Cell Survival Promoted by the Ras-MAPK Signaling Pathway by Transcription-Dependent and -Independent Mechanisms

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A mechanism by which the Ras–mitogen-activated protein kinase (MAPK) signaling pathway mediates growth factor–dependent cell survival was characterized. The MAPK-activated kinases, the Rsks, catalyzed the phosphorylation of the pro-apoptotic protein BAD at serine 112 both in vitro and in vivo. The Rsk-induced phosphorylation of BAD at serine 112 suppressed BAD-mediated apoptosis in neurons. Rsks also are known to phosphorylate the transcription factor CREB (cAMP response element–binding protein) at serine 133. Activated CREB promoted cell survival, and inhibition of CREB phosphorylation at serine 133 triggered apoptosis. These findings suggest that the MAPK signaling pathway promotes cell survival by a dual mechanism comprising the posttranslational modification and inactivation of a component of the cell death machinery and the increased transcription of pro-survival genes.

Polypeptide growth factors have a critical role in suppressing cell death during development and in homeostasis of multicellular organisms (*1*). The small guanosine triphosphatase protein Ras is a key mediator of growth factor–dependent cell survival (*2*). Of the various pathways that are activated by Ras, the pathway that comprises the series of sequentially activated protein kinases Raf, MEK (MAPK kinase), and MAPK has been found to promote cell survival and inhibit apoptosis (*3–5*). In *Drosophila*, the Ras-MAPK signaling pathway promotes cell survival by inhibiting the expression and activity of the pro-apoptotic protein Hid (*4*). In mammals, the mechanisms by which the Ras-MAPK signaling pathway promotes cell survival remain to be elucidated.

The Bcl-2 family of proteins consists of more than a dozen proteins that can have either pro-survival or pro-apoptotic functions and appear to act as gatekeepers of the cell death process (*6, 7*). Because the death machinery exerts a profound effect on the cell, its components are necessarily tightly regulated. To understand how the Ras-MAPK signaling pathway promotes cell survival, we sought to define the mechanisms by which this signaling cascade suppresses the components of the cell death machinery.

The neurotrophin BDNF (brain-derived neurotrophic factor) and its receptor TrkB regulate the survival of newly generated granule neurons within the developing cerebellum (*8*). BDNF promotes the survival of cultured rat cerebellar granule neurons, and upon BDNF withdrawal these neurons die by apoptosis (*9*).

We examined the importance of the MAPK pathway for BDNF-induced cerebellar neuron survival (*10*). Immunoblotting of lysates of untreated or BDNF-treated cerebellar granule cell cultures with an antibody to the phosphorylated, activated form of MAPK revealed that BDNF induces phosphorylation of MAPK (Fig. 1A) (*11*). Inhibition of MAPK activity by PD098059, a pharmacological agent that blocks MEK activity, diminished the effect of BDNF on the survival of cerebellar granule cells (Fig. 1B). Likewise, the introduction of a dominant interfering form of MEK (MEK-KA97) blocked BDNF-enhancement of neuronal survival (*12, 13*) (Fig. 1C). These results indicate that activation of MAPK is required for BDNF-induced survival of cerebellar granule neurons.

Like BDNF, insulin-like growth factor 1 (IGF-1) (or a high concentration of insulin that stimulates the IGF-1 receptor) promotes the survival of cerebellar granule neurons (*14, 15*). Both BDNF and IGF-1 activated phosphatidylinositol 3-kinase (PI-3K) and the protein kinase Akt (PKB) cascade in cerebellar granule neurons (*15, 16*). Although the PI-3K–Akt signaling pathway mediates the survival-promoting effects of BDNF and IGF-1 (*16–18*), inhibition of MAPK in cerebellar neurons had no effect on IGF-1 receptor–mediated cell survival (Fig. 1B). These

