Regulation of Bad Phosphorylation and Association with Bcl-xL by the MAPK/Erk Kinase*

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Phosphorylation of the Bcl-2 family protein Bad may represent an important bridge between survival signaling by growth factor receptors and the prevention of apoptosis. Bad phosphorylation was examined following cytokine stimulation, which revealed phosphorylation on a critical residue, serine 112, in a MEK-dependent manner. Furthermore, Bad phosphorylation also increased on several sites distinct from serine 112 but could not be detected on serine 136, previously thought to be a protein kinase B/Akt-targeted residue. Serine 112 phosphorylation was shown to be absolutely required for dissociation of Bad from Bcl-xL. These results demonstrate for the first time in mammalian cells the involvement of the Ras-MAPK pathway in the phosphorylation of Bad and the regulation of its function.

Apoptosis is a universal phenomenon whereby a damaged cell, a virally infected cell, or a cell that is no longer receiving a specific extracellular survival signal destroys itself. The process of apoptosis involves various discreet levels, ultimately leading to the activation of cysteine-aspartate specific proteases (caspases) 1 (1). An intensely studied family of proteins involved upstream of caspase activation share homology with Bcl-2, one of the first apoptosis regulating proteins identified as an oncogene. These proteins may function as a checkpoint for life and death decisions (2). Cytokines prevent the onset of apoptosis and caspase activation by activating both protein and lipid kinase cascades, which may converge on the Bcl-2 family. In this way, the cytoprotective actions of these signaling pathways may involve the up-regulation of death antagonists, as well as the post-translational modification of Bcl-2 family proteins, which alters their role in propagating the apoptotic signal.

Cytokine receptors of the hemopoietic superfamily activate a number of well studied signaling pathways following tyrosine kinase activation, including p21V(rf) and PI3K and their downstream targets, PI3K-generated 3'-phosphoinositides mediate activation of a family of phospholipid-dependent kinases, which includes PDK1 (3) and an incompletely characterized PDK2 (4–6). PDK1 is an important activation loop kinase and is responsible for the phosphorylation of PKB/c-Akt, p70 S6 kinase, and protein kinase Cδ (reviewed in Ref. 7). Ras-activated Raf operates in a parallel pathway, by phosphorylating and activating downstream targets including the dual specificity kinase MAPK/Erk kinase (MEK; also termed MKK)-1 and 2 (8–11). The ability of Ras to induce transformation is believed in part to involve sustained activation of Raf, MEK, and MAPK activity (12–14). MEK has a very narrow substrate specificity, restricted to the threonine and tyrosine residues of p44 and p42. Phosphorylation by MEK of this family of MAPKs dramatically increases their activity, resulting in phosphorylation of further downstream targets including p90 rsk (15), as well as translocation to the nucleus where they phosphorylate transcription factors involved in immediate/early gene expression (reviewed in Refs. 16 and 17). Two recently developed inhibitors of MEK1, PD98059 (18) and MEK1/2, U0126 (19), have been extensively characterized, and shown to be highly selective in their inhibition of the MAPK pathway.

One Bcl-2 family member shown recently to be a target of cytokine-stimulated signaling is Bad (20). Induction of Bad phosphorylation on multiple serine residues influences its subcellular distribution, from an association with Bcl-xL at the mitochondria, to a cytosolic location, associated with 14-3-3 (21). The association of Bad with Bcl-xL is mediated through dimerization of conserved BH3 domains (22, 23), characteristic for other BH3 domain containing proteins. Phosphorylation of residues in proximity to the BH3 domain of Bad may alter the affinity of Bad for Bcl-xL, promoting dissociation. This may relieve Bcl-xL of some negative influence, allowing protection of cells from apoptosis. The specific residues on Bad phosphorylated in response to survival factors are serine 112 and serine 136 (21). Mutation of either of these residues to alanine potentiates death following transient transfection with Bad, suggesting that both are critical in the disruption of Bad-Bcl-xL heterodimers. Dephosphorylation of Bad by specific phosphatases, such as calcineurin, reverses the cycle back to an unphosphorylated, death-promoting agonist during Ca\(^{2+}\)-induced apoptosis (24).

