Molecular Cell, Vol. 9, 1005–1016, May, 2002, Copyright ©2002 by Cell Press

Cdc2 Phosphorylation of BAD Links the Cell Cycle to the Cell Death Machinery

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Summary

A mechanism that triggers neuronal apoptosis has been characterized. We report that the cell cycle-regulated protein kinase Cdc2 is expressed in postmitotic granule neurons of the developing rat cerebellum and that Cdc2 mediates apoptosis of cerebellar granule neurons upon the suppression of neuronal activity. Cdc2 catalyzes the phosphorylation of the BH3-only protein BAD at a distinct site, serine 128, and thereby induces BAD-mediated apoptosis in primary neurons by opposing growth factor inhibition of the apoptotic effect of BAD. The phosphorylation of BAD serine 128 inhibits the interaction of growth factor-induced serine 136-phosphorylated BAD with 14-3-3 proteins. Our results suggest that a critical component of the cell cycle couples an apoptotic signal to the cell death machinery via a phosphorylation-dependent mechanism that may generally modulate protein-protein interactions.

Introduction

During the development of the mammalian nervous system, neurons are generated in excess, and their numbers are thus controlled by apoptotic cell death to ensure the proper wiring of the nervous system (Lewin and Barde, 1996). Electrical activity and polypeptide growth factors are thought to be major regulators of neuronal cell death and survival (Franklin and Johnson, 1992; Lewin and Barde, 1996). However, the mechanisms that drive neurons toward apoptosis in the absence of electrical activity or growth factors remain incompletely understood.

Within the developing brain, neuronal apoptosis and survival have been extensively investigated in the cerebellar cortex (Williams and Herrup, 1988). Cerebellar granule cell precursors differentiate into postmitotic neurons within the external granular layer (EGL) and then migrate inward within the cerebellar cortex into the internal granular layer (IGL) where they mature (Altman and Bayer, 1997). Newly generated granule neurons undergo apoptosis in the EGL and IGL (Wood et al., 1993). Apoptosis of cultured cerebellar granule neurons is suppressed by growth factors and neuronal activity, the latter mimicked by membrane-depolarizing concentrations of KCl (D'Mello et al., 1997; Miller and Johnson, 1996; Padmanabhan et al., 1999). Membrane depolarization activates voltage-sensitive calcium channels (VSCCs) in neurons, leading to the entry of calcium into neurons (Catterall, 2000).

Accumulating evidence suggests that activation of components of the cell cycle in postmitotic neurons might contribute to neuronal apoptosis (Liu and Greene, 2001). The induction of proteins that control the G1/S transition in proliferating cells occurs in dying neurons (Freeman et al., 1994; Kranenburg et al., 1996), and G1 cyclin-dependent kinases appear to trigger neuronal cell death (Park et al., 1997, 1998). While the role of G1 cell cycle proteins in neuronal apoptosis is beginning to be characterized, the role of G2/M cell cycle proteins, if any, in apoptosis of postmitotic neurons remained unknown. Similarities in the morphological appearance of apoptotic cells to proliferating cells undergoing mitosis suggest that the mechanisms that regulate mitosis play a role in apoptosis (King and Cidlowski, 1995). Consistent with this idea, the cell cycle-regulated protein kinase Cdc2 has been implicated in apoptosis of proliferating cells (Shi et al., 1994; Yu et al., 1998). However, the role of Cdc2 in promoting apoptosis of postmitotic neurons and the mechanisms by which Cdc2 might promote apoptosis in proliferating cells or postmitotic neurons remained unknown.

Studies of apoptosis suggest that members of the Bcl-2 family of proteins act as gatekeepers of the cell death machinery (Chao and Korsmeyer, 1998; Reed, 1997; Thompson, 1995). Proapoptotic members of the Bcl-2 family of proteins induce the release of cytochrome c from mitochondria, leading to the Apaf-1-dependent activation of Caspase 9 and consequent induction of a caspase cascade that executes the cell death program (Green and Reed, 1998; Thornberry and Lazebnik, 1998). In recent years, progress has been made in our understanding of the intracellular signaling mechanisms by which cell surface death receptors activate the cell death machinery and the mechanisms by which survival factors suppress the cell death machinery (Ashkenazi and Dixit, 1998; Downward, 1998). However, the mechanisms by which components of the cell cycle machinery might activate the cell death machinery remained to be characterized.

In this study, we have investigated the role of Cdc2 in neuronal apoptosis. We have found that Cdc2 is expressed in newly generated postmitotic granule neurons in the rat cerebellum. Neuronal activity deprivation stimulates the activity of Cdc2 in granule neurons, and once activated, Cdc2 triggers neuronal apoptosis. We have also characterized a mechanism by which Cdc2 mediates activity deprivation-induced neuronal apoptosis. Our results suggest that Cdc2 induces the phosphorylation of the BH3-only protein BAD at the distinct site of serine 128, which lies near the growth factor-regulated site of phosphorylation, serine 136. The Cdc2-induced phosphorylation of BAD at serine 128 inhibits the ability of 14-3-3 proteins to sequester growth factor-induced serine 136-phosphorylated BAD and thus promotes the apoptotic effect of BAD by opposing growth factor inhibition of BAD-mediated apoptosis. These results suggest that a critical component of the cell cycle directly

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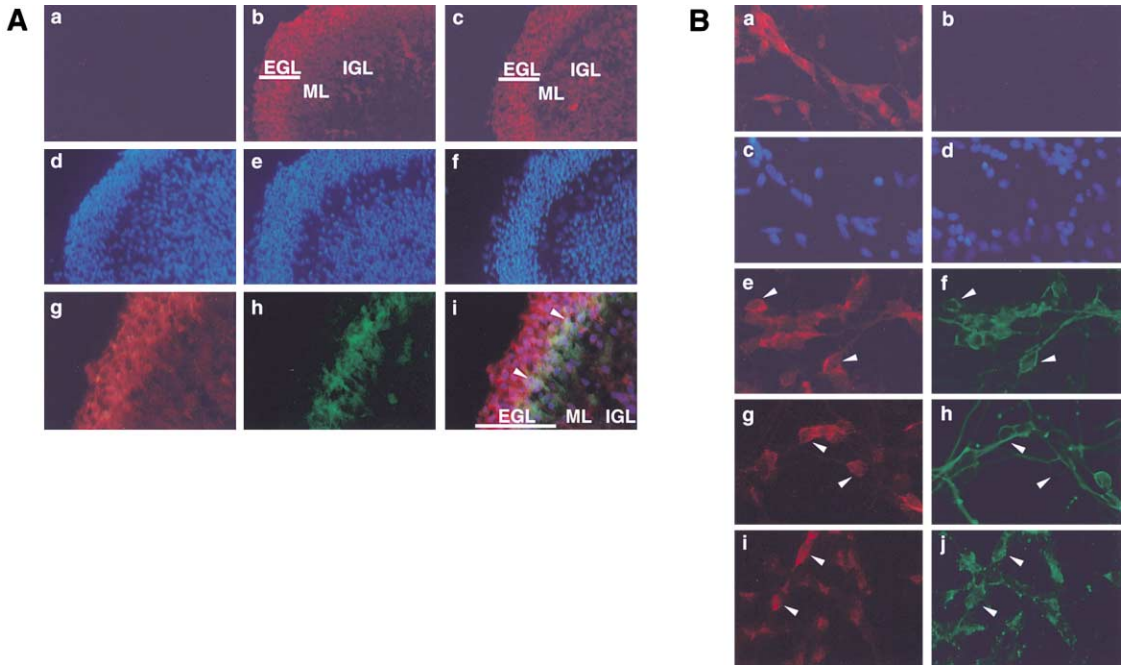

Figure 1. The Cell Cycle Regulatory Protein Cdc2 Is Expressed in Newly Generated Cerebellar Granule Neurons

(A) Cerebella of P6 rat pups were subjected to indirect immunofluorescence with no primary antibody (a), a mouse monoclonal antibody to Cdc2 (b), a rabbit antibody to Cdc2 (c and g), or a mouse monoclonal antibody to the protein TAG-1 (h). Sections were also stained with a DNA dye to visualize cells (d–f). (i) represents the merging of (g), (h), and an image of cells stained with the DNA dye.

