Evidence That Ser^{87} of Bim_{EL} Is Phosphorylated by Akt and Regulates Bim_{EL} Apoptotic Function*

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Xiao-Jun Qi¹, Gary M. Wildey¹, and Philip H. Howe²

From the Department of Cell Biology, Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, Ohio 44195

Bim, the Bcl-2 interacting mediator of cell death, is a member of the BH3-only family of pro-apoptotic proteins. Recent studies have demonstrated that the apoptotic activity of Bim can be regulated through a post-translational mechanism whereby ERK phosphorylation serves as a signal for Bim ubiquitination and proteasomal degradation. In this report, we investigated the signaling pathways leading to Bim phosphorylation in Ba/F3 cells, an interleukin-3 (IL-3)-dependent B-cell line. IL-3 stimulation induced phosphorylation of Bim_{EL}, one of the predominant isoforms of Bim expressed in cells, at multiple sites, as evidenced by the formation of at least three to four bands by Western blotting that were sensitive to phosphatase digestion. The appearance of multiple, phosphorylated species of Bim_{EL} correlated with Akt, and not ERK, activation. The PI3K inhibitor, LY294002, blocked IL-3-stimulated Akt activity and partially blocked Bim_{EL} phosphorylation. In vitro kinase assays showed that recombinant Akt could directly phosphorylate a GST-Bim_{EL} fusion protein and identified the Akt phosphorylation site in the Bim_{EL} domain as Ser^{87}. Further, we demonstrated that cytokine stimulation promotes Bim_{EL} binding to 14-3-3 proteins. Finally, we showed that mutation of Ser^{87} dramatically increases the apoptotic potency of Bim_{EL}. We propose that Ser^{87} of Bim_{EL} is an important regulatory site that is targeted by Akt to attenuate the pro-apoptotic function of Bim_{EL}, thereby promoting cell survival.

Bim, the Bcl-2 interacting mediator of cell death, is a member of the BH3-only family of pro-apoptotic proteins that also includes Bad and Bid (1–3). Bim proteins are expressed by a wide variety of tissues, but are most prominently expressed by cells of hematopoietic origin (4). Although multiple Bim mRNA transcripts have been described that are thought to be generated by alternative splicing (1, 5–8), most tissues express one predominant isoform of Bim as determined by Western blotting analysis, termed Bim_{EL}. Under conditions that promote cell growth, Bim is bound to dynein light chain (LC8), of the microtubular motor complex, and is sequestered away from other Bcl-2 family members (9). Following a pro-apoptotic stimulus, however, Bim localizes to the mitochondria, where it initiates the mitochondrial cell death pathway by either directly activating Bax-like proteins (7, 10) or by binding to pro-survival Bcl-2 family members and thereby releasing Bax-like proteins (11–13).

The apoptotic function of Bim is regulated by both pro-survival and pro-apoptotic cytokines. Withdrawal of IL-2 (14) or IL-3 (15–17) from cytokine-dependent hematopoietic cells results in an up-regulation of Bim expression with an associated induction of apoptosis. Similar results are obtained when nerve growth factor is withdrawn from cultured neuronal cells (18–21). However, as opposed to negative regulation of Bim expression levels by these pro-survival cytokines, we have recently demonstrated that addition of a pro-apoptotic cytokine, TGFβ, results in the up-regulation of Bim expression levels in two different B-cell lines, WEHI 231 and Ba/F3 (22). We further demonstrated that stimulation of the pro-survival CD40 receptor is capable of inhibiting TGFβ-mediated Bim expression and cell death in WEHI 231 cells concomitant with its rescue of the cells from apoptosis.

Several cell signaling pathways have been implicated in the control of Bim expression levels. In Ba/F3 cells, selective activation of either the Ras/MAPK or the Ras/PI3K pathway by inducible Ras mutants suppresses the increase in Bim expression normally observed during IL-3 deprivation (16). The inhibition of Bim expression by Ras/PI3K activation could be blocked by either the PI3K inhibitor wortmannin or the mTOR inhibitor rapamycin, but not by overexpression of a dominant negative form of Akt. Taken together, these results suggest that, although both Akt and mTOR are downstream effectors of PI3K activation, only mTOR regulates Bim expression levels.