The phosphorylation of Bad occurs by unknown, cytokine-stimulated pathways. Besides cytokines, many other receptors for growth and survival factors can activate pathways leading to phosphorylation of Bad, including the receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor-1, and Stem cell factor (21, 25–27). In this respect, oncogenes involved in the signal transduction of each of these receptors may bypass the requirement for extracellular stimuli to maintain protection from apoptosis, in part by in-

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1 The abbreviations used are: caspases, cysteine-aspartate specific proteases; Bad, Bcl-xL-associated death inducer; Erk, extracellular regulated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MAPK, mitogen-activated protein kinase; MEK, MAPK/Erk kinase; PKB, protein kinase B; PI3K, phosphatidylinositol 3'-OH kinase; PGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase.
increasing Bad phosphorylation. Therefore, the detailing of specific signaling pathways involved in the regulation of Bad is critical in our understanding of oncogenesis. In our previous report, we showed that the mobility shift of Bad, indicative of phosphorylation, could be blocked by a MEK inhibitor, PD 98059 (27). This lead us to perform a careful analysis of Bad phosphorylation following MEK inhibition. Here we show that Bad is targeted on serine 112 by a MEK-dependent pathway, whereas phosphorylation of two other residues occurs in a MEK-independent manner. Serine 112 phosphorylation was found to be required for dissociation of Bad from Bcl-xL. These results demonstrate for the first time the involvement of a Ras-controlled signaling pathway leading to the phosphorylation and inactivation of a pro-apoptotic Bcl-2 family member in mammalian cells. This ultimately may lead to better therapies designed to exploit the apoptosis machinery during diseases such as cancer.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies to Bad and Bcl-xL were from Transduction Laboratories (B36420 and B22630, Lexington, KY) and Santa Cruz Biotechnology (Minneapolis, MN). Recombinant murine GM-CSF and IL-3 were from R & D Systems (Minneapolis, MN). Synthetic IL-4 was a gift from Dr. James Wieler (University of British Columbia, Vancouver, BC, Canada). GM-CSF and IL-3 were used at 50 ng/ml, and IL-4 was used at 10 ng/ml (University of British Columbia, Vancouver, BC, Canada). GM-CSF and IL-3 were used at 50 ng/ml, and IL-4 was used at 10 ng/ml. These experiments were designed to exploit the apoptosis machinery during diseases such as cancer.

**Cell Lines and Tissue Culture—**MC/9 or FD-CP1 cells (American Type Culture Collection, Manassas, VA) were maintained at 37 °C and 5% CO₂ in a humidified incubator, in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% WEHI-3-conditioned medium as a source of IL-3. Cells were starved of cytokine by overnight incubation in medium in which 90% of the culture volume was replaced with RPMI 1640 plus 10% fetal calf serum but without added WEHI-3-conditioned medium. Alternatively, cells were washed three times and incubated in IL-3-free medium for at least 4 h prior to use in the experiment.