(B) Granule neurons (P6+1DIV) were subjected to indirect immunofluorescence with a rabbit antibody to Cdc2 in the absence (a) or presence of blocking peptide (Santa Cruz) (b), with the rabbit antiserum to Cdc2 (e and g) together with the antibody to TAG-1 (f) or a mouse monoclonal antibody to the neuron-specific β tubulin (TuJ1) (h), or with a mouse monoclonal antibody to Cyclin B1 (i) together with a rabbit antibody to the neuron-specific protein MAP2 (j). (c) and (d) are Hoechst images corresponding to (a) and (b). Arrowheads indicate examples of cells expressing both Cdc2 and TAG-1, both Cdc2 and the neuron-specific β-tubulin, or both Cyclin B1 and MAP2.

activates the cell death machinery in postmitotic neurons. In addition, our results point to a phosphorylation-dependent mechanism that may generally modulate protein-protein interactions.

Results

Cdc2 Mediates Apoptosis of Cerebellar Granule Neurons upon Activity Deprivation

To investigate the role of Cdc2 in cerebellar granule neuron apoptosis, we first characterized the expression of Cdc2 in the rat cerebellar cortex. Cdc2 immunoreactivity, as detected by two antibodies that recognize distinct epitopes on Cdc2, was found throughout the EGL in postnatal day 6 (P6) rat cerebellum, including cells that expressed the protein TAG1, a marker of newly generated granule neurons residing in the EGL (Figure 1A). Less intense Cdc2 immunoreactivity was also found in the IGL of P6 rat pups (Figure 1A). To confirm that Cdc2 is expressed in postmitotic neurons, we characterized the expression of Cdc2 in granule neurons that were cultured from P6 rat pups. Specific Cdc2 immunoreactivity was found in newly generated granule neurons that expressed the neuron-specific protein β-tubulin type III or the protein TAG-1 (Figure 1B). These results indicate that Cdc2 is expressed in postmitotic cerebellar granule neurons at the time that developmentally-regulated apoptosis peaks in vivo (Wood et al., 1993).

The expression of Cdc2 in postmitotic neurons led us to examine if Cdc2 becomes activated in neurons upon exposure to an apoptotic stimulus. Expression of the Cdc2 activator Cyclin B1 was found in granule neurons by immunoblotting and immunocytochemical analyses of P6 cerebellar cultures (Figures 1B and 2A). Cyclin B1 interacted physically with Cdc2 in neurons, as determined in coimmunoprecipitation experiments (Figure 2A). A larger amount of Cdc2 associated with Cyclin B1 in granule neuron cultures that were deprived of membrane depolarizing concentrations of KCl (5 mM KCl plus serum) than in cultures that were maintained in full medium (30 mM KCl plus serum), reflecting in part the increased amount of total Cdc2 in granule neurons upon KCl withdrawal (Figure 2A). Consistent with these results, KCl withdrawal induced the activity of Cdc2 in granule neurons, as determined by assaying the kinase activity that was immunoprecipitated from lysates of granule neuron cultures with an antibody to Cyclin B1 (Figure 2B) or with an antibody to Cdc2 (Figure 2C). Because the Cdc2-related kinase Cdk5 is expressed in postmitotic neurons, including cerebellar granule neurons (Ohshima et al., 1999; Tsai et al., 1993), we confirmed that the antibodies to Cdc2 used in our study recognize Cdc2 specifically and do not recognize Cdk5 by immunoblotting of lysates of 293T cells overexpressing Cdc2 or Cdk5 (Figure 2D). Together, these results indicate that the apoptosis-inducing stimulus of sup-

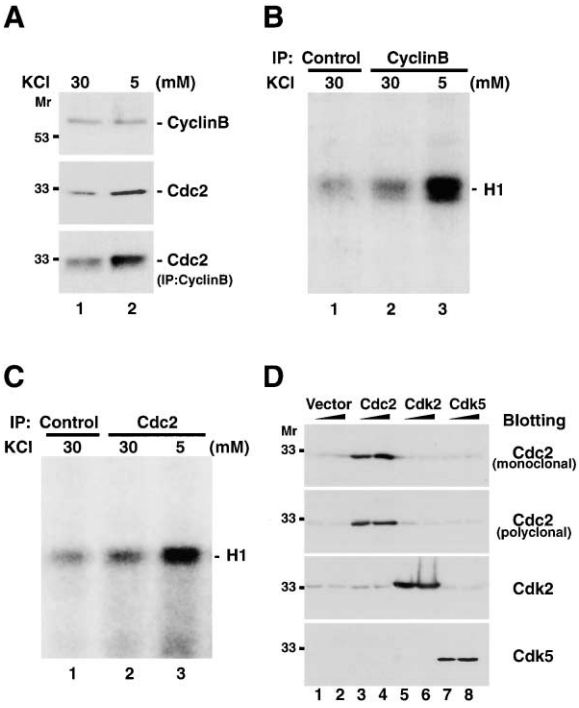

Figure 2. The Apoptosis-Inducing Stimulus of Neuronal Activity Deprivation Induces Cdc2 Expression and Activity

(A) Lysates of granule neuron cultures (P6+2DIV) that had been maintained in full medium (30 mM KCl plus serum) or deprived of KCl (5 mM KCl plus serum) for 24 hr were immunoblotted with a mouse monoclonal antibody to Cyclin B1 (top panel), the rabbit antibody to Cdc2 (middle panel), or immunoprecipitated with the antibody to Cyclin B1 followed by immunoblotting with the rabbit antibody to Cdc2 (bottom panel). Lysates of granule neuron cultures were immunoprecipitated with an antibody to Cyclin B1 (B) or with a rabbit antibody to Cdc2 (C) and then subjected to an in vitro kinase assay using histone H1 as substrate. The activity of Cdc2 in granule neurons was induced 2 $\pm$ 0.5-fold ($n = 7$, $p < 0.05$, t test) in (B) and was induced 1.6 $\pm$ 0.1-fold ($n = 5$, $p < 0.05$, t test) in (C). (D) Lysates of 293T cells that were transfected with an expression plasmid encoding Cdc2, Cdk2, or Cdk5, or their control plasmid were immunoblotted with the mouse monoclonal antibody to Cdc2, the rabbit antibody to Cdc2, a rabbit antibody to Cdk2, or a mouse monoclonal antibody to Cdk5.

pression of neuronal activity triggers the activation of endogenous Cdc2 in cerebellar granule neurons.

To determine the importance of KCl withdrawal-induced Cdc2 in mediating neuronal apoptosis, we blocked the function of endogenous Cdc2 in cerebellar granule neurons. Because Cdc2 is enriched in newly generated granule neurons (Figure 1), we analyzed cell survival in P6 plus 2 days in vitro (P6+2DIV) cultures that were exposed to full medium or that were deprived of membrane depolarization (5 mM KCl plus serum). KCl withdrawal stimulated the cleavage and activity of the protease Caspase 3 in granule neuron cultures and induced apoptosis of granule neurons (Figures 3A and 3B). However, incubation of granule neurons with the Cdc2 inhibitor roscovitine inhibited KCl withdrawal induction of Caspase 3 cleavage and activation and granule neuron apoptosis (Figures 3A and 3B). Together, these results suggest that Cdc2 mediates KCl withdrawal-induced granule neuron apoptosis.

Since roscovitine can also inhibit the Cdc2-related kinases Cdk2 and Cdk5, we determined in transfection experiments if a dominant interfering form of Cdc2 that specifically inhibits Cdc2, Cdc2-DN (van den Heuvel and Harlow, 1993), suppresses KCl withdrawal-induced neuronal apoptosis. We found that Cdc2-DN, when expressed in granule neurons, blocked KCl withdrawal-induced neuronal apoptosis (Figure 3C). KCl deprivation induced an ~70% reduction in the survival of vector-transfected neurons at 3 days following transfection (Figure 3C). However, KCl deprivation induced only a 10% reduction in the survival of Cdc2-DN-expressing neurons at 3 days after transfection (Figure 3C). By contrast to Cdc2-DN, a dominant interfering form of Cdk2, Cdk2-DN (van den Heuvel and Harlow, 1993), or a dominant interfering form of Cdk5, Cdk5-DN (Tsai et al., 1994), when expressed in granule neurons (Figure 3D), failed to protect neurons against KCl withdrawal-induced apoptosis (Figure 3D). Taken together, our results indicate that among Cdc2-related kinases, Cdc2 selectively mediates activity withdrawal-induced neuronal apoptosis.

We also examined the role of Cdc2 in neuronal apoptosis by reducing the expression of Cdc2 in cerebellar granule neurons. An antisense Cdc2 oligonucleotide, but not a corresponding control oligonucleotide, effectively reduced the expression of Cdc2 in granule neurons and blocked activity deprivation-induced apoptosis of granule neurons (Figure 3E). The antisense Cdc2 oligonucleotide reduced activity deprivation-induced apoptosis of granule neurons by 67%, whereas the corresponding control oligonucleotide reduced apoptosis by only 16% (Figure 3E). Therefore, on the basis of results from three distinct methods of Cdc2 blockage, our results suggest that endogenous Cdc2 plays a critical role in mediating apoptosis of newly generated neurons upon activity deprivation.