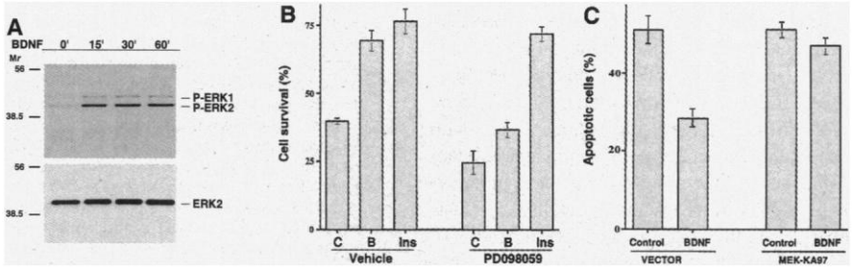

Fig. 1. Survival of cerebellar granule neurons promoted by BDNF in a MAPK-dependent manner. **(A)** Rapid and prolonged phosphorylation of MAPK in cerebellar granule neurons treated with BDNF. Cerebellar granule cell cultures (P6 + 5 DIV) were deprived of survival factors for 1 hour and then left untreated or treated with BDNF (100 ng/ml) for the indicated periods of time. Immunoblotting (*11*) was done with an antibody that recognizes the MAPKs ERK1 and ERK2 when they are phosphorylated on Tyr and Thr residues within the TEY motif (upper panel; Promega, dilution 1:10,000) or with a mouse mAb (B3B9) that recognizes ERK2 regardless of its phosphorylation state (lower panel; dilution 1:500). **(B)** BDNF-induced cell survival suppressed by pharmacological inhibition of MEK. Cerebellar granule cell cultures (P6 + 5 DIV) were deprived of survival factors and left untreated (C) or treated with BDNF (B, 100 ng/ml) or insulin (Ins, 10 μg/ml). Cultures were also incubated with the MEK inhibitor PD098059 (New England Biolabs, 30 μM) or its vehicle (DMSO). After 2 days, cultures were fixed and subjected to immunofluorescence (*13*). Percent cell survival is presented as mean ± SEM ($n = 3$). The MEK inhibitor significantly reduced BDNF enhancement of neuronal survival [analysis of variance (ANOVA), $P < 0.001$] but not insulin- or IGF-1–mediated cell survival. **(C)** Inhibition of BDNF-enhanced neuronal survival by a dominant interfering form of MEK. Cerebellar granule cell cultures (P6 + 5 DIV) were transfected (*12*) with a dominant interfering form of MEK that does not bind to ATP (MEK-KA97) or its control vector, together with a plasmid containing a gene encoding β-galactosidase. One day after the transfection, cultures were deprived of survival factors and left untreated or treated with BDNF (100 ng/ml). Two days later, cultures were fixed and subjected to indirect immunofluorescence (*13*). Percentage of cells remaining on the plate that showed evidence of apoptosis is presented as mean ± SEM ($n = 3$). BDNF suppressed apoptosis in neurons transfected with the control plasmid (ANOVA, $P < 0.01$) but not in neurons transfected with MEK-KA97.

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results suggest that BDNF and IGF-1 promote cell survival at least in part by distinct mechanisms.

Survival-promoting cytokines suppress the activity of the protein BAD, a pro-apoptotic member of the Bcl-2 family, by inducing the phosphorylation of BAD at two critical sites, Ser^{112} and Ser^{136}, which leads to the dissociation of BAD from pro-survival Bcl-2 proteins and the association of BAD with members of the 14-3-3 family of proteins (*19*). The regulation of BAD by these phosphorylation events suggests that BAD is a point of convergence for multiple signaling pathways that cooperate in promoting cell survival. The growth factor–activated protein kinase Akt phosphorylates BAD at Ser^{136} (*20, 21*). Although adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) can mediate cytokine-induced phosphorylation of BAD at Ser^{112} (*22*), the growth factor–induced kinases that phosphorylate BAD at Ser^{112} remain to be identified. Given that BDNF requires both the MAPK and PI-3K–Akt signaling pathways to promote cell survival (Fig. 1) (*16*), we considered the possibility that, whereas Akt induces the phosphorylation of BAD at Ser^{136}, the MAPK signaling pathway might induce the phosphorylation of BAD at Ser^{112}. The peptide sequence that surrounds BAD Ser^{112} corresponds to the sequence that can be phosphorylated by members of the MAPK-activated pp90 ribosomal S6 kinase family (Rsks), Rsk1, Rsk2, and Rsk3 (*23*). We used an in vitro kinase assay to assess the ability of Rsk2 to phosphorylate a BAD peptide containing Ser^{112} (Fig. 2A) (*24*). Activated Rsk2 was immunoprecipitated from lysates of 293T cells that were transfected with Rsk2 and a constitutively active MEK (MEK-1ca) that activates endogenous MAPK and thereby potentiates the activation of the transfected Rsk2. Activated Rsk2 phosphorylated the BAD Ser^{112} peptide. By contrast, an immunoprecipitated Rsk2 mutant (Rsk2-KN) that is catalytically inactive because of a mutation in its adenosine triphosphate (ATP) binding site did not induce phosphorylation. Akt and Rsk2 exhibited distinct preferences for the BAD Ser^{136} and Ser^{112} peptide substrates (Fig. 2A). BAD Ser^{112} was the preferred site of phosphorylation by Rsk2.