Other studies have also demonstrated an inhibitory effect of PI3K signaling on Bim expression. The PI3K inhibitor LY294002 induces Bim expression in B cells (Ba/F3) (15) and T cells (CTLL2) (14), concomitant with an increase in cell death. Thus, the biological effects of LY294002 were identical with those of cytokine withdrawal in these model systems. In these studies, however, it was proposed that an important downstream effect of LY294002 was to reduce the amount of Akt in its active, phosphorylated form. The link between reduced Akt activity and increased Bim expression levels was shown to be FOXO3 (previously termed FKHR-L1), a member of the forkhead family of transcriptional regulators that can directly elevate Bim expression levels and induce apoptosis in Ba/F3 and CTLL2 cells when overexpressed. FOXO3 is phosphorylated by Akt, leading to its cytoplasmic retention by 14-3-3 proteins and loss of target gene activation (23). Thus, the up-regulation of Bim expression induced by cytokine withdrawal or LY294002 addition correlated with a loss of the active, phosphorylated form of Akt and an increase in the active, non-phosphorylated form of FOXO3.

More recent studies have demonstrated that the apoptotic function of Bim is regulated by phosphorylation, followed by ubiquitination and degradation. Phosphorylated forms of Bim_{EL} and Bim_{E} were initially reported in Ba/F3 cells grown in the presence, but not the absence, of

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Cell Biology, NC-1, Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, OH 44195. Tel.: 216-445-9750; Fax: 216-444-9404; E-mail: howep@ccf.org.

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IL-3 (16). Subsequently, Bim\textsubscript{EL} phosphorylation has been demonstrated in nerve growth factor-stimulated PC12 cells (21) and superior cervical ganglion cells (24), serum-stimulated CCL39 fibroblasts (25), IgM-stimulated Ramos B cells (26), IL-2-maintained HT-2 T lymphocytes (27), and macrophage-colony stimulating factor-stimulated osteoclasts (28).

In the majority of these studies Bim phosphorylation could be blocked by the MEK/ERK pathway inhibitors PD98059 or U0126. Inducible expression of Raf, an upstream activator of the MEK/ERK pathway, in CCL39 fibroblasts (29), MCF10A mammary cells (30), and 293 cells (26) also resulted in Bim\textsubscript{EL} phosphorylation that could be blocked by U0126 and not by LY294002. A specific role for ERK in Bim phosphorylation was established using in vitro kinase assays, where it was demonstrated that purified ERK could phosphorylate only Bim\textsubscript{EL}, and not Bim\textsubscript{L} or Akt phosphorylation site in the Bim\textsubscript{EL} domain as Ser87. Akt activation by insulin was obtained from Invitrogen. pRL-TK was purchased from Promega (Madison, WI). Reagent chemicals and sodium vanadate were obtained from Sigma.

Cell Culture—Ba/F3 cells were maintained in T-75 flasks at a density of 2 × 10\textsuperscript{5} cells/ml (45-ml total) in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum, 30 μM 2-β-mercaptoethanol, and antibiotics/antimycotics (100 units/ml penicillin G, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B, Invitrogen). Conditioned medium from WEHI 3B cells was added to a final concentration of 10% to provide the IL-3 essential for Ba/F3 survival (32). Conditioned medium was prepared from WEHI 3B cells seeded at an initial density of 10 × 10\textsuperscript{5} cells/ml (45-ml total) and grown for 24 h in Ba/F3 medium.

To induce Bim expression, Ba/F3 cells were seeded at an initial density of 5–10 × 10\textsuperscript{5} cells/ml (45-ml total) and grown overnight in the absence of conditioned medium (IL-3- or cytokine-deprived). The next day, conditioned medium (10–20% final concentration) or recombinant mouse IL-3 (0.05–2.0 ng/ml, as indicated) was added for the indicated time period to induce Bim phosphorylation. At the end of the experiment, the cells were collected by centrifugation, washed twice with phosphate-buffered saline, and resuspended in the appropriate lysis buffer. Ba/F3 cells were kept at 4 °C during all the above procedures. Where indicated LY294002 (50 μM), wortmannin (100 nM), U0126 (10 μM), PD98059 (10 μM), SP600125 (10 μM), SB202190 (15 μM), or MG-132 (50 μM) were added to the culture medium 30–60 min before IL-3 stimulation and remained present during the IL-3 treatment.

Human Hep3B and rat FAO hepatoma cells were cultured in Dulbeccco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics/antimycotics. Where indicated, Hep3B cells were serum-starved for 48 h prior to the addition of insulin (10 μg/ml). MG-132 (50 μM) was added to the culture medium 60 min before insulin stimulation and remained present during the insulin treatment.