Cdc2 Promotes Neuronal Apoptosis via the Phosphorylation and Activation of BAD

Since Cdc2 is a protein kinase, we considered the possibility that Cdc2 might promote neuronal apoptosis upon activity deprivation by directly phosphorylating and thereby activating a component of the cell death machinery. The BH3-only protein BAD promotes apoptosis by inhibiting the prosurvival Bcl-2 proteins (Gross et al., 1999). Growth factors and neurotrophins promote cell survival in part by triggering the phosphorylation of BAD at serines 136 and 112, thereby inducing the sequestration of BAD with members of the 14-3-3 family of proteins (Blume-Jensen et al., 1998; Bonni et al., 1999; Datta et al., 1997; del Peso et al., 1997; Zha et al., 1996). We reasoned that in addition to linking survival signals to the cell death machinery, BAD might be directly regulated by protein kinases that propagate apoptotic signals.

Recombinant Cyclin B/Cdc2 induced the robust phosphorylation of recombinant BAD or GST-BAD fusion protein in in vitro kinase assays (Figure 4A). There are three potential sites of Cdc2-induced phosphorylation in mouse BAD, threonine 3, serine 128, and threonine 208. The Cdc2-induced phosphorylation of GST-BAD was reduced dramatically when BAD serine 128 was mutated to alanine (GST-BADS128A), and it was abolished when all three sites were mutated to alanine (GST-BAD2T/

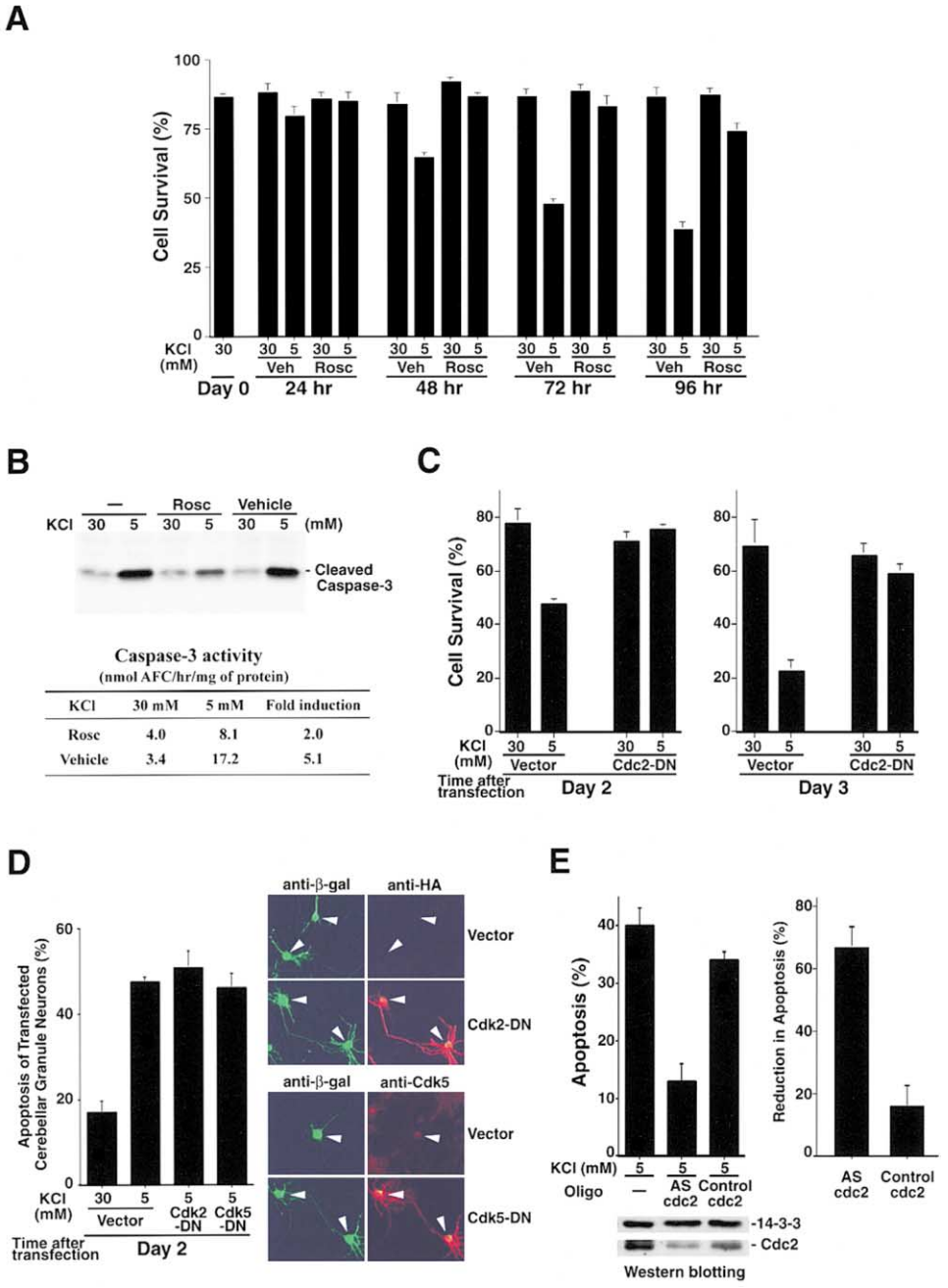

Figure 3. Cdc2 Mediates Apoptosis of Cerebellar Granule Neurons upon the Suppression of Neuronal Activity

(A) Survival of granule neurons (P6+2DIV) that were in full medium or deprived of activity in the presence of roscovitine (10 μM) or its vehicle (DMSO) for the indicated times. KCl deprivation significantly reduced neuronal survival in control cultures beyond 24 hr (mean ± SEM, n = 3; $p < 0.0001$, ANOVA), but not in roscovitine-treated cultures (n = 3).

(B) Lysates of granule neurons that were maintained in full medium or deprived of activity for 2 days were subjected to immunoblotting with an antibody that specifically recognizes the cleaved form of Caspase 3 or were subjected to a fluorometric Caspase 3 assay (Clonetech). Caspase measurements represent the average of two independent experiments.

(C) Cerebellar granule neurons (P6+2DIV) were transfected with a dominant interfering form of Cdc2 (Cdc2-DN) or its control expression vector together with an expression plasmid encoding β-galactosidase. Cultures were returned to full medium overnight, were switched to full medium or deprived of KCl, and 1 or 2 days later subjected to indirect immunofluorescence with a monoclonal antibody to β-galactosidase. Activity deprivation reduced significantly the survival of neurons transfected with the control plasmid (n = 3; ANOVA, $p < 0.001$) but not neurons transfected with the Cdc2-DN plasmid.

(D) Granule neurons were transfected with a dominant interfering form of Cdk2 (Cdk2-DN), a dominant interfering form of Cdk5 (Cdk5-DN), or their control vector. Transfected cultures were analyzed as in (C). Cdk2-DN and Cdk5-DN failed to protect neurons against apoptosis in activity-deprived cultures. Expression of Cdk2-DN in transfected granule neurons was confirmed by indirect immunofluorescence with an antibody to HA, as the Cdk2-DN is HA-tagged. Expression of Cdk5-DN was confirmed with an antibody to Cdk5.

(E) Granule neurons (P6+2DIV) were maintained in full medium or deprived of KCl in the absence or presence of an antisense Cdc2 oligonucleotide or a corresponding scrambled control oligonucleotide. The antisense Cdc2 oligonucleotide, but not the control oligonucleotide, significantly reduced granule neuron apoptosis upon KCl withdrawal (ANOVA, $p < 0.01$; n = 3). Immunoblotting with the mouse monoclonal antibody to Cdc2 confirmed that the antisense Cdc2 oligonucleotide specifically reduced Cdc2 expression in granule neurons.