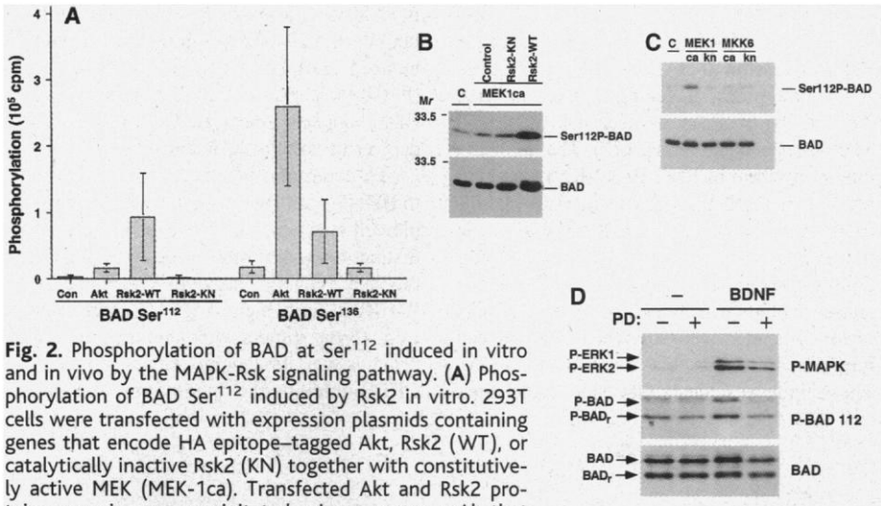

Fig. 2. Phosphorylation of BAD at Ser^{112} induced in vitro and in vivo by the MAPK-Rsk signaling pathway. (**A**) Phosphorylation of BAD Ser^{112} induced by Rsk2 in vitro. 293T cells were transfected with expression plasmids containing genes that encode HA epitope–tagged Akt, Rsk2 (WT), or catalytically inactive Rsk2 (KN) together with constitutively active MEK (MEK-1ca). Transfected Akt and Rsk2 proteins were immunoprecipitated using a mouse mAb that recognizes the HA tag, and the ability of immunoprecipitated kinases to phosphorylate a BAD Ser^{112} or a BAD Ser^{136} peptide was measured in an in vitro kinase assay (*24*). Values represent counts per minute of [γ-^{32}P]ATP incorporated into the peptide substrate (mean ± SEM, $n = 3$). (**B**) Phosphorylation of BAD Ser^{112} induced by Rsk2 in vivo. 293T cells were transfected with expression plasmids containing genes that encode Rsk2-WT, Rsk2-KN, MEK-1ca, and BAD (*25*). One day after transfection, cultures were deprived of serum for 7 hours. Immunoblotting (*11*) was done with an antibody that recognizes BAD phosphorylated at Ser^{112} (dilution 1:500) or with an antibody that recognizes BAD regardless of its phosphorylation state (Santa Cruz, dilution 1:500). Values for the intensity of the Ser^{112}-phosphorylated band relative to total BAD: control, 0.141; MEK-1ca with control plasmids for BAD, 0.253; MEK-1ca with Rsk2-KN, 0.299; MEK-1ca with Rsk2-WT, 0.712. Activated Rsk2, but not the catalytically inactive Rsk2, induced the phosphorylation of BAD at Ser^{112}. (**C**) 293T cells were transfected with an expression plasmid containing a constitutively active form or an inactive form of MEK1 or MKK6 at a higher amount (5 μg) than that in (B) (1 μg) together with an expression plasmid containing BAD. Transfected cultures were analyzed as in (B). (**D**) BDNF induces phosphorylation of endogenous BAD in a MAPK-dependent manner. Cerebellar granule neuron cultures (P6 + 5 DIV) were deprived of survival factors for 1 hour, then left untreated or treated with BDNF (100 ng/ml) for 15 min. Cultures were also treated with the MEK inhibitor PD098059 (PD, 75 μM) or with its vehicle, DMSO. Immunoblotting was done (*11*) with an antibody that recognizes the phosphorylated form of the MAPKs ERK1 and ERK2, an antibody that recognizes BAD phosphorylated at Ser^{112} (*26*) (dilution 1:500), or with an antibody that recognizes BAD regardless of its phosphorylation state (Santa Cruz, dilution 1:500). The antibodies to BAD recognized a full-length BAD and a BAD-related protein of lower molecular weight (BAD_r) that may represent a cleavage product of BAD. Values for the intensity of the Ser^{112}-phosphorylated band relative to total BAD: starved, 0.0556; starved plus PD098059, 0.0259; BDNF, 0.232; BDNF plus PD098059, 0.0617.

To determine whether MAPK-activated Rsks catalyze the phosphorylation of BAD Ser^{112} in cells, we expressed Rsk2, MEK1, and BAD in 293T cells (*25*). Immunoblotting of 293T cell lysates with an antibody that specifically recognizes BAD phosphorylated at Ser^{112} revealed that expression of Rsk2 caused phosphorylation of BAD at Ser^{112}, whereas expression of the catalytically inactive Rsk2 mutant did not (Fig. 2B) (*11, 26*). Expression of large amounts of MEK-1ca triggered the phosphorylation of BAD Ser^{112} in the absence of exogenous Rsk2, which suggests that the activation of endogenous Rsks can induce phosphorylation of BAD Ser^{112} (Fig. 2C). In contrast to MEK-1ca, expression of large amounts of the constitutively active form of the related kinase MKK6 did not induce the phosphorylation of BAD at Ser^{112} (Fig. 2C).

