hand, we also found that Cdk5 activity is required for mediating neuronal apoptosis in cerebellar granule neurons through modulation of the acetylation status of histone H3, thus leading to the re-expression of cyclin proteins. Taken together, our findings provide evidence that active Cdk5 regulates phosphorylation of the component(s) of the HDAC complex and its integrity, as well as the histone acetylation status during neuronal apoptosis.

Materials and Methods

Constructs and Antibodies

Expression constructs encoding mSds3 were generated as described [17]. Roscovitine was purchased from Millipore. Antibodies specific for mSin3A, mSin3B, Cdk5 and p35 were purchased from Santa Cruz Biotechnology. Custom antibodies against p39, mSds3 (a.a. 59–170) and phosphorylated-mSds3 were generated. All the reagents for cell cultures were purchased from Invitrogen.

Cell Cultures and Transfection

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics. HEK 293T cells were transiently transfected with different combination of plasmids as indicated using LipofectAMINE PLUS reagents according to the manufacturer's instructions.

Primary cultures of cerebellar granule neurons were prepared from postnatal day-7 mouse pups and plated on culture dishes coated with poly-L-lysine (Sigma-Aldrich). Granule neurons were incubated with basal Eagle's medium (BME) containing 10% HBS and 25 mM KCl in the presence of 2 mM glutamate. Apoptosis of granule neurons was induced at 7 days *in vitro*. The neurons were washed twice with BME to remove serum and the excess potassium, and then incubated with BME containing 5 mM KCl (activity deprivation) or 25 mM KCl (control) for different periods of time as indicated.

Protein Extraction and Coimmunoprecipitation Analysis

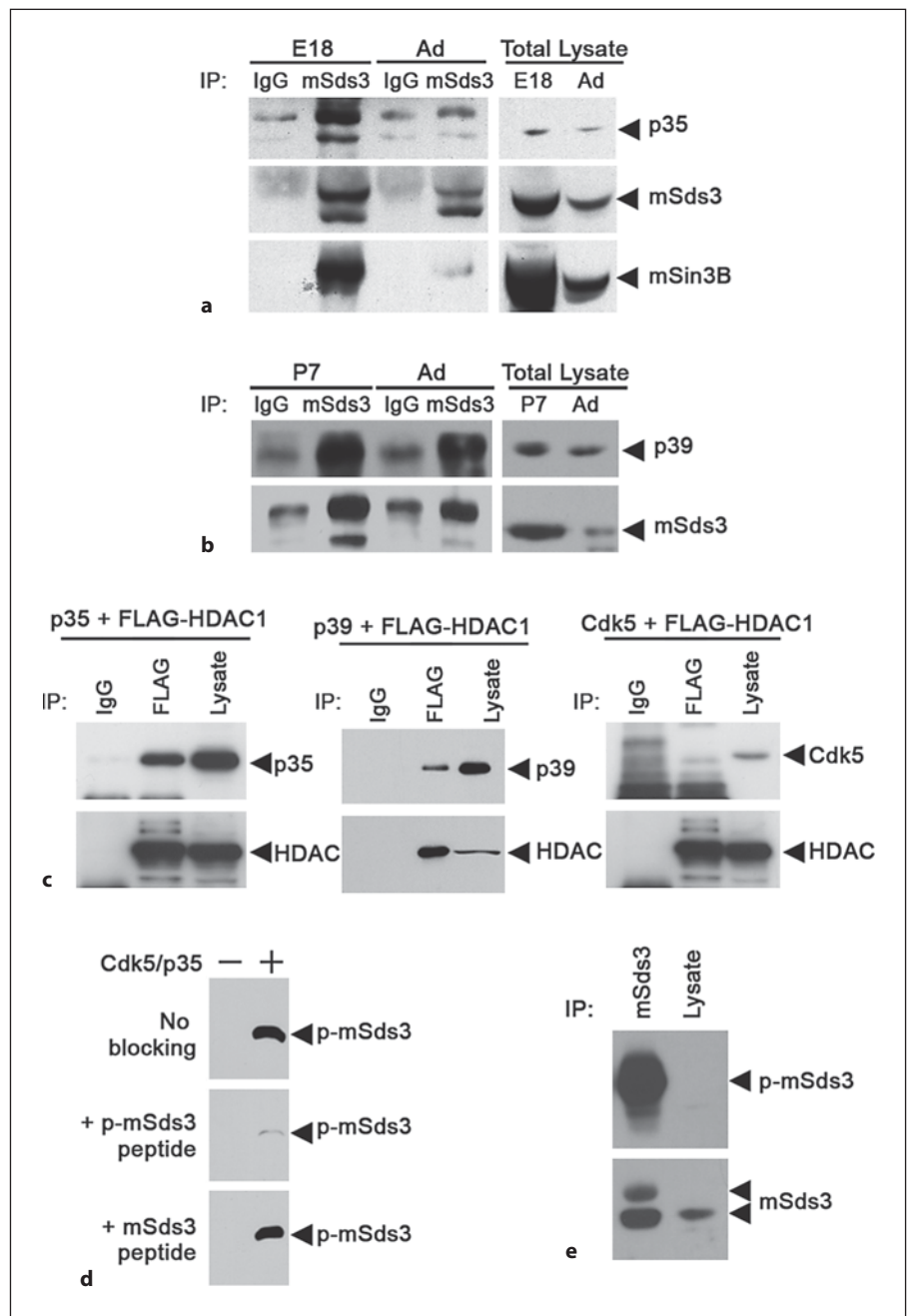
Proteins were extracted using different lysis buffers supplemented with various protease inhibitors. HEK 293T cells and cultured neurons were lysed in lysis buffer A (Tris, pH 8.5, 100 mM; NaCl, 100 mM; EDTA, 1 mM) with 0.5% Nonidet P-40 and various protease inhibitors. Mouse brain tissues were homogenized in lysis buffer B (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with various protease inhibitors. The nuclear fractions of brain tissues were prepared as previously described [12]. Five hundred micrograms of nuclear fractions of brain lysates were incubated with the corresponding antibody (2 µg) at 4°C overnight and then incubated with 40 µl of protein G-Sepharose at 4°C for 1 h. The samples were washed and resuspended in SDS sample buffer, and coimmunoprecipitated proteins were detected using Western blot analysis.