**Immunoprecipitation and Blotting of Bad—**For determining the electrophoretic mobility shift of Bad, MC/9 or FD-CP1 cells stimulated under various conditions were lysed with ice-cold solubilization buffer (20 mM Tris·HCl, pH 7.4, 137 mM NaCl, 0.25% Nonidet P-40, 10 mM NaF, 0.2 mM Na₃VO₄, 1 mM Na₂MoO₄, 1 μg/ml microcin-LR, 0.25 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor) and incubated on ice for 10 min. Samples were centrifuged (20,000 × g, 1 min), and supernatants were transferred to clean tubes. 2 μg of anti-Bad monoclonal antibody (B36420; Transduction Laboratories) was added, and the samples were rotated overnight at 4 °C. Bad immunocomplexes were captured with 20 μl of protein G-Sepharose beads at 4 °C for 1 h. Beads were washed three times with fresh solubilization buffer and resuspended in 1× reducing sample buffer followed by boiling for 5 min. Samples were fractionated in a 12.5% polyacrylamide gel with a 18:1 acrylamide/bisacrylamide ratio and transferred to nitrocellulose. Blots were blocked with 3% skim milk solution for 1 h and then incubated with 1 μg/ml anti-Bad antibody (either SC-943 from Santa Cruz or B36420 from Transduction Laboratories) overnight at room temperature. Primary antibody was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. These same immunoprecipitating and blotting conditions were used to detect Bad co-immunoprecipitation with Bcl-xL, with the following exceptions. In these experiments, 2 μg of anti-Bcl-xL antibody were added for 1 h at 4 °C and captured for an additional hour with 20 μl of protein G-Sepharose beads at 4 °C for 1 h. Beads were washed and fractionated by SDS-PAGE as described above.

**Metabolic Labeling—**MC/9 cells were starved of cytokine as described above, washed in phosphate-free medium, and then placed in phosphate-free RPMI 1640 medium buffered with 10 mM Heps, pH 7.4, with 1 μCi/ml 32P-labeled orthophosphate at 37 °C for 2 h. Bad was immunoprecipitated from detergent-solubilized lysates as described above. Immunoprecipitates were fractionated on a 12.5% gel with an acrylamide/bisacrylamide ratio of 118:1 and dried under heat and vacuum. 32P-Labeled Bad was detected by autoradiography and quantified by liquid scintillation counting or by using a Molecular Imager (Bio-Rad).

**Tryptic Digestion, Two-dimensional Phosphopeptide Mapping, and Phosphoamino Acid Analysis—**32P metabolically labeled Bad from various conditions and isolated above was excised from the gel and digested with 10 μg/ml tryosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in 50 mM (NH₄)HCO₃, pH 7.8, overnight at 37 °C. Gel fragments were pelleted by centrifugation, and the remaining supernatant was transferred to clean tubes and dried under vacuum. Peptides were washed with diminishing volumes of water and resuspended in 5 μl of electrophoresis buffer (1% (NH₄)₂CO₃, pH 8.8). Electrophoresis was performed on 200-μm microcrystalline cellulose plates (Kodak) at 600 V, 7 °C for 45 min. The plates were chromatographed in the second dimension in chromophorography buffer (n-butanol/ pyridine/acetic acid/water, 32.5:25:5:20). Plates were dried, and phosphopeptides were visualized by autoradiography. In earlier experiments, two sided Kodak BioMS film with intensifying screens was used, but a sharper image was obtained using single sided Kodak BioMR film without intensifying screens. If cold synthetic phosphopeptides were also run, these were visualized with ninhydrin staining. Phosphoamino acid analysis was performed by scraping the visualized phosphopeptides into glass reaction vessels, and treatment with 500 μl of 6 N HCl heated to 105 °C for 60 min. The HCl was removed under vacuum, and the phosphoamino acids were washed with diminishing volumes of water. Separation was performed on cellulose plates using buffer consisting of 0.5% pyridine and 5% acetic acid at 1000 V, 10 °C for 45 min. 32P-Labeled phosphoamino acids were detected by autoradiography. In each of the samples, a 1 μg of a mixture of phospho-lysine, phospho-t-threonine, and phospho-t-tyrosine was also added that was visualized by ninhydrin staining.

