Activation of JNK1, RSK2, and MSK1 Is Involved in Serine 112
Phosphorylation of Bad by Ultraviolet B Radiation*

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The Bcl-2 family member Bad is a pro-apoptotic protein and phosphorylation of Bad by cytokines and growth factors promotes cell survival in many cell types. Induction of apoptosis by UV radiation is well documented. However, little is known about UV activation of cell survival pathways. Here, we demonstrate that UVB induces Bad phosphorylation at serine 112 in JNK1, RSK2, and MSK1-dependent pathways. Inhibition of MAP kinases including ERKs, JNKs, and p38 kinase by the use of their respective dominant negative mutant or a specific inhibitor for MEK1 or p38 kinase, PD98059 or SB202190, resulted in abrogation of UVB-induced phosphorylation of Bad at serine 112. Incubation of active MAP kinase members with Bad protein showed serine 112 phosphorylation of Bad by JNK1 only. However, activated RSK2 and MSK1, downstream kinases of ERKs and p38 kinase, respectively, also phosphorylated Bad at serine 112 \textit{in vitro}. Cells from a Coffin-Lowry syndrome patient (deficient in RSK2) or expressing an N-terminal or C-terminal kinase-dead mutant of MSK1 were defective for UVB-induced serine 112 phosphorylation of Bad. Furthermore, MAP kinase pathway-dependent serine 112 phosphorylation was shown to be required for dissociation of Bad from Bcl-X\textsubscript{L}. These data illustrated that UVB-induced phosphorylation of Bad at serine 112 was mediated through MAP kinase signaling pathways in which JNK1, RSK2, and MSK1 served as direct mediators.
The development and maintenance of healthy tissues is critically dependent on a balance between cell survival and cell death (apoptosis). Alterations of both pathways contribute to the clonal expansion of cancer cells. The Bcl-2 family of related proteins contains protein-protein interaction domains that facilitate homo- and heterodimerization. Some members, Bcl-2, Bcl-XL, Mcl-1, and A1, promote cell survival whereas others, Bad, Bid, Bax, and Bak, promote cell death. A possible mechanism exists whereby the interactions resulting in homo- or heterodimerization of the various proteins define the fate of a cell (1, 2). Bad, for example, has been shown to heterodimerize with Bcl-XL through interaction with its BH3 domain at the mitochondrial membrane (3, 4). The complex formation of Bad with Bcl-XL may cause Bcl-XL to release Apaf1 or regulate other Bcl-XL activities resulting in a caspase 9-initiated cascade of proteolysis and induction of apoptosis (5, 6). Survival factors such as interleukin (IL) \(^1\)-3 can inhibit the apoptotic activity of Bad by activating intracellular signaling pathways that result in the phosphorylation of Bad at two critical sites, serine 112 and serine 136 (7). Akt has been shown to promote cell survival through its ability to phosphorylate Bad specifically at serine 136 (8, 9). Recent studies showed that RSK2 (p90 ribosomal S6 kinase 2), mitochondria-associated protein kinase A (PKA), and \(\alpha\)- or \(\gamma\)-p21-activated protein kinase (PAK) can mediate cytokine or growth factor-induced phosphorylation of Bad at serine 112 (10-12). Phosphorylation of Bad at serine residues 112 and 136 leads to the dissociation of Bad from pro-survival Bcl-XL protein (7). Mutation of either of these residues to alanine potentiates cell death following transient transfection with Bad, suggesting that both are critical in the disruption of Bad-Bcl-XL heterodimers.

Ultraviolet (UV) radiation, especially in the UVB range (290-320 nm), is an important environmental factor of inducible health hazards for mankind, which include the induction of
skin cancer (15), suppression of the immune system (16), and chronic skin damage including premature skin aging (17). Similar to chemical agents, UV has the ability to activate various signal transduction pathways and to induce the expression of specific genes (18-20). A great deal of progress has been made recently in elucidating the mechanisms of the UV-induced apoptotic signaling transduction pathways (21, 22). However, much less is known about the UV-induced survival-signaling pathway, especially during the immediate time following UV radiation.

One of the major UV responsive pathways is the ras/mitogen-activated protein (MAP) kinases cascade (23). MAP kinases belong to a large family of serine/threonine protein kinases comprised of three distinct components: extracellular-signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinase. Generally, JNKs and p38 kinase are known to be activated by various forms of stress, such as UV radiation, heat shock, and inflammation (24-26). Our studies and those of others have shown that ERKs are critical for UV-induced signal transduction (27-29). Although UVB radiation has been shown to induce cytokine production (30-33) and to activate growth factor and cytokine receptors (26), whether UVB radiation induces Bad phosphorylation and the signaling pathways that are involved in the phosphorylation remain largely unknown. MAP kinases have been implicated in both apoptosis and survival signaling (34-37). Therefore, we investigated the possible role of MAP kinase signaling pathways in the regulation of Bad phosphorylation and its function following UVB radiation. In this study, we demonstrated that UVB radiation induces Bad phosphorylation at serine 112, but not serine 136. Using a dominant negative mutant of ERK2, JNK1, p38 kinase, or an N-terminal or C-terminal kinase-dead mutant of mitogen- and stress-activated protein kinase 1 (MSK1), RSK2-deficient cells, and a specific inhibitor of mitogen-activated protein kinase kinase 1 (MEK1) or p38 kinase, we conclude that UVB-induced phosphorylation of Bad at serine 112 is
mediated through MAP kinase signaling pathways in which JNK1, RSK2, and MSK1 have a direct role in the regulation of Bad phosphorylation and its function.

