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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_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_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 α-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_L) and induces cell death (9), perhaps by blocking the pro-survival function of Bcl-X_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_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-

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[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% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were passaged at 80–90% confluence. The MC/9-Bcl-X_L clone 4 was constructed using retroviral infection (the plasmid CTV 83 expressing human Bcl-X_L was a kind gift from Dr. Rob Kay), and G-418 was used for selection. These cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 20 μM 2-mercaptoethanol, and 10% WEHI-3 conditioned medium as a source of IL-3.

Bad Plasmid Cloning and Mutagenesis—The full-length murine Bad cDNA in the pBluescript vector was digested with *Bam*HI and *Eco*RI 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 primer pairs corresponding to 5′-GGACTTCCTCGCCCAAAGGCCGC-AGGCACTGCAACACAG-3′. Ser residue 170 was substituted with aspartate using the primer pairs corresponding to 5′-GGACTTCCTC-GCCCAAAGGACGCAGGCACTGCAACACAG-3′. Ser residue 155 was substituted with alanine using the primer pairs corresponding to 5′-C-GTGAGCTCCGAAGGATGGCCGATGAGTTTGAGGGTTCC-3′. Ser residue 155 was substituted with aspartate using the primer pairs corresponding to 5′-CGTGAGCTCCGAAGGATGGACGATGAGTTTGA-GGGTTCC-3′. To generate the double mutant S112A/S170D, an internal *Esp*I-*Eco*RI fragment encoding the last 78 amino acids of Bad was swapped from Bad S170D into Bad S112A. FLAG-tagged wild-type Bad in the pCDNA.3 expression vector was a generous gift from Dr. G. Mills. To generate the various FLAG-tagged Bad mutants in pCDNA.3, an internal *Bsp*EI-*Eco*RI fragment containing most of the Bad sequence (amino acids 24–204) was swapped from the different pMX-pie derivatives into pCDNA.3 FLAG-Bad. All plasmids were sequenced to verify the integrity of the constructs.

Expression of GST-Bad and His-Bcl-X_L in Escherichia coli—A cDNA encoding full-length murine Bad was cloned into pGEX 2T with *Bam*HI-*Eco*RI followed by *Bam*HI fill-in. The Bad wild-type and S170A constructs were transformed into BL21 DE3 *E. coli*, and the GST fusion proteins were purified on GSH-Sepharose as recommended by the manufacturer. For His-Bcl-X_L, the first 211 amino acids were amplified from a cDNA encoding the full-length Bcl-X_L and cloned into pET28a with *Nde*I and *Eco*RI sites. This was also expressed in *E. coli* and purified on a Ni^{2+}-agarose column.

Cell Transfection—HEK293 or COS-7 were plated in 6-well plates and transfected using LipofectAMINE reagent (Invitrogen) or Effectene (Qiagen) according to the manufacturer's protocol. In co-transfection experiments, the molar ratio of Bad-expressing plasmid to that of GFP-expressing plasmid (pGFP-N2 from CLONTECH) was adjusted to 5:1 to ensure co-expression of Bad with GFP. After transfection, cells were analyzed for green fluorescence by microscopy. Lower GFP fluorescence in these assays reflects apoptosis and elimination of the transfected cells. The pan-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) was purchased from Biomol. Stable MC/9 Bcl-X_L transfectants expressing various FLAG-tagged Bad constructs in the pMX-puro vector were selected in complete medium containing 2 μg/ml puromycin, and stable clones were isolated using Methocult (StemCell Technologies, Vancouver, Canada).

