Bad is a pro-apoptotic member of the Bcl-2 family of proteins that is thought to exert a death-promoting effect by heterodimerization with Bcl-X\textsubscript{L}, nullifying its anti-apoptotic activity. Growth factors may promote cell survival at least partially through phosphorylation of Bad at one or more of Ser-112, -136, or -155. Our previous work showed that Bad is also phosphorylated in response to cytokines at another site, which we now identify as Ser-170. The functional role of this novel phosphorylation site was assessed by site-directed mutagenesis and analysis of the pro-apoptotic function of Bad in transiently transfected HEK293 and COS-7 cells or by stable expression in the cytokine-dependent cell line, MC/9. In general, mutation of Ser-170 to Ala results in a protein with increased ability to induce apoptosis, similar to the S112A mutant. Mutation of Ser-170 to Asp, mimicking a constitutively phosphorylated site, results in a protein that is virtually unable to induce apoptosis. Similarly, the S112A/S170D double mutant does not cause apoptosis in HEK293 and MC/9 cell lines. These data strongly suggest that phosphorylation of Bad at Ser-170 is a critical event in blocking the pro-apoptotic activity of Bad.

Bcl-2 family proteins have emerged as key players in the regulation of apoptosis in many types of cells (for review, see Refs. 1 and 2). Whereas some members prevent cell death (Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, Mcl-1), others exhibit pro-apoptotic activity (Bax, Bak, Bok). The balance between these two groups can be disrupted by a third group of proteins that share at least one common structural motif, a short \(\alpha\)-helical region termed the BH3 domain. BH3-only members include Bad, Bim, Bik, Bid, Noxa, and Hrk (3, 4). The model that prevails suggests that BH3-only proteins act in response to death-inducing signals and function to neutralize pro-survival Bcl-2 proteins by heterodimerization, freeing the Bax-like proteins to execute cell death (2, 5–8). Bad is a "BH3-only" pro-apoptotic Bcl-2 member (9) that seems to be unique in that its function is regulated by phosphorylation at several sites in response to survival factors. Initial studies by Zha et al. (10) identify Ser-112 and Ser-136 as two major sites that, when phosphorylated in response to IL-3,\(^1\) blocked the pro-apoptotic function of Bad (10). Recently, a third site, Ser-155, located at the center of the Bad BH3 domain (amino acids 151–163), was shown to be phosphorylated in response to growth factors and prevented the cytotoxic effects of Bad (11–15). Although the precise mechanisms by which these proteins induce apoptosis are still unclear, the proposed model is that, in its unphosphorylated state, Bad binds to anti-apoptotic proteins (primarily Bcl-X\textsubscript{L}) and induces cell death (9), perhaps by blocking the pro-survival function of Bcl-X\textsubscript{L}. When Bad is phosphorylated on one or more of three serine residues Ser112, Ser-136, or Ser-155 in response to survival factors, it is sequestered in the cytosol, complexed to 14-3-3 proteins, and fails to interact with pro-survival Bcl-2 members in the outer mitochondrial membranes (10–12, 16–18). By definition, phosphatases that dephosphorylate Bad also play an important role in regulating its activity (19–21).

Although numerous reports have shown the involvement of Bad in induction of apoptosis, recent studies suggest that the Bad protein can also have growth-promoting activity (22) and can be involved in promoting cell cycle progression (23). At present the molecular mechanism by which Bad can mediate such functions is unknown, and the role of Bad phosphorylation in cell cycle regulation is controversial. In one study, phosphorylation at Ser-136 and association with 14-3-3 was found to be essential for its growth-promoting effect (22), whereas in the other study only association with Bcl-X\textsubscript{L} was shown to be required, independent of the phosphorylation state of Bad (23).