**RESULTS**

**Bad Is Phosphorylated on Multiple Serine Residues, but Not Serine 136—**To examine the phosphorylation of Bad in detail, we first performed two-dimensional tryptic phosphopeptide mapping. Bad isolated from 32P-labeled cell stimulated with phorbol esters, a general kinase activator, produced a two-dimensional map of numerous labeled phosphopeptides (Fig. 1A). Two of the observed phosphopeptides co-migrated exactly with synthetic tryptic peptides containing phosphorylated Ser 112 and Ser 136 (Fig. 1B). We next stimulated cells with GM-CSF. Compared with unstimulated cells (Fig. 1C), GM-CSF caused an increase in phosphorylation of two residues distinct from either Ser 112 or Ser 136, as well as the appearance of a third phosphopeptide corresponding to the phospho-Ser 112-containing peptide (Fig. 1D). Unlike stimulation with phorbol esters, we were unable to detect Ser 136 phosphorylation following GM-CSF stimulation. Identical results were obtained when cells were stimulated with IL-3 or Stem cell factor (data not shown). The three phosphopeptides, including the one containing Ser 112, were isolated and subjected to phosphoamino acid analysis, which demonstrated exclusive serine phosphorylation (Fig. 1D).

**Bad Phosphorylation on Serine 112 Is MEK-dependent—**Our previous studies have shown that Bad phosphorylation induced by the hemopoietic cytokines IL-3 and GM-CSF is dependent on MEK activation (27). This set of experiments monitored the mobility shift of Bad on SDS-PAGE, a phenomenon common to phosphorylated proteins. To further characterize the phosphorylation of Bad downstream of cytokine receptors, Bad isolated from 32P-labeled cells was examined following treatment with the MEK inhibitor PD 98059 (Fig. 2A). Treatment with this compound significantly reduced the ability of GM-CSF or IL-3 to induce Bad phosphorylation compared with unstimulated cells, but it did not completely abrogate phosphorylation. As further evidence for MEK as an upstream regulator of Bad phosphorylation, a second, unrelated
inhibitor of MEK was employed. Treatment of cells with U0126 (19) also partially reduced the phosphorylation of Bad induced by GM-CSF (Fig. 2B). We also tested the effect of raising intracellular cAMP levels with forskolin to activate PKA, because Bad can be phosphorylated in vitro by PKA (21). We have previously shown that forskolin induces a large increase in cAMP in MC/9 cells, as well as promoting CREB phosphorylation on Ser133 in a PKA-dependent manner (49). Under these conditions, forskolin increased Bad phosphorylation measured by [32P] labeling only slightly compared with GM-CSF. In contrast to GM-CSF stimulation, inhibition of MEK did not inhibit the slight increase in Bad phosphorylation promoted by forskolin. MEK-dependent Bad phosphorylation was also observed in another cell line, FD-CP1 (Fig. 2C). In these cells, both PD 98059 and U0126 effectively reduced the phosphorylation of Bad, which matched exactly the reduction in phosphorylation of p42/44. We next asked which sites of phosphorylation were downstream of MEK. Cells were metabolically labeled with 32P and pretreated with PD98059, followed by stimulation with GM-CSF. The 32P-labeled Bad was digested by trypsin and separated by two-dimensional phosphopeptide mapping. The 32P-labeled phosphopeptides were visualized by autoradiography. The 32P-labeled Bad was cut from the gel and digested with 10 μg/ml of tosylphenylalanyl chloromethyl ketone-treated trypsin overnight in (NH4)HCO3 (50 mM, pH 7.8) at 37 °C. The digested peptides were separated by two-dimensional peptide mapping. 32P-Labeled phosphopeptides containing phosphoserine 136 or phosphoserine 112 were separated as described in A and visualized by ninhydrin staining. C and D, MC/9 cells were 32P-labeled for 2 h and then treated with either vehicle or recombinant murine GM-CSF (50 ng/ml). Bad was isolated, tryptically digested, and chromatographed as in A. E, the three major spots visualized in D were scraped from the TLC plate and separated by electrophoresis. Cold phosphotyrosine, phosphoserine, and phosphothreonine (1 μg each) were run concurrently. Amino acids were visualized by autoradiography and ninhydrin staining.