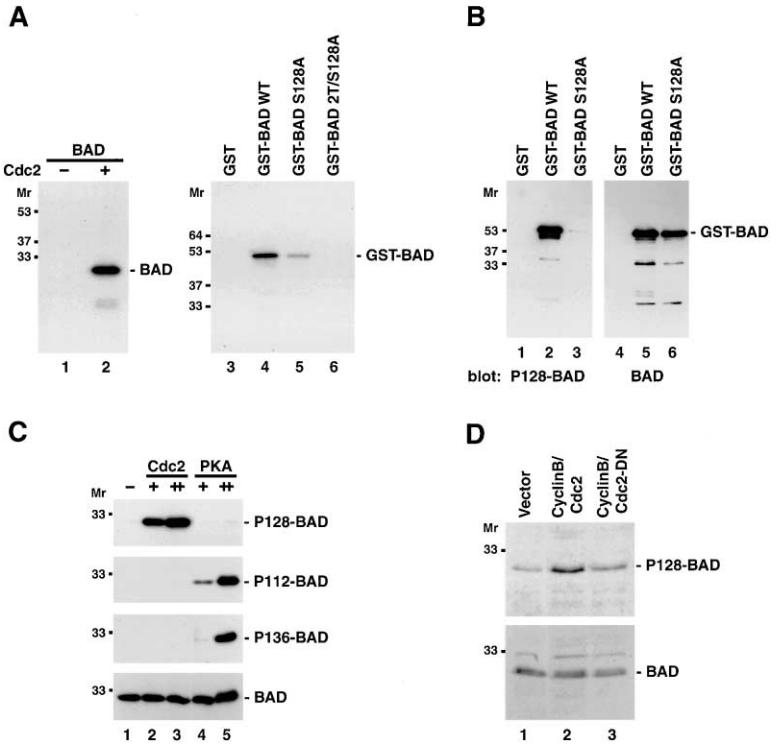

Figure 4. Cdc2 Catalyzes the Phosphorylation of BAD at Serine 128 In Vitro and In Vivo

(A) Recombinant BAD was subjected to an in vitro kinase assay in the absence (lane 1) or presence of recombinant Cyclin B/Cdc2 (lane 2). Recombinant GST, GST-BAD, or GST-BAD mutants, in which potential Cdc2 phosphorylation sites were replaced with alanine, were subjected to an in vitro kinase assay with Cyclin B/Cdc2 (lanes 3–6). Phosphorylated BAD or GST-BAD was separated by PAGE and analyzed by autoradiography.

(B) GST, GST-BAD, or GST-BADS128A was subjected to an in vitro kinase assay with Cyclin B/Cdc2 and immunoblotted with the phospho128-BAD antibody (lanes 1–3) or with a rabbit antibody that recognizes BAD regardless of its phosphorylation state (C-20; lanes 4–6).

(C) Recombinant BAD was subjected to an in vitro kinase assay with Cyclin B/Cdc2 or PKA, and immunoblotted with the phospho128-BAD antibody, a phospho112-BAD antibody, a phospho136-BAD antibody, or the C-20 BAD antibody.

(D) Lysates of COS cells that were transfected with BAD together with Cyclin B and Cdc2 or Cdc2-DN or their control expression vectors were immunoblotted with the phospho128-BAD antibody or the C-20 BAD antibody.

S128A) (Figure 4A). We focused on the Cdc2-induced phosphorylation of serine 128, because serine 128 is conserved across several species and the amino acid sequence encompassing serine 128 conforms most to the sequence preferred by Cdc2 as a substrate (Holmes and Solomon, 1996).

We raised a rabbit antiserum that specifically recognizes BAD when BAD is phosphorylated at serine 128. The phospho128-BAD antibody recognized BAD or GST-BAD that was phosphorylated by Cyclin B/Cdc2, but did not recognize unphosphorylated BAD, unphosphorylated GST-BAD, or GST-BADS128A that was incubated with Cyclin B/Cdc2 (Figures 4B and 4C). In addition, the phospho128-BAD antibody did not recognize BAD that was phosphorylated in vitro at serine 112 or 136 by PKA (Figure 4C), and Cdc2 did not induce the phosphorylation of BAD at serine 112 or serine 136 (Figure 4C). These results establish the specificity of the phospho128-BAD antibody and that Cdc2 catalyzes the phosphorylation of BAD at the distinct site of serine 128 in vitro. In other experiments, we found that Cyclin B and Cdc2 but not Cdc2-DN, when expressed in COS cells together with BAD, induced the phosphorylation of BAD at serine 128 (Figure 4D), demonstrating the ability of Cdc2 to induce the phosphorylation of BAD at serine 128 in cells.

We next investigated if Cdc2 triggers the phosphorylation of BAD that is expressed at normal levels in primary neurons. Consistent with the hypothesis that BAD might be a physiological substrate of Cdc2, endogenous Cdc2 and endogenous BAD in cerebellar granule neurons were found to interact physically (Figure 5A). Immunoblotting experiments revealed that KCl withdrawal induced the robust phosphorylation of BAD at serine 128 in granule neurons (Figure 5B). The KCl withdrawal-induced BAD serine 128 phosphorylation was blocked by roscovitine (Figure 5B), suggesting that Cdc2 mediates KCl withdrawal-induced phosphorylation of BAD serine 128. We also found that blockage by nimodipine of L-type VSCCs in membrane-depolarized granule neurons (Catterall, 2000) induced the expression of Cdc2 and the phosphorylation of BAD at serine 128 (Figure 5C), suggesting that the inhibition of calcium entry in granule neurons activates the Cdc2-BAD pathway.

A specific phospho128-BAD signal was also detected immunocytochemically in granule neurons that were deprived of membrane depolarization (Figure 5D), thus allowing, in transfection experiments, the direct assessment of Cdc2's role in the phosphorylation of BAD serine 128 in granule neurons. KCl withdrawal induced serine 128 phosphorylation of endogenous BAD in a large fraction of vector-transfected granule neurons (Figures 5E and 5F). By contrast, the dominant interfering form of Cdc2, Cdc2-DN, when expressed in granule neurons, blocked KCl withdrawal-induced serine 128 phosphorylation of endogenous BAD (Figures 5E and 5F). Taken together, our results suggest that endogenous Cdc2 mediates the phosphorylation of endogenous BAD at serine 128 in cerebellar granule neurons upon activity deprivation.

To determine the functional effect of the Cdc2-induced phosphorylation of BAD at serine 128 in neurons, we first used an assay established to measure the apoptotic effect of BAD that is exogenously expressed in granule neurons (Bonni et al., 1999; Datta et al., 1997). We transfected newly generated granule neurons with an expression plasmid encoding BAD alone or together with expression plasmids encoding Cyclin B and Cdc2. One day after transfection, cultures were deprived of survival medium or were treated with a high concentration of

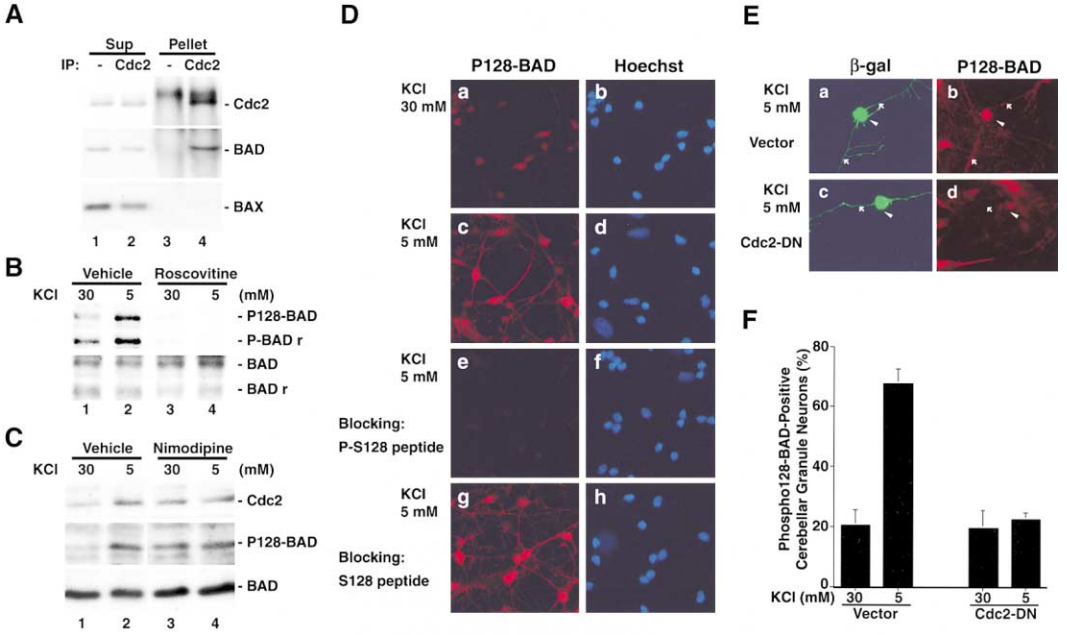

Figure 5. Cdc2 Mediates the Phosphorylation of Endogenous BAD at Serine 128 in Neurons

(A) Lysates of P6+2DIV granule neurons were immunoprecipitated with the monoclonal antibody to Cdc2 or no antibody, and then were immunoblotted with the rabbit antibody to Cdc2, a rabbit antibody to BAD that recognizes BAD regardless of its phosphorylation state (N-20), or with a rabbit antibody to Bax. Cdc2 interacts with BAD but not with Bax in granule neurons.