To determine whether activation of the MAPK signaling pathway triggers the phosphorylation of BAD when BAD is expressed in normal amounts within neurons, we assessed the ability of BDNF to trigger phosphorylation of endogenous BAD in cerebellar granule neurons. Lysates of untreated or BDNF-treated cerebellar granule cell cultures were immunoblotted with the antibody that recognizes BAD phosphorylated at Ser^{112}. BDNF induced the phosphorylation of endogenous BAD at Ser^{112} within minutes of BDNF addition (Fig. 2D). BDNF-induced phosphorylation of BAD at Ser^{112} was diminished when cerebellar granule cells were first incubated with the MEK inhibitor PD098059 (Fig. 2D). PKA mediates cytokine-induced phosphorylation of BAD at Ser^{112} (*22*). Inhibition of PKA in cerebellar granule neurons using the pharmacological agent H89 blocked cAMP-induced phosphorylation of BAD at Ser^{112} but had little effect on BDNF-induced phosphorylation of endogenous BAD at Ser^{112} (*16*). Thus, various extracellular factors may activate distinct signaling pathways to induce the phosphorylation of BAD at Ser^{112} in cerebellar granule neurons, and the MAPK-Rsk signaling pathway appears to mediate BDNF-induced phosphorylation of endogenous BAD.

To assess the functional consequences of the MAPK-Rsk–induced phosphorylation of BAD at Ser^{112}, we transfected cerebellar granule cell cultures with expression plasmids encoding BAD and MEK-1ca. The expression of BAD induced the death of 60% of

transfected neurons (Fig. 3A). However, the activation of the MAPK signaling pathway by the expression of MEK-1ca did not inhibit the apoptotic effect of BAD (Fig. 3A).

MEK-1ca did inhibit BAD-mediated apoptosis in cultures that were exposed to IGF-1 (Fig. 3A). In the absence of MEK-1ca expression, IGF-1 reduced the apoptotic effect of BAD in cerebellar granule neurons, albeit to a lesser extent than that achieved by the combination of MEK-1ca expression and IGF-1 receptor activation (Fig. 3A). Because IGF-1 inhibits the apoptotic effect of BAD by inducing the phosphorylation of BAD at Ser^{136} (*20*), our results raise the possibility that MAPK suppression of BAD-mediated cell death requires that BAD be phosphorylated at Ser^{136}. Consistent with this possibility, in IGF-1–treated cerebellar granule neurons MEK-1ca failed to inhibit the apoptotic effect of a BAD protein in which Ser^{136} was converted to Ala (*16*). Together, these results suggest that the MAPK and PI-3K–Akt signaling pathways converge at BAD to suppress the apoptotic effect of BAD.

We next examined the ability of the MAPK signaling pathway to inhibit the apoptotic effect of a BAD mutant in which Ser^{112} was replaced by Ala (BADS112A). BADS112A was as effective as wild-type BAD in inducing apoptosis of transfected neurons (Fig. 3A). In the absence of transfected MEK-1ca, IGF-1 inhibited the apoptotic effect of wild-type BAD and BADS112A to a similar extent (Fig. 3A). However, when cultures were transfected with MEK-1ca and with wild-type or mutant BAD and then treated with IGF-1, expression of MEK-1ca inhibited the apoptotic effect of wild-type BAD but not of BADS112A (Fig. 3A). These results suggest that in the presence of IGF-1, MEK-1ca (via MAPK activation) suppresses BAD-mediated apoptosis by inducing the phosphorylation of BAD at Ser^{112}.

To directly assess the ability of Rsks to inhibit the apoptotic effect of BAD, we tested whether Rsk2 when overexpressed can inhibit the BAD-mediated death of cerebellar granule neurons. In cultures in which the IGF-1 receptor was activated, BAD when expressed alone induced the death of 40% of transfected neurons (Fig. 3B). However, the expression of Rsk2, together with smaller amounts of MEK-1ca relative to BAD than those described in Fig. 3A, led to a 50% reduction of BAD-mediated cell death (Fig. 3B). The suppression of BAD-mediated death required the kinase activity of Rsk2 because the catalytically inactive Rsk2 mutant did not inhibit BAD-mediated cell death (Fig. 3B). In addition, activated Rsk2 did not suppress the apoptotic effect of BADS112A (Fig. 3B). These results suggest that Rsks mediate MAPK inhibition of the apoptotic effect of BAD, and that Rsk-induced phosphorylation of BAD at Ser^{112} is necessary for Rsk suppression of BAD-mediated cell death.

Neurotrophins, including BDNF, increase the activity of Rsks in neuronal cells in a MAPK-dependent manner (*27*). The dominant interfering form of Rsk2 (Rsk2-KN) when expressed in cerebellar granule neurons significantly reduced the ability of BDNF to promote the survival of these neurons (Fig. 3C). Inhibition of endogenous Rsk function with specific antisense oligonucleotides also blocked growth factor–dependent cell survival of several cell types, including fibroblasts and neurons (*28*). These findings indicate that endogenous Rsks play a critical role in mediating growth factor–dependent cell survival.