Previous work in our laboratory characterized the phosphorylation of Bad in response to cytokine treatment in the murine mast cell line, MC/9 (24, 25). It was demonstrated that phosphorylation of Bad at Ser-112 was dependent upon a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-dependent phosphorylation event (24), and other reports suggest that this phosphorylation is mediated by a kinase downstream of MEK, p90Rsk (16, 26–28). In contrast to several reports showing phosphorylation of Bad at Ser-136 by the serine/threonine kinase protein kinase B (Akt) in response to survival factors (17, 29, 30), we have not detected any significant change in phosphorylation of endogenous Bad at Ser-136 in response to cytokine treatments (24). Furthermore, blocking protein kinase B activation had no effect on GM-CSF-induced phosphorylation of Bad. Others also report that activation of protein kinase B does not automatically confer a survival signal or result in phosphorylation of Bad in hemopoi-

---

\(1\) The abbreviations used are: IL-3, interleukin 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; GST, glutathione S-transferase; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase.
etic cells (31, 32). It should be noted that our experiments were carried out in GM-CSF-stimulated MC/9 mast cells that express endogenous levels of Bad and protein kinase B, and thus, the possible connection between protein kinase B activation and Ser-136 may depend on cell type or the particular growth factor being studied. In our characterization of Bad phosphorylation by two-dimensional tryptic peptide mapping, we also demonstrated at least two additional novel phosphorylation sites distinct from either Ser-112 or Ser-136 (24). Here we report the identity of one of these phosphorylation sites as Ser-170, which is a novel site of phosphorylation in murine Bad. We have probed its potential function by the use of mutants altered at Ser-170, and our results strongly suggest that the phosphorylation of Bad at this site may serve a crucial role in regulating its pro-apoptotic activity. Furthermore, phosphorylation of Bad at Ser-170 may also play a role in growth promotion in the cytokine-dependent MC/9 mast cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** HEK293 and COS-7 cells were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, a kind gift from Dr. Rob Kay), and G-418 was used for selection. These experiments were carried out in GM-CSF-stimulated MC/9 mast cells that express endogenous levels of Bad and protein kinase B. We have probed the potential function of the use of mutants altered at Ser-170, and our results strongly suggest that the phosphorylation of Bad at this site may serve a crucial role in regulating its pro-apoptotic activity. Furthermore, phosphorylation of Bad at Ser-170 may also play a role in growth promotion in the cytokine-dependent MC/9 mast cells.

**Bad Plasmid Cloning and Mutagenesis—** The full-length murine Bad cDNA in the pBluescript vector was digested with BamHI and EcoRI and subcloned into the polylinker of the retroviral expression vector pMX-pie. Ser residue 170 was substituted with alanine using PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA) and the GST fusion protein Bad in the pCDNA.3 expression vector was a generous gift from Dr. G. G. Mills. The GST fusion protein was purified on glutathione-Sepharose and washed twice in resuspension buffer containing 1% Nonidet P-40 and once in kinase buffer (20 mM Hepes, pH 7.4, 2 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM pepstatin, 0.5 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor) and incubated on ice for 1 min. Samples were centrifuged (2000 × g, 1 min) and supernatants were transferred to microcentrifuge. Blots were blocked with 3% skim milk solution for 1 h and then incubated with anti-Bad antibody (either SC-943 from Santa Cruz or 1/100 dilution). The same immunoprecipitation and blotting conditions were used to detect Bad co-precipitation with Bcl-XL, with the following exceptions. In these experiments, 5 µg of purified His-Bcl-XL was added for 2 h at 4 °C and captured for an additional 2 h with 30 µg of Ni2+-agarose beads at 4 °C. Beads were washed and fractionated by SDS-PAGE as described above.

**Preparation of Cell Lysates for PARP Analysis—** Thirty-six hours post-transfection with FLAG-tagged Bad derivatives, COS-7 cells were harvested in phosphate-buffered saline with 2 mM EDTA, recovered by centrifugation, and lysed in 0.25 ml of sample buffer (50 mM Tris-Cl, pH 7.4, 20% glycerol, 1 mM β-glycerophosphate, 1 mM dithiothreitol, 5 mM EGTA, pH 7.2, 75 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 µg/ml microcystin-LR). Beads coupled to GST-Bad fusion proteins were resuspended in 10 µl of kinase buffer to which was added 10 µl of lysate from 106 GM-CSF-stimulated MC/9 cells (25), 50 µl ATP, and 10 µCi of [γ-32P]ATP. kinase reactions were incubated for 20 min at 30 °C stopped with 20 mM EDTA, pH 8.0, and washed twice with resuspension buffer containing 1% Nonidet P-40. The beads were boiled in sample buffer, and proteins were separated by SDS-PAGE. Bands on the dried gel corresponding to the GST fusion proteins were prepared for two-dimensional peptide analysis exactly as described (24), except that electrophoresis in the first dimension used 7.8% acetic acid, 2.2% formic acid, pH 1.9.