**EXPERIMENTAL PROCEDURES**

*Materials*—Dominant negative (DN) mutants of ERK2, JNK1, and p38 kinase were generous gifts from Dr. Melanie H. Cobb (38), Dr. Roger J. Davis (39), and Dr. Mercedes Rincon (40), respectively; plasmids of cytomegalovirus (CMV) 5 vector, N-terminal, or C-terminal kinase-dead mutant of MSK1 were kindly provided by Dr. Dario R. Alessi (41); active recombinant ERK1 (100 units/mg), JNK1 (85 units/mg), JNK2 (100 units/mg), p38 kinase (157 units/mg), RSK2 (350 units/mg), MSK1 (167 units/mg), mitogen activated protein kinase-activated protein kinase 2 (MAPKAPK-2) (442 units/mg), Bad fusion proteins, polyclonal RSK2 antibody, and RSK2 immunoprecipitation kinase assay kit were from Upstate Biotechnology, Inc. (Lake Placid, NY); and active recombinant ERK2 (10,000 units/mg), phospho-specific Bad (Ser112) and Bad (Ser136) antibodies, phosphoPlus p44/42 MAP kinase, JNK, and p38 kinase antibody kits, p44/42 MAP kinase, JNK, and p38 kinase assay kits were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The monoclonal Bad antibody (B36420) was from Pharmingen Laboratories (Los Angeles, CA); the polyclonal Bcl-XL antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and the monoclonal β-actin antibody was from Sigma (St. Louis, MO). MEK1-specific inhibitor, PD98059, was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA); p38 kinase inhibitor, SB202190, was from Calbiochem (LaJolla, CA); Eagle’s minimum essential medium (MEM) and RPMI 1640 medium were from Life Technologies, Inc. (Baltimore, MD); and fetal bovine serum (FBS) was from Biowhittaker, Inc (Baltimore, MD).
UV Radiation—UVB radiation was performed on serum-starved monolayer cultures utilizing a transluminator emitting UVB (42). The source of UVB was a bank of four Westinghouse F520 Lamps (National Biological, Twinsburg, OH) at 6 J/S/m light in the UVB range. Approximately 10% of the remaining radiation from the F520 lamp is in the UVA region (320 nm). Although almost no UVC leakage occurred, the UVB radiation was carried out in a UVB exposure chamber fitted with a Kodak Kodacel K6808 filter that eliminates all wavelengths below 290 nm. This lamp is one of the most frequently used UVB sources for the study of carcinogenesis. The International Agency for Research on Cancer refers to this lamp as a source emitting mainly UVB radiation for the study of cancer induction in animals. UVB radiation was measured using the UVX radiometer from UVP (UVX-31).

Generation of Stable Cotransfectants—JB6 Cl 41 cells were transfected with CMV-neo or CMV5 vector with or without the plasmids of dominant negative mutant of ERK2, JNK1, p38 kinase, or N-terminal or C-terminal kinase-dead mutant of MSK1 by using LipofectAMINE following the manufacturer's instructions. The stable transfectants were obtained by selection for G418 resistance (400 µg/ml) and further confirmed by assay of respective activity as described (27, 43-46).

Cell Culture—The JB6 mouse epidermal cell line Cl 41 and its stable transfectants, CMV-neo, DN-ERK2, DN-JNK1, DN-p38 kinase, CMV5, N-MSK1, and C-MSK1 were cultured in monolayers at 37 °C and 5% CO2 using MEM containing 5% FBS, 2 mM L-glutamine, and 25 µg/ml gentamicin (27, 43-46). Lymphoblast cells that originated from a Coffin-Lowry syndrome (CLS) patient (deficient in RSK2) and a clinically unaffected person (Normal) (Coriell Institute for Medical Research, Camden, NJ) were cultured in RPMI 1640 medium with 15% FBS, 2 mM L-glutamine, and 25 µg/ml gentamicin. The CLS patient was an
8-year-old male. Clinically unaffected lymphoblasts were obtained from an age- and race-
matched male.

**Immunoblotting and Immunoprecipitation**—Immunoblotting for phosphorylated proteins of ERKs, JNKs, and p38 kinase was carried out using phospho-specific antibodies against phosphorylated sites of ERKs, JNKs, or p38 kinase, respectively (28, 45). To study the effect of UVB radiation on the induction of Bad phosphorylation, the Bad protein was first immunoprecipitated with a specific antibody. The immunocomplex was then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the phospho-specific antibodies against Bad at serine 112 and serine 136. Briefly, cells were cultured in 100-mm dishes until they reached 80-90% confluence. Then, the cells were starved by culturing them in 0.1% FBS MEM or 0.5% RPMI 1640 medium for 24 h. The cells were exposed to UVB radiation to induce Bad phosphorylation and then were disrupted on ice for 30 min in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates containing 500 µg of protein were immunoprecipitated using a monoclonal antibody against Bad and then protein A/G plus agarose to capture the complex. The beads were washed extensively to eliminate nonspecific binding and levels of phosphorylated proteins of Bad at serine 112 and serine 136 and total Bad were selectively measured by Western immunoblotting using a specific antibody and a chemifluorescence detection system (ECF; Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

**Co-immunoprecipitation of Proteins**—To study the effect of UVB radiation on the interaction of Bad with Bcl-XL *in vivo*, cell lysates were first immunoprecipitated with an
antibody against Bad as described above. Immunoprecipitates of Bad were immunoblotted with the antibodies against Bcl-X₁.

