Brief Communications

Cyclin-Dependent Kinase 5 Supports Neuronal Survival through Phosphorylation of Bcl-2

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Accumulating evidence indicates that deregulation of cyclin-dependent kinase 5 (Cdk5) activity is associated with apoptosis in various neurodegenerative disease models. Interestingly, recent studies suggest that Cdk5 may also favor neuronal survival. Nonetheless, whether Cdk5 is directly required for neuronal survival during development remains enigmatic. In the current study, we established the pivotal role of Cdk5 in neuronal survival during development by demonstrating that reduction or absence of Cdk5 activity markedly exacerbated neuronal death in cultures and *in vivo*. Interestingly, the antiapoptotic protein Bcl-2 (B-cell lymphoma protein 2) was identified as a novel substrate of Cdk5. We found that Cdk5-mediated phosphorylation of Bcl-2 at Ser70 was required for the neuroprotective effect of Bcl-2. Intriguingly, inhibition of this phosphorylation conferred proapoptotic property to Bcl-2. Furthermore, overexpression of a Bcl-2 mutant lacking the Cdk5 phosphorylation site abolished the protective effect of Cdk5 re-expression in *Cdk5*$^{-/-}$ neurons, suggesting that Ser70 phosphorylation of Bcl-2 contributed to Cdk5-mediated neuronal survival. Our observations revealed that Cdk5-mediated Bcl-2 phosphorylation is pivotal for the antiapoptotic effect of Bcl-2 and contributes to the maintenance of neuronal survival by Cdk5. Our study has also identified Cdk5 as a regulator of Bcl-2 function in neuronal apoptosis.

Key words: **apoptosis; p35; Bcl-2; Cdk5; neuronal survival; retinal ganglion cells**

Introduction

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that is implicated in the regulation of neuronal migration, neuronal survival, and synaptic functions. Unlike other Cdks, activation of Cdk5 requires association with two noncyclin neural-specific activators p35 and p39, thus restricting the activity of Cdk5 to the nervous systems (Dhavan and Tsai, 2001). Recent studies reveal that precise regulation of Cdk5 activity is critical for the maintenance of neuronal survival (Cheung and Ip, 2004). Deregulated Cdk5 activity, for example, is observed in postmortem brains of Alzheimer's disease patients and is accompanied by hyperphosphorylated Tau and accumulation of p25, a cleaved fragment of p35 (Patrick et al., 1999). Furthermore, Cdk5/p25-mediated phosphorylation of NDMA receptors and prosurvival transcription factor MEF2 (myocyte enhancer factor 2) contribute to ischemia-induced neuronal death and dopaminergic neuronal loss in models of Parkinson's disease, respectively (Gong et al., 2003; Wang et al., 2003; Tang et al., 2005; Smith et al., 2006). These observations collectively indicate that prolonged activation of Cdk5 attributable to p25 production favors neuronal death in various pathological conditions and neurodegenerative diseases.

Interestingly, recent studies also suggest that Cdk5 may play a role in promoting neuronal survival. For example, ballooned neurons are evident in the spinal cord and brainstem of Cdk5-deficient mice (Ohshima et al., 1996). Indeed, Cdk5 activity is important for mediating the trophic effects of neuregulin (Li et al., 2003). In addition, Cdk5 counters apoptotic signals through phosphorylation and inhibition of c-Jun N-terminal protein kinase 3 (JNK3) (Li et al., 2002). Recently, reduction of Cdk5 activity is associated with neuronal death after treatment with DNA damage agent (O'Hare et al., 2005). Although these findings suggest a crucial role of Cdk5 in the maintenance of survival signals, whether Cdk5 activity is directly required for neuronal survival during development has not been thoroughly investigated. In addition, the mechanisms by which Cdk5 support neuronal survival remains essentially unknown. We thus aim to further characterize the role of Cdk5 in the life/death decision of developing neurons.

Materials and Methods

Antibodies, neuronal cultures, and transfection. Antibodies against Bcl-2 (B-cell lymphoma protein 2), Cdk5, and p35 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine antibody (16B4) was from Calbiochem (La Jolla, CA). Actin and β-tubulin type III antibodies were from Sigma (St. Louis, MO). Expression vectors of p35, Cdk5, and dominant-negative Cdk5 (dnCdk5) were prepared as described previously (Fu et al., 2001). Flag-tagged, green fluorescent protein (GST)-tagged Bcl-2 and Stealth RNA interference molecules for Cdk5 were prepared as described previously (Ng et al., 2006). Selective Cdk inhibitor, roscovitine (Ros) (Calbiochem) was used to inhibit Cdk5 activity.