**Mass Spectrometry—** After two-dimensional tryptic peptide maps and autoradiography to visualize the phosphopeptides, the cellulose matrix corresponding to the area of the spot was extracted (33), concentrated, and analyzed by micropipillar high performance liquid chromatography electrospray ionization tandem mass spectrometry using data-dependent ion selection (34).

**AP100 Assays and Flow Cytometry Analysis—** After COS-7 transfection using the different Bad mutants, cells were stained for propidium iodide. Briefly, cells were harvested, collected by centrifugation, and fixed in 70% ethanol overnight at 4 °C. On day 2, the cells were pelleted and resuspended in phosphate-buffered saline containing 0.1% glucose, 100 µg/ml RNaseA, and 50 µg/ml propidium iodide and left in the dark at room temperature for 45 min. The samples were analyzed using an Epics XL flow cytometer (Coulter).

**RESULTS**

**Ser-170 Is a Fourth Phosphorylation Site on Bad—** In our recent report demonstrating mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-dependent phosphorylation of Bad at Ser-112, two-dimensional tryptic phosphopeptide mapping revealed two novel sites of phosphorylation, distinct from either Ser-112 or Ser-136 (24). One of these sites, referred to as unknown peptide 2, was shown to be

---

Downloaded from http://www.jbc.org/ by guest on July 22, 2018
phosphorylated to some extent in unstimulated cells, and phosphorylation was further increased in response to GM-CSF. To identify this novel site, GST-Bad was phosphorylated in vitro using cell extracts from GM-CSF-stimulated MC/9 cells. The phosphopeptide corresponding to unknown peptide 2 was identified by tandem mass spectrometry and molecular weight using the known amino acid sequence of murine Bad. The other unknown site (site 1) was not phosphorylated under these conditions in vitro. Analysis of the microparticulate high performance liquid chromatography electrospray ionization tandem mass spectrometry data for one of the ions selected, [M + 2H]^{2+} = 501.5 m/z, identified a phosphorylated Bad peptide with the sequence Ser-Ala-Gly-Thr-Ala-Thr-Gln-Met-Arg in the sample. Furthermore, a strong y-ion series within this tandem mass spectrum for the sequence Thr-Ala-Thr-Gln confirmed the location of the phosphate on the amino-terminal serine at position 170 on Bad (Ser-170). In separate experiments, the unknown phosphopeptide 2 was subjected to manual Edman degradation, which supported the mass spectrometry data, since the ^{32}P-labeled position was at the first amino acid residue (data not shown). Ser-170 is one of the few conserved Ser sites in murine Bad that would be the first residue in a tryptic peptide, with one of the others being Ser-136. These data demonstrate that Bad can be phosphorylated in vitro and in vivo in response to GM-CSF on a novel site that we now identify as Ser-170. We hypothesize that the second unknown site from our tryptic peptide maps is the Ser-155 that was reported previously (11), since the ^{32}P-labeled position was at the first amino acid residue. Ser-170 of Bad is phosphorylated in vitro and in vivo. A, comparison of murine (mBAD) and human (hBAD) protein sequences surrounding the Ser-155 and Ser-170 sites. B, two-dimensional maps of tryptic peptides from GST-Bad and GST-Bad S170A phosphorylated with GM-CSF-stimulated MC/9 cell extracts. C, two-dimensional maps of tryptic peptides from HEK293 cells expressing wild-type (WT) Bad or Bad S170A. Regulation of Bad by Phosphorylation at Ser-170—Phosphorylation of Bad at Ser-170 occurs in vitro. When HEK293 cells stably transfected with FLAG-tagged wild-type Bad or Bad S170A were labeled with ^{32}Porthophosphate and immunoprecipitated with anti-FLAG antibody, there was also one less tryptic peptide observed in the S170A mutant compared with wild-type Bad (Fig. 1C). It should be noted that additional phosphopeptides are always detected in Bad phosphorylated in vitro compared with the protein phosphorylated in cells. Also, migration of the peptides in the two-dimensional maps shown in Fig. 1 is not the same as shown previously (24), since an acidic, as opposed to basic, electrophoresis buffer was used. Phosphorylation of Bad on Ser-170 Correlates with Cell Survival—The functional role of phosphorylation at the Ser-170 site was investigated by site-directed mutagenesis and analysis of the pro-apoptotic function of Bad in transiently transfected HEK293 cells. As shown in Fig. 2A, transfection with wild-type Bad resulted in fewer cells expressing GFP compared with cells transfected with the vector alone, as expected. Mutation of Ser-170 to Ala (S170A), a non-phosphorylatable residue, enhanced the pro-apoptotic function of Bad similar to that of the Bad Ser-112 to Ala mutant (S112A). Substitution of Ser-170 with an aspartate residue (S170D) to mimic the negatively charged phospho-Ser-170 residue resulted in the abrogation of
Activity of Bad S170A—To determine whether phosphorylation at Ser-112 was phosphorylated under the conditions tested in the various mutants, immunoblotting was performed using the phospho-Bad antibody on HEK293 cells stably transfected with the different Bad constructs. As shown in Fig. 4, upper panel, all the transfectants expressed equivalent amounts of Bad protein. When the cell lysates were reprobed with the phospho-Ser-112 antibody (Fig. 4, lower panel), it was observed that wild-type Bad, Bad S170A, and Bad S170D were phosphorylated at the Ser-112 site. These data indicate that the enhanced pro-apoptotic effect of Bad S170A is not mediated by a decrease in phosphorylation of Ser-112 and that phosphorylation at Ser-112 is not sufficient to inhibit the pro-apoptotic activity of Bad. As expected, Bad S112A and the double mutant Bad S112A/S170D are not phosphorylated on Ser-112, confirming the specificity of the phospho-Ser-112 antibody.

