JNK Phosphorylation and Activation of BAD Couples the Stress-activated Signaling Pathway to the Cell Death Machinery*

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The c-Jun N-terminal kinase (JNK) signaling pathway plays a critical role in mediating apoptosis in the developing and mature organism. The JNK signaling pathway is thought to induce apoptosis via transcription-dependent and transcription-independent mechanisms that remain to be elucidated. In this study, we report a novel mechanism by which the JNK signaling pathway directly activates a component of the cell death machinery. We have found that JNK catalyzes the phosphorylation of the BH3-only protein BAD at the distinct site of serine 128 in vitro. Activation of the JNK signaling pathway induces the BAD serine 128 phosphorylation in vivo, including in primary granule neurons of the developing rat cerebellum. The JNK-induced BAD serine 128 phosphorylation promotes the apoptotic effect of BAD in primary neurons by antagonizing the ability of growth factors to inhibit BAD-mediated apoptosis. These findings indicate that BAD is a novel substrate of JNK that links the stress-activated signaling pathway to the cell death machinery.

Regulation of cell death is critical to the normal development and homeostasis of multicellular organisms. In the developing nervous system, neurons are produced in excess, and naturally occurring neuronal cell death is thus critical in ensuring that neurons form the appropriate connections (1). In the mature nervous system, abnormally occurring neuronal cell death contributes to the pathogenesis of a variety of diseases including stroke, epilepsy, and neurodegenerative diseases (2–4). Not surprisingly, the mechanisms that underlie neuronal cell death have been the subject of intense interest.

Studies of cell death in a variety of organisms have revealed that members of the Bcl-2 family of proteins act as gatekeepers of the cell death machinery (5–7). Under conditions in which cells are destined to undergo programmed cell death (apoptosis), the proapoptotic multidomain Bcl-2 proteins, including Bax, induce the release of cytochrome c from mitochondria leading to the activation of a caspase cascade that executes the cell death program (8, 9). The prosurvival multidomain Bcl-2 proteins, including Bcl-2 and Bcl-xl, interact with and inhibit the proapoptotic multidomain Bcl-2 proteins (10).

The BH3-only subfamily of Bcl-2 proteins appears to play an important role in regulating the function of the multidomain Bcl-2 protein in response to signals from the cell surface or the cytoskeleton (11). The BH3-only protein BAD promotes cell death by interacting with and inhibiting the prosurvival multidomain Bcl-2 proteins (10). Survival factors suppress BAD-mediated apoptosis by inducing the phosphorylation of BAD at serine 136, serine 112, or both, culminating in the interaction and sequestration of phosphorylated BAD by members of the 14-3-3 family of proteins (12). The protein kinases Akt, p21-activated kinase 1, and p70S6 kinase appear to mediate survival factor-induced phosphorylation of BAD serine 136 (13–17). On the other hand, the protein kinases Rsk, protein kinase A, and p21-activated kinase 1 are thought to mediate survival factor-induced phosphorylation of BAD serine 112 (16, 18–21).

Recently, we reported that BAD is also the target of an apoptotic signaling pathway (22). We found that the protein kinase Cdc2, previously established as controlling the transition of proliferating cells through mitosis, mediates apoptosis of newly generated postmitotic granule neurons of the developing rat cerebellum (22). Cdc2 triggers the phosphorylation of BAD at the novel site of serine 128, which lies near the growth factor-induced site of phosphorylation of serine 136 (22). The Cdc2-induced phosphorylation of BAD at serine 128 inhibits the interaction of growth factor-induced serine 136-phosphorylated BAD with 14-3-3 proteins and thereby activates the apoptotic effect of BAD (22). A major question that is raised by these findings is whether BAD, in particular BAD serine 128, is the target of other protein kinases that propagate apoptotic signals.

The c-Jun N-terminal kinase (JNK)1 represents a group of mitogen-activated protein kinases (MAPKs) that mediate stress-induced cellular responses including programmed cell death (23). JNK is thought to induce apoptosis via transcription-dependent and transcription-independent mechanisms that remain incompletely understood. Studies of JNK-induced neuronal apoptosis suggest that JNK-induced phosphorylation of the transcription factor c-Jun and the consequent expression of c-Jun-induced genes mediate JNK-induced apoptosis (24–29). In addition to transcription-dependent mechanisms, JNK promotes cell death by directly regulating the cell death machinery (30). JNK has been reported to catalyze the phosphorylation of BAD with 14-3-3 proteins and thereby activates the apoptotic effect of BAD (12). A major question that is raised by these findings is whether BAD, in particular BAD serine 128, is the target of other protein kinases that propagate apoptotic signals.