Western Blot Analysis—Western blot analysis was performed by standard SDS-PAGE, as described previously (22). Whole cell lysates were prepared in TMNG lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5.0 mM MgCl\textsubscript{2}, 0.5% Nonidet P-40, and 10% glycerol) containing protease inhibitors and 1 mM Na\textsubscript{3}VO\textsubscript{4}. Lysates were sonicated and clarified by centrifugation at 10,000 g, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B, Invitrogen). Conditioned medium from WEHI 3B cells was added to a final concentration of 10% to provide the IL-3 essential for Ba/F3 survival (32). Conditioned medium was prepared from WEHI 3B cells seeded at an initial density of 10 × 10\textsuperscript{5} cells/ml (45-ml total) and grown for 24 h in Ba/F3 medium.

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Alkaline Phosphatase Digestion of Bim—Triplicate samples of cell lysates (150 μg) were individually digested with either a low (20 units) or high (50 units) dose of calf alkaline phosphatase for 1 h at 37 °C in buffer supplied by the manufacturer. For one set of digests, the calf alkaline phosphatase was heat-inactivated at 95 °C for 5 min prior to adding the cell lysate. For the third set of digests, the phosphatase inhibitor Na\textsubscript{3}VO\textsubscript{4}.

MATERIALS AND METHODS

Reagents—TGFβ was a generous gift from Genzyme Inc. (Cambridge, MA) and was used at a final concentration of 5 ng/ml. Rabbit anti-Bim antibody was obtained from BD Pharmingen (San Diego, CA). Rabbit anti-phospho-Akt (Ser-473) and rabbit anti-total Akt were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-phospho-ERK (E4), rabbit anti-ERK 1 (C-16), mouse anti-14-3-3β (H-8), rabbit anti-HSP 90 (H-114), and normal rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Active recombinant mouse MAPK/ERK 2, recombinant human Akt1/PKBα kinase, recombinant Gsk-3β, and ribosomal S6 kinase 1/MPKAP kinase 1α were purchased from Upstate (Charlottesville, VA). Secondary antibodies were purchased from the following vendors: anti-mouse-IgG-HRP from Accurate Antibodies (San Diego, CA) and anti-rabbit-IgG-HRP from Bio-Rad (Hercules, CA). MG-132, LY294002, PD98059, SP600125, SB202190, and wortmannin were obtained from Calbiochem. U0126 was purchased from Promega (Madison, WI). Murine recombinant IL-3 was purchased from BioSource International (Camarillo, CA), and human recombinant insulin was from Sigma. Protease inhibitor mixture tablets were purchased from Roche Diagnostics (Indianapolis, IN). Tris-buffered saline-casein for Western blotting was obtained from Pierce, and Rainbow low molecular weight markers were from Amersham Biosciences. Calf alkaline phosphatase was purchased from New England Biolabs (Beverly, MA). Lipofectamine Plus transfection reagent was obtained from Invitrogen. pRL-TK–Renilla luciferase and the Dual-Luciferase Reporter Assay system were purchased from Promega (Madison, WI). Reagent chemicals and sodium vanadate were obtained from Sigma.

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lysates. Whole cell lysates for immunoprecipitation (IP) were prepared both purified enzymes and kinases immunoprecipitated from whole cell protein. GST-BimEL fusion proteins containing single serine to alanine substitution (GST-BimEL<sup>S87A</sup>) was made using the top primer 5′-CTTATGAGAATGATCCGCCCTGCTGTCCGATC-3′ and its complement in PCR reactions. The serine 87 to alanine substitution (GST-BimEL<sup>S87A</sup>) was made using the top primer 5′-CTTATGAGAATGATCCGCCCTGCTGTCCGATC-3′ and its complement in PCR reactions. The serine 94 to alanine substitution (GST-BimEL<sup>S94A</sup>) was made using the top primer 5′-CTTATGAGAATGATCCGCCCTGCTGTCCGATC-3′ and its complement in PCR reactions.