Results

Cdk5 Interacts with and Phosphorylates mSds3 in the Mouse Brain

We have previously reported that Cdk5 phosphorylates mSds3 at Ser228, and regulates mSin3-HDAC1 transcriptional repressor activity *in vitro* [17]. Here, we demonstrate that mSds3 indeed exists as a signaling complex with the Cdk5 activators p35 or p39 and that the interaction is regulated upon development. While p35 is prominently expressed in embryonic mouse brains, p39 protein

Fig. 1. mSds3/HDAC1 complex interacts with Cdk5 activators in vivo and detection of Ser228 phosphorylated mSds3 in mouse brain nuclei. **a** Association of mSds3/mSin3B with p35 in mouse brain nuclei. Nuclear fractions of embryonic day 18 (E18) and adult (Ad) mouse brains were coimmunoprecipitated (IP) with mSds3 antibody, and immunoblotted with p35 and mSin3B antibodies. The slower migrating band of mSds3 is likely the phosphorylated mSds3. **b** mSds3 interacts with p39 in mouse brains. Nuclear proteins of postnatal day 7 (P7) and adult mouse brains were subjected to coimmunoprecipitation analysis. **c** HDAC1 specifically interacts with p35 and p39 but not Cdk5. HEK293T cells were transfected with FLAG-HDAC1 together with p35, p39 or Cdk5 as indicated. The lysate was coimmunoprecipitated with FLAG antibody, and then subjected to Western blot analysis as indicated. **d, e** Cdk5-dependent phosphorylated mSds3 at Ser228 is present in the mouse brain. **d** Characterization of the p-mSds3 antibody. HEK293T cells were transfected with mSds3 together with Cdk5 and p35 expression constructs. Phosphorylation of mSds3 was examined by Western blot analysis using p-mSds3 antibody. The specificity of the p-mSds3 antibody was confirmed by the drastic reduction in immunoreactivity after blocking with the p-mSds3 peptide (middle), as compared to the incubation with non-phosphorylated mSds3 peptide (bottom). **e** mSds3 is phosphorylated at Ser228 in adult mouse brain nucleus. Nuclear fraction of adult mouse brains was immunoprecipitated with mSds3 antibody, and then immunoblotted with p-mSds3 antibody.