Phosphorylation of Bad on Ser-170 Correlates with Cell Survival in COS-7 Cells—Further analysis was done in a different cellular system to verify the role of phosphorylation at the Ser-170 site and to compare changes at the Ser-155 phosphorylation site. COS-7 cells were transiently transfected with FLAG-tagged wild-type Bad, or S155A, S155D, S170A, or S170D mutant forms, harvested at 36 h post-transfection, and the amount of apoptosis was determined by propidium iodide staining of DNA followed by flow cytometry analysis. As shown in Fig. 5A, transfection with Bad S170A enhanced apoptosis compared with vector alone. Wild-type Bad did not cause a significant increase in apoptosis in COS-7 cells, as determined by propidium iodide staining of sub-diploid DNA. However, the morphology of the transfected cells, detected by co-expression of GFP, was altered by Bad expression compared with transfection with empty vector such that the Bad-expressing cells had a very rounded morphology (Fig. 5B). Mutation of Ser-155 to Ala also resulted in enhanced levels of apoptosis. As expected, transfection with Bad S170D did not cause apoptosis, and similar results were seen with the Bad S155D mutant. It should be noted that the morphology of the cells transfected with either S170D or S155D mutants were not different from control cells (data not shown), indicating that the presence of a negatively charged residue at either of these two sites alters the activity of Bad. Immunoblot analysis confirmed expression of FLAG-Bad in transiently transfected COS-7 cells, with lesser amounts in S155A- and S170A-transfected cells that were undergoing apoptosis (Fig. 5C). Quantification of the viable adherent cells by trypan blue exclusion revealed a striking effect of S170A in inducing death in COS-7 cells, whereas Bad S170D was unable to cause cell death (Fig. 5D). Finally, Bad S170A also induced cleavage of PARP similar to that induced by Bad S155A (Fig. 5E). Mutations of either Ser-155 or Ser-170 to the phospho-mimicking Asp resulted in proteins unable to induce PARP cleavage, as expected.