Immunoprecipitation and Blotting—Cells were lysed with ice-cold solubilization buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.25% Nonidet P-40, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO4, 1 mg/ml microcystin-LR, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 0.5 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor) and incubated on ice for 1 min. Samples were centrifuged (20,000 × *g*, 1 min), and supernatants were transferred to clean tubes. 2 μg of anti-FLAG monoclonal antibody (Sigma) was added, and the samples were rotated for 2 h at 4 °C. FLAG-tagged Bad immunocomplexes were captured with 25 μl of a 50% slurry of protein G-agarose beads (Amersham Biosciences, Inc.) for 1 h at 4 °C. Beads were washed 5 times with solubilization buffer and resuspended in 25 μl of 2× reducing sample buffer followed by boiling for 5 min. Samples were fractionated on 12.5% polyacrylamide gels and transferred to nitrocellulose. Blots were blocked with 3% skim milk solution for 1 h and then incubated with anti-Bad antibody (either SC-943 from Santa Cruz 1/200 dilution or phospho-Ser-112 from Cell Signaling Technology, 1/1000 dilution). The same immunoprecipitation and blotting conditions were used to detect Bad co-precipitation with Bcl-X_L, with the following exceptions. In these experiments, 5 μg of purified His-Bcl-X_L was added for 2 h at 4 °C and captured for an additional 2 h with 30 μl of Ni^{2+}-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-HCl, pH 6.8, 6 M urea, 6% 2-mercaptoethanol, 3% SDS, and 0.003% bromphenol blue) for separation by SDS-PAGE. In cases in which non-adherent cells were present in the culture medium, floating cells were also harvested by centrifugation and combined with the adherent cell pellet before lysis. The anti-PARP monoclonal antibody was obtained from Pharmingen.

Two-dimensional Peptide Analysis of Bad Phosphopeptides—To identify novel sites of phosphorylation on the Bad protein, a GST-Bad fusion protein was constructed by subcloning a *Bam*HI filled-in/*Eco*RI Bad fragment into the pGEX-2T vector. Similarly, a Bad gene fragment containing a point mutation of Ser-170 to Ala was also inserted into pGEX-2T for the generation of fusion protein. Briefly, GST-Bad or GST-Bad S170A was isolated from bacterial lysates with glutathione-Sepharose and washed twice in resuspension buffer containing 1% Nonidet P-40 and once in kinase buffer (20 mM Hepes, pH 7.4, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 5 mM EGTA, pH 7.2, 75 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 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 10^7 GM-CSF-stimulated MC/9 cells (25), 50 μM ATP, and 10 μCi of [γ-^{32}P]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 microcapillary high performance liquid chromatography electrospray ionization tandem mass spectrometry using data-dependent ion selection (34).

Apoptosis 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

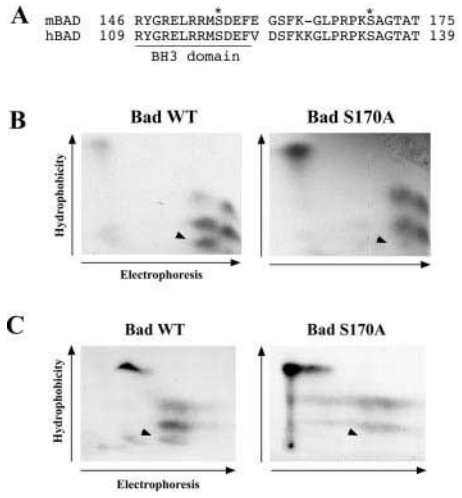

FIG. 1. **Ser-170 of Bad is phosphorylated *in vitro* and *in vivo*.** *A*, comparison of murine (*mBAD*) and human (*hBAD*) protein sequences surrounding the Ser-155 and Ser-170 sites. *B*, two-dimensional maps of tryptic peptides from GST-Bad and GST-Bad S170A phosphorylated with GM-CSF-stimulated MC/9 cell extracts. *C*, two-dimensional maps of tryptic peptides from HEK293 cells expressing wild-type (*WT*) Bad or Bad S170A.