**RSK2 Immunoprecipitation Kinase Assay**—The assay of RSK2 was carried out as described by the protocol of Upstate Biotechnology, Inc. Briefly, cells were exposed to UVB (4 kJ/m²) and then the cells were disrupted in 300 µl of the lysis buffer as described above. The lysates were sonicated and centrifuged. The supernatant fraction containing 500 µg of protein was incubated with 4 µg of anti-RSK2 antibody for 6-10 h at 4 °C, followed by incubation with protein A/G plus-agarose for another 4 h. The enzyme immune complex was washed three times with 0.5 ml of lysis buffer and once with 100 µl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, pH 7.0, 1 mM sodium orthovanadate, 1 mM DTT). Then, the enzyme immune complex was added to 10 µl of assay dilution buffer, 10 µl of 50 µM RSK2 substrate peptide, 10 µl of inhibitor cocktail and 10 µCi [γ-32P]ATP. The reaction was incubated for 10 min at 30 °C and centrifuged and then 30 µl of the supernatant fraction was transferred onto P81 phosphocellulose paper and allowed to bind for 30 s. The P81 papers were washed four times in 0.75% phosphoric acid and then washed once in acetone and γ-32P incorporation was measured by scintillation counting.

**Phosphorylation of Bad by UVB-activated RSK2**—Cells were exposed to UVB (4 kJ/m²) and cultured for an additional 30 min. Lysates were prepared from the cells and the immunoprecipitation of RSK2 was described above. The enzyme immune complex was washed twice with 500 µl of the lysis buffer and twice with 500 µl of kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 10 mM MgCl₂). The kinase reactions were carried out in the presence of 200 µM ATP at 30 °C for 30 min using 3 µg
of Bad as substrate. The phosphorylated proteins were detected by immunoblotting using phospho-specific antibodies.

**Phosphorylation Assay of Bad Protein In Vitro**—Phosphorylation of Bad by active recombinant ERK1, ERK2, JNK1, JNK2, p38 kinase, RSK2, MSK1, or MAPKAPK-2 was carried out at 30 °C for 30 min in the presence of the kinase buffer with 200 µM ATP and 3 µg of Bad as substrate. The phosphorylated Bad protein was detected by immunoblotting using a phospho-specific antibody.

**RESULTS**

*Induction of Bad Phosphorylation at Serine 112, but not Serine 136, by UVB Radiation*—Because the phosphorylation of serine 112 and serine 136 is critical for Bad function (7), we first investigated whether exposure of cells to UVB radiation results in phosphorylation of Bad at these sites. We used a specific antibody to detect Bad phosphorylation at serine 112 or serine 136 by Western blot analysis (Cell Signaling Technology, Inc.) (11). The specificity of the antibody to Bad phosphorylation at serine 112 or serine 136 was confirmed previously by showing its reactivity to recombinant Bad protein, which was phosphorylated by PKA or RSK2, but not to unphosphorylated Bad, and by its recognition of TPA- or forsklin-induced phosphorylated wild-type Bad, but not of a Bad mutant with substitution of serine 112 or serine 136 with alanine that was expressed in 293T cells (Cell Signaling Technology, Inc.) (11). As shown in Fig. 1A, phosphorylation of Bad at serine 112 occurred 15 min after cells were exposed to UVB radiation and reached a maximum at 30 min. A dose-response study indicated that 4 kJ/m² was the optimal dosage for induction of Bad phosphorylation at serine 112 (Fig. 1B). In contrast, phosphorylation of Bad at serine 136 was not observed in either experiment (Fig. 1A and B). These results
indicated that UVB radiation only induces Bad phosphorylation at serine 112 in a time- and dose-dependent manner.