Phosphorylation of Bad on Ser-170 Does Not Prevent Bad/Bcl-X<sub>L</sub> Heterodimerization—One mechanism by which Bad S170D may rescue cells from apoptosis induced by Bad is by disrupting Bad/Bcl-X<sub>L</sub> association, as clearly shown previously.

**Fig. 3.** Increased levels of transfected Bad wild-type, Bad S112A, and Bad S170A promote apoptosis, whereas Bad S170D and Bad S112A/S170D do not cause apoptosis. HEK293 cells were co-transfected with GFP and either vector alone, wild-type Bad, or the indicated Bad mutants (S112A, S170A, S170D, S112A/S170D) and analyzed for GFP fluorescence 24–48 h post-transfection. The total amount of DNA transfected in all cases was adjusted to 0.5 μg with the vector (pMX-pie), and 0.1 μg of GFP vector was co-transfected in all cases. The result obtained with vector alone (not shown) was similar to the images obtained with Bad S170D.

**Fig. 4.** Bad S170A and S170D are phosphorylated on Ser-112. Immunoblot analysis of HEK293 cells stably transfected with the various Bad mutants. Cell lysates corresponding to ~7 × 10<sup>6</sup> cells were probed with the polyclonal anti-Bad antibody (SC-943, upper panel) or anti-phospho Bad Ser-112 (pSer112, lower panel). The bands indicated by arrows above and below the Bad band in the upper panel were nonspecifically recognized by the antibody. wt, wild type.
for phosphorylation at Ser-155 (12–14). Pull-down assays with purified recombinant His-Bcl-XL were performed in HEK293 cells stably expressing various mutants. Cells were treated with or without serum to test whether Bad was being phosphorylated and whether this affected its association with Bcl-XL. As shown in Fig. 6, Bad S170D as well as Bad S112D and wild-type Bad associated with Bcl-XL, as indicated by co-precipitation. Only in the case of the Bad S155D mutant was there a loss of association with Bcl-XL. In most cases, treatment with serum resulted in slower-migrating forms of Bad, as expected if the protein was phosphorylated, and there was a reduction in association with Bcl-XL.

Substitution of Ser-170 to Asp Protects Cytokine-dependent Mast Cells from Bad Death-promoting Activity and Promotes Cell Growth—The effect of Bad expression in the cytokine-dependent MC/9 cells, in which we have shown phosphorylation of endogenous Bad in response to cytokines (24), was tested. We first infected MC/9 cells with a Bcl-XL-encoding retrovirus. MC/9 Bcl-XL clones stably expressing different levels of wt Bad or the various mutants were deprived of IL-3 and assessed for cell viability by trypan blue exclusion (Fig. 7). In this assay, the presence of Bcl-XL allows for survival of the cells for at least 4 days after cytokine withdrawal (see vector alone cells). In comparison, the parental MC/9 cells undergo apoptosis and result in very few viable cells within 24 h of cytokine withdrawal (24, 35). As expected, expression of wild-type Bad was able to block the pro-survival effect of Bcl-XL, resulting in a substantial reduction in cell numbers within 48 h. Bad S170A clone 8 resulted in death activity that was very similar to that of wild-type Bad. On the other hand, Bad S170A clone 12, which expressed higher amounts of protein, could not be tested in the cytokine starvation assay since the majority of the cells were undergoing cell death, even in the presence of IL-3. The mutant Bad S170D was unable to cause cell death and in fact resulted in an increase in cell number over the 7 day time course. This was even observed in Bad S170D clone 9, which expresses as much mutant Bad protein as the Bad S170A clone 12. The double mutant Bad S112A/S170D clones 10 and 11 also had the same phenotype as the S170D clones. Despite large differences in expression levels of the double mutant protein in the two clones, there was a dramatic increase in cell survival and cell number in both cases.