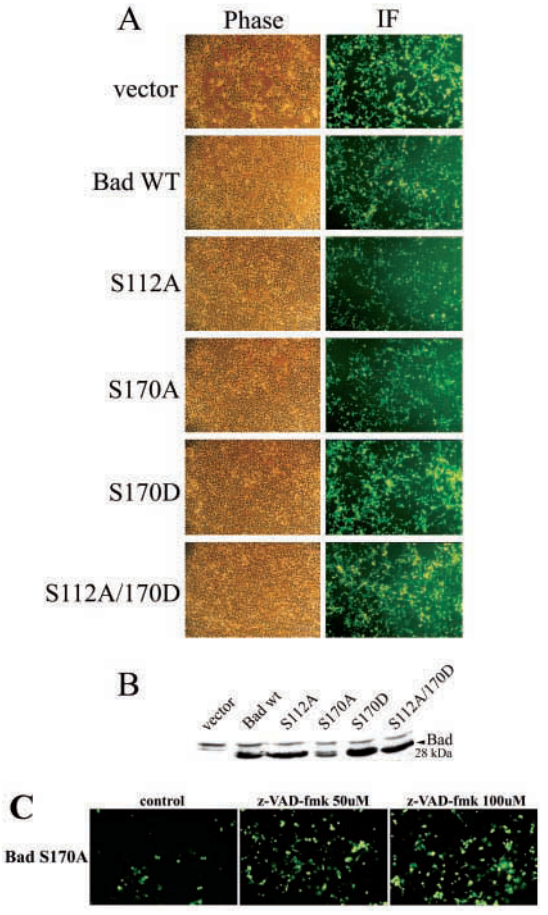

FIG. 2. **Phosphorylation of Bad on Ser-170 correlates with cell survival.** *A*, HEK293 cells were co-transfected with GFP and either vector alone, wild-type (*WT*) Bad, or the indicated Bad mutants (S112A, S170A, S170D, S112A/S170D) and analyzed for GFP fluorescence 24–48 h post-transfection. The results shown are representative of at least five separate experiments. *IF*, immunofluorescence. *B*, expression levels of Bad variants in transfected cell lysates (corresponding to $\sim 1 \times 10^6$ cells) were detected with anti Bad antibody (SC-943). *C*, HEK293 cells co-transfected with GFP and Bad S170A in the presence of different amounts of z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) and analyzed for GFP fluorescence 48 h post-transfection.

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 microcapillary 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 recently (11–15), although this has not been proven. Sequence alignment of mouse and human Bad (Fig. 1*A*) shows that human Bad Ser-134 corresponds to murine Bad Ser-170, and the surrounding residues are perfectly conserved, which also suggests that phosphorylation at this site could be important in regulation of Bad function. In all experiments presented, residue numbers refer to positions in murine Bad.

Phosphorylation of Bad at Ser-170 Occurs in Vitro and in Vivo—To further verify the identity of the Ser-170 containing peptide on the two-dimensional phosphopeptide maps, the GST-Bad Ser-170 was mutated to Ala. As expected, one spot was observed to be absent on GST-Bad S170A maps compared with wild-type GST-Bad, confirming that Ser-170 can be phosphorylated *in vitro* using GM-CSF-stimulated cell extracts (Fig. 1*B*). We next demonstrated that phosphorylation of Ser-170 occurs *in vivo*. When HEK293 cells stably transfected with FLAG-tagged wild-type Bad or Bad S170A were labeled with [^{32}P]orthophosphate and immunoprecipitated with anti-FLAG antibody, there was also one less tryptic peptide observed in the S170A mutant compared with wild-type Bad (Fig. 1*C*). 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. 2*A*, 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

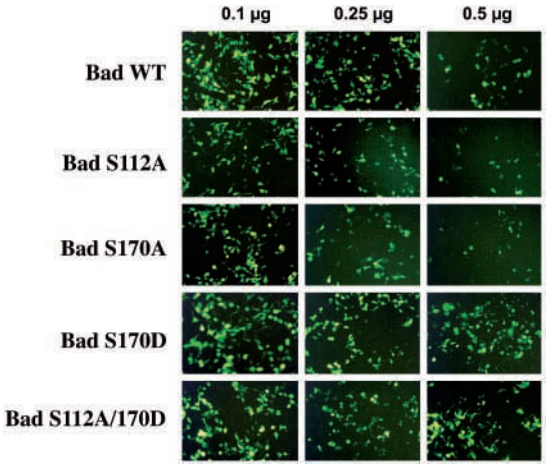

FIG. 3. **Increased levels of transfected Bad wild-type, Bad S112A, and Bad S170A promote apoptosis, whereas Bad S170D and Bad S112A/S170D do not cause apoptosis.** HEK293 cells were co-transfected with GFP and either vector alone, wild-type Bad, or the indicated Bad mutants (S112A, S170A, S170D, S112A/S170D) 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.