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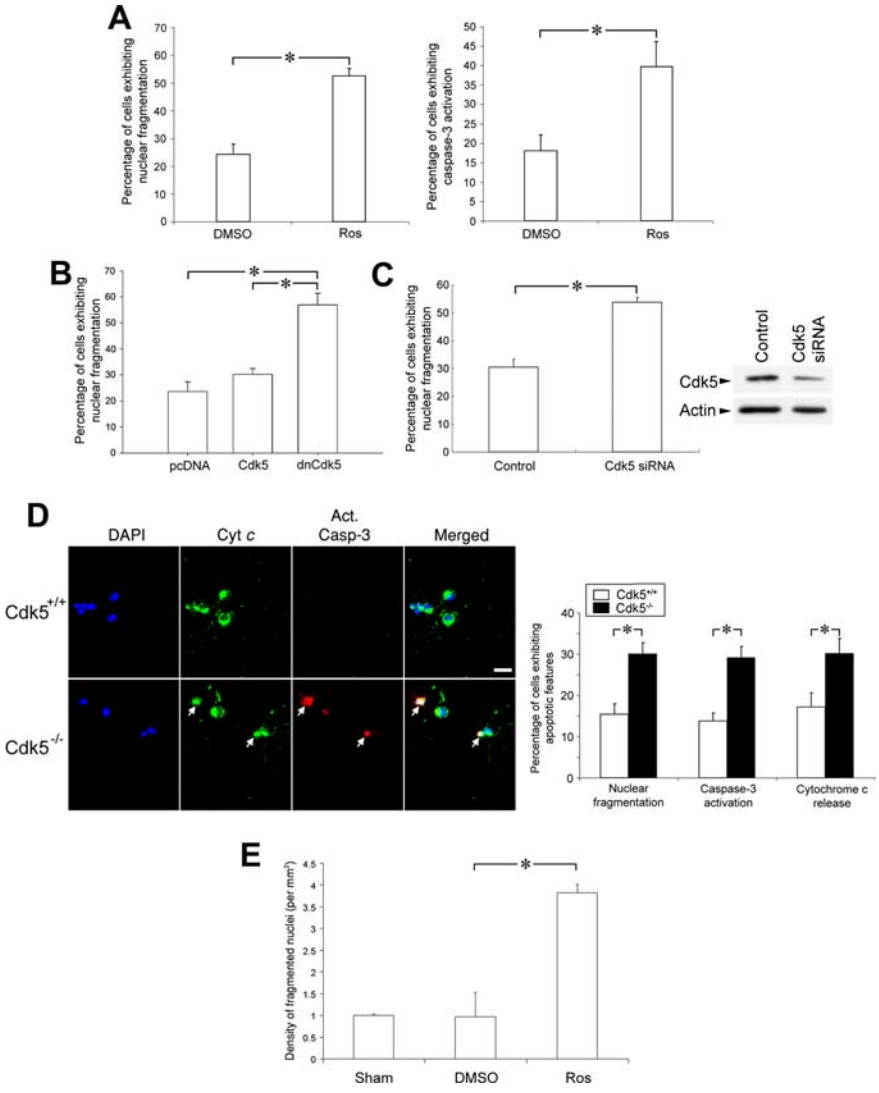

Figure 1. Cdk5 was required for neuronal survival. ***A***, Treatment with Cdk5-selective inhibitor Ros (25 μM) induced nuclear fragmentation and caspase-3 activation in pure RGC culture. ***B***, Overexpression of dnCdk5 led to a marked increase in nuclear fragmentation in retinal neurons. ***C***, Western blot analysis demonstrated that transfection of Cdk5 siRNA, but not scrambled siRNA (control), markedly reduced Cdk5 expression in retinal neurons. Transfection of Cdk5 siRNA increased the percentage of retinal neurons exhibiting nuclear fragmentation. ***D***, Retinal neurons taken from *Cdk5*$^{-/-}$ mice displayed a higher incidence of nuclear fragmentation, caspase-3 activation, and cytochrome *c* (Cyt *c*) release (arrows), as indicated by diffuse cytochrome *c* staining. ***E***, Intravitreal injection of Ros significantly increased the incidence of nuclear fragmentation in the ganglion cell layer of adult rat retina. Scale bar, 20 μm. *$p < 0.05$.

Cdk5 knock-out mice were kindly provided by A. B. Kulkarni (National Institutes of Health, Bethesda, MD) and T. Curran (School of Medicine, University of Pennsylvania, Philadelphia, PA). Pure retinal ganglion cell (RGC) cultures were prepared according to the protocol provided by B. Barres (Barres et al., 1988). Transfection of primary cultures was performed using calcium phosphate precipitation. Small interfering RNA (siRNA) transfection was performed with Lipofectamine 2000 transfection reagent according to the protocols of the manufacturer (Invitrogen, Carlsbad, CA). COS7 cells and HEK293T cells were maintained and transfected as described previously (Ng et al., 2006). Western blotting and immunoprecipitation was performed as described previously (Ng et al., 2006).

In vitro *kinase assay, intravitreal injection, and statistical analysis.* Recombinant Cdk5/p35 was kindly provided by Shin-Ichi Hisanaga (Tokyo Metropolitan University, Tokyo, Japan). *In vitro* kinase assay was performed as described previously (Fu et al., 2001). Intravitreal injection was performed using glass micropipettes as described previously (Cheung et al., 2004). Vehicle or Ros (estimated intravitreous concentration, ~25 μM) were injected once every 3 d for 6 d. Retinas were dissected and fixed on day 7. Cell death in flat-mounted retina was quantified as described previously (Cheung et al., 2004).

Data were expressed as mean ± SEM. Statistical significance was determined by Student's *t* test or one-way ANOVA, followed by Newman–Keuls *post hoc* test with 95% confidence. $p < 0.05$ was considered statistically significant.