**In Vitro Kinase Assays**—In vitro kinase assays were performed using both purified enzymes and kinases immunoprecipitated from whole cell lysates. Whole cell lysates for immunoprecipitation (IP) were prepared in IP lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitors and 1 mM Na<sub>2</sub>VO<sub>4</sub>. Lysates were sonicated and clarified by centrifugation at 4 °C for 10 min in a Beckman microcentrifuge at maximum speed. Cell lysates (250 μg) were incubated for 4 h at 4 °C with 2.5 μg of either rabbit anti-p-Akt antibody or anti-Akt antibody in 200 μl of IP lysis buffer containing Protein G-agarose (Amersham Biosciences). The immune complexes were collected by centrifugation, washed five times in IP lysis buffer, and resuspended in 30 μl of kinase buffer, and ligated into pGEX-2T to obtain the truncated BimEL fusion protein. BimEL<sup>S69A</sup> fusion proteins containing single serine to alanine substitutions were produced using a QuiChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s directions. The template for mutations was BimEL<sup>S69A</sup>-ligated into pGEX-2T. The serine 87 to alanine substitution (GST-BimEL<sup>S87A</sup>) was made using the top primer 5′-CTTATGAGAATGATCCGCCCTGCTGTCCGATC-3′ and its complement in PCR reactions. The serine 94 to alanine substitution (GST-BimEL<sup>S94A</sup>) was made using the top primer 5′-CGATCCGCCCTGCTGTCCGATC-3′ and its complement in PCR reactions.

**RESULTS**

**Bim<sub>EL</sub> Is Phosphorylated and Proteasomally Degraded in Ba/F3 Cells**—Ba/F3 cells require IL-3 in the tissue culture medium to survive and proliferate, and withdrawal of IL-3 leads to increased Bim expression and DNA ladder formation, a hallmark of apoptosis (15, 16). Because of the recent interest in Bim as a target for ERK phosphorylation and proteosomal degradation, it was of interest to determine whether or not the suppression of Bim expression in Ba/F3 cells by IL-3 involved Bim phosphorylation.

To this end, Ba/F3 cells were incubated overnight in the presence or absence of WEHI 3B-conditioned medium, which contains IL-3 (32). The next day, conditioned medium was added to the culture medium of “starved” cells for 30 min prior to collecting the cells. Western blot analysis of the cell lysates with anti-Bim antibody revealed the presence of 25- to 30-kDa, high molecular mass forms of Bim, presumably BimEL<sub>EL</sub> (Fig. 1A, top panel). As expected, withdrawal of conditioned medium dramatically increased Bim<sub>EL</sub> expression in Ba/F3 cells (lane 2, −CM) relative to cells grown in the presence of conditioned medium (lane 1, + CM). Addition of conditioned medium led to the formation of at least three species of Bim<sub>EL</sub> protein (lanes 3 and 4). To prove that it was the IL-3 in the WEHI 3B conditioned medium that stimulated Bim<sub>EL</sub> phosphorylation, starved Ba/F3 cells were incubated for 30 min with recombinant IL-3. IL-3 stimulation led to a dose-dependent increase in the appearance of multiple, phosphorylated Bim<sub>EL</sub> bands (Fig. 1A, lanes 5–9), and this stimulation was greater than that observed with conditioned medium (lanes 3 and 4). There also appeared to be a loss of immunoreactive Bim<sub>EL</sub> protein at higher doses (1–2 ng/ml) of IL-3.
Ser87 Phosphorylation of Bim

**FIGURE 1.** IL-3-induced Akt activation in Ba/F3 cells correlates with BimEL hyper-phosphorylation and proteasomal degradation. **A**, Ba/F3 cells were incubated overnight in the presence or absence of conditioned medium. The next day starved cells were stimulated with either 10% or 20% conditioned medium (+ CM, 10 and 20%) or with increasing doses of recombinant IL-3 (IL-3, 0.05–2.0 ng/ml), as indicated. After a 30-min incubation, cells were collected, lysed, and analyzed by Western blotting. Cells were also collected at the beginning (+ CM and − CM) of the experiment to serve as controls. **B**, Ba/F3 cells were starved overnight, and the next day were stimulated with IL-3 (1.0 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting. **C**, cell lysates were prepared from Ba/F3 cells stimulated with CM for 15 min and divided into eight aliquots. Three aliquots were digested with either a low (lanes 4 and 5) or high (lanes 6 and 7) dose of calf alkaline phosphatase for 1 h. One set of alkaline phosphatase digestions was heat-inactivated (CIP/Heat) prior to digestion while another set was digested in the presence of the phosphatase inhibitor sodium vanadate (CIP/Nav). As controls, one aliquot of cell lysate was untreated and the other was incubated for 1 h in buffer only. The cell lysates were analyzed by Western blotting. **D**, Ba/F3 cells were incubated overnight in the presence or absence of conditioned medium. The next day conditioned medium was added back to the cytokine-deprived cells for 1 h. Prior to this, cells were incubated for 1 h in the absence (+ CM and − MG) or presence (+ CM and + MG) of MG-132. Cell lysates were prepared and analyzed by Western blotting. Cells were also collected at the beginning (+ CM and − CM) of the experiment to serve as controls. The location of molecular mass markers is shown at the left.