The MAPK-activated Rsks also have an important role in mediating the ability of growth factors and neurotrophins to induce transcription (*29*). A critical target of Rsks is the transcription factor CREB (cAMP response element–binding protein) (*30*). In cells exposed to neurotrophins and growth factors, Rsks catalyze the phosphorylation of CREB at a site, Ser^{133}, that leads to CREB activation (*30, 31*). We tested whether BDNF led to the increased phosphorylation of CREB Ser^{133} in cerebellar granule neurons, and whether the phosphorylation and activation of CREB contributed to the enhancement of neuronal survival. Immunoblotting of cerebellar granule lysates with an antibody that specifically recognizes CREB phosphorylated at Ser^{133} revealed that BDNF induced this phosphorylation (Fig. 4A). However, BDNF-induced phosphorylation of CREB at Ser^{133} was diminished when cerebellar granule neurons were first incubated with the MEK inhibitor PD098059 (Fig. 4A). By contrast, the PI-3K inhibitor LY294002 had little effect on BDNF-induced phosphorylation of CREB at Ser^{133} (Fig. 4A). Thus, in cerebellar granule neurons, BDNF appears to activate CREB in a MAPK-dependent and PI-3K–independent manner.

To determine whether CREB contributes to BDNF's ability to enhance cerebellar granule cell survival, we tested the effects of two distinct dominant interfering forms of CREB on the BDNF survival response (*32*). K-CREB, in which Arg^{287} is converted to Leu, forms dimers with endogenous CREB proteins via its leucine zipper domain. K-CREB inhibits the binding of endogenous

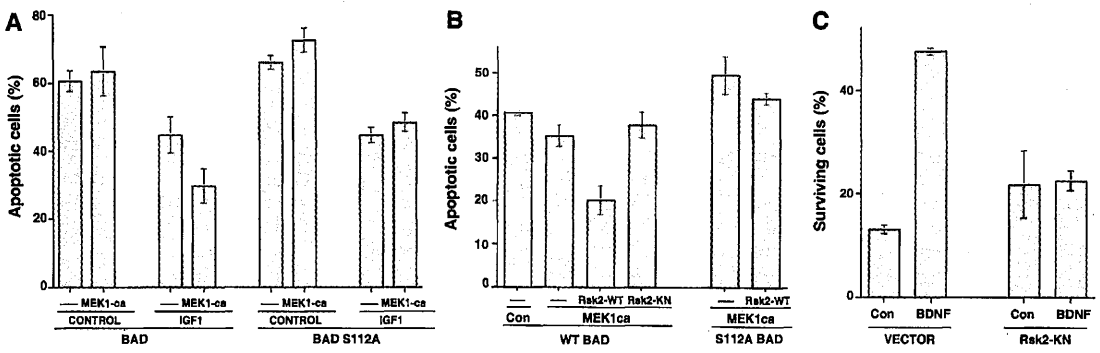

Fig. 3. Inhibition of BAD-mediated apoptosis by Rsk2. (**A**) Inhibition of BAD-mediated apoptosis by the MAPK signaling pathway in cooperation with the PI-3K–Akt signaling pathway. Cerebellar granule neurons were transfected (*12*) with expression plasmids encoding MEK-1ca or its control vector together with BAD or BADS112A and the β-galactosidase expression plasmid. One day after transfection, cultures were switched from full survival medium to basal medium with or without IGF-1 (50 ng/ml). After 8 hours, cultures were fixed and subjected to indirect immunofluorescence (*13*). MEK-1ca significantly reduced BAD-mediated cell death in IGF-1–treated cultures (ANOVA, $P < 0.001$; $n = 3$) but not in control cultures. MEK-1ca did not inhibit the apoptotic effect of BADS112A in control or IGF-1–treated cultures. (**B**) Activated Rsk2 suppresses BAD-mediated cell death by inducing its phosphorylation at Ser^{112}. Cerebellar granule neuron cultures (P6 + 5 DIV) were transfected (*12*) with expression plasmids encoding BAD, BADS112A, MEK-1ca, Rsk2-WT, or Rsk2-KN or their vector controls and an expression plasmid containing a gene encoding β-galactosidase. The ratio of transfected MEK-1ca to BAD was lower than that used in (A) (*12*). One day after transfection, cultures were switched from full survival medium to basal medium supplemented with insulin (10 μg/ml) and then analyzed as in (A). Activated wild-type Rsk2 (WT), but not the catalytically inactive Rsk2 (KN), significantly reduced BAD-mediated apoptosis [ANOVA with Fisher's post hoc test between Rsk2-WT and vector (–) together with MEK-1ca and BAD, $P < 0.001$; $n = 3$]. (**C**) Requirement of endogenous Rsks for BDNF enhancement of cell survival. Cerebellar granule neuron cultures (P6 + 6 DIV) were transfected (*12*) with an expression plasmid containing the catalytically inactive form of Rsk2 (Rsk2-KN) or its vector control together with the β-galactosidase expression plasmid. Cultures were then analyzed as in Fig. 1C. Percent cell survival is presented as mean ± SEM ($n = 3$). BDNF significantly enhanced the survival of neurons transfected with the vector control (ANOVA with Fisher's post hoc test between control and BDNF; $P < 0.001$) but not in neurons transfected with Rsk2-KN.