**FIG. 6. Heterodimerization of Bad with Bcl-XL is not affected by Asp substitutions at either Ser-112 or Ser-170.** Stable HEK293 cells overexpressing various FLAG-tagged Bad mutants were analyzed for association with Bcl-XL by pull-down assay with purified His-Bcl-XL. Cells were starved for 18 h without serum (−) and then stimulated for 30 min with 15% serum (+). The upper panel shows Bad protein associated with Bcl-XL, whereas the lower panel shows expression of the FLAG-tagged Bad (wild type (wt) or mutants) in the stable cell lines. FBS, fetal bovine serum.

**FIG. 5. Bad S170A expression is sufficient to induce apoptosis in COS-7.** A, COS-7 cells were transfected with pCDNA3 derivatives expressing various FLAG-tagged Bad mutants. After 36 h, the cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry. Histograms corresponding to 10,000 cells are shown. The data are representative of four independent experiments. wt, wild type. B, cells transfected with vector alone or wild-type Bad were detected by co-expression of GFP. C, immunoblot with an antibody directed against the FLAG epitope (Sigma) of cell lysates from transfected COS-7 cells. The blot was reprobed with antibodies against p85 (the regulatory subunit of protein iodide 3-kinase), which showed that the amount of total protein in S155A and S170A lanes was lower due to the recovery of a fewer number of cells (not shown). D, quantification of viable adherent COS-7 cells as determined by trypan blue exclusion at 36 h post-transfection (mean ± S.D., n = 3). A constant number of cells was used within each experiment. The results were normalized within each experiment, with the vector alone as 100% (an average of 3 × 10⁵ cells counted per experiment). E, PARP cleavage was induced by expression of Bad S170A and not by Bad S170D in COS-7 cells; the immunoblot analysis was performed to detect PARP (upper panel). The blot was probed with anti β-actin (Sigma) to demonstrate the relative level of total COS-7 whole-cell lysates loaded.

**DISCUSSION**

Phosphorylation of Bad at several distinct sites has been shown to be an important link connecting at least some of the effects of extracellular survival factors on the intrinsic cell death machinery regulated by Bcl-2 family members. In this study we have identified a novel site of phosphorylation, Ser-170, which can serve to modulate the pro-apoptotic activity of Bad. We have concentrated on extensive mutational analysis to...
Clones, and the blot was probed with the monoclonal anti-Bad antibody. Bad was immunoprecipitated from cell extracts from the corresponding upper two arrows indicate cross-reacting bands.

Changes in the case of HEK293, the wild type Bad protein had undergone dramatic changes in phosphorylation at the Ser-170 site. In the case of HEK293, the wild type Bad protein had undergone dramatic changes in phosphorylation at the Ser-170 site. However, at the Ser-170 site lies close to the BH3 region (amino acids 151–163), which was shown to be necessary and sufficient for heterodimerization with Bcl-X<sub>L</sub> (18). Mutational studies provide strong evidence that Bad promotes apoptosis by heterodimerizing with Bcl-X<sub>L</sub> and thus, we attempted to address the question of whether phosphorylation at the Ser-170 site would affect this association. Recent work supports the view that Ser-155 phosphorylation directly prevents hetero-dimerization.

However, at the Ser-170 site lies close to the BH3 region (amino acids 151–163), which was shown to be necessary and sufficient for heterodimerization with Bcl-X<sub>L</sub> (18). Mutational studies provide strong evidence that Bad promotes apoptosis by heterodimerizing with Bcl-X<sub>L</sub> and thus, we attempted to address the question of whether phosphorylation at the Ser-170 site would affect this association. Recent work supports the view that Ser-155 phosphorylation directly prevents hetero-dimerization.