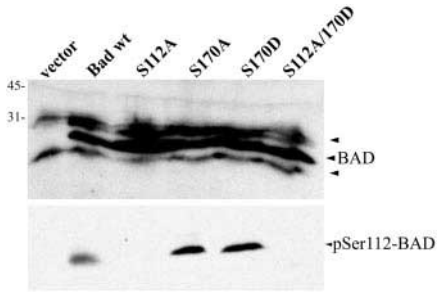

FIG. 4. **Bad S170A and S170D are phosphorylated on Ser-112.** Immunoblot analysis of HEK293 cells stably transfected with the various Bad mutants. Cell lysates corresponding to $\sim 7 \times 10^6$ cells were probed with the polyclonal anti Bad antibody (SC-943, *upper panel*) or anti-phospho Bad Ser-112 (*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.

pro-apoptotic activity compared with wild-type Bad. Similar results were obtained with a glutamate substitution (data not shown). Immunoblot analysis of transiently transfected cells demonstrated that the variant Bad proteins were expressed and that the enhanced cytotoxicity of wild-type Bad, Bad S112A, and Bad S170A was not due to elevated levels of protein expressed in these transfectants (Fig. 2*B*). The human HEK293 cells express very low levels of endogenous Bad protein that cannot be detected with the polyclonal antibody used in Fig. 2*B*, which is specific for murine Bad (data not shown). In the presence of the broad spectrum caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone), apoptosis induced by the mutant Bad S170A was completely suppressed, which also supports the conclusion that in these assays, loss of GFP expression reflects apoptosis (Fig. 2*C*).

Phosphorylation of Bad at Ser-170 Inhibits the Pro-apoptotic Activity of Bad S112A—To determine whether phosphorylation at both Ser-112 and Ser-170 was required for inhibiting the pro-apoptotic activity of Bad, the double mutant Bad S112A/S170D was constructed. Upon expression in HEK293 cells, Bad S112A/S170D demonstrated no pro-apoptotic activity compared with Bad S112A; results were similar to those observed for the vector alone or the Bad S170D mutant (Fig. 2*A*). These data indicate that Bad phosphorylation at Ser-170 or substitution with a negatively charged residue at this site is sufficient to block the death function of Bad and is independent of the phosphorylation state of Ser-112. A dose-response analysis was also performed with wild-type Bad and mutants. As shown in Fig. 3, increased amounts of transfected Bad lead to increased apoptosis. Again, Bad S170A as well as Bad S112A demonstrated an enhanced pro-apoptotic activity (decreased GFP expression) compared with Bad wild type. In contrast, cells transfected with Bad S170D and Bad S112A/S170D expressed GFP to the same extent as vector alone.

Bad S170A and S170D Are Phosphorylated on Ser-112—To determine whether Ser-112 was phosphorylated under the conditions tested in the various mutants, immunoblotting was performed using the phospho-Bad Ser-112 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. 5*A*, 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. 5*B*). 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. 5*C*). 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. 5*D*). Finally, Bad S170A also induced cleavage of PARP similar to that induced by Bad S155A (Fig. 5*E*). 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_L Heterodimerization—One mechanism by which Bad S170D may rescue cells from apoptosis induced by Bad is by disrupting Bad/Bcl-X_L association, as clearly shown previously