Results

Cdk5 is required for neuronal survival

To verify whether Cdk5 directly affects neuronal survival, pure RGC cultures were prepared from postnatal day 8 (P8) rats using immunopanning. Interestingly, treatment of RGCs with Cdk5-selective inhibitor Ros for 4 h markedly enhanced nuclear fragmentation and caspase-3 activation that was evident 72 h after the initial treatment (Fig. 1*A*), indicating that suppression of Cdk5 activity for 4 h induced delayed neuronal apoptosis in RGCs. To further substantiate the role of Cdk5 in neuronal survival, retinal neurons were transfected with wild-type or dnCdk5. Similar to the induction of apoptosis by Ros, overexpression of dnCdk5 led to a marked increase in nuclear fragmentation compared with those expressing wild-type Cdk5 (Fig. 1*B*). Furthermore, reduction of endogenous Cdk5 expression using siRNA approach also resulted in enhanced nuclear fragmentation (Fig. 1*C*). More importantly, retinal neurons isolated from *Cdk5*$^{-/-}$ embryos exhibited elevated incidence of cytochrome *c* release, caspase-3 activation, and nuclear fragmentation (Fig. 1*D*). These observations strongly suggest that Cdk5 activity was required for the survival of retinal neurons.

To verify that a decrease in Cdk5 activity is sufficient to induce neuronal death *in vivo*, Ros was injected into the vitreous chamber of adult rats to reduce Cdk5 activity in the retina. We found that Ros injection markedly increased nuclear fragmentation in the ganglion cells layers (Fig. 1*E*), indicating that a reduction of Cdk5 activity was sufficient to induce apoptosis in the retina *in vivo*.

Bcl-2 is a substrate of Cdk5

We next proceeded to examine the potential mechanisms by which Cdk5 support neuronal survival. Because cytochrome *c* release is markedly elevated in *Cdk5*$^{-/-}$ neurons, we wanted to examine whether Cdk5 affects cytochrome *c* release through interacting with members of the Bcl-2 family. Cytochrome *c* release is tightly regulated by Bcl-2 family members, which includes proapoptotic members, such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2-antagonist/killer), and antiapoptotic members, such as Bcl-2 and Bcl-xL (Reed, 2006). Interestingly, sequence analysis revealed that Bcl-2 contains multiple proline-directed serine and threonine residues and one Cdk5 consensus site, S/TPXK/H/R (see Fig. 3*A*). This suggests that Bcl-2 may serve as a Cdk5 sub-

strate. To examine this possibility, we first investigated whether p35, Cdk5, and Bcl-2 were localized to the same subcellular fractions. Bcl-2 is localized to the outer membrane of the mitochondria (Blagosklonny, 2001). We also found that Bcl-2 was predominantly expressed in the mitochondrial fraction of cortical neurons. Interestingly, p35 was also abundantly expressed in the mitochondrial fraction, whereas Cdk5 was detected in both the mitochondrial and cytosolic fractions (Fig. 2*A*). Furthermore, immunocytochemical staining revealed that p35 colocalized extensively with both Bcl-2 and mitochondrial marker subunit IV of cytochrome *c* oxidase (COX4) in developing neurons, thus confirming the colocalization of Bcl-2 and p35 to the mitochondria (Fig. 2*B*). We next examined whether Bcl-2 associated with p35 or Cdk5 using lysates from HEK293T cells overexpressing p35 or Cdk5. Interestingly, whereas GST–Bcl-2 pulled down p35, and to a lesser extent Cdk5, in a GST pull-down assay (Fig. 2*C*), Bcl-2 only coimmunoprecipitated with p35 when both p35 and Bcl-2 were overexpressed in HEK293T cells (Fig. 2*D*). We further verified the interaction between Bcl-2 and p35 using membrane fractions of postnatal rat brains. We found that Bcl-2 coimmunoprecipitated with p35 and Cdk5, indicating that Bcl-2 associated with p35 and Cdk5 endogenously (Fig. 2*E*).