Inhibition of Bad Phosphorylation on Serine 112 Promotes Increased Association with Bcl-xL—Having established the role of Ser112 in Bad phosphorylation, we asked whether MEK-dependent phosphorylation of Ser112 was important for disrupting Bad-Bcl-xL heterodimerization. During apoptosis, Bad heterodimerization may play a significant role in promoting the death signal by inactivating Bcl-xL (21, 23). In our model, a significant fraction of Bad co-immunoprecipitated with Bcl-xL from cells that had been deprived of cytokine for 8 h, demonstrating the physical interaction of these two proteins during the early stages of apoptosis (Fig. 4A). Stimulation with GM-CSF for 5, 10, or 20 min resulted in an immediate and dramatic loss of Bad-Bcl-xL association, consistent with phosphorylation-induced dissociation. To test whether Ser112 phosphorylation was essential for this dissociation, cells were stimulated with GM-CSF for 5 min while in the presence of U0126. Blocking the activation of MEK completely restored Bad-Bcl-xL association to levels equivalent to unstimulated cells (Fig. 4A). In another experiment, similar conditions were tested as well as another hemopoietic factor, IL-4, which has been shown previously to activate PKB (27) but not Ras or MAPK (29). Consistent with a requirement for MEK-MAPK activation, IL-4 was unable to
Serine 112 Phosphorylation of Bad by a MEK-dependent Pathway

**Figure 3.** BAD Ser112 phosphorylation requires MEK activity. A, cells were 32P-labeled for 2 h and pretreated with PD98059 for 10 min, followed by stimulation with GM-CSF for 5 min. Bad was tryptically digested and two-dimensional chromatography was performed as described in the legend to Fig. 1 and under “Experimental Procedures.” The arrowheads indicate the position of phospho-Ser-112 peptide as determined by co-migration with cold synthetic phospho-Ser-112 peptide. B, cell were pretreated with PD98059 (50 μM) for 10 min and stimulated with IL-3 for 5 min. Equal quantities of detergent-solubilized nuclear-free extracts were fractionated by SDS-PAGE and immunoblotted with anti-phospho-Ser112 antibody (12921). C, similar to B, except that U0126 (25 μM) was used instead of PD98059 and cells were stimulated with GM-CSF, Bad was immunoprecipitated with B36420, fractionated by SDS-PAGE, and immunoblotted with anti-Bad antibody (SC-943; top panel). The blot was stripped and reprobed with anti-phospho-Ser112 antibody (middle panel). Lysates from these conditions were also immunoblotted with anti-phospho-MAPK (bottom panel). These results are representative of at least four independent experiments.

**D I C U S S I O N**

This study demonstrates the phosphorylation of the Bcl-2 family member Bad by the MEK-MAPK pathway on a single residue, Ser112. This phosphorylation was found to be essential for Bad dissociation from Bcl-xL, providing an important link between growth and survival signaling pathways and protection from apoptosis. Because MEK has so far only been shown to phosphorylate and activate p44erk1 and p42erk2, it is likely that these kinases are also involved in BAD Ser112 phosphorylation.

The Erks are not the only kinases to phosphorylate BAD at Ser112 in vitro (data not shown), and the site surrounding Ser112 does not conform to the consensus phosphorylation site for these enzymes (30). Downstream targets of the Erks include p90rsk (15), and it is likely that one of these serine kinases is responsible for BAD phosphorylation on Ser112.

The unique properties of MEK have allowed the development of two cell-permeable drugs that potently block the activation of the Erks, PD98059 (2-[(2′-amino-3′-methoxyphenyl)-ox-anaphthalen-4-one; Ref. 18) and U0126 (1, 4-diamo-no-2,3-dicyano-1, 4-bis[2-amino phenylthio]butadiene; Ref. 19). Both compounds are very specific for MEK. For example, U0126 has been shown not to inhibit molecules such as protein kinase C, Raf, MEKK, Erk, JNK, MKK3, 4, and 6, and Cdk2 and 4. Except for MEK1, PD 98059 displays no inhibitory actions toward 18 known serine/threonine kinases (31).