level increases in the mouse brain during postnatal stages and reaches its maximal level in the adult brain [3]. Coimmunoprecipitation analysis revealed that mSds3 interacted strongly with p35 and mSin3B in the nuclear fractions of E18 mouse brains, but the association was drastically reduced at the adult stage (fig. 1a). In contrast, mSds3 bound strongly with p39 in mouse brains at both postnatal and adult stages (fig. 1b). Furthermore, HDAC1

in the transcriptional repressor complex was shown to interact with both p35 and p39 but not with Cdk5 (fig. 1c), suggesting that the active Cdk5 complex regulates the functions of the mSds3-mSin3-HDAC1 repressor complex at multiple levels. To study whether Cdk5-dependent phosphorylation of mSds3 occurs in vivo, we generated a phosphospecific antibody against the phosphorylated mSds3 at Ser228 (p-mSds3). We first confirmed the spec-

ificity of the newly generated p-mSds3 antibody by Western blot analysis. The p-mSds3 antibody was able to recognize a specific protein band whose size corresponded to mSds3 protein in the lysates of 293T cells that overexpress mSds3 together with Cdk5/p35 (fig. 1d). Blocking the p-mSds3 antibody with the phosphorylated mSds3 (Ser228) peptide, but not the nonphosphorylated mSds3 peptide, abolished the observed immunoreactivity of the p-mSds3 antibody (fig. 1d). Using this phosphospecific antibody, we found that mSds3 is specifically phosphorylated at the Ser228 residue in the mouse brain nuclear fractions (fig. 1e). Taken together, our findings demonstrate that mSds3/mSin3B/HDAC1 exists as a signaling complex with the active Cdk5 complex in mouse brain nuclei and is phosphorylated by Cdk5 at Ser228, implicating a functional role of Cdk5 in the regulation of gene transcription through modulating chromatin structural integrity in neurons.

mSds3 Expression Is Developmentally Regulated in Mouse Brain Nuclei

We then studied the temporal expression profiles of mSds3 and mSin3 proteins, and correlated their regulation with that of p35 and p39 proteins in mouse brain nuclei. mSds3 and mSin3 proteins were prominently expressed in the nuclear fractions of embryonic mouse brains, and their level were reduced gradually upon postnatal development (fig. 2). While the total level of Cdk5 and p35 proteins remained relatively unchanged in mouse brains during development, their protein expression increased steadily in the nuclei along the same time course (fig. 2). In contrast, temporal regulation of nuclear p39 in the mouse brain is different from that of p35 or Cdk5; the protein was barely detectable in mouse brain nuclei during early development, and then increased prominently at the postnatal stages. The interaction of Cdk5 and its activators with mSds3 and mSin3 proteins and their nuclear localization were consistent with our notion that active Cdk5 regulates the functions of the mSds3/mSin3 complex during brain development.

Cdk5 Regulates Histone Acetylation in Activity Deprivation-Induced Cerebellar Granule Neuron Apoptosis

Cdk5 has been implicated in the regulation of different molecular pathways that underlie neuronal apoptosis, e.g. through the reactivation of cell cycle proteins although the exact roles of nuclear Cdk5 in histone acetylation are still unclear. To examine the functional role of nuclear Cdk5 in neuronal apoptosis, we triggered depri-

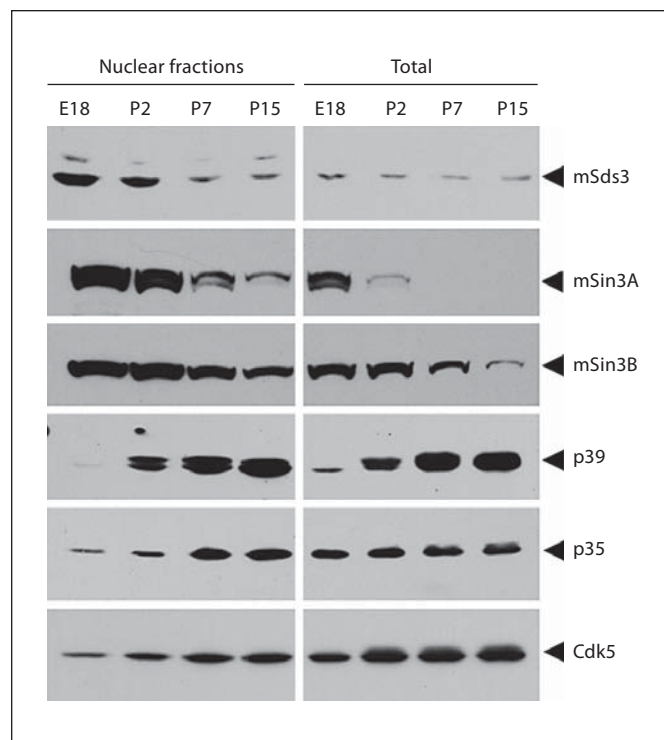


Fig. 2. Temporal expression profiles of mSds3 and its interacting components mSin3A and mSin3B, and Cdk5 and its activators in the nuclear fractions of mouse brains. Lysates from mouse brain nuclear fractions at different developmental stages were collected, and subjected to Western blot analysis using mSds3, mSin3A, mSin3B, p35, p39 and Cdk5 antibodies (total protein lysates were collected as controls).