1 The abbreviations used are: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-related kinase kinase; MEKK1, MEK kinase 1; MKK3, MAPK kinase 3; MOPS, 4-morpholino-1-propanesulfonic acid; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; IGF1, insulin-like growth factor 1; LC, liquid chromatography; MS, mass spectrometry; P6, postnatal day 6 rats; DIV, days in vitro.

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tion of Bcl-2 (31–35). The JNK-induced phosphorylation of Bcl-2 appears to suppress the prosurvival function of Bcl-2 (33, 35). However, under certain circumstances, the phosphorylation of Bcl-2 at the site of the JNK-induced phosphorylation of Bcl-2 has been suggested to be required for the prosurvival function of Bcl-2 (34).

In this study, we have characterized a novel mechanism by which JNK directly activates the cell death machinery in neurons. We have found that JNK catalyzes the phosphorylation of the BH3-only protein BAD in vitro at the distinct site of serine 128. Activation of the JNK signaling pathway in vivo was found to induce the serine 128 phosphorylation of BAD, including endogenous BAD in primary cerebellar granule neurons. We also found that JNK signaling pathway-induced phosphorylation of BAD at serine 128 promotes the apoptotic effect of BAD in granule neurons by opposing growth factor-inhibition of BAD-mediated apoptosis. These findings define a new mechanism by which the stress-activated signaling pathway triggers apoptosis. In addition, together with our recent characterization of the Cdc2-BAD apoptotic pathway (22), our results suggest that BAD serine 128 represents a common link for apoptosis-inducing protein kinases with the cell death machinery.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The BAD plasmids (pCDNA3-BAD wild type and pCDNA3-BADs128A) (22), activated MEKK1 (38), and activated MKK2 (Stratagene) have been described. The N-20 BAD (Santa Cruz Biotechnology), phospho128-BAD (New England Biolabs), phospho136-BAD (New England Biolabs), antibodies were purchased. The phospho128-BAD antibody was generated as described (22). The JNK inhibitor II (SP600125) and the phospho-136 BAD antibody (SB203580) were purchased from Calbiochem.

In Vitro Kinase Assays—In vitro kinase assays were carried out as described in the Upstate Biotechnology protocol for the JNK1/1 protein kinase product. In the experiments in Fig. 1A, the final kinase assay reaction containing 0.05 units of JNK1/1 (Upstate Biotechnology), 1 μg of substrate (recombinant GST-BAD), 100 μM ATP, and 5 μCi of [γ-32 P]ATP in 2 mM MOPS, pH 7.2, 2.5 mM Mg-glycero-phosphate, 0.5 mM EGTA, 1 mM sodium orthovanadate, 0.1 mM dithiothreitol, and 15 mM magnesium chloride was incubated at 30 °C for 30 min. In the experiment depicted in Figs. 1B and 2, 200 μM ATP but no radiolabeled ATP was used. Cyclin B1/Cdc2 (New England Biolabs) was used at 1 unit, and protein kinase A (Upstate Biotechnology) was used at 0.3 unit per reaction.

Mass Spectrometry—Coomasie-stained GST-BAD that was phosphorylated by JNK was digested with trypsin in gel. Peptides were separated by nanoscale micropipillary high performance liquid chromatography as described (37). Eluting peptides were ionized by electrospray ionization and analyzed by an LCQ-DECA ion trap mass spectrometer as described (37).

Western Analysis—Western analyses were carried out as described (22). Briefly, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in a Tris-buffered saline solution containing 0.5% Tween and 3% bovine serum albumin (USB). Following the blocking step, membranes were incubated with primary antibody (dilution of 1:1000) for 1 h at room temperature followed by the appropriate horseradish peroxidase-conjugated secondary antibody at 1:20,000 for 45 min. All Westerns were developed by enhanced chemiluminescence.