**UVB-induced Bad Phosphorylation at Serine 112 Is MAP Kinase-dependent**—Our previous studies indicated that in JB6 Cl 41 cells, UVB leads to activation of the MAP kinase superfamily, composed of ERKs, JNKs, and p38 kinase, even at 15 min after UVB radiation (28, 45-47). Therefore, we investigated the possible role of the MAP kinase family in mediating the phosphorylation of Bad at serine 112. To test this, we used two approaches to inactivate MAP kinases. First, PD98059, a specific inhibitor of MEK1 that acts by inhibiting activation of ERKs (48), and SB202190, a specific inhibitor of p38 kinase (49), were tested for their effect on UVB-induced serine 112 phosphorylation of Bad. Pretreatment with 12.5 µM of PD98059 or 0.5 µM of SB202190, which specifically inhibited UVB-induced phosphorylation of ERKs (Fig. 2A) or p38 kinase (Fig. 2B), but not JNKs (Fig. 2C), markedly impaired the phosphorylation of Bad at serine 112 (Fig. 2D). These data suggested that both ERKs and p38 kinase may be involved in UVB-induced serine 112 phosphorylation of Bad. The second strategy used to inactivate MAP kinases was to use dominant negative mutants of ERK2, JNK1, or p38 kinase. Previous studies have shown that overexpression of DN-ERK2, DN-JNK1, or DN-p38 kinase specifically blocked UVB-induced activation of ERKs, JNKs, or p38 kinase, respectively (27, 28, 43-45, 47). Compared with Cl 41 cells expressing CMV-neo vector, the expression of DN-ERK2, DN-JNK1, or DN-p38 kinase markedly suppressed Bad phosphorylation at serine 112 after exposure of cells to UVB for up to 60 min (Fig. 3A-C). These two experiments demonstrated that UVB-induced phosphorylation of Bad at serine 112 is mediated through MAP kinases, including ERKs, JNKs, and p38 kinase.
Phosphorylation of Bad at Serine 112 by Activated JNK1, RSK2, and MSK1 In Vitro—To test whether MAP kinases are direct mediators of UVB-induced phosphorylation of Bad at serine 112, we incubated Bad fusion protein with one of several pure and active recombinant MAP kinase family members in the presence of ATP. The results showed that Bad at serine 112 was phosphorylated strongly by active JNK1, but not by active JNK2, ERK1, ERK2, or p38 kinase (Fig. 4A). Although ERKs and p38 kinase could not directly phosphorylate Bad at serine 112, inactivated ERKs and p38 kinase significantly inhibited serine 112 phosphorylation in vivo (Fig. 2 and Fig. 3). This prompted us to test whether downstream members of ERKs or p38 kinase, such as RSK2 (50), MSK1 (41, 51) or MAPKAPK-2 (52), catalyze the phosphorylation of Bad at serine 112. In JB6 Cl 41 cells, UVB induced RSK2 activity and reached a maximum at 15-30 min (Fig. 4B). The activation of RSK2 induced by UVB was markedly inhibited by pretreatment with 12.5-25 µM PD98059 or using cells expressing DN-ERK2 (Fig. 4B), indicating that RSK2 is a downstream kinase of the UVB-activated ERK signaling pathway. We performed an immune complex phosphorylation assay of UVB-activated RSK2, which was immunoprecipitated from cells exposed to UVB, using a Bad fusion protein as the substrate. The result showed that UVB-activated RSK2 phosphorylated Bad at serine 112, whereas pretreatment with 12.5 µM PD98059 blocked the phosphorylation (Fig. 4C). In addition, phosphorylation of Bad at serine 112 was further confirmed by using pure and active recombinant RSK2 (Fig. 4D). Very recently, we have shown that MSK1 was also activated in JB6 Cl 41 cells at 5-30 min after UVB radiation and inhibition of ERKs or p38 kinase markedly repressed UVB-induced MSK1 activities (45, 46). Therefore, we performed a phosphorylation assay of Bad protein by active MSK1 in vitro and found that Bad could also be phosphorylated at serine 112 by active MSK1, but not by active
MAPKAPK-2 (Fig. 4E), another downstream kinase of p38 kinase (52). These results suggested that Bad at serine 112 phosphorylation is mediated via JNK1, RSK2, and MSK1.

**Inactivated RSK2 or MSK1 Abrogates UVB-induced Bad Phosphorylation at Serine 112**

*In Vivo*—To further confirm that RSK2 and MSK1 have specific roles in UVB-induced phosphorylation of Bad at serine 112, we used RSK2-deficient lymphoblasts derived from a CLS patient or established a stable transfectant with an N-terminal kinase-dead mutant of MSK1 (N-MSK1) or a C-terminal kinase-dead mutant of MSK1 (C-MSK1). Western blot analysis confirmed a complete loss of RSK2 protein in the CLS patient (Fig. 5A). Compared to the normal lymphoblasts, UVB-induced activation of RSK2 was totally blocked in CLS lymphoblasts (Fig. 5B). As a result, the phosphorylation of Bad at serine 112 induced by UVB radiation was shown to be profoundly suppressed in CLS lymphoblasts (Fig. 5C). Overexpression of N-MSK1 or C-MSK1 markedly inhibited UVB-induced MSK1 activity as described previously (45, 46). Using these stable transfectants, we found that both N-terminal mutant MSK1 and C-terminal mutant MSK1 attenuated UVB-induced serine 112 phosphorylation of Bad, compared with a CMV5 vector *in vivo* (Fig. 6). Together, these results indicated that both RSK2 and MSK1 indeed mediate phosphorylation of Bad at serine 112 in response to UVB radiation.