IL-3. Taken together, these observations suggest that IL-3 induces a dose-dependent phosphorylation of BimEL at multiple sites in Ba/F3 cells, leading to its degradation. A time-course study of IL-3 stimulation of Ba/F3 cells showed that the apparent phosphorylation and degradation of BimEL was rapid in onset (Fig. 1B).

The results of Fig. 1 (A and B) are consistent with the idea that the addition of IL-3 to Ba/F3 cells leads to the production of multiple forms of phosphorylated BimEL. To prove that the observed mobility shift in BimEL species was due to phosphorylation, cell lysates from Ba/F3 cells stimulated for 15 min with conditioned medium were digested with calf alkaline phosphatase (CIP) (Fig. 1C). Digestion of the cell lysates with a low (lane 3, L) or high (lane 6, H) dose of CIP resulted in a dose-dependent increase in the mobility of BimEL species compared with the undigested control sample (lane 1, control). Blocking CIP activity by either heat inactivation of the enzyme (lanes 4 and 7, CIP/Heat) or addition of the phosphatase inhibitor sodium vanadate (lanes 5 and 8, CIP/Nav) prevented the CIP-induced increase in BimEL mobility. Incubation of the lysate with buffer in the absence of CIP had no effect (lane 2, buffer). Thus, these results demonstrate that the multiple species of BimEL are produced by phosphorylation.

If the addition of IL-3 to Ba/F3 cells leads to the phosphorylation of BimEL, then the apparent loss of BimEL protein observed in Fig. 1 (A and B) may be due to proteasomal degradation of phosphorylated BimEL. To address this question, Ba/F3 cells were starved overnight and treated with conditioned medium the next morning for 1 h in the presence or absence of the proteasomal inhibitor MG-132 (Fig. 1D). As expected, BimEL protein levels were increased by withdrawal of the conditioned medium (lane 2, − CM versus lane 1, + CM). Once again, addition of conditioned medium stimulated BimEL phosphorylation and loss of BimEL total protein in the absence of MG-132 (lane 3). In the presence of MG-132, however, a higher molecular weight, phosphorylated species of BimEL accumulated (lane 4). Taken together, the results of Fig. 1 demonstrate that IL-3 stimulates BimEL phosphorylation in starved Ba/F3 cells and signals the proteasomal degradation of BimEL.

**II-3 Stimulates BimEL Phosphorylation by Akt**—The results in Fig. 1A suggest that IL-3 stimulates BimEL phosphorylation at multiple sites, particularly at higher (0.5–2 ng/ml) doses of IL-3. Because BimEL is a known target of ERK phosphorylation, then it is possible that the different levels of BimEL phosphorylation observed in Fig. 1A resulted from different levels of IL-3-induced ERK activation. To address this question, the level of ERK activation was determined using an anti-active phospho-ERK antibody. The results (Fig. 1A, middle panels) demonstrated that most doses of IL-3 and CM gave a robust stimulation of ERK.
FIGURE 2. LY294002 inhibits Akt activation and BimEL phosphorylation induced by IL-3. A, Ba/F3 cells were incubated overnight in the presence or absence of conditioned medium. The next day starved cells were stimulated for 30 min with either of two doses of recombinant IL-3, as indicated. Stimulated cells were preincubated for 30 min with either 50 μM LY294002 (LY) or Me2SO prior to the addition of IL-3. The LY294002 remained present throughout the IL-3 stimulation. Cell lysates were analyzed by Western blotting. Cells were also collected at the beginning (−CM and −CM) of the experiment to serve as controls. B, similar to panel A except that cells were preincubated in the absence or presence of 100 nM wortmannin (Wort) prior to IL-3 stimulation for 5, 15, or 30 min. C, similar to panel A except that cells were preincubated with 30 μM LY294002 (LY) prior to being stimulated with IL-3 for 1–30 min. D, Ba/F3 cells were stimulated for 30 min with 1.0 ng/ml of recombinant IL-3. Stimulated cells were preincubated for 30 min with either 50 μM LY294002 (LY), 10 μM PD98059 (PD), 10 μM SP600125 (SP), 15 μM SB202190 (SB), 10 μM U0126 (U0), or Me2SO prior to the addition of IL-3. The inhibitors remained present throughout the IL-3 stimulation. Cell lysates were analyzed by Western blotting. Cells were also collected at the beginning (~CM) of the experiment to serve as controls. E, the results (Fig. 1A, bottom panels) demonstrated a dose-dependent increase in phospho-Akt immunoreactivity with both IL-3 and CM that paralleled the dose-dependent increase in BimEL phosphorylation. The level of total Akt protein was equal in all lanes. This result indicated that Akt may be involved in IL-3-induced BimEL phosphorylation.