(B) Lysates of granule neurons (P6+2DIV) that had been maintained in full medium or deprived of KCl and incubated with roscovitine (10 μM) or its vehicle (DMSO) for 24 hr were immunoblotted with the phospho128-BAD antibody or the C-20 BAD antibody. The BAD antibodies recognized full-length and BAD-related proteins of lower molecular weight (BADr) that may represent cleavage products of BAD.

(C) Lysates of granule neurons that were in full medium or deprived of KCl and incubated with nimodipine (20 μM) or its vehicle (DMSO) were subjected to immunoblotting with the mouse monoclonal antibody to Cdc2, the phospho128-BAD antibody, or the N-20 BAD.

(D) Granule neurons that were in full medium (a and b) or deprived of KCl for 24 hr (c–h) were subjected to indirect immunofluorescence with the phospho128-BAD antibody (left panels) and Hoechst (right panels). The KCl withdrawal-induced phospho128-BAD signal (c) was competed by phosphorylated BAD serine 128 peptide (e) but not by unphosphorylated BAD serine 128 peptide (g).

(E) Granule neurons (P6+2DIV) were transfected with Cdc2-DN or its control expression plasmid together with β-galactosidase. After overnight incubation with full medium, cultures were switched to full medium or deprived of KCl, and 24 hr later, they were subjected to indirect immunofluorescence with the phospho128-BAD antibody and the mouse monoclonal antibody to β-galactosidase. Shown from KCl-deprived cultures are examples of a phospho128-BAD-positive granule neuron transfected with control vector (a and b) and a phospho128-BAD-negative neuron transfected with Cdc2-DN (c and d).

(F) Quantitation of the experiment described in (E). KCl withdrawal induced a large increase in the percentage of transfected neurons that were phospho128-BAD positive in vector-transfected ($n = 3$; $p < 0.0001$, ANOVA) but not in Cdc2-DN-transfected neurons. For each condition, at least 200 transfected neurons were counted.

insulin, which activates the IGF1 receptor, and 8 hr later, cultures were analyzed for the presence of apoptosis in transfected neurons. The measurement of apoptosis 8 hr following survival factor deprivation allowed the assessment of the apoptotic effect of exogenous BAD in neurons, because activation of the endogenous Cdc2-BAD apoptotic pathway begins peaking 1–2 days following activity deprivation (Figures 3A and 5, and data not shown). The expression of BAD induced apoptosis of transfected newly generated granule neurons that were deprived of survival factors, and activation of the IGF1 receptor inhibited BAD-mediated apoptosis (Figure 6A). Cyclin B/Cdc2, when coexpressed with BAD, did not augment BAD-mediated apoptosis in cultures that were deprived of survival factors. However, we found that Cyclin B/Cdc2 blocked the ability of activated IGF1 receptor to inhibit the apoptotic effect of BAD (Figure 6A).

We next determined the effect of Cdc2 on growth factor inhibition of the apoptotic effect of a BAD mutant in which serine 128 was replaced with an alanine (BADS128A). While Cyclin B/Cdc2 antagonized IGF1 receptor inhibition of wild-type BAD, Cyclin B/Cdc2 failed to block the ability of the IGF1 receptor to inhibit the apoptotic effect of BADS128A (Figure 6A). Taken together, these results suggest that the Cdc2-induced phosphorylation of BAD at serine 128 promotes the apoptotic effect of BAD in cerebellar granule neurons by opposing growth factor inhibition of BAD.

To determine if the Cdc2-induced serine 128 phosphorylation of BAD that is expressed endogenously in granule neurons promotes apoptosis, we tested if a mutant allele of BAD serine 128, when expressed in granule neurons, can act in a dominant-negative manner to inhibit activity deprivation-induced neuronal apoptosis. We reasoned that full-length BAD mutants, when overexpressed at levels that are required to dominantly inhibit the activity of endogenous BAD, might be toxic because of the potential of full-length BAD to titrate

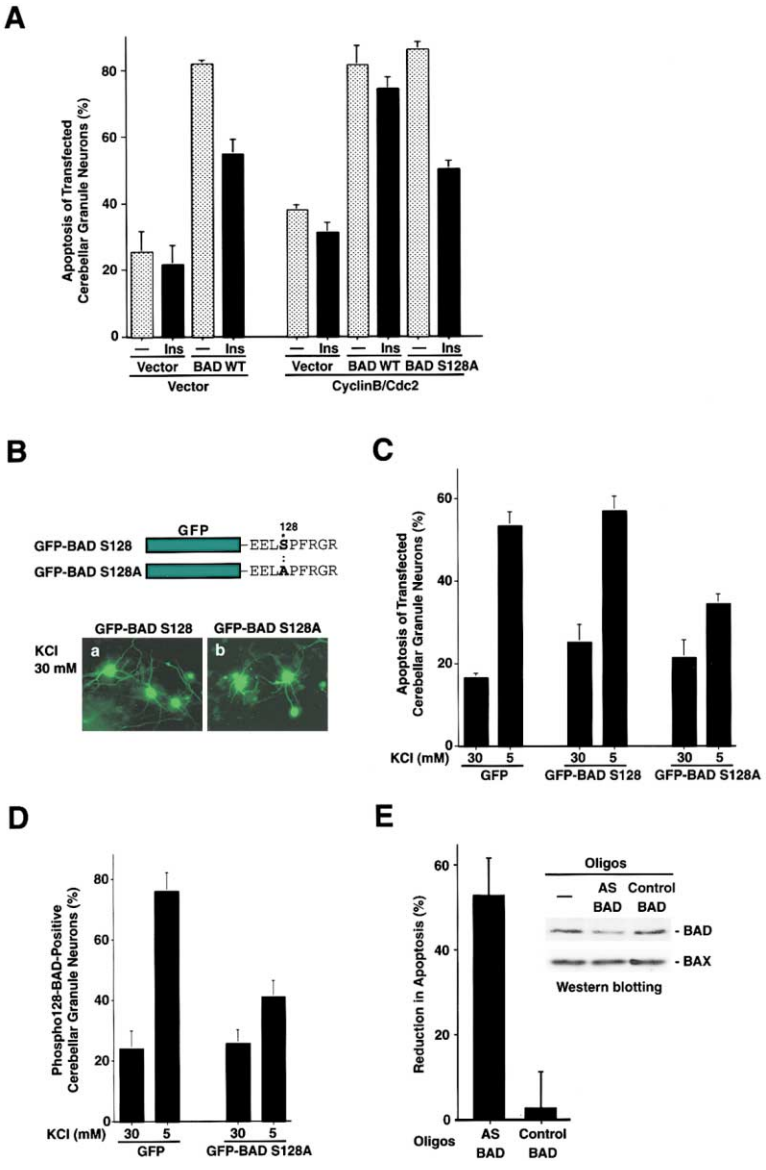

Figure 6. Cdc2-Induced Phosphorylation of BAD Serine 128 Promotes BAD-Mediated Apoptosis by Antagonizing Growth Factor Inhibition of BAD

(A) Granule neurons (P6+2DIV) were transfected with Cyclin B and Cdc2 or their control vector together with wild-type BAD or BADS128A and β-galactosidase. One day after transfection, cultures were switched from full medium to basal medium in the absence or presence of insulin (10 μg/ml) to activate the IGF1 receptor. After 8 hr, cultures were subjected to indirect immunofluorescence. Percent apoptosis is represented as mean ± SEM ($n = 4$). Activation of the IGF1 receptor reduced BAD-mediated apoptosis in neurons transfected with the control (ANOVA, $p < 0.001$) but not in neurons transfected with Cyclin B and Cdc2 plasmids. Cyclin B/Cdc2 failed to reduce IGF1 receptor suppression of the apoptotic effect of BADS128A (ANOVA, $p < 0.001$).