**MAP Kinases Signaling-dependent Serine 112 Phosphorylation Dissociates Bad from Bcl-X<sub>L</sub>**—Having established the role of MAP kinases and their downstream kinases in mediating Bad phosphorylation at serine 112 following UVB radiation, we determined whether MAP kinase pathway-dependent phosphorylation of serine 112 was important for disrupting Bad-Bcl-X<sub>L</sub> heterodimerization. During apoptosis, Bad heterodimerization may play a significant role in promoting death signal by inactivating Bcl-X<sub>L</sub> (4, 7). Our results showed that Bcl-X<sub>L</sub> was not co-precipitated with Bad at 30 min after UVB radiation, consistent with serine 112 phosphorylation-
induced dissociation (Fig. 7A). Inhibition of Bad phosphorylation at serine 112 by pretreatment with PD98059 or SB202190 (Fig. 7A) or using cells expressing DN-JNK1 (Fig. 7B), restored Bad-Bcl-X<sub>L</sub> association. Phosphorylation of Bad at serine 136 has also been shown to play an important role in disassociation of Bad from Bcl-X<sub>L</sub> (7, 8). However, our previous study showed that inhibition of ERKs or p38 kinase blocked UVB-induced activation of Akt (45), which is responsible for serine 136 phosphorylation of Bad (8, 9). Furthermore, phosphorylation of Bad at serine 136 could not still be induced by UVB radiation following inactivation of MAP kinases (Fig. 7, A and B). These data demonstrated that MAP kinase pathway-dependent serine 112 phosphorylation of Bad is critical for the dissociation of Bad-Bcl-X<sub>L</sub> dimers in the early response to UVB radiation.

DISCUSSION

The phosphorylation of Bad, a Bcl-2 family protein, may represent an important bridge between survival signaling by growth factor receptors and the prevention of apoptosis. Oncogenes involved in the signal transduction of growth factor receptors may mediate the requirement for extracellular stimuli to maintain protection from apoptosis, in part by increasing Bad phosphorylation. Therefore, identifying the specific signaling pathways involved in the regulation of Bad is crucial in our understanding of oncogenesis. In this study, we demonstrated that Bad is phosphorylated at serine 112, but not serine 136, early after UVB radiation. Furthermore, we found that UVB-induced serine 112 phosphorylation of Bad depends on MAP kinase signaling pathways in which JNK1 directly mediates serine 112 phosphorylation, whereas RSK2 and MSK1 transduce ERKs and p38 kinase signals by phosphorylating Bad.
Exposure of cells to UV radiation elicits a complex set of acute cellular responses called “UV responses.” The initial signal triggering the UV response is in large part independent of DNA damage, but it instead appears to be mediated by a membrane-associated component of the Ras pathway and activation of MAP kinases (23). ERKs are involved in survival signaling in response to a variety of growth factors (10, 35, 53), whereas activation of JNKs or p38 kinase is suggested to play decisive roles in the control of cell death (34). The early activation of JNKs and p38 kinase by tumor necrosis factor-α and overexpression of MAP kinase kinase 6, an upstream kinase of p38 kinase, also have been reported to contribute to survival signaling (36, 37). Our recent study demonstrates the requirement of JNK activation for tumor necrosis factor-α-induced JB6 cell transformation (44). Embryos with disruption of Jnk1 and Jnk2 genes exhibit increased apoptosis in the development of forebrain (54, 55). In addition, integrin-mediated survival signaling has been shown to be mediated by the JNK pathway (56). However, little direct evidence has been obtained to show that the MAP kinase family regulates survival-signaling components in response to UV radiation. Very recently, we reported that ERK- and p38 kinase-dependent MSK1 activation, in addition to the phosphatidylinositol 3-kinase (PI3-K) pathway, is required for Akt activation early after UVB radiation (45). In the present study, we further found that MAP kinases mediate UVB-induced Bad phosphorylation at serine 112. The results of our study indicated that JNK1 is a direct mediator of UVB-induced phosphorylation of Bad at serine 112 (Fig. 3B and Fig. 4A). Although ERKs and p38 kinase did not directly phosphorylate Bad at serine 112, RSK2, a downstream kinase of ERKs (Fig. 4B) (50), and MSK1, a downstream kinase of ERKs and p38 kinase (41, 45, 46, 51), were shown to be responsible for the phosphorylation in vitro and in vivo (Figs. 4-6). Furthermore, the MAP kinase-dependent phosphorylation of Bad at serine 112 was found to be required for Bad dissociation from Bcl-XL.
(Fig. 7). Therefore, these data suggest a novel role for MAP kinases and their downstream kinases in the regulation of survival signal transduction pathways immediately following UV radiation. However, the significance of the members of MAP kinases being required for UVB-induced serine 112 phosphorylation of Bad and regulation of its function is not presently known. Some evidence indicates that cross-talk among ERKs, JNKs and p38 kinase signaling may play an important role in determining cell survival and death (57, 58). Further study will be required to confirm this hypothesis.