Transfections of Neuro2A Cells—Neuro2A cells were grown in DMEM containing 10% fetal bovine serum. Cells were split the day before transfection onto 6-well plates and transfected when cells became 60% confluent using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. The total amount of DNA (2 μg) was added to 100 μl of OptiMEM (Invitrogen), mixed gently with 6 μl of LipofectAMINE in 100 μl of OPTIMEM, allowed to incubate at room temperature for 45 min, and then 1 ml of serum-free DMEM was added to the mixture. The DMEM/LipofectAMINE mixture was then added to the cells for 3 h. Following transfection, cells were returned to DMEM containing 10% fetal bovine serum. In the experiments in which inhibitors were added, the inhibitors SP600125 and SB203580 were used at 10 and 5 μM, respectively, and were added at the time that DMEM (plus 10% FBS) was added to cells. Lysates were prepared from transfected cultures 20 h after transfection.

Transfections of Cerebellar Granule Neurons—Cerebellar granule neurons were prepared as described (22) and transfected using a calcium phosphate transfection method. For the experiments depicted in Fig. 5, cultures from postnatal day 6 that were in culture for 7 or 8 days were transfected with 0.05 μg of BAD or BADs128A and 0.5 μg of activated MEKKI or the control vector together with 0.25 μg of a plasmid encoding β-galactosidase. The calcium phosphate precipitate was placed on granule neurons (50 μl/well in a 24-well plate) for 20 min, and cultures were then returned to conditioned medium (basal medium Eagle containing 10% calf serum and 30 μM KC1). After overnight incubation with conditioned medium, cultures were deprived of conditioned medium and left in basal medium Eagle in the presence or absence of insulin (10 μg/ml) for 8 h. Cultures were fixed and subjected to indirect immunofluorescence using a mouse monoclonal antibody to β-galactosidase. Cell survival and death were assessed in β-galactosidase-expressing neurons based on the integrity of the neurites and the nucleus as determined by the DNA dye bisbenzimide (Hoechst 33258). Cell counts were carried out in a blinded manner.

RESULTS

JNK Phosphorylates BAD at Serine 128 in Vitro—We investigated the mechanism by which JNK might directly regulate the cell death machinery via the BH3-only protein BAD. In a recent study (22) we found that Cdc2 directly couples the cell cycle to the cell death machinery by inducing the phosphorylation of BAD at the novel site of serine 128. Because JNK, like Cdc2, is a proline-directed kinase, and serine 128 conforms to the type of sequence that might be phosphorylated by JNK, we investigated the possibility that JNK might couple the stress-induced signaling pathways to the cell death machinery via the phosphorylation of BAD at serine 128.

We first examined whether JNK phosphorylates BAD in vitro. In an in vitro kinase assay, we found that recombinant JNK robustly catalyzed the phosphorylation of a recombinant GST-BAD fusion protein (Fig. 1A). To determine whether JNK induces the phosphorylation of BAD at serine 128, we subjected recombinant GST-BAD that was phosphorylated by JNK to mass spectrometry. Following preparative SDS-polyacrylamide gel electrophoresis and Coomasie staining, the JNK-phosphorylated BAD band was cut from the gel, digested with trypsin, and subjected to LC-MS/MS. Trypptic peptides were identified that matched the sequence of BAD. Analysis of the peptide encompassing serine 128 (His-110 to Arg-131) revealed that serine 128 within this peptide is modified by a phosphate group (Fig. 1B). These results indicate that JNK induces the phosphorylation of BAD at serine 128 in vitro.

To further support the conclusion that JNK phosphorylates BAD at serine 128, we employed a phosphospecific antibody that we raised to recognize BAD only when BAD is phosphorylated at serine 128 (phospho128-BAD antibody) (22). Recombinant BAD protein was subjected to an in vitro kinase reaction with JNK or Cdc2 and then immunoblotted with the phospho128-BAD or the N-20 BAD antibody that recognizes BAD regardless of its phosphorylation state. In these experiments, we found that JNK induced the phosphorylation of BAD at serine 128 as robustly as Cdc2 (Fig. 2A). We also determined whether JNK induces the phosphorylation of BAD at the nearby sites of serine 112 and serine 136, sites that are phosphorylated by kinases propagating survival signals (10). Cyclic AMP-dependent kinase (protein kinase A) phosphorylated BAD in vitro at serine 112 and serine 136 but not at serine 128 (Fig. 2B). By contrast, JNK induced the phosphorylation of BAD at serine 128 but failed to phosphorylate BAD effectively at serine 122 or serine 136 (Fig. 2B). Together, these results establish that JNK phosphorylates BAD specifically at serine 128 in vitro.