If IL-3 stimulates BimEL phosphorylation by Akt, then LY294002, a PI3K inhibitor that acts as an upstream inhibitor of Akt activation, should block IL-3-induced BimEL phosphorylation. Cytokine-deprived Ba/F3 cells were stimulated with two different doses of IL-3 for 30 min in the absence or presence of LY294002 and analyzed for BimEL phosphorylation (Fig. 2A, top panels). As expected, IL-3 induced BimEL phosphorylation in a dose-dependent manner (lanes 3 and 4). The addition of LY294002 inhibited the phosphorylation of BimEL to higher molecular weight forms (lanes 5 and 6). Control blots demonstrated that LY294002 blocked the formation of active, phospho-Akt (Fig. 2A, lanes 5 and 6, lower panels). Further experiments demonstrated that 100 nM wortmannin, another PI3K inhibitor, was also capable of inhibiting IL-3-stimulated BimEL phosphorylation and Akt activation, similar to LY294002 (Fig. 2B).

There are reports that LY294002 can inhibit ERK activation in Ba/F3 cells (33). Because this would complicate the interpretation of our BimEL phosphorylation results, control experiments were performed to investigate the effect of LY294002 on IL-3 stimulated ERK activity (Fig. 2C). The results demonstrated that LY294002 specifically blocked the IL-3-stimulated formation of active, phospho-Akt (Fig. 2C, bottom panels) and had no effect on the formation of active, phospho-ERK (Fig. 2C, top panels). Time-course analysis also indicated that LY294002 had no effect on the rapid kinetics of ERK activation by IL-3 (Fig. 2D).

Bim has also been reported to be a target for JNK phosphorylation (34). Therefore, other MAPK pathways, besides ERK, may play a role in IL-3-induced BimEL phosphorylation. To explore this possibility, specific inhibitors of the ERK (PD98059, U0126), JNK (SP600125), and p38 (SB202190) pathways were tested to determine their effect on BimEL phosphorylation. The results showed that neither SP600125 nor SB202190 inhibited IL-3-induced BimEL phosphorylation. Similarly, the results showed that neither PD98059 nor U0126 inhibited BimEL phosphorylation, with U0126 being the most effective inhibitor. Taken together, our results suggested that the ERK and Akt pathways were involved in IL-3-induced BimEL phosphorylation.

Akt Can Phosphorylate GST-BimEL. —The results of Figs. 1 and 2 suggest that Akt is involved in IL-3-stimulated BimEL phosphorylation. To activity, as measured by anti-phospho-ERK immunoreactivity, with little change in total ERK protein.

We next considered whether or not other kinases might be responsible for the multiple phosphorylated forms of BimEL induced by IL-3 stimulation. We initially chose to investigate a potential role for Akt in IL-3-induced BimEL phosphorylation because of numerous studies demonstrating the ability the PI3K inhibitor LY294002 to induce Bim expression and apoptosis in Ba/F3 cells (15, 17). The level of Akt activation was determined using anti-active phospho-Akt (Ser-473) antibody. The results (Fig. 1A, bottom panels) demonstrated the ability the PI3K inhibitor LY294002 to induce Bim phosphorylation in a dose-dependent manner (lanes 3 and 4). As expected, IL-3 induced Bim phosphorylation in a dose-dependent manner (lanes 3 and 4). The addition of LY294002 inhibited the phosphorylation of Bim to higher molecular weight forms (lanes 5 and 6). Control blots demonstrated that LY294002 blocked the formation of active, phospho-Akt (Fig. 2A, lanes 5 and 6, lower panels). Further experiments demonstrated that 100 nM wortmannin, another PI3K inhibitor, was also capable of inhibiting IL-3-stimulated Bim phosphorylation and Akt activation, similar to LY294002 (Fig. 2B).
Ser87 Phosphorylation of Bim

A.