UV-induced Bad phosphorylation at a single residue, serine 112, and activation of the PI3-K/Akt survival pathway have also recently been observed in human skin cells by Wan et al. (59). They showed that the UV-induced serine 112 phosphorylation of Bad occurred in a PI3-K-dependent manner by using PI3-K inhibitors such as LY294002 and wortmannin. However, Akt, a downstream kinase of PI3-K, has been shown to catalyze the phosphorylation of Bad specifically at serine 136, but not serine 112 (8, 9). Furthermore, previous studies have demonstrated the inhibitory effect of both LY294002 and wortmannin on ERKs activation in several cell types after various modes of stimulation (60-62). Our recent results also showed that pretreatment with LY294002 or overexpression of a dominant negative mutant of PI3-K subunit p85 blocks UVB-induced ERKs and MSK1 activation (45) as well as activation of RSK2 (data not shown), indicating the requirement for PI3-K upstream of ERKs following UVB radiation. The phosphorylation of Bad at serine 112 induced by UVB was also confirmed to be partially inhibited by pretreatment with 12.5-25 µM LY294002 in our experiments (data not shown). However, in light of the above findings and in context with our results here, inhibition of Bad serine 112 phosphorylation by LY294002 most likely occurs through inhibition of the ERKs/RSK2 and ERKs/MSK1 pathways, but not Akt.
PKA has been shown to mediate IL-3-induced phosphorylation of Bad at serine 112 in a cAMP-dependent manner, offering an explanation for the survival-promoting effects of cAMP in some cell types (12). However, recent studies have reported that the level of cAMP was not affected after stimulation with cytokines, suggesting that PKA activation by cAMP is not the principle means for Bad phosphorylation at serine 112 (63, 64). Very recently, we showed that PKA was not activated following UVB radiation (46). Therefore, we conclude that PKA may not be involved in UVB-induced Bad phosphorylation at serine 112.

Recently, α- and γ-PAK have also been shown to phosphorylate Bad at serine 112 in vitro and in vivo (13, 14). Activation of α-PAK was shown to be induced by IL-3 in FL5.12 lymphoid progenitor cells (13), but not by tumor necrosis factor-α (TNF-α) in BALB3T3 fibroblasts (14). These results indicate that activation of α-PAK may depend on cell type differences or different extracellular stimuli. Furthermore, whether α-PAK is involved in IL-3-induced endogenous Bad phosphorylation at serine 112 has not been determined (13). Overexpression of constitutively active γ-PAK stimulates cell survival of BALB3T3 fibroblasts in response to TNF-α, growth factor withdrawal, and UVC radiation (14). The authors suggested that phosphorylation of Bad at serine 112 by γ-PAK may be one of mechanisms for protection from cell death. However, whether UVC radiation induction of endogenous Bad phosphorylation and γ-PAK is required for the phosphorylation has not yet been investigated. Interestingly, expression of active γ-PAK increases the early activation of ERKs, JNKs and p38 kinase induced by TNF-α (14). Currently, we are also identifying the upstream effectors of MAP kinases involved in UVB-induced phosphorylation of Bad at serine 112. Whether γ-PAK is one of the candidate effectors will need to be determined.
A number of published works have suggested that serine 136 phosphorylation of Bad is physiologically important. Expression of a mutant Bad in which serine 136 was changed to alanine potentiates apoptosis, arguing that the inability of Akt to phosphorylate this altered residue promotes association with Bcl-X\textsubscript{L}, thus leading to cell death (7, 8, 65). However, singly-phosphorylated Bad at serine 112 also proved incapable of binding to Bcl-X\textsubscript{L} (7). Our studies here with endogenous Bad argue that Bad-Bcl-X\textsubscript{L} association is disrupted independently of serine 136 phosphorylation. Rather, the dissociation is primarily dependent upon the phosphorylation state of serine 112. The phosphorylation of Bad at serine 136 could not be detected following UVB radiation (Fig. 1). Furthermore, Bad was not shown to be phosphorylated at serine 136 by active MAP kinases (data not shown) and inhibition of MAP kinases did not result in activation of other protein kinases such as Akt (45) to phosphorylate Bad at serine 136 after UVB radiation (Fig. 7). On the other hand, loss of serine 112 phosphorylation of Bad, through inhibition of MAP kinases including ERKs, JNKs, and p38 kinase, completely restores the association of Bad with Bcl-X\textsubscript{L} (Fig. 7). Therefore, these results suggest that serine 136 may not necessarily be phosphorylated for cell survival in response to UVB radiation.

In summary, our studies demonstrate that the phosphorylation of Bad at serine 112 induced by UVB radiation is mediated by the signaling of MAP kinases and their downstream kinases (Fig. 8). In addition to serine 112 phosphorylation of Bad by RSK2 as demonstrated previously (10, 11), our results further identify JNK1 and MSK1 as novel and direct signal mediators of serine 112 phosphorylation in response to UVB radiation. The phosphorylation of Bad at serine 112 by MAP kinase-dependent pathways may cooperate with the PI3-K/Akt pathway (45) to balance UV-induced apoptotic signals, thereby preventing widespread cell death. Conversely, activation of these survival pathways by UV radiation may enhance inappropriate
cell survival leading to skin cancer, as has been found in several other types of cancer (29, 66). Therefore, understanding the cascade of molecular signals in the UV-induced survival pathway may be helpful in designing therapeutic targets for prevention of skin cancer induced by UV radiation.

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REFERENCES


FOOTNOTES

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1 The abbreviations used are: IL, interleukin; UV, ultraviolet; CMV, cytomegalovirus; DN, dominant negative; CLS, Coffin-Lowry syndrome; JNKs, c-Jun N-terminal kinases; ERKs, extracellular-signal-regulated protein kinases; MAP, mitogen-activated protein; MEK1, mitogen-activated protein kinase kinase 1; RSK2, p90 ribosomal S6 kinase 2; MSK1, mitogen- and stress-activated protein kinase 1; MAPKAPK-2, mitogen-activated protein kinase-activated protein kinase 2; PI3-K, phosphatidylinositol 3-kinase; PAK, p21-activated protein kinase; PKA, protein kinase A; MEM, Eagle’s minimum essential medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis.
FIGURE LEGENDS

FIG. 1. **UVB radiation induces Bad phosphorylation at serine 112, but not serine 136.** JB6 Cl 41 cells were starved by replacing the medium with 0.1% FBS MEM and culturing for 24 h. The cells were exposed to UVB (4 kJ/m²) and then cultured for the indicated times (A) or were treated with different doses of UVB and then cultured for 30 min (B). Lysates were prepared from the cells and Bad was immunoprecipitated using monoclonal Bad antibodies. The levels of phosphorylated Bad at serine 112 or serine 136 and total Bad were selectively measured as described under “Experimental Procedures.”