JNK Phosphorylates BAD at Serine 128 in Vivo—We next determined whether activation of the JNK signaling pathway

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induces the phosphorylation of BAD at serine 128 within cells. We expressed BAD alone or together with an expression plasmid encoding the activated form of the kinase MEKK1 in the mouse neuroblastoma cell line Neuro2A. MEKK1 is the prototypical MAPK kinase kinase that activates the JNK signaling pathway. One day after transfection, we subjected Neuro2A cell lysates to immunoblotting using the phospho128-BAD antibody (fig. 3A). The lower band is a degradation product of BAD. Together, these results suggest that JNK mediates the ability of stress-induced signals to induce the phosphorylation of BAD at serine 128.

FIG. 1. JNK catalyzes the phosphorylation of BAD in vitro. A, recombinant GST-BAD was incubated with no kinase (lane 2) or with recombinant JNK1α1 (lane 3) and subjected to an in vitro kinase assay using [-γ-32P]ATP. Phosphorylated GST-BAD was separated by SDS-PAGE and visualized by autoradiography (upper panel). Coomassie Brilliant Blue (CBB) staining of GST-BAD is also shown (lower panel). B, mass spectrometric analysis of JNK-phosphorylated GST-BAD. GST-BAD was subjected to an in vitro kinase reaction using JNK1α1. JNK-phosphorylated GST-BAD was separated by SDS-PAGE, cut from the gel, digested with trypsin, and subjected to LC-MS/MS. BAD peptides were identified including one (His110 to Arg131) displaying a phosphorylated Ser128 and an oxidized Met123. Shown are the predicted b- and y-type fragment ions (singly charged) and acquired tandem mass spectrum containing prominent y-ions bearing the phosphate group at serine 128.

To determine whether the JNK signaling pathway induces the phosphorylation of endogenous BAD in vivo, we tested whether anisomycin, a stimulus known to induce the JNK signaling pathway in cells (23), induces the phosphorylation of BAD at serine 128 in Neuro2A cells. We found that exposure of NIH-3T3 cells to anisomycin induced the phosphorylation of BAD at serine 128. The expression of activated MEKK3 in Neuro2A cells induced the phosphorylation of p38MAPK but not JNK (fig. 3A). We found that the p38MAPK inhibitor failed to reduce the ability of activated MEKK1 to induce the phosphorylation of BAD at serine 128. The expression of activated MEKK1 in Neuro2A cells induced the phosphorylation of p38MAPK but not JNK (fig. 3C). We found that activated MEKK1 failed to induce the phosphorylation of BAD in vivo (fig. 3C), suggesting that p38MAPK does not mediate BAD serine 128 phosphorylation. In other experiments, we determined the ability of activated MEKK1 to induce BAD phosphorylation in Neuro2A cells that were incubated with the p38MAPK inhibitor SB203580 or the JNK inhibitor SP600125 (38). We found that the p38MAPK inhibitor failed to reduce the ability of activated MEKK1 to induce the phosphorylation of BAD Serine 128 phosphorylation (fig. 3D). By contrast, the JNK inhibitor reduced significantly the MEKK1-induced BAD serine 128 phosphorylation (fig. 3D). Together, these data suggest that JNK mediates the ability of stress-induced signals to induce the phosphorylation of BAD at serine 128.

To determine whether the JNK signaling pathway induces the phosphorylation of endogenous BAD in vivo, we tested whether anisomycin, a stimulus known to induce the JNK signaling pathway in cells (23), induces the phosphorylation of BAD at serine 128 in NIH-3T3 cells. We found that exposure of NIH-3T3 cells to anisomycin induced the phosphorylation of endogenous BAD at serine 128 (fig. 4A). Inhibition of JNK activity by the inhibitor SP600125 reduced effectively anisomycin-induced phosphorylation of BAD at Serine 128 (fig. 4A). These results suggest that JNK mediates the phosphorylation of BAD at Serine 128 in vivo.