vation-induced apoptosis in cerebellar granule neurons by serum withdrawal and potassium ion deprivation. Upon activity deprivation, the level of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP), a proteolytic substrate of the activated caspase, two critical indicators of apoptosis, increased significantly in granule neurons (fig. 3a). Inhibition of Cdk5 by a specific inhibitor, roscovitine, attenuated the specific induction of cleaved caspase-3 and cleaved PARP in the potassium ion deprivation condition (fig. 3a), suggesting that Cdk5 activity is required for neuronal apoptosis following activity deprivation.

It is believed that the terminally differentiated neurons reenter the cell cycle before undergoing apoptosis [9]. Reentry into the cell cycle requires reexpression of cell cycle proteins. Whereas the protein expression of cyclin D1 and cyclin E isoforms were both induced in granule neurons 24 h after activity deprivation, the increase in

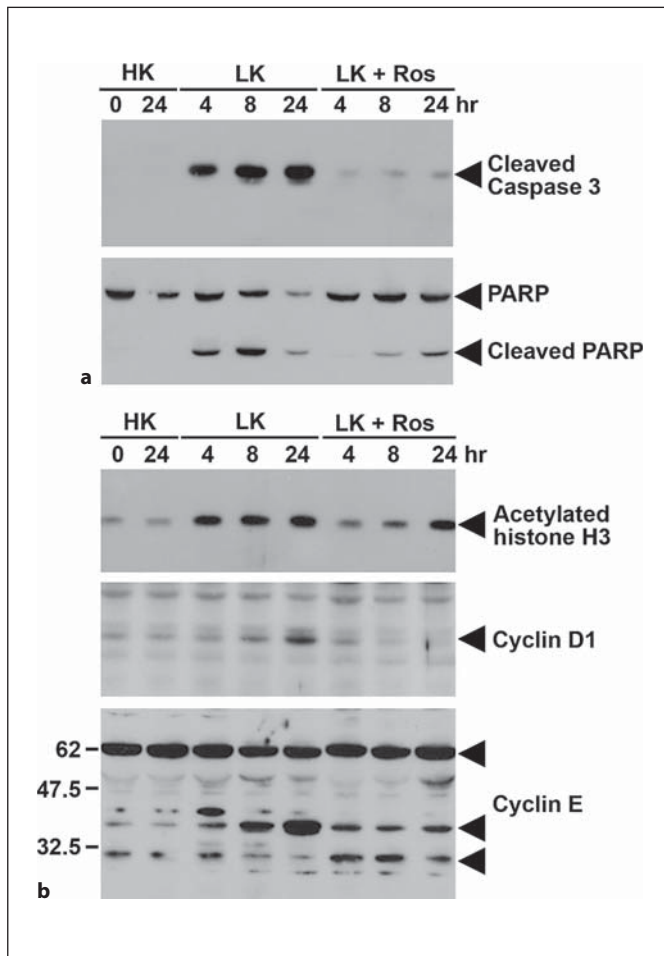


Fig. 3. Blockade of Cdk5 prevents activity deprivation-induced histone acetylation and protects the neurons from apoptosis. **a** Treatment of neurons with roscovitine (Ros) suppressed the induction of cleaved-caspase 3 and cleaved-PARP upon activity deprivation. Cerebellar granule neurons were switched from 25 mM KCl (HK, control) to 5 mM KCl (LK)-containing medium with or without roscovitine as indicated. Protein lysate was collected and subjected to Western blot analysis. **b** Roscovitine treatment reduced the induction of acetylated histone H3 and the up-regulation of cyclin D1 and cyclin E proteins in cerebellar granule neurons after activity deprivation.

histone H3 acetylation observed after activity deprivation preceded the reexpression of cyclins (fig. 3b). Importantly, suppression of Cdk5 activity by roscovitine effectively attenuated both histone H3 acetylation and reexpression of cyclin proteins in granule neurons under the potassium ion deprivation condition (fig. 3b). These findings suggest that Cdk5 activity regulates neuronal apoptosis through a modification of the chromatin structural integrity, thereby regulating gene expression.