In this study, we first compared the physiological effects of Bad on two different cell lines into which the mutated forms of Bad were introduced. The Ser-170 site lies close to the BH3 region (amino acids 151–163), which was shown to be necessary and sufficient for heterodimerization with Bcl-X<sub>L</sub> (18). Mutational studies provide strong evidence that Bad promotes apoptosis by heterodimerizing with Bcl-X<sub>L</sub> and thus, we attempted to address the question of whether phosphorylation at the Ser-170 site would affect this association. Recent work supports the view that Ser-155 phosphorylation directly prevents hetero-dimerization.
by abolishing the affinity of Bad BH3 for Bcl-xL (11–15), whereas phosphorylation on Ser-112 and Ser-136, which are located outside the BH3 domain, may inhibit dimerization with Bcl-xL indirectly, perhaps by promoting associations with 14-3-3 proteins. Here we have shown that mimicking phosphorylation at either Ser-170 or at Ser-112 by mutation to Asp is not sufficient to disrupt Bad/Bcl-xL complexes. Thus, although the Bad S170D mutants do not induce apoptosis, the protein can still associate with Bcl-xL. Analysis of the subcellular distribution of the different Bad mutants using confocal microscopy did not reveal striking differences between wild-type Bad, Bad S170A, and S170D (data not shown). The significance of Bad phosphorylated at Ser-170 remaining associated with Bcl-xL remains to be determined, but other possible models will have to be considered. For example, phosphorylation at Ser-170 may serve as a novel docking site for another protein that may modulate pro-survival activity of the protein complex.

The other factor that remains to be characterized is the kinase responsible for phosphorylation at the Ser-170 site. Our previous results suggest that neither activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/MAPK nor the phosphatidylinositol 3-kinase pathways is responsible for phosphorylation at the site that we have now shown to be Ser-170 (24). A search for potential kinases that can phosphorylate Ser-170 (using NetPhos) reveals protein kinase A or calmodulin kinase II as candidates. Protein kinase A has been implicated in phosphorylation of Bad (12, 14). The consensus sequence for protein kinase A phosphorylation is RXXS, which fits with the sequence PRPKS at Ser-170, which fits with the sequence PRPKS at which Ser-170 could play a similar role, although our results with the newly characterized Ser-170 site had a profound effect on the function of Bad. In our initial characterization of this phosphorylation event, even cytokine-dependent MC/9 cells starved of cytokine retained some phosphorylation at the Ser-170 site. Phosphorylation at this site was enhanced with cytokine stimulation, and together with our mutational analysis, the results suggest that phosphorylation at Ser-170 plays a key role in the function of Bad. Regulation of the various phosphorylation sites on Bad reveals a degree of complexity that was unexpected. All these sites may be regulated differently depending on the cell type, and the role of one particular site with respect to the others may vary as well, which could explain the differences in the effect of Bad expression in various cell types. Furthermore, the various sites appear to have very different effects. Phosphorylation at Ser-112 or Ser-136 promotes Bad association with 14-3-3. The Ser-155 site serves to prevent association of Bad with Bcl-xL. Finally, we speculate that phosphorylation at Ser-170 on Bad, although it is still associated with Bcl-xL, may enhance the survival effect of Bcl-xL and also impart a growth-promoting effect. It is clear that the many sites of phosphorylation on Bad serve to make this protein a key player in the complex cellular events involving apoptosis and perhaps in regulation of cell cycle progression.

Acknowledgments—We thank Dr. A. Mui for providing the pMX-pie and pMX-puro plasmids and Jennifer Kong for helping with the flow cytometry analysis. We also thank Dr. J. D. Watts for advice on phosphoprotein analysis.