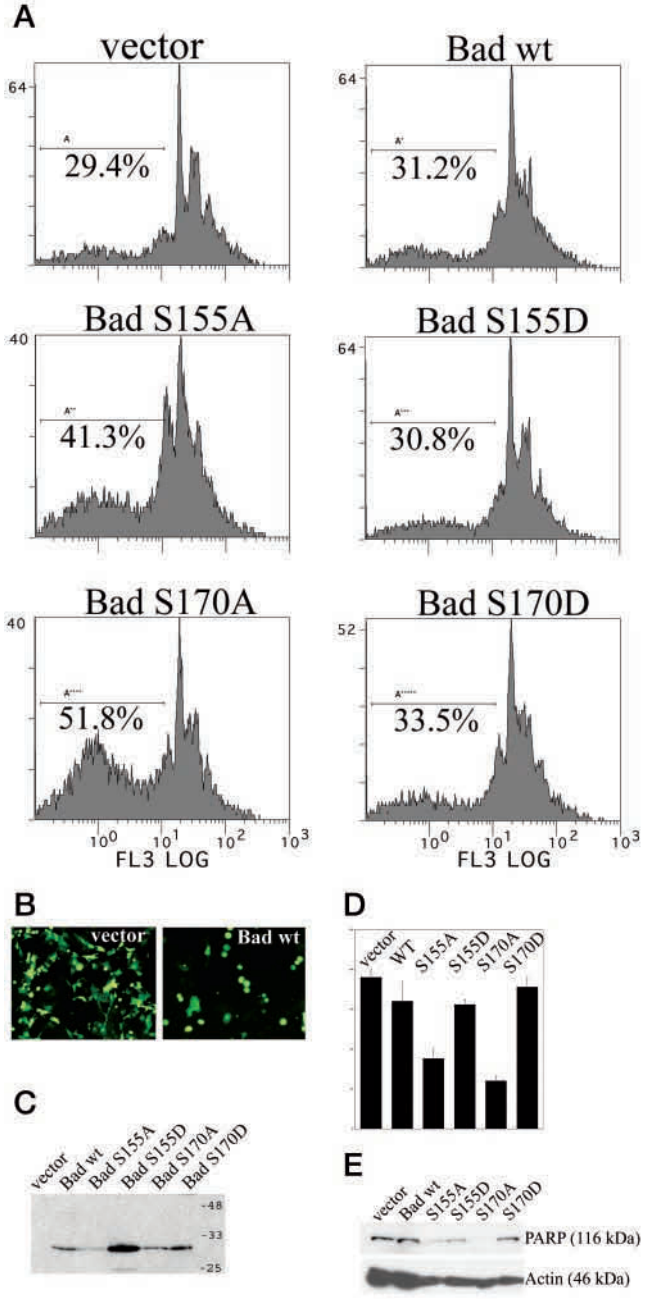

FIG. 5. **Bad S170A expression is sufficient to induce apoptosis in COS-7.** *A*, COS-7 cells were transfected with pCDNA.3 derivatives expressing various FLAG-tagged Bad mutants. After 36 h, the cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry. Histograms 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 propidium 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 3×10^5 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.

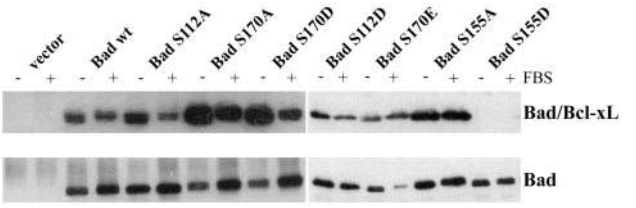

FIG. 6. **Heterodimerization of Bad with Bcl-X_L is not affected by Asp substitutions at either Ser-112 or Ser-170.** Stable HEK293 cells overexpressing various FLAG-tagged Bad mutants were analyzed for association with Bcl-X_L by pull-down assay with purified His-Bcl-X_L. Cells were starved for 18 h without serum (−) and then stimulated for 30 min with 15% serum (+). The *upper panel* shows Bad protein associated with Bcl-X_L, 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.

for phosphorylation at Ser-155 (12–14). Pull-down assays with purified recombinant His-Bcl-X_L 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-X_L. As shown in Fig. 6, Bad S170D as well as Bad S112D and wild-type Bad associated with Bcl-X_L, as indicated by co-precipitation. Only in the case of the Bad S155D mutant was there a loss of association with Bcl-X_L. 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-X_L.

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-X_L-encoding retrovirus. MC/9 Bcl-X_L 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-X_L 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-X_L, 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 clone12. 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.