(B) Schematic of dominant-negative BAD serine 128A and its control. Green fluorescent protein is fused at its C terminus to a 9 amino acid BAD peptide encompassing serine 128, in which serine 128 is wild-type (GFP-BADS128) or in which serine 128 is replaced by an alanine (GFP-BADS128A). GFP-BADS128 and GFP-BADS128A were expressed at equivalent levels in transfected neurons.

(C) Granule neurons were transfected with the GFP expression plasmid, GFP-BADS128, or GFP-BADS128A together with an expression plasmid encoding β-galactosidase. Transfected cultures were processed as in Figure 3C. KCl withdrawal induced a significant increase in apoptosis of granule neurons transfected with the control GFP or GFP-BADS128 ($n = 3$; $p < 0.0001$, ANOVA) but not in granule neurons transfected with GFP-BADS128A.

(D) Granule neurons were transfected with GFP-BADS128A or its control GFP expression plasmid together with β-galactosidase. Transfected cultures were then processed as in Figures 5E and 5F. KCl withdrawal induced a large increase in the percentage of transfected neurons that were phospho128-BAD positive in GFP-transfected neurons ($n = 3$; $p < 0.0001$, ANOVA) but not in GFP-BADS128A-transfected neurons. For each condition, at least 200 transfected neurons were counted.

(E) Granule neurons were maintained in full medium or deprived of neuronal activity in the absence or presence of an antisense BAD oligonucleotide or a corresponding scrambled control oligonucleotide. The antisense BAD oligonucleotide, but not the control oligonucleotide, significantly reduced granule neuron apoptosis upon KCl withdrawal (ANOVA, $p < 0.01$; $n = 3$). Immunoblotting with the N-20 BAD antibody revealed that the antisense BAD oligonucleotide specifically reduced the expression of BAD.

prosurvival proteins via its BH3 domain and BAD serines 112 and 136. Therefore, in designing a dominant-negative BAD serine 128 mutant allele, we constructed a gene encoding green fluorescent protein fused at its C terminus to a 9 amino acid BAD peptide encompassing serine 128 (GFP-BADS128) or an identical peptide in which serine 128 was replaced with an alanine (GFP-BADS128A) (Figure 6B).

In transfection experiments, GFP-BADS128 and GFP-BADS128A were expressed at equivalent levels in granule neurons, as determined by visualization of GFP (Figure 6B). GFP-BADS128A, but not GFP-BADS128, when expressed in granule neurons, reduced significantly activity deprivation-induced apoptosis (Figure 6C). In other experiments, GFP-BADS128A was found to significantly reduce the KCl withdrawal-induced phosphorylation of endogenous BAD (Figure 6D). Together, these results suggest that GFP-BADS128A acts in a dominant-negative manner to inhibit the endogenous Cdc2-BAD apoptotic pathway in neurons upon the suppression of neuronal activity. However, in addition to inhibiting endogenous BAD, GFP-BADS128A might prevent neuronal apoptosis by inhibiting Cdc2 activation of other substrates that remain to be identified.

The dominant-negative GFP-BADS128A findings were supported by additional experiments in which we found that an antisense oligonucleotide to BAD, but not a corresponding control oligonucleotide, reduced both the level of BAD expression in granule neurons and KCl withdrawal-induced granule neuron apoptosis (Figure

6E). Taken together, our findings suggest that the Cdc2-induced phosphorylation of endogenous BAD at serine 128 is required for the ability of the suppression of neuronal activity to promote the apoptotic effect of BAD.

The Serine 128 Phosphorylation of BAD Inhibits the Interaction of Serine 136-Phosphorylated BAD with 14-3-3 Proteins

We next investigated the mechanism by which the BAD serine 128 phosphorylation promotes apoptosis. BAD lies at the junction of prosurvival protein kinase cascades and the cell death machinery. The protein kinases Akt, p21-activated kinase 1 (PAK1), and pp70S6 kinase mediate survival factor-induced phosphorylation of BAD serine 136 (Blume-Jensen et al., 1998; Datta et al., 1997; del Peso et al., 1997; Harada et al., 2001; Schurmann et al., 2000). On the other hand, the protein kinases Rsk, PKA, and PAK1 mediate survival factor-induced phosphorylation of BAD serine 112 (Bonni et al., 1999; Harada et al., 1999; Schurmann et al., 2000; Shimamura et al., 2000; Tan et al., 1999). The phosphorylation of BAD at serines 136 and 112 culminates in the sequestration of phosphorylated BAD by members of the 14-3-3 family of proteins (Zha et al., 1996).

We focused on how the Cdc2-induced serine 128 phosphorylation of BAD antagonizes growth factor inhibition of BAD. Growth factor inhibition of BAD in granule neurons appears to be mediated by the Akt-induced phosphorylation of BAD at serine 136 (Datta et al., 1997). In in vitro kinase assays, the prior Cdc2-induced phosphorylation of BAD at serine 128 did not reduce the Akt-induced phosphorylation of BAD at serine 136 (Figure 7A), suggesting that the phosphorylation of BAD at serine 128 does not interfere with the ability of Akt to access BAD serine 136 as a substrate.

We next determined if the Cdc2-induced phosphorylation of BAD at serine 128 inhibits the sequestration of serine 136-phosphorylated BAD by 14-3-3 proteins. In a GST pull-down assay, recombinant BAD, when unphosphorylated or when phosphorylated at serine 128 by Cdc2, did not coprecipitate with a GST-14-3-3ζ fusion protein (Figure 7B). However, BAD, when phosphorylated at serine 136 by Akt, coprecipitated with GST-14-3-3ζ (Figure 7B). By contrast, BAD, when phosphorylated at both serines 128 and 136 by Cdc2 and Akt, respectively, coprecipitated less effectively with GST-14-3-3ζ (Figure 7B). In other experiments, His-tagged 14-3-3ζ, when captured on Ni^{+2}-NTA agarose beads, coprecipitated effectively both GST-BAD and GST-BADS128A that were phosphorylated by Akt (Figure 7B). However, 14-3-3ζ coprecipitated less effectively GST-BAD that was phosphorylated with both Cdc2 and Akt (Figure 7B). By contrast, Cdc2 had little effect on the ability of Akt-phosphorylated GST-BADS128A to coprecipitate with 14-3-3ζ (Figure 7B). Together, these in vitro results suggest that the Cdc2-induced phosphorylation of BAD at serine 128 interferes with the interaction of 14-3-3 proteins with serine 136-phosphorylated BAD.

To facilitate our investigations of the effect of the BAD serine 128 phosphorylation on the interaction of serine 136-phosphorylated BAD with 14-3-3 proteins in vivo, we replaced BAD serine 128 with aspartate (BADS128D) to mimic serine 128-phosphorylated BAD. In granule neurons, while IGF1 receptor activation inhibited the apoptotic effect of BAD or BADS128A, IGF1 receptor activation failed to suppress the apoptotic effect of BADS128D (Figure 7C), indicating that BADS128D mimics the function of the Cdc2-induced phosphorylated BAD in vivo. Consistent with the idea that BAD acts downstream of Cdc2, the dominant interfering form of Cdc2 (Cdc2-DN), when expressed in granule neurons together with BADS128D, failed to protect neurons from BADS128D-induced apoptosis (data not shown). Replacement of BAD serine 128 by aspartate had little effect on the phosphorylation of BAD at serine 136 or serine 112 in vitro (Figure 7D), suggesting that BADS128D opposes growth factor inhibition of BAD at a step that follows the phosphorylation of BAD at serine 136. In other control experiments, wild-type BAD, BADS128D, and BADS128A were found to be expressed at equivalent levels in transfected neurons or COS cells (Figure 7D). We next compared in transfected COS cells the ability of BAD, BADS128A, or BADS128D to interact with 14-3-3ζ that was tagged with the FLAG epitope (FLAG-14-3-3ζ). We found that wild-type BAD or BADS128A coimmunoprecipitated with FLAG-14-3-3ζ (Figure 7E). However, BADS128D failed to effectively coimmunoprecipitate with FLAG-14-3-3ζ (Figure 7E), suggesting that the phosphorylation of BAD serine 128 inhibits the interaction of BAD with 14-3-3ζ. In other experiments, BAD but not BADS128A, when coexpressed with Cyclin B and Cdc2 in COS cells, interacted less effectively with 14-3-3 proteins (Figure 7F). Taken together, these results suggest that the Cdc2-induced phosphorylation of BAD at serine 128 inhibits the ability of 14-3-3 proteins to interact with serine 136-phosphorylated BAD in vivo.