Discussion

Cdk5 is an important nervous system kinase that plays crucial roles in regulating the life and death decision of neurons [2, 6]. Increased neuronal apoptosis in Cdk5 knockout mice suggests that Cdk5 is required for maintaining the survival of developing neurons [19]. Cdk5 has been suggested to mediate its pro-survival functions through various signaling pathways [6]. For example, a recent study suggests that Cdk5/p35 suppresses the reentry of neurons into the cell cycle by disrupting the cell cycle regulatory transcriptional complex [8]. On the other hand, hyperactivation of Cdk5 by p25, a proteolytic cleavage product of p35 during neuronal apoptosis, leads to abnormal activation of cell cycle reentry [20, 21]. Thus, tight control of Cdk5 activity is important for the gene regulation of cell cycle proteins in both neuronal survival and apoptosis. Transcriptional regulation is mediated via multiple mechanisms, including regulation of the activities of specific transcription factors, and the control of chromatin integrity by histone acetylation and histone methylation. While Cdk5 activity is well characterized to regulate cell cycle proteins through a basal transcriptional machinery [14, 15], emerging evidence suggests that Cdk5 also regulates transcription through the modification of histones by acetylation [16, 19].

Phosphorylation of mSds3 by Cdk5 facilitates the homodimerization of mSds3, and overexpression of p35 enhances mSds3-mediated transcriptional repression *in vitro* [17]. Our findings in the current study show that mSds3 binds to Cdk5 activators p35 or p39, thereby enabling active Cdk5 to phosphorylate mSds3 at Ser228 in the mouse brain. Thus, it is interesting to speculate that the Cdk5-dependent phosphorylation of mSds3 may promote HDAC1 activity in the mSin3-HDAC complex through modulation of the integrity of this transcriptional complex in neurons. HDAC1 activity is shown to repress cell cycle-related genes, such as cyclin D, during neural development [22]. Thus, regulation of the binding between mSds3 and p35 or p39 in the mouse brain (fig. 1) may contribute to an alternative regulatory pathway by which Cdk5 suppresses cell cycle genes in neurons during development. Nonetheless, the detailed mechanism underlying the action of Cdk5-dependent phosphorylation of mSds3 in normal neuronal functions awaits further study.

In contrast to observations in normal developing neurons, the action of active Cdk5 complex on regulation of mSin3/HDAC complex during neuronal apoptosis may be different. Upon neuronal apoptosis, calpain-dependent proteolytic cleavage of Cdk5 activators (p35 to p25 or p39

to p29) occurs [20]. Interestingly, p25 interacts with and phosphorylates HDAC1 directly [16]. Although HDAC1 is able to interact with p35 (fig. 1), the binding affinity between HDAC1 and p25 is much stronger, suggesting that HDAC1 is a pathological substrate for Cdk5/p25 [16]. The phosphorylation of HDAC1 results in inhibition of its activity, which leads to enhanced transcription of cell cycle genes and abnormal cell cycle reentry. Consistent with this notion, we found that while acetylation of histone H3 increases upon neuronal apoptosis, inhibition of Cdk5 attenuated this increase, and hence cell cycle reentry.

Our findings suggest that the interaction of Cdk5 activators with components of the HDAC complex and their Cdk5-dependent phosphorylation is important for HDAC functions, either by regulating the integrity of the HDAC complex or directly modulating the activity of HDAC. To control the accessibility of Cdk5 activators to the HDAC complex, nuclear localization of these activators is one of the major determining factors. Nuclear im-

port and export pathways for p35 have been identified [4 and Zhao and Ip, unpubl. observation]. Further understanding of the molecular control(s) that regulate the nucleocytoplasmic shuttling of p35 and p25 may provide insights into how the active Cdk5 complex differentially regulates histone acetylation and transcriptional regulation during neuronal development and upon neuronal apoptosis.

Acknowledgements

We are grateful to William Chau, Cara Kwong, Busma Butt, Alberto Ng and Winnie Chien for their excellent technical assistance. We also thank Dr. Kwok-On Lai and Zeldia H. Cheung for critical reading of the manuscript and members of N.Y.I.'s laboratory for many helpful discussions. This study was supported in part by the Research Grants Council of Hong Kong (HKUST6444/06M) and the Area of Excellence Scheme of the University Grants Committee (AoE/B-15/01). N.Y.I. is a Croucher Foundation Senior Research Fellow.

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