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

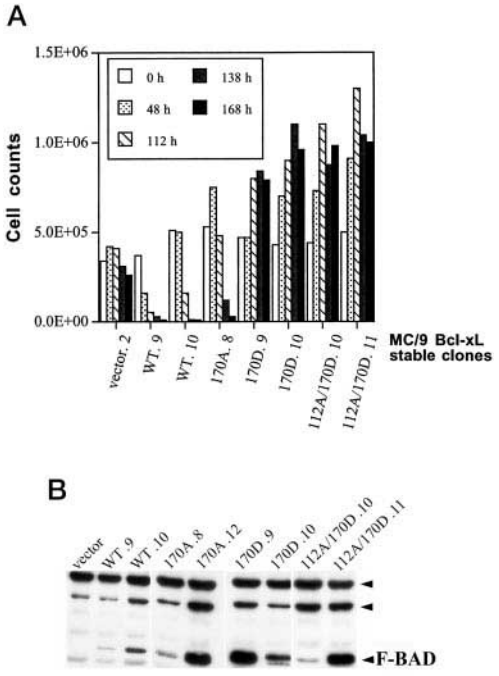

FIG. 7. **Viability of MC/9-Bcl-X_L clones expressing wild type (*WT*) and mutant forms of Bad.** *A*, indicated clones were deprived of IL-3, and the relative fraction of viable cells at the indicated time points was assessed by trypan blue exclusion. Similar results were obtained using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolim colorimetric assay (not shown). *B*, FLAG-tagged Bad was immunoprecipitated from cell extracts from the corresponding clones, and the blot was probed with the monoclonal anti-Bad antibody. *F-Bad* indicates the band corresponding to FLAG-tagged Bad, and the *upper two arrows* indicate cross-reacting bands.

determine the function of phosphorylation at the Ser-170 site and its relative importance compared with other known sites of phosphorylation on Bad. The cells used for such experiments are important to note, since we have found differences in the effects of Bad expression that depend on cell type. One explanation for such variability in Bad function may be the differences in endogenous levels of Bcl-2, Bcl-X_L, and perhaps other Bcl-2 family members. However, another factor to consider is the differential expression of specific kinases that can phosphorylate Bad at the various sites. More analyses in various systems testing the role of various kinases in phosphorylation at all sites of Bad will be necessary to fully understand how Bad is inducing apoptosis and how survival pathways may either block or modulate its action.

In this study, we first compared the physiological effects of Bad on two different cell lines into which the mutated forms of Bad can be readily introduced by transfection strategies, COS-7 and HEK293. The COS-7 cells were found to be transfected much more efficiently, allowing the analysis of these cells by flow cytometry. COS-7 cells did not appear to be undergoing apoptosis in the presence of wild type Bad, based on the detection of sub-diploid DNA using propidium iodide. However, we did find that the cells expressing Bad were affected by the presence of the protein, undergoing dramatic changes in cell morphology. There was a clear contrast in the effects of introducing Bad with mutations S155A or S170A, both of which caused increased apoptosis as assessed by propidium iodide staining, cell viability, caspase 3 activation,[2] and PARP cleavage, compared with the Bad S155D or S170D mutants, which did not cause apoptosis and did not produce any morphologic changes. In the case of HEK293, the wild type Bad protein had a more pronounced effect on the cells that were transfected. However, it was still possible to select stable transfectants that expressed Bad. When cells were transfected with Bad mutants having the major sites of phosphorylation, Ser-112, Ser-155, and as we have now shown, Ser-170, mutated to Ala, there was clearly an enhanced level of apoptosis. Furthermore, changing Ser-170 to the negatively charged Asp had a dramatic effect in limiting the ability of Bad to cause apoptosis, supporting the hypothesis that phosphorylation of Bad at Ser-170 is a key event in controlling its ability to induce apoptosis.

