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 Rsk, 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. Rsk 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.

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).

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
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, Ser112 and Ser136, 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 Ser136 (20, 21). Although adenosine 3',5'-monophosphate (cAMP)–dependent protein kinase (PKA) can mediate cytokine-induced phosphorylation of BAD at Ser112 (22), the growth factor–induced kinases that phosphorylate BAD at Ser112 or a BAD Ser 136 peptide was measured in an in vitro kinase assay (24). Values for the intensity of the Ser112-phosphorylated band relative to total BAD: control, 0.141; MEK-1ca with Rsk2-KN, 0.299; MEK-1ca with Rsk2-WT, 0.712. The peptide sequence that surrounds BAD Ser112 corresponds to the sequence that can be phosphorylated by members of the MAPK-activated p90 ribosomal S6 kinase family (Rsk3), Rsk3, Rsk1, and Rsk3 (23). We used an in vitro kinase assay to assess the ability of Rsk2 to phosphorylate a BAD peptide containing Ser112 (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 Ser112 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 Ser112 and Ser136 peptide substrates (Fig. 2A). BAD Ser112 was the preferred site of phosphorylation by Rsk2.

To determine whether MAPK-activated Rsk2s catalyze the phosphorylation of BAD Ser112 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 Ser112 revealed that expression of Rsk2 caused phosphorylation of BAD at Ser112, 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 Ser112 in the absence of exogenous Rsk2, which suggests that the activation of endogenous Rsk2s can induce phosphorylation of BAD Ser112 (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 Ser112 (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 Ser112. BDNF induced the phosphorylation of endogenous BAD at Ser112 within minutes of BDNF addition (Fig. 2D). BDNF-induced phosphorylation of BAD at Ser112 was diminished when cerebellar granule cells were first incubated with the MEK inhibitor PD098059 (Fig. 2D). PKA mediates cytokine-induced phosphorylation of BAD at Ser112 (22). Inhibition of PKA in cerebellar granule neurons using the pharmacological agent H89 blocked cAMP-induced phosphorylation of BAD at Ser112 but had little effect on BDNF-induced phosphorylation of endogenous BAD at Ser112 (16). Thus, various extracellular factors may activate distinct signaling pathways to induce the phosphorylation of BAD at Ser112 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 Ser112, 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 (BAD-S112A). BAD-S112A 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 BAD-S112A 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 BAD-S112A (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 Rsk mediates 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
CREB to the promoters of CREB-responsive genes. M1-CREB, in which Ser
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
(C). 

CREB-VP16 is a constitutively active mutant form of CREB in which the full-length CREB protein is fused at its NH2-terminus to the transcriptional coactivator VP16. In transient transfection assays, CREB-VP16 significantly enhanced the survival of cerebellar granule neurons (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 inactivating 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 at different times in developing neurons.

Note added in proof: Ser
-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).

References and Notes
-neuronin (10 pmol) to prevent cell proliferation. For the assessment of cell survival in the
absence or presence of inhibitors, PS-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 sumo factor (BODIPY, 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, 10% FBS + 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 (DMSO) was added.

Immunoblots were 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, Amer sham) with a secondary antibody conjugated to horse radish peroxidase (dilution 1:2000).

24. We transfected 100-mm plates of 293T cells as de scribed ([26] X. Xia et al., unpublished data).

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-Ser112 [11, 26].

26. A rabbit antiserum to BAD phosphorylated at Ser 112 was generated by injecting New Zealand rabbits with the phosphopeptide C-METRSRpSSYPAG (20). The specificity of the antibody to BAD phosphorylated at Ser112 was confirmed by its reactivity to reconstituted 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.


28. J. Xing and M. E. Greenberg, unpublished data.


37. A. Shimamura et al., in preparation.

38. A. Brunet et al., Cell 96, 857 (1999).

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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 immuno-depleted 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 described 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 MEK3 (6), or thiophosphate-activated, 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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