Although the biochemical pathways activated by cytokines to protect apoptosis are not yet fully understood, recent work has illustrated the importance of PI3K-dependent activation of protein kinase B (PKB/Akt) (28). PKB has recently been shown to mediate survival through phosphorylation of numerous cellular proteins, including the Forkhead family of transcription factors (which regulates Fas expression; Refs. 32–34), caspase-9 (35), GSK-3 (36), NF-κB (37), and endothelial nitric-oxide synthase (38, 39). PKB has also been shown to catalyze the phosphorylation of Bad at serine 136 (26, 40), although the in vitro rate of phosphorylation is much slower when compared with another PKB target, FKHR (34). Consistent with this, the degree of phosphorylation of Bad in vivo on serine 136 by PKB may be dependent upon the degree of expression of Bad. For example, del Peso et al. (25) demonstrated that phosphorylation of expressed Bad was entirely blocked by PI3K inhibitors, indicating that Bad was phosphorylated to the greatest degree on PKB-targeted residues. Datta (26) and Blume-Jensen (40) showed in different systems that expressed Bad was only partially blocked by PI3K inhibitors or dominant negative PKB constructs, isolated to serine 136, demonstrating that other sites were targeted by PI3K-independent pathways.

These later studies were consistent with the original report by Zha and co-workers (21), who demonstrated that phosphorylation of expressed Bad induced by IL-3 occurred primarily on serine 136 and serine 112. Our recent study has suggested that PI3K-dependent PKB activation does not lead to the phosphorylation of Bad (27). This is based on the finding that IL-4 can activate PI3K and PKB but does not promote Bad phosphorylation. Also, GM-CSF-stimulated Bad phosphorylation occurs independently of PI3K and PKB. Similar findings have recently been reported in several other cell types (41). In agreement with our earlier report, two-dimensional phosphopeptide analysis revealed that cytokine-stimulated Bad phosphorylation occurs primarily at serine 112 as well as at two additional residues. Serine 136 phosphorylation was not detected but could be observed following stimulation with phorbol esters, a nonphysiological kinase activator.

Our data thus raise the question of whether serine 136 phosphorylation is physiologically important. A significant body of published work would suggest that it is. For example, expression of mutant Bad in which Ser136 has been mutated to alanine potentiates apoptosis, arguing that the inability of PKB to phosphorylate this altered residue promotes association with Bcl-xL, thus leading to cell death (21, 26, 40). However, our studies here with endogenous Bad argue that Bad-Bcl-xL association is disrupted independently of Ser136 phosphorylation. Rather, dissociation is primarily dependent upon the phosphorylation state of Ser112. Loss of Ser112 phosphorylation, through inhibition of MEK, fully restores the association of Bad with Bcl-xL, even in the presence of PI3K and PKB activation. It is currently unclear whether association of Bad with 14-3-3 proteins requires Ser136 phosphorylation or whether phosphorylation of Ser112 or the other sites we have detected are sufficient. It also remains a possibility that Bad mediates anti-apoptotic signaling through its interaction with 14-3-3 molecules, via Ser136 phosphorylation, and independent
of Bcl-xL interaction, but this appears not to be a dominant pathway in our model. Efforts in our laboratory are currently underway to resolve this issue.

Treatment of cells with MEK inhibitors caused a slow but gradual appearance of nonviable cells (data not shown). The induction of cell death was considerably slower than following removal of a survival factor, indicating that inhibiting Bad phosphorylation at Ser^{112} does not promote apoptosis at the same rate as cytokine-withdrawal. However, we may not expect them to be the same, for several reasons. Bad phosphorylation by cytokines probably is not the only mechanism in place to protect cells from apoptosis. As well, phosphorylation of Bad on the other residues besides Ser^{112} may also provide protection, although we have shown here that this is independent of association with Bcl-xL. For example, there may also be additional proteins that interact with Bad dependent upon phosphorylation at these other residues. Finally, cell death induced by MEK inhibition may be through mechanisms completely independent of Bad phosphorylation. Our current results cannot distinguish between these various possibilities.