FIG. 2. **Inhibition of UVB-induced Bad phosphorylation at serine 112 by MEK1 inhibitor, PD98059, and p38 kinase inhibitor, SB202190.** JB6 Cl 41 cells were starved by replacing the medium with 0.1% FBS MEM and culturing for 24 h. The cells were then pretreated with PD98059 or SB202190 at the indicated concentrations for 30 min. The cells were exposed to UVB (4 kJ/m²) and subsequently cultured for 30 min. Lysates were prepared from the cells and one-tenth of the lysates was used for immunoblotting with phospho-specific or total antibodies against ERKs (A), p38 kinase (B), or JNKs (C). The rest of lysates was used for immunoprecipitation with monoclonal antibodies against Bad. The levels of phosphorylated Bad at serine 112 and total Bad (D) were assessed by immunoblotting.

FIG. 3. **Expression of a dominant negative mutant of ERK2, JNK1, or p38 kinase impairs UVB-induced Bad phosphorylation at serine 112.** JB6 Cl 41 cell stable transfectants, CMV-neo, DN-ERK2 (A), DN-JNK1 (B), and DN-p38 kinase (C) were starved by replacing the
medium with 0.1% FBS MEM and culturing for 24 h. The cells were exposed to UVB (4 kJ/m²) and subsequently cultured for the indicated times. Lysates were prepared from the cells and Bad was immunoprecipitated using monoclonal Bad antibodies. The levels of phosphorylated Bad at serine 112 and total Bad were assessed by immunoblotting.

FIG. 4. **Bad is phosphorylated at serine 112 in vitro by activated JNK1, RSK2, and MSK1.** (A) Bad serine 112 is phosphorylated by active JNK1, but not by active JNK2, ERK1/2, and p38 kinase. Phosphorylation of Bad at serine 112 by active recombinant kinases was carried out at 30 °C for 30 min in the presence of Bad fusion protein, kinase buffer, 200 µM ATP, and one of the MAP kinases family. Phosphorylation was immunodetected with antibodies against phosphorylation of Bad at serine 112. (B) RSK2 is a downstream kinase of the UVB-activated ERKs signaling pathway. JB6 Cl 41 cells (B1) and their stable transfectants, CMV-neo and DN-ERK2 (B2), were starved by replacing the medium with 0.1% FBS MEM and culturing for 24 h. The JB6 Cl 41 cells were either pretreated or not with PD98059 for 30 min at the indicated concentrations. The cells were exposed to UVB (4 kJ/m²) and subsequently cultured for the indicated times. Lysates (500 µg of protein) were prepared from the cells and RSK2 was immunoprecipitated using RSK2 antibodies. The activity of RSK2 was determined as described under “Experimental Procedures.” Data from three independent experiments were averaged and are presented as mean ± S.E. (C and D) UVB-activated or -active RSK2 phosphorylates Bad at serine 112. JB6 Cl 41 cells were treated as in B and lysed. The immunoprecipitated (IP) RSK2 was assayed for kinase activity by adding Bad fusion protein as substrate (C). Phosphorylation of Bad at serine 112 by active recombinant RSK2 was carried out as described in A, except for using active RSK2 to replace the MAP kinases in the reaction mixture (D). The levels of
phosphorylated Bad at serine 112 were assessed by immunoblotting. (E) Bad serine 112 is phosphorylated by active MSK1, but not by active MAPKAPK-2. Phosphorylation of Bad at serine 112 by active recombinant MSK1 or MAPKAPK-2 was carried out as described in A, except for using active MSK1 or MAPKAPK-2 to replace the MAP kinases in the reaction mixture.