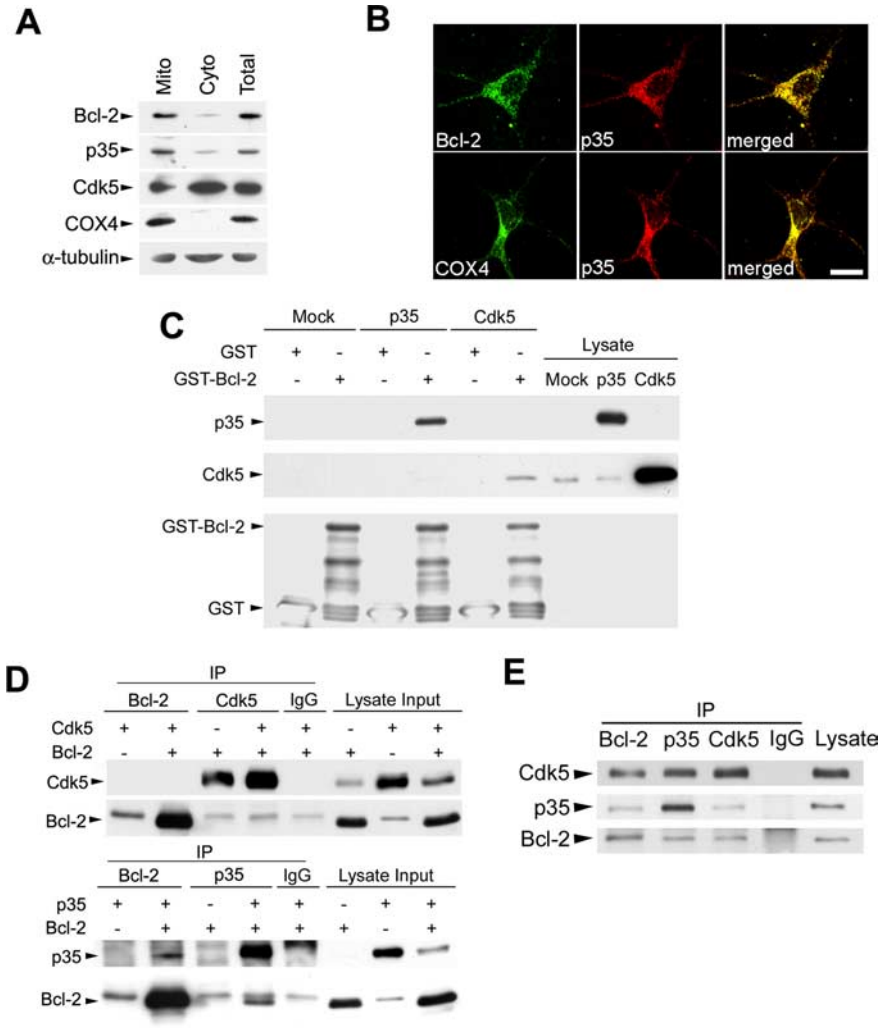

Figure 2. Bcl-2 interacted with Cdk5 in adult brain. ***A***, Bcl-2 and p35 were concentrated in the mitochondrial fraction of cortical neurons, whereas Cdk5 was ubiquitously expressed. COX4 serves as a marker for the mitochondrial fraction, and α-tubulin serves as an equal loading control. ***B***, p35 colocalized with both Bcl-2 and COX4 in developing cortical neurons. Scale bar, 10 μm. ***C***, GST–Bcl-2, but not GST, pulled down p35 and to a lesser extent Cdk5 in HEK293T cell lysates overexpressing p35 or Cdk5. ***D***, p35 (bottom), but not Cdk5 (top), associated with Bcl-2 when overexpressed in HEK293T cells. IgG was included as a control. ***E***, Bcl-2 associated endogenously with Cdk5 and p35 in the membrane fraction of P7 rat brain. IP, Immunoprecipitation.

To verify whether Bcl-2 serves as a Cdk5 substrate, GST–Bcl-2 was subjected to *in vitro* kinase assay using Cdk5/p35 as the kinase. Consistent with the presence of potential Cdk5 phosphorylation sites, GST–Bcl-2 was increasingly phosphorylated by Cdk5/p35 with increasing concentrations of the kinase (Fig. 3*B*), indicating that Bcl-2 is a substrate of Cdk5/p35. We next identified the potential Cdk5 phosphorylation site(s) on Bcl-2. Phosphorylation of Bcl-2 at the loop region has been demonstrated to modulate the neuroprotective effect of Bcl-2 (Ruvolo et al., 2001). Bcl-2 exhibits multiple proline-directed serine/threonine residues in the loop region, including Thr55, Ser70, and Ser84. In particular, a strong Cdk5 consensus site is found at Ser70 with the fourth amino acid requirement fulfilled (Fig. 3*A*). To identify the Cdk5 phosphorylation site(s), we generated three mutants in which Thr55 (T55A), Ser70 (S70A), or Ser84 (S84A) was mutated to alanine and examined whether Cdk5-mediated phosphorylation of Bcl-2 was retained. Bcl-2–wild-type, Bcl-2–T55A, Bcl-2–S70A, and Bcl-2–S84A were overexpressed in COS7 cells and lysates were immunoprecipitated and subjected to *in vitro* kinase assay using Cdk5/p35. Interestingly, Cdk5-mediated phosphorylation of Bcl-2 was almost completely abolished when Ser70 was mutated to alanine (Fig. 3*C*) but not when Thr55 or Ser84 was mutated to alanine. Furthermore, when Bcl-2 and Bcl-2–S70A were overexpressed together with Cdk5 and p35 in HEK293T cells, Bcl-2, but not Bcl-2–S70A, was serine phosphorylated as detected by an antibody against phosphoserine (Fig. 3*D*). These observations collectively indicate that Cdk5 phosphorylated Bcl-2 at Ser70.