Cytokines may be able to activate survival pathways that operate independently of Bad phosphorylation. This seems likely, because IL-4 is unable to promote Ser^{112} phosphorylation or prevent Bad association with Bcl-xL, but it can protect cells from apoptosis by activating PI3K (42). A possible mechanism may be the phosphorylation of caspase-9 by PKB in a PI3K-dependent manner (35). This would be consistent with findings by Parrizas and co-workers (43), who showed that MEK inhibition on its own could not induce apoptosis in insulin-like growth factor-1-stimulated PC12 cells but resulted in synergistic apoptosis when PI3K was also inhibited. Therefore, cell survival may be regulated by multiple signaling pathways, at the level of the mitochondria by the Ras-PAK and Bad phosphorylation (Bad phosphorylation), as well as downstream targets involved in the execution of apoptosis by the PI3K/PKB pathway. This possibility would also explain why under some circumstances PI3K inhibition does not always lead to cell death, and it will be critical to test whether under these conditions Bad phosphorylation by MEK plays an important role.

PKA has also recently been proposed to catalyze phosphorylation of Ser^{112}, in a cAMP-dependent manner, offering an explanation for the survival-promoting effects of cAMP in some cell types (44). This observation is complicated by the recent finding that cAMP can activate the guanine-nucleotide exchange factor Epac, independently of PKA (45). Epac in turn can activate Rap1, a small G-protein similar to Ras located at internal membranes, which may stimulate components upstream of MAPK, including B-Raf (46). In different cell types, forskolin can both activate and attenuate MAPK activity (47, 48), possibly because of the level of expression and degree of cross-talk between Rap1 and other signaling molecules, including PKA, MEKK, and Raf. In our model, we have previously reported that forskolin treatment slightly attenuates p44/p42 activation, potently induces CREB phosphorylation, and suppresses apoptosis induced by PI3K inhibition, ceramide treatment, or cytokine withdrawal (49). In the present study, forskolin could only slightly promote Bad phosphorylation, which was independent of MEK activity. In addition, we have previously been unable to detect any rise in cAMP levels following stimulation with cytokines (49). Therefore, we conclude that PKA activation by adenylate cyclase-generated cAMP is not the principle means for Bad phosphorylation on Ser^{112} and suggest that Bad phosphorylation is not a major route for cAMP-promoted survival.

Hinton and Welham (41) have recently described the phosphorylation of Bad Ser^{112} in a PI3K-dependent manner. In the factor-dependent cell lines used, LY-294002 abolished Ser^{112} phosphorylation in response to IL-3. The requirement for PI3K upstream of MAPK is controversial, based on the apparent cell type-specific inhibition of MAPK by wortmannin, which may be through targets other than PI3K (50). However, recent studies (51, 52) have demonstrated the inhibitory actions of both wortmannin and LY294002 on MAPK at low agonist concentrations. Although the role that PI3K plays upstream of MAPK requires further investigation, in light of these findings and in context with our results here, it is likely that inhibition of Bad Ser^{112} phosphorylation by LY-294002 is through inhibition of the MAPK pathway and not PKB.
In summary, our results have described the role of the MEK-MAPK pathway in the phosphorylation of the pro-apoptotic protein Bad. This may form one of several survival pathways activated by cytokines that together support the survival of mammalian cells. Our data suggest that additional pathways, possibly PI3K-mediated, can compensate for increased Bad association with Bcl-xL. Therapies designed to target elements of these pathways may be useful in the treatment of diseases characterized by inappropriate cell survival.

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