**FIG. 5. UVB-induced Bad phosphorylation at serine 112 is blocked in RSK2-deficient cells.** (A) RSK2 is deficient in the lymphoblasts derived from a CLS patient. Extracts were prepared from normal and CLS lymphoblasts. Expression of RSK2 was immunodetected with RSK2 antibodies. β-Actin was used as an internal control to monitor equal protein loading. (B) *In vitro* RSK2 assay. Normal and CLS lymphoblasts were starved by replacing the medium with 0.5% FBS RPMI 1640 medium and culturing for 24 h. The lymphoblasts were exposed to UVB (4 kJ/m²) and subsequently cultured for the indicated times. Lysates (500 µg of protein) were prepared from the lymphoblasts and RSK2 was immunoprecipitated using RSK2 antibodies. The activity of RSK2 was determined as described under “Experimental Procedures.” Data from three independent experiments were averaged and are presented as mean ± S.E. (C) CLS cells are defective in UVB-induced Bad phosphorylation at serine 112. Normal and CLS lymphoblasts were starved by replacing the medium with 0.5% FBS RPMI 1640 medium and culturing for 24 h. The lymphoblasts were exposed to UVB (4 kJ/m²) and subsequently cultured for the indicated times. Lysates were prepared from the lymphoblasts and Bad was immunoprecipitated using monoclonal Bad antibodies. The levels of phosphorylated Bad at serine 112 and total Bad were assessed by immunoblotting.
FIG. 6. Blocking UVB-induced phosphorylation of Bad at serine 112 by overexpression of N-terminal or C-terminal kinase-dead mutant of MSK1. JB6 Cl 41 cell stable transfectants, CMV5, N-MSK1, and C-MSK1 were starved by replacing the medium with 0.1% FBS MEM and culturing for 24 h. The cells were exposed to UVB (4 kJ/m²) and subsequently cultured for the indicated times. Lysates were prepared from the cells and Bad was immunoprecipitated using monoclonal Bad antibodies. The levels of phosphorylated Bad at serine 112 and total Bad were assessed by immunoblotting.

FIG. 7. UVB-induced serine 112 phosphorylation disrupts association of Bad with Bcl-XL. JB6 Cl 41 cells (A) and their stable transfectants, CMV-neo and DN-JNK1 (B), were starved by replacing the medium with 0.1% FBS MEM and culturing for 24 h. The JB6 Cl 41 cells were either pretreated or not with PD98059 or SB202190 at the indicated concentrations for 30 min. The cells were exposed to UVB (4 kJ/m²) and subsequently cultured for 30 min. Lysates were prepared from the cells and Bad was immunoprecipitated using monoclonal Bad antibodies. The Bad immunoprecipitates were immunoblotted with Bcl-XL phospho-Bad (Ser136) or total Bad antibodies.

FIG. 8. Schematic diagram showing the involvement of MAP kinases and their downstream kinases in the phosphorylation of Bad at serine 112 induced by UVB radiation. UVB radiation induces activation of ERKs, JNKs, p38 kinase, and their downstream kinases in which JNK1, RSK2, and MSK1 are direct signal mediators of Bad phosphorylation at serine 112. The arrows indicate activation and ⊥ indicates inhibition.
Qing-Bai She et al., Fig. 1

A

Time after UVB (min)  -  15  30  60

- p-Bad (Ser112)

- p-Bad (Ser136)

- Bad

B

UVB (kJ/m²)  -  2  4  6

- p-Bad (Ser112)

- p-Bad (Ser136)

- Bad
Qing-Bai She et al., Fig. 2

<table>
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<tr>
<th>Treatment</th>
<th>SB202190 (0.5 μM)</th>
<th>PD98059 (12.5 μM)</th>
<th>UVB (kJ/m²)</th>
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**A**
- p-ERKs
- ERKs

**B**
- p-p38 kinase
- p38 kinase

**C**
- p-JNKs
- JNKs

**D**
- p-Bad (Ser112)
- Bad
Qing-Bai She et al., Fig. 3

A

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<th>Time after UVB (min)</th>
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<td>60</td>
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B

<table>
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<td>60</td>
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C

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<td>60</td>
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Qing-Bai She et al., Fig 4

A

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<th>-</th>
<th>-</th>
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B

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<tr>
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</table>

PD98059 (µM)
- - - - - - - - 12.5 25

C

<table>
<thead>
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<th>PD98059 (12.5 µM)</th>
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<tr>
<td>IP: anti-RSK2</td>
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<tr>
<td>Bad (3 µg)</td>
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<tr>
<td>UVB (4 kJ/m²)</td>
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D

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CMV-neo DN-ERK2
- - - - - - - - - -

E

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<tr>
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<td>MSK1 (units)</td>
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<td>Bad (µg)</td>
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</table>

p-Bad (Ser112) →
Bad →
Qing-Bai She et al., Fig. 6

<table>
<thead>
<tr>
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<th>CMV5</th>
<th>N-MSK1</th>
<th>C-MSK1</th>
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</table>

- p-Bad (Ser112)
- Bad
Qing-Bai She et al., Fig. 7

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.5 μM</th>
<th>12.5 μM</th>
<th>UVB (kJ/m²)</th>
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<td>SB202190</td>
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</tr>
<tr>
<td>PD98059</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>UVB (kJ/m²)</td>
<td>-</td>
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</table>

B

IP: anti-Bad

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CMV-neo</th>
<th>DN-JNK1</th>
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</thead>
<tbody>
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<td>UVB (kJ/m²)</td>
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<td>+</td>
</tr>
</tbody>
</table>

- Bcl-X<sub>L</sub>
- p-Bad (Ser136)
- Bad
Qing-Bai She et al., Fig. 8

UVB

Membrane

Cytoplasm

Mutant

JNK1 → JNK2 → MEK1 → ERK1/2 → RSK2 → MSK1 → p38

PD98059 → SB202190

Mutant

Ser112

Bad

Bad → Bcl-X₁

Mutant
Activation of JNK1, RSK2, and MSK1 Is Involved in Serine 112 Phosphorylation of Bad by Ultraviolet B Radiation
Qing-Bai She, Wei-Ya Ma, Shuping Zhong and Zigang Dong

J. Biol. Chem. published online April 30, 2002

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