CREB to the promoters of CREB-responsive genes. M1-CREB, in which Ser^{133} is converted to Ala, competes with endogenous CREB proteins for binding to the promoters of CREB-responsive genes. However, once bound to DNA, M1-CREB does not activate transcription. When transfected into cerebellar granule neurons, either K-CREB or M1-CREB inhibited the effect of BDNF on cell survival (Fig. 4B). However, the dominant interfering forms of CREB did not inhibit IGF-1–mediated cerebellar granule cell survival (Fig. 4B); this finding suggests that these proteins act specifically to block the BDNF response. In addition, M1-CREB did not lead to inhibition of Rsk function because its expression in 293T cells did not inhibit the MEK-induced phosphorylation of BAD at Ser^{112} (*16*).

CREB-VP16 is a constitutively active mutant form of CREB in which the full-length CREB protein is fused at its NH_2-terminus to the transactivation domain of the viral transcriptional coactivator VP16. In transient transfection assays, CREB-VP16 significantly enhanced the survival of cerebellar granule cells in the absence of extracellular survival factors such as IGF-1 or BDNF (Fig. 4C). The ability of CREB-VP16 to promote cell survival required an intact DNA binding region of CREB (Fig. 4D), which indicates that the CREB-VP16 protein functions by specifically binding to the promoters of CREB-regulated genes that mediate cell survival. Our results and those of others (*33*) indicate that the pro-survival gene *bcl-2* is a target of CREB in cerebellar granule neurons (*16*). BDNF increased transcription of a *bcl-2* promoter–driven reporter construct in cerebellar granule neurons. A mutation of the CREB binding sequence within the *bcl-2* promoter diminished the ability of BDNF to activate the *bcl-2* promoter. In addition, the dominant interfering forms of CREB, when expressed in cerebellar granule neurons, blocked BDNF-induced transcription of the *bcl-2* promoter (*16*).

CREB has been implicated in mediating adaptive responses of neurons to trans-synaptic stimuli (*34*). Our findings indicate that CREB may also have a function in the regulation of neuronal survival in the developing central nervous system. Mice in which the CREB gene has been disrupted die perinatally before the majority of cerebellar granule neurons are generated (*35*). However, analysis of the $CREB^{-/-}$ mouse embryos revealed a number of abnormalities in brain development that may reflect the contribution of CREB to the regulation of the survival of neurons.

Our findings suggest that the MAPK signaling pathway promotes cell survival by a dual mechanism that modulates the cell death machinery directly by phosphorylating and thereby inhibiting the pro-apoptotic protein BAD, and by inducing the expression of pro-survival genes in a CREB-dependent manner. Suppression of BAD-mediated cell death by Rsk occurred relatively early after the removal of extracellular survival factors, whereas the contribution of CREB-mediated cell survival was detected significantly later. Therefore, the two arms of the MAPK-Rsk–regulated mechanism might act with different kinetics or at different times in developing neurons.

Note added in proof: Ser^{133}-phosphorylated CREB has been suggested to mediate follicle-stimulating hormone–induced survival of rat granulosa cells (*36*). John Blenis and colleagues have recently implicated Rsks in cytokine suppression of BAD-mediated apoptosis (*37*).