- CM 5 15 30 60 (min)

IP: α-Akt
In Vitro Kinase: Gst/Bim

B.

- CM 5 15 30 60 (min)

blot: α-p-Akt

C.

- CM IL3 (15 min) Ly+IL3

IP: α-IgG α-Akt
In Vitro Kinase: Gst/Bim

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Phospho-Akt antibody (Fig. 3B, top panel). The results demonstrated that the time course of IL-3-induced Akt activity measured with the in vitro GST-BimEL kinase assay paralleled that measured by using the anti-phospho-Akt antibody. Collectively, these data support the idea that Ba/F3 stimulation by IL-3 induces the phosphorylation of BimEL by Akt.

Akt Phosphorylates BimEL on Ser87—To confirm that Akt phosphorylates BimEL, we examined the ability of recombinant Akt to phosphorylate GST-BimEL using the in vitro kinase assay (Fig. 4A). As a positive control, we also tested equal units of activity of pure ERK. As controls, we used pure Rsk, a member of the AGC kinase family (protein kinase A, G, and C families) like Akt, as well as pure Gsk-3β, a member of the CMGC kinase family (cyclin-dependent kinase, MAPK, Gsk-3, and CDC-like kinase families) like ERK, in the in vitro kinase assay. Recombinant Akt phosphorylated GST-BimEL but to a lesser extent than ERK (Fig. 4A, top left panel). The phosphorylation of GST-BimEL by recombinant Akt was dose-dependent (data not shown). In contrast, both Rsk and Gsk-3β demonstrated little phosphorylation of GST-BimEL (Fig. 4A, top left panel). All of the pure kinases phosphorylated their model substrates (Fig. 4A, right panel). Control experiments demonstrated that none of the purified kinases phosphorylated GST by itself (Fig. 4A, left bottom panel) and that Coomassie Blue staining revealed equal amounts of substrate used in all of these experiments (data not shown).

We next wanted to determine the Akt phosphorylation site(s) of GST-BimEL. The reported consensus site for Akt phosphorylation is RXRXX(S/T) (35–38). Although this exact site is not found in the protein domain specific to human BimEL, there are two copies of the sequence "RXXS" found in its C-terminal region (see schematic in Fig. 4B). We therefore generated two mutants of GST-BimEL that contained serine to alanine substitutions at positions 87 and 94, termed GST-BimEL[S87A] and GST-BimEL[S94A], respectively. In addition, we generated a mutant GST-BimEL protein from which the last 16 C-terminal amino acids (Ser88 to Thr101) were deleted, termed GST-BimEL[C16]. We then tested the ability of recombinant Akt to phosphorylate these three mutant GST-BimEL fusion proteins using the in vitro kinase assay (Fig. 4C, middle panel). Akt phosphorylation of GST-BimEL[S87A] (lane 3) was similar to that of wild-type GST-BimEL (lane 1). Akt was unable, however, to phosphorylate either GST-BimEL[S94A] (lane 2) or GST-BimEL[C16] (lane 4). As a control, all three mutant GST-BimEL fusion proteins were tested as substrates for in vitro phosphorylation by recombinant ERK (Fig. 4C, top panel) because the reported ERK phosphorylation site, Ser89 (25, 26), is preserved in all of the GST-BimEL mutants (see schematic in Fig. 4B). As expected, all three GST-BimEL mutants were phosphorylated by recombinant ERK (Fig. 4C). Identical results were obtained when the Akt used in the in vitro kinase assay was obtained by immunoprecipitation from IL-3-stimulated Ba/F3 lysates (Fig. 4D). These results, therefore, identify Ser87 as the Akt phosphorylation site.