To further verify whether Cdk5 phosphorylates Bcl-2 *in vivo*, Bcl-2 phosphorylation was examined in $Cdk5^{+/+}$ and $Cdk5^{-/-}$ brains. Importantly, Bcl-2 phosphorylation was markedly reduced in $Cdk5^{-/-}$ brain, whereas Bcl-2 total levels remained rather constant, suggesting that Cdk5 phosphorylated Bcl-2 *in vivo* (Fig. 3*E*). It should be noted that a number of kinases have also been observed to phosphorylate Bcl-2, including extracellular signal-regulated kinase (Erk1/2), JNK, and p38 mitogen-activated protein kinase (MAPK) (Blagosklonny, 2001; Ruvolo et al., 2001). We thus examined the activation of these kinases in $Cdk5^{+/+}$ and $Cdk5^{-/-}$ brains to verify that the lack of Cdk5 did not affect Bcl-2 phosphorylation through indirect modulation of these kinases. We found that activation of Erk and p38 MAPK were both enhanced in $Cdk5^{-/-}$ brain, whereas JNK activation was comparable between $Cdk5^{+/+}$ and $Cdk5^{-/-}$ brains (Fig. 3*E*). This indicates that Bcl-2 phosphorylation was not attenuated because of inactivity of other known Bcl-2 kinases in Cdk5-deficient brain.

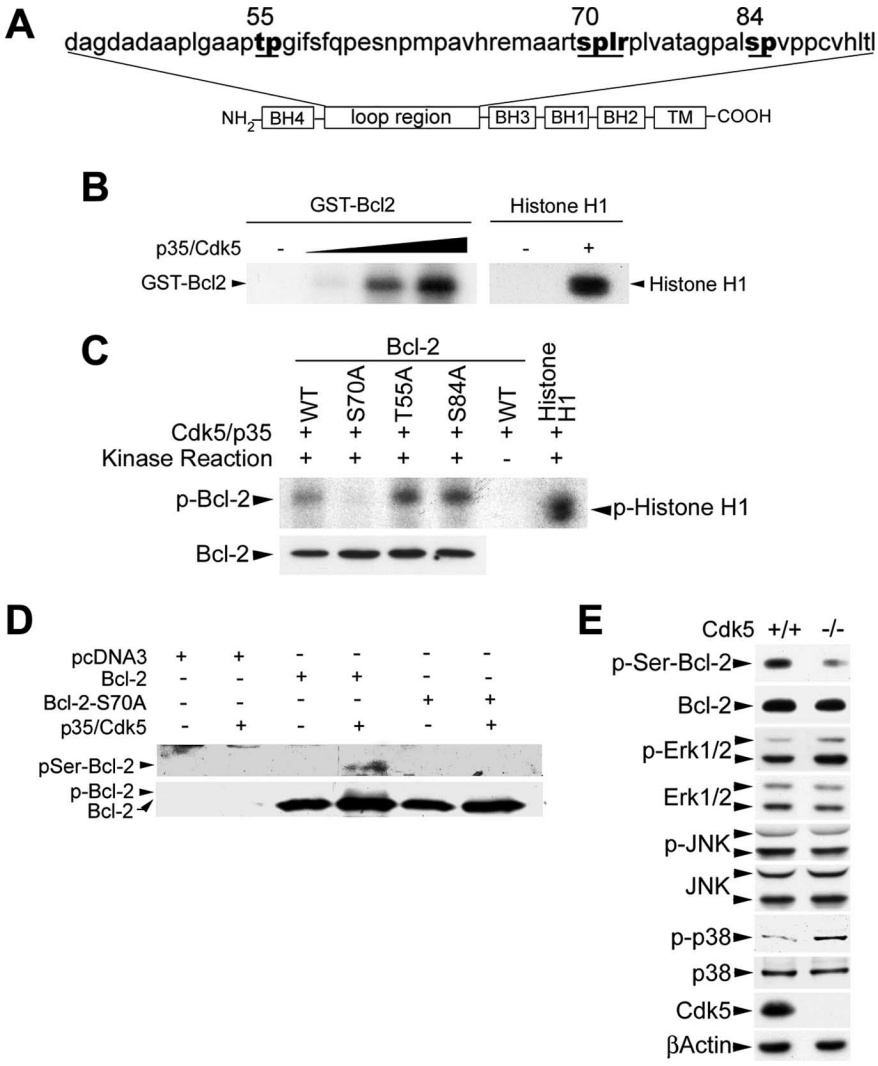

Figure 3. Cdk5 phosphorylated Bcl-2 at Ser70. ***A***, Potential Cdk5 phosphorylation sites are found at Thr55, Ser70, and Ser84 of the loop region of mouse Bcl-2. ***B***, GST–Bcl-2 was phosphorylated by Cdk5/p35 in a dose-dependent manner in *in vitro* kinase assay. Histone H1 served as a control to demonstrate activity of the Cdk5/p35 complex. ***C***, Wild-type (WT) Bcl-2, Bcl-2–S70A, Bcl-2–T55A, or Bcl-2–S84A were subjected to *in vitro* kinase assay. Mutation of Ser70, but not Thr55 or Ser84, to alanine abolished phosphorylation of Bcl-2 by Cdk5/p35. ***D***, Bcl-2, but not Bcl-2–S70A, was serine phosphorylated (pSer–Bcl-2) in the presence of Cdk5/p35. The serine-phosphorylated band corresponded to an upward shift in mobility (pBcl-2) when the blot was reprobed with Bcl-2 antibody. ***E***, Bcl-2 phosphorylation (p-Bcl-2) was markedly reduced in *Cdk5*$^{-/-}$ brain. Activation of Bcl-2 kinases, such as Erk1/2, JNK, and p38 MAPK, was not reduced in *Cdk5*$^{-/-}$ brain.