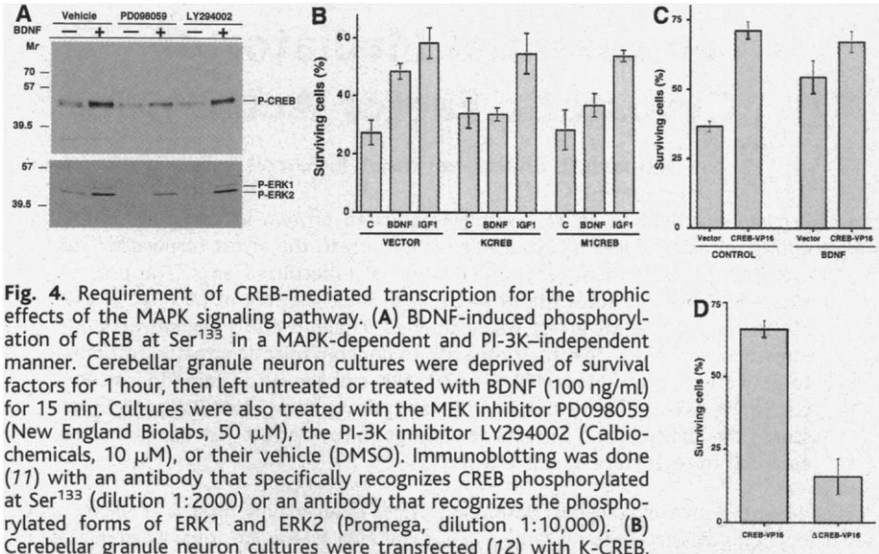

Fig. 4. Requirement of CREB-mediated transcription for the trophic effects of the MAPK signaling pathway. (**A**) BDNF-induced phosphorylation of CREB at Ser^{133} in a MAPK-dependent and PI-3K–independent manner. Cerebellar granule neuron cultures were deprived of survival factors for 1 hour, then left untreated or treated with BDNF (100 ng/ml) for 15 min. Cultures were also treated with the MEK inhibitor PD098059 (New England Biolabs, 50 μM), the PI-3K inhibitor LY294002 (Calbiochemicals, 10 μM), or their vehicle (DMSO). Immunoblotting was done (*11*) with an antibody that specifically recognizes CREB phosphorylated at Ser^{133} (dilution 1:2000) or an antibody that recognizes the phosphorylated forms of ERK1 and ERK2 (Promega, dilution 1:10,000). (**B**) Cerebellar granule neuron cultures were transfected (*12*) with K-CREB, M1-CREB, or a control vector plasmid together with an expression plasmid containing a gene encoding β-galactosidase. Cultures were then analyzed as in Fig. 1C. Each dominant interfering form of CREB significantly reduced BDNF-mediated (ANOVA with Fisher's post hoc test between vector and K-CREB or between vector and M1-CREB, $P < 0.01$; $n = 4$) but not IGF-mediated neuronal survival. (**C**) CREB-dependent transcription is sufficient to promote cell survival in the absence of survival factors. Cerebellar granule neuron cultures were transfected (*12*) with an expression plasmid containing a gene encoding a CREB mutant in which the full-length CREB was fused to the robust transcriptional coactivator VP16 (CREB-VP16) together with an expression plasmid containing a gene encoding β-galactosidase. Cultures were then analyzed as in Fig. 1C. Relative to its vector control, CREB-VP16 promoted the survival of cerebellar granule neurons in the absence of survival factors (ANOVA, $P < 0.001$; $n = 3$). (**D**) Cerebellar granule neuron cultures were transfected (*12*) with an expression plasmid containing the gene encoding the CREB-VP16 fusion protein or a mutant CREB-VP16 protein in which the DNA binding and leucine zipper regions of CREB were mutated (ΔCREB-VP16) together with the β-galactosidase expression plasmid. Cultures were then analyzed as in (C). Cell survival was significantly reduced in cells transfected with ΔCREB-VP16 relative to cells transfected with CREB-VP16 (paired *t* test, $P < 0.01$; $n = 3$).

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10. Cerebellar granule cultures were prepared as described (*15*). Cerebellar granule neurons were cultured from Long-Evans rats on postnatal day 6. Neurons were placed on polyornithine-coated plates and grown in basal medium Eagle (BME, Sigma) supplemented with calf serum (10%) (Hyclone), 25 mM KCl, 2 mM glutamine, penicillin, and streptomycin. One day after cultures were prepared (P6 + 1 DIV), they were treated with the antimitotic agent cytosine-β-D-arabinofuranoside (10 μM) to prevent cell proliferation. For the assessment of cell survival in the

absence or presence of inhibitors, P6 + 5 DIV cultures were washed twice with medium (BME) containing 5 mM KCl and no serum (starvation medium) and then placed in this medium in the presence or absence of the survival factor (BDNF, insulin, or IGF-1) and in the presence of MAPK, PI-3K inhibitors, or their vehicle, dimethyl sulfoxide (DMSO). In experiments in which biochemical analyses were done, the medium [BME + calf serum (10%) + 25 mM KCl] was switched to starvation medium 1 hour before stimulation with the survival factor. In the last 30 min of the 1-hour starvation period, the inhibitor or its vehicle control (DMSO) was added.