We have also tested the Bad mutants in a model system that is more physiologically relevant, the cytokine-dependent MC/9 cells, in which we first characterized phosphorylation of Bad at Ser-170 (24). We introduced wild type Bad and the various mutants in parental MC/9 cells or MC/9 cells overexpressing either Bcl-2 or Bcl-X_L using retroviral infection strategies and selected stable clones expressing the Bad protein. We did not find any effect of Bad in parental MC/9 cells or in MC/9 overexpressing Bcl-2 (data not shown), in agreement with the results obtained in FL5.12 cells in the first paper describing Bad (9). In MC/9 Bcl-X_L cells, we found that wild-type Bad as well as Bad with the S170A mutation induced apoptosis upon cytokine starvation, whereas changing Ser-170 to the charged Asp residue had a dramatic inhibitory effect on the function of Bad. In each of the clones in which S170D was present, including the S112A/S170D double mutants, there was greatly enhanced survival of the MC/9 cells, and in fact there was also an increase in cell numbers. This could suggest that phosphorylation of Bad at Ser-170 may not only function to block the pro-apoptosis effect of Bad, but it could have additional growth-promoting effects or enhance the pro-survival function of Bcl-X_L. At present, we also cannot rule out the possibility that Bad S170D could have other functions that are independent of its association with Bcl-X_L. Further experiments will be required to investigate these possible novel functions of Bad.

Several recent reports have also raised the possibility that Bad can have effects on cell cycle progression. Mok *et al.* (36) show that in mouse thymocytes expressing Bad, both the percentage of cells in S phase and the production of IL-2 in those cells were increased substantially. Because Bad also increased thymocyte apoptosis, it was suggested that the effect on cell cycle progression may be attributed to a feedback mechanism, which may be explained as an attempt by these cells to overcome the pro-apoptotic effect of Bad. However, two more recent reports provide intriguing data also showing that Bad can have effects on promoting cell growth and progression through the cell cycle (22, 23). Maslyar *et al.* (22) suggest that Bad phosphorylation at Ser-136 and association with 14-3-3 is required for growth promotion in chick embryo fibroblasts (22). On the other hand, Chattopadhyay *et al.* (23) find that Bad association with Bcl-X_L promoted cell cycle progression through the G_0/G_1 checkpoint in mouse embryo fibroblasts, but this effect was independent of Bad phosphorylation (23). Although these studies came to opposite conclusions regarding the requirement for Bad phosphorylation, only the two originally reported sites of phosphorylation on Bad, Ser-112 and Ser-136, were tested. In light of our results, the role of Ser-170 phosphorylation must also be considered.

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_L (18). Mutational studies provide strong evidence that Bad promotes apoptosis by heterodimerizing with Bcl-X_L, 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

[2] S. Dramsi and V. Duronio, unpublished information.

by abolishing the affinity of Bad BH3 for Bcl-X_L (11–15), whereas phosphorylation on Ser-112 and Ser-136, which are located outside the BH3 domain, may inhibit dimerization with Bcl-X_L 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-X_L complexes. Thus, although the Bad S170D mutants do not induce apoptosis, the protein can still associate with Bcl-X_L. 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-X_L 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 R*XX*S, which fits with the sequence PRPKS at Ser-170, but the two prolines make this an unlikely protein kinase A site. Preliminary experiments have not revealed any role for cAMP in stimulating Bad phosphorylation in MC/9 cells (data not shown). The possibility of a calmodulin-dependent kinase acting in the hemopoietic cell system in response to cytokines is also unlikely since cytokines are not known to induce a calcium flux. Clearly, identification of the kinase(s) that phosphorylates Bad at Ser-170 will enhance our understanding of cytokine-dependent survival signals. It will also be important to determine the temporal sequence of the phosphorylation events in various cell types expressing Bad. Datta *et al.* (13) propose that phosphorylation of the Ser-136 site may precede phosphorylation of Ser-155 and perhaps affects Bad so that it can be phosphorylated at Ser-155. Phosphorylation at Ser-170 could play a similar role, although our results with the Bad S170D mutant suggest that phosphorylation at Ser-170 still allows Bad association with Bcl-X_L. Efforts to identify the kinase that phosphorylates Ser-170 as well as other possible studies investigating the role of Ser-170 in the function of Bad will be greatly facilitated by the availability of a phospho-specific antibody, which is currently in preparation.

In summary, we have characterized a fourth site of phosphorylation on the BH3-only Bcl-2 family member, Bad. In assays using three different cell lines, it was clear that mutations at the newly characterized Ser-170 site had a profound effect on the pro-apoptotic effect 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-X_L. Finally, we speculate that phosphorylation at Ser-170 on Bad, although it is still associated with Bcl-X_L, may enhance the survival effect of Bcl-X_L 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.

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