Phosphorylation of Bcl-2 at Ser70 by Cdk5 regulates if Bcl-2 exhibits antiapoptotic or proapoptotic property

To examine whether Cdk5-mediated phosphorylation of Bcl-2 affects the antiapoptotic functions of Bcl-2, Bcl-2 and Bcl-2–S70A were transfected into P8 retinal neurons to examine their effects on neuronal survival. Developmental apoptosis of retinal neurons occurs within the first 2–3 weeks after birth (Young, 1984). In agreement with this observation, 20–30% of P8 retinal neurons exhibited apoptotic features (Fig. 4*A*) (also in Fig. 1*A–C*). Overexpression of Bcl-2 reduced the percentage of retinal neurons undergoing nuclear fragmentation by ~50%. Interestingly, overexpression of Bcl-2–S70A exhibited no neuroprotective effect and even slightly enhanced nuclear fragmentation in P8 retinal neurons (Fig. 4*A*). This suggests that phosphorylation of Bcl-2 at Ser70 is required for the antiapoptotic effect of Bcl-2.

To further characterize the involvement of Cdk5 in Bcl-2-mediated regulation of neuronal survival, we examined the effect of Bcl-2 or Bcl-2–S70A in *Cdk5*$^{+/+}$ and *Cdk5*$^{-/-}$ retinal neurons. Because of the exhibition of perinatal death by *Cdk5*$^{-/-}$ embryos, retinal neurons were isolated from embryonic day 18 embryos instead of P8 pups. Interestingly, overexpression of Bcl-2 failed to further decrease nuclear fragmentation in *Cdk5*$^{+/+}$ neurons (Fig. 4*B*), possibly because of a lower level of developmental apoptosis and different endogenous Bcl-2 levels in retinal neurons at different developmental stages (Sharma, 2001). Surprisingly, we found that overexpression of Bcl-2–S70A resulted in a marked elevation of nuclear fragmentation in *Cdk5*$^{+/+}$ neurons (Fig. 4*B*). Together with the slight elevation in neuronal apoptosis observed in Bcl-2–S70A-expressing P8 retinal neurons, these findings suggest that a Bcl-2 mutant that is not phosphorylated at Ser70 may exhibit proapoptotic effect and suggest that Cdk5 may contribute to determining whether Bcl-2 exhibits antiapoptotic or proapoptotic property through phosphorylation at Ser 70.

To investigate this possibility, we adopted a model in which transient overexpression of Bcl-2 triggers apoptosis in HEK293T cells. Despite the well established role of Bcl-2 as an antiapoptotic molecule, several studies have demonstrated that transient overexpression of Bcl-2 in cell lines such as HEK293T cells induces apoptosis (Uhlmann et al., 1998; Portier and Taglialatela, 2006). Because Cdk5 activity is absent in HEK293T cells attributable to the lack of p35 expression (Dhavan and Tsai, 2001), we hypothesized that Bcl-2 may exhibit apoptosis-inducing effect in HEK293T cells because it exists mainly as an unphosphorylated form at Ser70 in these cells. Indeed, pSer–Bcl-2 was not detected in the absence of p35/Cdk5 overexpression in HEK293T cells (Fig. 3*D*). To verify our hypothesis, we examined whether p35 overexpression, which leads to Cdk5 activation, can attenuate Bcl-2-triggered cell death in HEK293T cells. As reported previously (Uhlmann et al., 1998; Portier and Taglialatela, 2006), overexpression of Bcl-2 in HEK293T cells induced massive nuclear fragmentation (Fig. 4*C*). Remarkably, overexpression of p35 significantly reduced Bcl-2-induced cell death (Fig. 4*C*), indicating that Bcl-2 phosphorylation by Cdk5 attenuated the apoptosis-inducing property of Bcl-2 in HEK293T cells. Bcl-2–S70A, conversely, induced similar extent of apoptosis with or without p35 (Fig. 4*C*). Our findings therefore revealed that Cdk5 played an important role in modulating the precise role of Bcl-2 in apoptosis regulation. Through phosphorylation at Ser70, Cdk5 possibly regulates whether Bcl-2 exhibits proapoptotic or antiapoptotic function. Our findings also suggest that the lack of Bcl-2 phosphorylation at Ser70 in cell lines such as HEK293T cells may play a role in the induction of apoptosis by Bcl-2.

Bcl-2 phosphorylation at Ser70 contributes to the maintenance of neuronal survival by Cdk5