We next determined if the Cdc2-induced phosphorylation of BAD that is endogenously expressed in granule neurons triggers the dissociation of BAD from endogenous 14-3-3 proteins. The association of endogenous BAD and endogenous 14-3-3 proteins was observed by coimmunoprecipitation in granule neurons that were maintained in full medium (Figure 7G). However, the amount of 14-3-3 that coimmunoprecipitated with BAD was reduced in lysates of granule neurons that were deprived of neuronal activity (Figure 7G). Importantly, roscovitine prevented the reduction in the amount of BAD-14-3-3 protein complex in granule neurons upon KCl withdrawal (Figure 7G), suggesting that activity deprivation induces the Cdc2-mediated dissociation of the BAD-14-3-3 protein complex.

We next determined if activity deprivation might induce the dephosphorylation of BAD at serine 136 or serine 112, an event that might be expected to further contribute to the dissociation of the BAD-14-3-3 protein complex. Interestingly, KCl withdrawal had little effect on the phosphorylation of endogenous BAD at serine 136 and serine 112 (Figure 7H), reflecting the continued presence of growth factors in the medium. In addition, the inhibition of BAD serine 128 phosphorylation by roscovitine had little effect on the BAD serine 136 or serine 112 phosphorylation (Figure 7H). Together, these results suggest that the Cdc2-induced phosphorylation of BAD at serine 128 mediates the activity deprivation-induced dissociation of the endogenous BAD-14-3-3 protein complex in granule neurons.

The activity deprivation-induced dissociation of BAD from 14-3-3 proteins raised the possibility that a larger amount of BAD might be available to interact with the

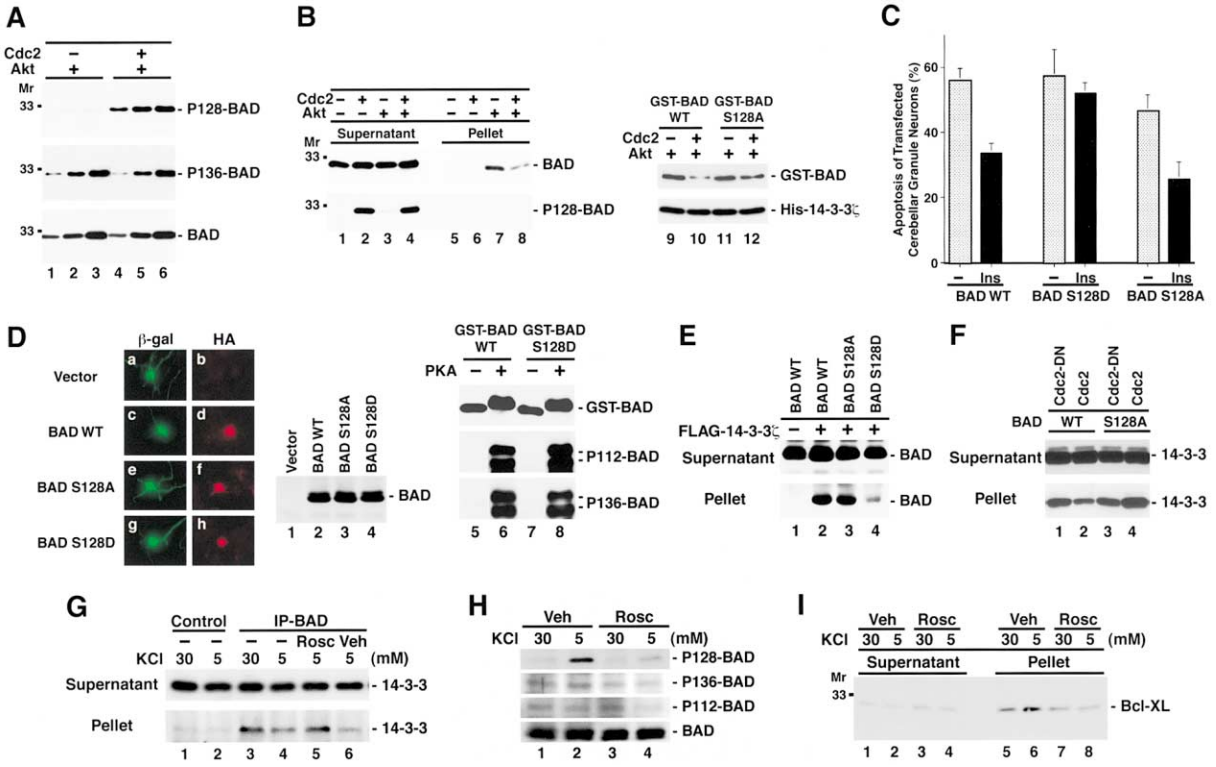

Figure 7. The Cdc2-Induced Phosphorylation of BAD Serine 128 Inhibits the Interaction of Growth Factor-Induced Serine 136-Phosphorylated BAD with 14-3-3 Proteins

(A) Prior phosphorylation of BAD at serine 128 does not reduce Akt-induced phosphorylation of BAD at serine 136. Increasing amounts of BAD (0.05 μg, lanes 1 and 4; 0.1 μg, lanes 2 and 5; 0.25 μg, lanes 3 and 6) were subjected to an in vitro kinase assay with Cyclin B/Cdc2 followed by Akt. In vitro kinase reaction products were immunoblotted with a phospho128-BAD antibody, a phospho136-BAD antibody, or the C-20 BAD antibody.

(B) BAD was subjected to an in vitro kinase reaction with Cdc2, Akt, or both kinases, and then was incubated with GST-14-3-3. Supernatant and precipitated fractions were immunoblotted with the C-20 BAD or phospho128-BAD antibody. In the experiments shown in the right panel, GST-BAD or GST-BADS128A was incubated with or without Cdc2 and then phosphorylated by Akt and incubated with His-14-3-3ζ and Ni-NTA agarose beads. Precipitated proteins were immunoblotted with the BAD-C20 antibody or a mouse monoclonal antibody to 14-3-3.

(C) Granule neurons were transfected with wild-type BAD, BADS128D, or BADS128A together with β-galactosidase. Cultures were then processed as in Figure 6A. Activation of the IGF1 receptor reduced the apoptotic effect of BAD and BADS128A (ANOVA, $p < 0.01$) but not of BADS128D.

(D) Left panel: granule neurons were transfected with HA-tagged BAD, HA-BADS128A, HA-BADS128D, or their control expression plasmid (0.5 μg/well) together with β-galactosidase and Bcl-2 to protect neurons against apoptosis. Transfected neurons were subjected to indirect immunofluorescence with the antibody to β-galactosidase and a rabbit antibody to HA. Middle panel: lysates of COS cells that were transfected with BAD, BADS128A, BADS128D, or their control vector were immunoblotted with the C-20 BAD antibody. Right panel: GST-BAD or GST-BADS128D was subjected to an in vitro kinase assay with PKA and immunoblotted with the C-20 BAD, phospho112-BAD, or phospho136-BAD antibody.

(E) COS cells were transfected with BAD alone or together with 14-3-3ζ tagged with the FLAG epitope, or BADS128A, or BADS128D together with FLAG-14-3-3ζ. Lysates of transfected cells were immunoprecipitated with an antibody to FLAG followed by immunoblotting with the N-20 BAD antibody.

(F) COS cells were transfected with BAD or BADS128A together with FLAG-14-3-3ζ and cyclin B and Cdc2 or the kinase-inactive Cdc2-DN. Lysates of transfected cells were immunoprecipitated with the C-20 BAD antibody and then immunoblotted with the 14-3-3 antibody.

(G) Lysates of granule neurons were immunoprecipitated with no antibody or with the C-20 BAD antibody followed by immunoblotting with the 14-3-3 antibody.

(H) Lysates of granule neurons were immunoblotted with the phospho128-BAD, phospho136-BAD, phospho112-BAD, or the N-20 BAD antibody.

(I) Lysates of granule neurons were immunoprecipitated with the C-20 BAD antibody followed by immunoblotting with an antibody to Bcl-xl.

prosurvival Bcl-2 proteins in granule neurons upon activity deprivation. In coimmunoprecipitation experiments, a pool of endogenous BAD was found in a protein complex with endogenous Bcl-xl in granule neurons that were maintained in full medium (Figure 7I). In contrast to the KCl withdrawal-induced dissociation of the BAD-14-3-3 protein complex in neurons, KCl withdrawal induced a modest roscovitine-sensitive increase in the amount of Bcl-xl coprecipitating with BAD in granule neurons (Figure 7I). Taken together, our findings suggest that Cdc2 mediates the activity deprivation-induced dissociation of the BAD-14-3-3 protein complex and the concomitant increased association of the BAD-Bcl-xl protein complex.