11. Immunoblotting was carried out as described (*31, 38*). Briefly, proteins from lysates were separated by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and immunoblotted with the appropriate primary antibody. Antibody binding was detected by enhanced chemiluminescence (ECL, Amersham) with a secondary antibody conjugated to horseradish peroxidase (dilution: 1:20,000).
12. Transfections of cerebellar granule cultures were done with a calcium phosphate transfection method as described [Z. Xia *et al.*, *J. Neurosci.* **16**, 5425 (1995)]. In the experiments shown in Fig. 1C and in Fig. 4, B to D, cultures were transfected with test plasmid (2 μg per well) together with the β-galactosidase expression plasmid (0.5 μg per well) in a 24-well plate. Each well contained 6×10^5 cells. In the experiments shown in Fig. 3C, cultures were transfected with 0.5 μg of the test plasmid and 0.5 μg of the β-galactosidase expression plasmid per well of a 24-well plate. In Fig. 3A, each well was transfected with 1.5 μg of MEK-1ca, 0.05 μg of the BAD or BADS112A expression plasmid, and 0.5 μg of the β-galactosidase expression plasmid. In Fig. 3B, each well was transfected with 1.5 μg of MEK-1ca with 1 μg of Rsk2-WT or Rsk2-KN [Rsk2KR100 (*32*)], 0.125 μg of the BAD or BADS112A expression plasmid, and 0.5 μg of the β-galactosidase expression plasmid.
13. Indirect immunofluorescence was done as described [A. Bonni, D. A. Frank, C. Schindler, M. E. Greenberg, *Science* **262**, 1575 (1993)]. Cultures were fixed in 4% paraformaldehyde and subjected to indirect immunofluorescence with a mouse monoclonal antibody (mAb) to β-galactosidase (Promega, dilution 1:500) and the DNA dye bisbenzimide (Hoechst 33258). Determination of cell death in neurons was made on the basis of integrity of neurites and nuclear morphology. Cell counts were carried out in a blinded manner by two independent observers.
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24. We transfected 100-mm plates of 293T cells as described (*20*) with expression plasmids encoding hemagglutinin (HA) epitope–tagged constitutively active Akt [myrΔPH Akt (*15*)] (15 μg), Rsk2-WT (5 μg), or Rsk-KN (5 μg) together with MEK-1ca [MEK-S218D/S222D; A. Brunet *et al.*, *Oncogene* **9**, 3379 (1994)] (10 μg). Two days after transfection, cells were lysed and the HA epitope–tagged proteins were immunoprecipitated as described (*15*). Immunoprecipitated proteins were subjected to an in vitro kinase assay as described (*15, 30*) using BAD peptide substrates (14 amino acids in length encompassing Ser^{112} or Ser^{136}).
25. Six-well plates of 293T cells containing 7.5×10^5 cells per well were transfected by a calcium phosphate transfection method as described (*38*). In Fig. 2B, cells were transfected with Rsk2-WT or Rsk2-KN (2 μg) together with MEK-1ca (1 μg) and BAD (1 μg). In Fig. 2C, cells were transfected with 5 μg of the MEK-1ca expression plasmid together with 0.75 μg of the BAD. One day after transfection, cultures were starved for 7 hours. Cell lysates were prepared and immunoblotted with the antibody to phospho-Ser^{112} (*11, 26*).
26. A rabbit antiserum to BAD phosphorylated at Ser^{112} was generated by injecting New Zealand rabbits with the phosphopeptide C-METRSRHpSSYPAG (*20*). The specificity of the antibody to BAD phosphorylated at Ser^{112} was confirmed by its reactivity to recombinant BAD protein that was phosphorylated by PKA but not to unphosphorylated BAD, and by its recognition of Rsk2- or PKA-induced phosphorylated wild-type BAD but not S112A BAD that was expressed in 293T cells.
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The Protein Kinase p90 Rsk as an Essential Mediator of Cytostatic Factor Activity

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Persistent activation of p42 mitogen-activated protein kinase (p42 MAPK) during mitosis induces a "cytostatic factor" arrest, the arrest responsible for preventing the parthenogenetic activation of unfertilized eggs. The protein kinase p90 Rsk is a substrate of p42 MAPK; thus, the role of p90 Rsk in p42 MAPK-induced mitotic arrest was examined. *Xenopus laevis* egg extracts immunodepleted of Rsk lost their capacity to undergo mitotic arrest in response to activation of the Mos–MEK-1–p42 MAPK cascade of protein kinases. Replenishing Rsk-depleted extracts with catalytically competent Rsk protein restored the ability of the extracts to undergo mitotic arrest. Rsk appears to be essential for cytostatic factor arrest.

Masui identified two hypothetical M-phase regulators in his classic studies of *Rana pipiens* oocyte maturation. The first, maturation-promoting factor (MPF), was an activity present in mature oocytes that was able to induce immature oocytes to mature even in the absence of protein synthesis (*1*). MPF ultimately proved to be a complex of the universal M-phase regulators Cdc2 and cyclin B (*2*). Cytostatic factor (CSF) was defined as an activity present in mature oocytes that induced mitotic arrest when injected into cleaving embryos (*1*). The underlying hypothesis was that CSF activity is responsible for the maintenance of mature oocytes in their normal metaphase arrest state.

Studies over the past decade have identified the proto-oncoprotein Mos as CSF and the protein kinases MEK and p42 MAPK as essential mediators of CSF activity (*3*). The introduction of Mos mRNA (*4*) or protein (*5*), constitutively active MEK (*3*), or thiophosphorylated, active p42 MAPK (*6*) into *Xenopus laevis* embryos or cell-free cycling extracts (*7, 8*) causes a metaphase arrest. Depletion of Mos from extracts of mature

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