We next examined whether the lack of Bcl-2 phosphorylation at Ser70 contributed to augmented neuronal death in *Cdk5*$^{-/-}$ mice by comparing the effect of Bcl-2 and Bcl-2–S70A overexpression in *Cdk5*$^{-/-}$ and *Cdk5*$^{+/+}$ retinal neurons. Interestingly, Bcl-2 overexpression failed to exhibit any neuroprotective effect despite the extensive neuronal death observed in *Cdk5*$^{-/-}$ neurons (Fig. 4*B*). Furthermore, in contrast to the marked induction of apoptosis by Bcl-2–S70A in *Cdk5*$^{+/+}$ neurons, Bcl-2–S70A overexpression had negligible effect on neuronal survival in *Cdk5*$^{-/-}$ neurons (Fig. 4*B*). These observations suggest that Bcl-2 loses its ability to regulate neuronal survival when Cdk5 activity is absent, thus underscoring the importance of Cdk5 in the apoptosis-regulatory role of Bcl-2. In addition, it is interesting to note that lack of Cdk5 activity (mock-transfected *Cdk5*$^{-/-}$ neurons) and removal of the Cdk5 phosphorylation site in Bcl-2 (Bcl-2–S70A-transfected *Cdk5*$^{+/+}$ neurons) triggered comparable levels of neuronal apoptosis, but the concurrent inhibition of Cdk5 activity and Bcl-2 phosphorylation at Ser70 (Bcl-2–S70A-transfected *Cdk5*$^{-/-}$ neurons) had no additive effect on the induction of neuronal death (Fig. 4*B*). These observations are in support of the scenario in which lack of Cdk5 activity and inhibition of Bcl-2 Ser70 phosphorylation contributed to neuronal death through the same pathway. In light of the observed phosphorylation of Bcl-2 at Ser70 by Cdk5 and the identification of Cdk5 as an important kinase of Bcl-2 *in vivo* (Fig. 3*E*), we believe that the lack of Bcl-2 phosphorylation at Ser70 in *Cdk5*$^{-/-}$ neurons contributed, at least in part, to the compromised neuronal survival in *Cdk5*$^{-/-}$ neurons.

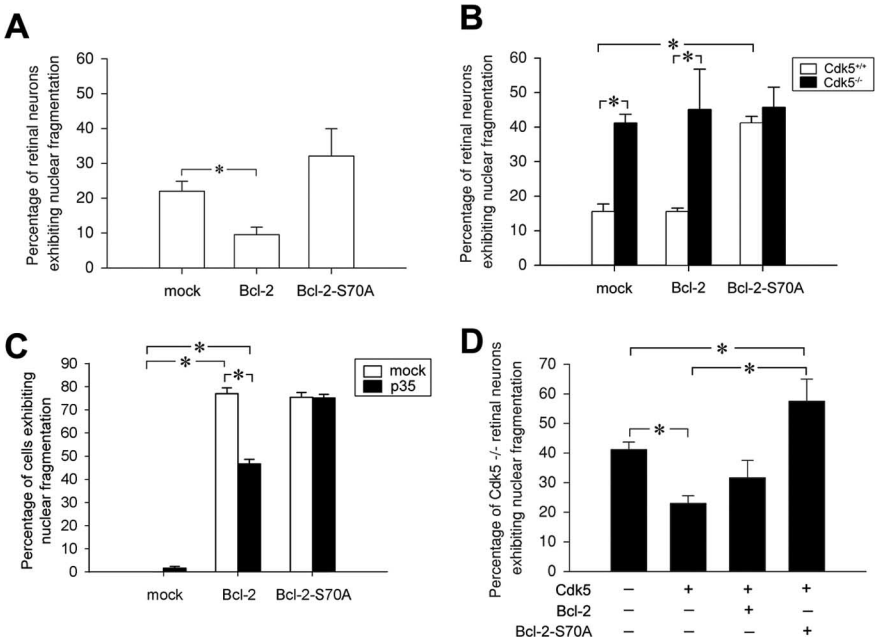

Figure 4. Phosphorylation of Bcl-2 at Ser70 determined whether Bcl-2 exhibited proapoptotic or antiapoptotic function and was essential for the maintenance of neuronal survival by Cdk5. ***A***, Overexpression of Bcl-2 markedly reduced nuclear fragmentation in P8 retinal neurons, but no protection was offered by Bcl-2–S70A. ***B***, Incidence of nuclear fragmentation was markedly higher in *Cdk5*$^{-/-}$ neurons. Overexpression of Bcl-2–S70A induced nuclear fragmentation in *Cdk5*$^{+/+}$ neurons, but overexpression of Bcl-2–S70A failed to further exacerbate neuronal death in *Cdk5*$^{-/-}$ neurons. ***C***, Overexpression of p35 attenuated Bcl-2 induced apoptosis in HEK293T cells. Overexpression of Bcl-2 and Bcl-2–S70A induced massive nuclear fragmentation. Overexpression of p35 attenuated cell death triggered by Bcl-2 but not Bcl-2–S70A. ***D***, Nuclear fragmentation was markedly reduced by Cdk5 overexpression in *Cdk5*$^{-/-}$ neurons. Coexpression of Bcl-2–S70A abolished the neuroprotective effect of Cdk5 and further enhanced nuclear fragmentation in *Cdk5*$^{-/-}$ neurons. $^*p < 0.05$.