REFERENCES
1. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev.* 13, 1899–1911
2. Chao, D. T., and Korsmeyer, S. J. (1998) *Ann. Rev. Immunol.* 16, 395–419
3. Huang, D. C. S., and Strauss, A. (2000) *Cell* 103, 839–842
4. Oda, E., Okhi, R., Muraasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (1993) *Science* 258, 1053–1055
5. Chinnayyan, A. M., and Dixit, V. M. (1996) *Curr. Biol.* 6, 556–562
6. Jacobson, M. D. (1997) *Curr. Biol.* 7, 277–281
7. Reed, J. C. (1998) *Oncogene* 17, 3225–3236
8. Zong, W. X., Lindsten, T., Rutkowski, P., MacGregor, G. R., and Thompson, C. B. (1981) *Genes Dev.* 15, 1481–1486
9. Yang, E., Zha, J., Jockel, J., Boiese, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) *Cell* 80, 285–291
10. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* 87, 619–628
11. Tan, Y., Demeter, M. R., Ruan, H., and Combi, M. J. (1999) *J. Biol. Chem.* 274, 25866–25869
12. Zhou, X. M., Liu, Y. M., Payne, G., Lutz, R. J., and Chittenden, T. (2000) *J. Biol. Chem.* 275, 25046–25051
13. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) *Mol. Cell* 6, 41–51
14. Lizzano, J. M., Morrice, N., and Cohen, P. (2000) *Biochem. J.* 349, 547–557
15. Virdie, K., Parone, P. A., and Tolkovsky, A. M. (2000) *Curr. Biol.* 10, 1151–1154
16. Tan, Y., Ruan, H., Demeter, M. R., and Combi, M. J. (1999) *J. Biol. Chem.* 274, 34859–34867
17. Datta, S. R., Dudek, H. T., Tao, X., Masters, S., Fu, H., Gehot, Y., and Greenberg, M. E. (1997) *Cell* 91, 231–241
18. Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G., and Korsmeyer, S. J. (1997) *J. Biol. Chem.* 272, 24101–24104
19. Ayllon, V., Martinez, A. C., Garcia, A., Cayla, X., and Rebollo, A. (2000) *EMBO J.* 19, 2237–2246
20. Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, P., Bobo, T., Franke, T. F., and Reed, J. C. (1999) *Science* 284, 339–343
21. Chiang, C., Harris, G., Ellig, C., Masters, S. C., Subramanian, R., Shenolikar, S., Wadzinski, B. E., and Yang, E. (2001) *Blood* 97, 1299–1307
22. Masljar, D. J., Aoki, M., and Vogt, P. K. (2001) *Oncogene* 20, 5087–5092
23. Chattopadhyay, A., Chiang, C. W., and Yang, E. (2001) *Oncogene* 20, 4507–4518
24. Scheid, M. P., Schubert, K. M., and Duronio, V. (1999) *J. Biol. Chem.* 274, 31108–31113
25. Scheid, M. P., and Duronio, V. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 7439–7444
26. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Tsuchi, M., and Greenberg, M. E. (1999) *Science* 286, 1358–1362
27. Fang, X. J., Yu, S. X., Eder, A., Mao, M. L., Bast, R. C., Boyd, D., and Mills, G. B. (1999) *Oncogene* 18, 645–650
28. Shimamura, A., Ballif, B. A., Richards, S. A., and Blelloch, R. (2000) *Curr. Biol.* 10, 127–135
29. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) *Science* 275, 687–689
30. Blume-Jensen, P., Janknecht, R., and Hunter, T. (1998) *Curr. Biol.* 8, 779–782
31. Hinton, H. J., and Welham, M. J. (1999) *J. Immunol.* 162, 7002–7009
32. Craddock, B. L., Orchiston, K. A., Hinton, H. J., and Welham, M. J. (1999) *J. Biol. Chem.* 274, 10633–10640
33. Affolter, M., Watts, J. D., Krebs, D. L., and Aebersold, R. (1994) *Anal. Biochem.* 223, 74–81
34. Gallie, B., Corkhah, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figey, D., Harrison, D. G., Berk, B. C., Aebersold, R., and Corson, M. A. (1999) *J. Biol. Chem.* 274, 30101–30108
35. Scheid, M. P., Lauener, R. W., and Duronio, V. (1995) *Biochem. J.* 312, 159–162
36. Mok, C. L., Gil-Gomez, G., Williams, O., Coles, M., Taga, S., Tsalmani, M., Norton, T., Kioussis, D., and Brady, H. J. (1999) *J. Exp. Med.* 189, 575–586
Identification of a Novel Phosphorylation Site, Ser-170, as a Regulator of Bad Pro-apoptotic Activity
Shaynoor Dramsi, Michael P. Scheid, Arpita Maiti, Payman Hojabrpour, Xianming Chen, Kathryn Schubert, David R. Goodlett, Ruedi Aebersold and Vincent Duronio

J. Biol. Chem. 2002, 277:6399-6405.
doi: 10.1074/jbc.M109990200 originally published online November 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109990200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 19 of which can be accessed free at http://www.jbc.org/content/277/8/6399.full.html#ref-list-1