To assess whether the JNK signaling pathway induces the phosphorylation of BAD that is endogenously expressed within
neurons, we examined the phosphorylation of endogenous BAD in cerebellar granule neurons that were exposed to anisomycin. We found that anisomycin induces the phosphorylation of endogenous BAD at serine 128 in cerebellar granule neurons (Fig. 4B). We also determined the effect of activated MEKK1 on the phosphorylation of endogenous BAD in cerebellar granule neurons. We carried out immunocytochemical analyses of cerebellar granule neurons that were transfected with the activated MEKK1 expression plasmid or the control expression plasmid. We found that a small fraction (14.3 ± 4.2%) of granule neurons transfected with the control vector plasmid displayed phospho128-BAD immunoreactivity (Fig. 4C). However, phospho128-BAD immunoreactivity was detected in a large fraction (71.4 ± 3.6%) of granule neurons expressing activated MEKK1 (Fig. 4C). These results suggest that activation of the JNK signaling pathway induces the phosphorylation of endogenous BAD at serine 128 in granule neurons.

**JNK-induced Phosphorylation of BAD at Serine 128 Activates BAD**—To determine the functional effect of the JNK-induced phosphorylation of BAD at serine 128 on the function of BAD, we expressed BAD alone or together with activated MEKK1 in primary cerebellar granule neurons. After transfection, granule neurons were deprived of survival factors or treated with a high concentration of insulin (10 μg/ml) that activates the IGF1 receptor for 8 h. The expression of BAD alone induced apoptosis of cerebellar granule neurons, and IGF1 receptor activation inhibited BAD-mediated apoptosis (Fig. 5). The expression of activated MEKK1 together with BAD did not increase BAD-mediated apoptosis in survival factor-deprived granule cell cultures (Fig. 5). However, IGF1 receptor activation failed to inhibit the apoptotic effect of BAD when BAD was co-expressed with activated MEKK1 (Fig. 5). These results suggest that activated MEKK1 induces a signal that opposes growth factor inhibition of BAD.

We next determined the role of MEKK1-induced phosphorylation of BAD at serine 128 in MEKK1 induction of BAD-mediated apoptosis. We tested the effect of activated MEKK1 on the apoptotic effect of a BAD mutant in which serine 128 was replaced with alanine (BAD128A). In contrast to the ability of activated MEKK1 to promote the apoptotic effect of wild type BAD in insulin-treated granule neuron cultures, activated MEKK1 failed to antagonize the ability of insulin to inhibit the apoptotic effect of BAD128A (Fig. 5). Together, these results suggest that activation of the JNK signaling pathway induces the phosphorylation of BAD at serine 128 and thereby promotes the apoptotic effect of BAD by antagonizing growth factor inhibition of BAD.

**DISCUSSION**

In this study, we have characterized a novel mechanism by which the JNK signaling pathway promotes neuronal apopto-
phosphorylation of BAD, whether it is induced by Cdc2 or by JNK, activates the apoptotic function of BAD. We have found that the JNK-induced serine 128 phosphorylation of BAD promotes the apoptotic effect of BAD by antagonizing growth factor-inhibition of BAD-mediated apoptosis. The serine 128 phosphorylation of BAD opposes growth factor suppression of BAD by inhibiting the interaction of growth factor-inhibited serine 136-phosphorylated BAD with 14-3-3 proteins (22).

The finding that JNK-induced phosphorylation of BAD at serine 128 opposes growth factor inhibition of BAD supports the idea that BAD is a point of convergence for both survival and apoptotic signals. Survival-promoting kinases including Akt, Rsk, p21-activated kinase 1, p70S6 kinase, and mitochondrially anchored protein kinase A induce the phosphorylation of BAD at serine 112 or serine 136 leading to the sequestration of BAD with 14-3-3 proteins (12–21), whereas the apoptosis-inducing kinases JNK and Cdc2 induce the phosphorylation of BAD at serine 128 (this study and Ref. 22). Thus, BAD might serve as a protein that integrates pro-survival and pro-apoptotic signals with the net effect contributing to the decision of the cell to survive or undergo apoptosis.

JNK-induced apoptosis is thought to play an important role in the development of the nervous system (39, 40). In future studies, it will be important to determine whether the JNK-induced phosphorylation of BAD plays a role in neuronal cell death during normal brain development. Neuronal apoptosis is also thought to contribute to neuronal cell loss upon exposure to pathogenic stimuli, including those that occur in ischemia, epilepsy, and neurodegenerative diseases of the brain (2–4). It will be interesting to determine whether JNK-induced BAD serine 128 phosphorylation contributes to neuronal cell loss in these neuropathological conditions.

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