To further consolidate the involvement of Ser70 phosphorylation of Bcl-2 in the maintenance of neuronal survival by Cdk5, we attempted to reverse neuronal death in *Cdk5*$^{-/-}$ neurons through the overexpression of Cdk5. As expected, overexpression of Cdk5 markedly reduced nuclear fragmentation in *Cdk5*$^{-/-}$ neurons (Fig. 4*D*). Interestingly, although cotransfection of Bcl-2 did not offer additional neuroprotection, concomitant overexpression of Bcl-2–S70A abolished the neuroprotective effect of Cdk5 and even further enhanced neuronal apoptosis compared with mock-transfected *Cdk5*$^{-/-}$ neurons (Fig. 4*D*). Our observation therefore revealed that Ser70 phosphorylation of Bcl-2 was crucial for Cdk5 overexpression-mediated neuronal survival in *Cdk5*$^{-/-}$ neurons. In addition, similar to what was observed in Figure 4, *A* and *B*, mutation of the Cdk5 phosphorylation site conferred proapoptotic ability to Bcl-2 (Fig. 4*D*), further supporting the notion that phosphorylation of Bcl-2 by Cdk5 modulates the apoptosis regulatory role of Bcl-2. Collectively, our findings suggest that the lack of Bcl-2 Ser70 phosphorylation likely contributes to the enhanced developmental apoptosis exhibited by *Cdk5*$^{-/-}$ neurons and reveal an unexpected role of Cdk5 as a regulator of the antiapoptotic property of Bcl-2, where lack of Cdk5 phosphorylation appears to turn Bcl-2 into a proapoptotic protein.

Discussion

Numerous reports have documented phosphorylation of Bcl-2 by a number of kinases, such as receptor tyrosine kinase and MAPK kinases (Blagosklonny, 2001; Ruvolo et al., 2001). Here we have identified Cdk5 as a novel Bcl-2 kinase that phosphorylates Bcl-2 at Ser70. Importantly, marked reduction of Bcl-2 phosphorylation was observed in *Cdk5*$^{-/-}$ brain, thus underscoring the importance of Cdk5 in Bcl-2 phosphorylation *in vivo*. Indeed, activation of other kinases that have been demonstrated to phosphorylate Bcl-2 at Ser70, such as Erk1/2, JNK, and p38 MAPK, were elevated or relatively unchanged in *Cdk5*$^{-/-}$ brains compared with *Cdk5*$^{+/+}$ brains. Nonetheless, the *in vivo* involvement of these kinases in Bcl-2 phosphorylation in the developing brain remains primarily enigmatic. Our observations suggest that Cdk5 serves as one of the important kinases for Bcl-2 phosphorylation in the developing nervous system *in vivo*. Furthermore, inhibiting Cdk5-mediated Bcl-2 phosphorylation abolished the maintenance of neuronal survival by exogenous expression of Cdk5 in *Cdk5*$^{-/-}$ neurons, suggesting that the reduced Bcl-2 phosphorylation likely contributes to the compromised survival of *Cdk5*$^{-/-}$ neurons.

Despite the myriad of kinases that have been demonstrated to phosphorylate Bcl-2, whether phosphorylation of Bcl-2 enhances or diminishes the antiapoptotic roles of Bcl-2 remains controversial. Recent literature suggests that, although phosphorylation of Bcl-2 at Ser70 alone is required for the antiapoptotic effect of Bcl-2, phosphorylation of Bcl-2 at multiple sites (Ser70, Ser87, and Thr69 of human Bcl-2) turns Bcl-2 into a proapoptotic mol-

ecule (Ruvolo et al., 2001). In the current study, we found that Bcl-2 was phosphorylated by Cdk5 only at Ser70. Consistent with previous observations, inhibition of Bcl-2 phosphorylation at Ser70 essentially abolished the antiapoptotic effect of Bcl-2. Unexpectedly, we also revealed that a mutant lacking the Cdk5 phosphorylation site not only exhibited no neuroprotective effect, but instead exacerbated neuronal death. Our observations therefore suggest that Cdk5 may take part in determining whether Bcl-2 exhibits antiapoptotic or proapoptotic role through Ser70 phosphorylation of Bcl-2. Indeed, previous studies have demonstrated that Bcl-2 can exhibit proapoptotic function under certain conditions. Aside from phosphorylation of Bcl-2 at multiple sites (Ruvolo et al., 2001), localization of Bcl-2 to the nucleus also triggers apoptosis (Portier and Taglialatela, 2006). Furthermore, transient overexpression of Bcl-2 and caspase-mediated cleavage of Bcl-2 have both been observed to induce cell death (Cheng et al., 1997; Uhlmann et al., 1998). Association of Bcl-2 with orphan nuclear receptor Nur77 also turns Bcl-2 into a proapoptotic protein (Lin et al., 2004). Whether Cdk5-mediated Bcl-2 phosphorylation acts together with these known pathways to trigger conversion of Bcl-2 into a killer or via a currently unknown mechanism remains to be determined. It is plausible that Cdk5-mediated phosphorylation of Bcl-2 at Ser70 may affect its subcellular localization or its cleavage by caspases. Alternatively, this phosphorylation may directly affect its association with other Bcl-2 family members, thereby favoring or hindering apoptosis. Additional studies will be required to delineate these possibilities.

Together, our data reveal that Bcl-2 phosphorylation by Cdk5 is important for the antiapoptotic property of Bcl-2 and that this phosphorylation contributes to Cdk5-mediated maintenance of neuronal survival. Identification of Bcl-2 as a Cdk5 substrate provides an additional mechanism by which Cdk5 affects neuronal survival. Furthermore, our findings support the notion that precise regulation of Cdk5 activity is critical for neuronal survival. In light of the pivotal role of Cdk5 in neurodegenerative diseases and the essential involvement of Bcl-2 in regulating neuronal survival, it will be interesting to further delineate the significance of this crosstalk during traumatic injury and neurodegenerative diseases *in vivo*.

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