Discussion

In this study, we have identified a direct link between a component of the cell cycle and the cell death machinery in postmitotic neurons. Our results suggest that the criti-

cal component of the cell cycle machinery Cdc2 is coopted by newly generated postmitotic neurons to activate cell death upon the suppression of neuronal activity. We have found that Cdc2 catalyzes the phosphorylation of the BH3-only protein BAD at the distinct site of serine 128, thereby activating BAD-mediated apoptosis by inhibiting the sequestration of growth factor-induced serine 136-phosphorylated BAD by members of the 14-3-3 family of proteins. Thus, our results suggest that Cdc2 promotes neuronal apoptosis by antagonizing growth factor-induced signals that suppress the cell death machinery.

The established role of Cdc2 is to drive proliferating cells through the G2/M transition of the cell cycle. Surprisingly, we found that Cdc2 is expressed in newly generated postmitotic neurons and that Cdc2 plays a critical role in triggering neuronal apoptosis. These findings are consistent with the hypothesis that reactivation of components of the cell cycle in postmitotic cells induces cell death (Liu and Greene, 2001). In proliferating cells, the regulation of Cdc2 expression and activity has been well characterized (Coleman and Dunphy, 1994; Lees, 1995). How Cdc2 expression and activity are induced in postmitotic neurons by apoptosis-inducing stimuli and whether such regulation recapitulates aspects of Cdc2's regulation in proliferating cells will be the subject of future studies.

The identification of the BH3-only protein BAD as a physiological substrate of Cdc2 provides a direct link between a component of the cell cycle and the cell death machinery. BAD appears to be the target of several protein kinases that suppress BAD's apoptotic function (reviewed in Gross et al., 1999) (Bonni et al., 1999; Tan et al., 1999; Shimamura et al., 2000; Schurmann et al., 2000; Lizcano et al., 2000). On the other hand, death-inducing stimuli have been suggested to promote BAD-mediated apoptosis by inducing the dephosphorylation of BAD at these sites (Ayllon et al., 2000; Wang et al., 1999). Our finding that Cdc2 mediates neuronal apoptosis by inducing the phosphorylation of BAD at serine 128 suggests that the apoptotic function of BAD can be activated, rather than just suppressed, in a phosphorylation-dependent manner. In future studies, it will be important to determine if Cdc2 directly activates other BH3-only proteins or other components of the cell death machinery in a phosphorylation-dependent manner. It will be also important to determine if BAD serine 128 is the target of other protein kinases that propagate apoptotic signals.

That the Cdc2-induced phosphorylation of BAD at serine 128 regulates the interaction of growth factor-induced serine 136-phosphorylated BAD with 14-3-3 proteins illuminates a specific mechanism that underlies the dynamic interplay of proapoptotic and prosurvival signaling pathways in cells. Structural data suggest that 14-3-3 protein dimers engage in a bidentate interaction with phosphorylated serines 136 and 112 of BAD (Yaffe et al., 1997). The location of serine 128 in the vicinity of serine 136 together with our in vitro data suggest that the phosphorylation of serine 128 interferes sterically with the interaction of 14-3-3 proteins with serine 136-phosphorylated BAD. However, the possibility remains that within cells, serine 128-phosphorylated BAD interacts with another protein that then disrupts the interaction of 14-3-3 proteins with their phosphoserine 136 ligand. Beyond BAD, our observations point to a mechanism by which the phosphorylation of proteins in proximity to the phosphorylated serine ligands of 14-3-3 proteins may negatively modulate the interaction of 14-3-3 proteins with their binding proteins.

In newly generated neurons, we have found that neuronal activity inhibits the Cdc2-induced phosphorylation of BAD at serine 128, while growth factors and neurotrophins induce the phosphorylation of BAD at the neighboring serines 136 and 112 (Bonni et al., 1999; Datta et al., 1997). Together, these modifications promote BAD's interaction with 14-3-3 proteins. These findings illustrate how neuronal activity, growth factors, and neurotrophins cooperate to effectively suppress a component of the cell death machinery. In the future, it will be important to characterize signaling mechanisms by which neuronal activity suppresses the Cdc2-BAD apoptotic pathway.

Recent studies suggest that in addition to the role of the cell cycle in developmentally regulated neuronal apoptosis, components of the cell cycle machinery are activated in degenerating neurons in the adult brain (Vincent et al., 1997). It will be interesting to determine if the Cdc2-BAD apoptotic pathway contributes to neuronal cell death in neurodegenerative diseases of the brain.

Experimental Procedures

Antibodies

Antibodies to Cdc2 (mouse monoclonal and rabbit polyclonal), Cyclin B1, Cdk2, Cdk5 (rabbit polyclonal), BAD (C-20 and N-20), Bax, and 14-3-3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to Bcl-xl (UBI, Lake Placid, NY), the mouse monoclonal antibody to β-galactosidase (Promega, Madison, WI), FLAG (Sigma, St. Louis, MO), and cleaved Caspase 3 (Cell Signaling, Beverly, MA) were also purchased. The rabbit antibody to phosphorylated serine 128 BAD was generated by injecting New Zealand rabbits with the phosphopeptide antibody C-EGMEEELpSPFRGRS as described (Bonni et al., 1999).

Transfections

Cerebellar granule neurons were prepared and transfected as described (Bonni et al., 1999), with the following modifications: 35 μl of the DNA-calcium phosphate precipitate was added per well in a 24-well plate containing 7×10^5 cerebellar granule cells and left on the cells for 15 min. In the experiments shown in Figure 3C, cultures were transfected with 0.5 μg of test plasmid together with 0.25 μg of the β-galactosidase expression plasmid per well of a 24-well plate. In the experiments shown in Figure 6C, each well was transfected with 1.0 μg of GFP-BAD or GFP-BADS128A and 0.25 μg of the β-galactosidase expression plasmid. In the experiments shown in Figure 6A, each well was transfected with 0.75 μg of Cyclin B, 0.75 μg of Cdc2, 0.05 μg of BAD or BADS128A, and 0.25 μg of the β-galactosidase expression plasmid. Cell survival and death were assessed in β-galactosidase-expressing neurons based on the integrity of neurites and integrity of the nucleus as determined by the DNA dye bisbenzimide (Hoechst 33258). Indirect immunofluorescence was carried out as described (Bonni et al., 1999). Cell counts were carried out in a blinded manner by two observers. In our transfection experiments, Tuj-1-positive granule neurons accounted for 99.1% ± 0.3%, and GFAP-positive astrocytes accounted for only 0.7% ± 0.7% of transfected β-galactosidase-expressing cells (n = 3).

COS cells were transfected by a calcium phosphate method as described (Bonni et al., 1999).

Biochemical Assays

Immunoprecipitation and immunoblotting were carried out as described (Bonni et al., 1999). In vitro kinase assays were carried out

as described in the New England Biolabs protocol for the Cdc2 kinase product. GST pull-down assays were done as described (Zha et al., 1996).

Antisense Oligonucleotides

A phosphorothioate antisense Cdc2 oligonucleotide designed to hybridize to nucleotides −10 to +10 relative the translation initiation site of rat Cdc2 or a corresponding scrambled control oligonucleotide, or a phosphorothioate antisense BAD oligonucleotide designed to nucleotides −11 to +8 or a corresponding scrambled control oligonucleotide was synthesized (Invitrogen, Carlsbad, CA) and added to granule neurons cultures at 10 μM (Cdc2) or 5 μM (BAD). At P6+2DIV, cultures were deprived of neuronal activity (5 mM KCl plus serum). After 2 days, cultures were fixed and cell death was assessed.

Acknowledgments

We thank G. Gill and Y. Shi for critical reading of the manuscript, D. Piscopo and P. Hinds for providing the Cdc2 plasmids, M.-S. Lee and L.-H. Tsai for providing Cdk5 reagents, R. Vallee for the MAP2 antibody, and S. Vasquez for technical assistance. The TAG-1 antibody was obtained from the Developmental Studies Hybridoma Bank (DSHB) under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Science (Iowa City, IA). This work was supported by a Burroughs Wellcome Career Development Award (A.B.), a Taplin postdoctoral research fellowship (Y.K.), an NSF graduate research fellowship (M.L.), and by an NIH grant to A.B. (R01-NS41021-01). A.B. is the recipient of a fellowship from the Alfred P. Sloan Foundation, a Robert H. Ebert Clinical Scholar Award from the Esther A. and Joseph Klingenstein Fund, an EJLB Foundation award, and a Sidney Kimmel Foundation Award.

Received: September 25, 2001
Revised: February 22, 2002

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