Phosphorylated BimEL Binds 14-3-3 Proteins in Vivo—It was of interest to determine if BimEL phosphorylation by Akt served some regulatory function. Several studies have demonstrated that Akt phosphorylates Bad on Ser136, resulting in a down-regulation in the apoptotic function of Bad via sequestration by 14-3-3 proteins (39–41). Therefore, we explored whether or not Akt phosphorylation of Bim promoted 14-3-3 binding in co-immunoprecipitation experiments (Fig. 5A, top panel). Our results demonstrated that IL-3 stimulated a rapid and sustained increase in the amount of 14-3-3 protein that co-immunoprecipitated with BimEL (lanes 2–5) compared with untreated, control levels (lane 1). Control, rabbit IgG was not able to immunoprecipitate 14-3-3 protein (data not shown), demonstrating the specificity of the BimEL-14-3-3 interaction. Although this association could be demon-
cell lines. Initially, we tested the ability of overexpressed, wild-type BimEL to induce apoptosis in FAO cells. As shown in Fig. 6A, transient transfection of BimEL into FAO cells resulted in the dose-dependent appearance of a high molecular weight form of immunoreactive Bim that was not observed in cells transfected with GFP (top right panel). This result is consistent with the fact that overexpressed BimEL contains an epitope tag. Interestingly, there appeared to be multiple bands of overexpressed BimEL. Western blotting for HSP-90 demonstrated equal protein loading (bottom right panel). As expected, overexpression of BimEL led to a dose-dependent increase in cell death in FAO cells as measured by DNA ladder formation (Fig. 6A, left panel). The extent of cell death was also demonstrated more quantitatively as a loss of TK-RENilla activity with increasing concentrations of co-expressed BimEL (Fig. 6B). This type of cell survival assay has been used previously to determine the relative apoptotic potency of BimEL mutants using either CMV-Juciferase (26) or GFP fluorescence (21, 24) as a readout assay.

We next tested the apoptotic potency of three mutants of BimEL compared with wild-type BimEL. The mutants contained single serine to alanine substitutions at residue 69, the ERK phosphorylation site, or residue 87, the Akt phosphorylation site, or residue 94, a non-phosphorylated site. Western blotting demonstrated that all mutants could be expressed, but their patterns of expression were different (Fig. 6C, bottom panels). The expression of the S94A mutant was similar to wild-type BimEL. Interestingly, neither the S69A or S87A mutant showed a dose-dependent increase in expression with increased DNA, in contrast to wild-type BimEL and the S94A mutant. The S87A mutant demonstrated the lowest expression level of all BimEL proteins. The S69A mutant was the only BimEL protein that did not demonstrate multiple immunoreactive bands. Western blotting for HSP-90 demonstrated equal protein loading.

When the ability of the four BimEL proteins to induce apoptosis was measured (Fig. 6, C (top panel) and D), we found that the S69A mutant...
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A.

B.

FIGURE 5. Akt stimulation promotes BimEL binding to 14-3-3 proteins. A, Ba/F3 cells were starved overnight and the next day were stimulated with IL3 (1.0 ng/ml) for the times indicated. Bim was immunoprecipitated from cell lysates and the amount of 14-3-3 protein co-immunoprecipitated with Bim was determined by Western blotting (top panel). The amount of Bim (middle panel) and 14-3-3 (bottom panel) protein in the original cell lysates was also determined by Western blotting. B, Hep3B cells were serum-starved for 48 h prior to the addition of insulin for the times indicated. Bim was immunoprecipitated from cell lysates, and the amount of 14-3-3 protein co-immunoprecipitated with Bim was determined by Western blotting (top panel). The amount of phospho-Akt (second panel), phospho-Erk (third panel), and Hsp-90 (bottom panel) protein in the original Hep3B cell lysates was also determined by Western blotting.

was more potent than wild-type BimEL, as expected. However, we were surprised to find that the S87A mutant was clearly more potent than the S69A mutant. The S94A mutant was about equal to wild-type BimEL in its ability to induce cell death. Taken together, the results of Fig. 6 suggest that the low expression of the S87A mutant is likely, because it is a very potent inducer of apoptosis and cannot be overexpressed without killing the cell.

DISCUSSION

Expression levels of BH3-only proteins are key determinants of cellular survival and are therefore under stringent control. Transcriptional and post-translational regulatory mechanisms have previously been described in the control of Bim expression levels. Cytokine-regulated phosphorylation of Bim and its ubiquitin-mediated degradation through the proteasomal pathway has recently emerged as a key regulatory mechanism controlling cellular Bim levels. In this study we show that the kinases responsible for IL-3-induced BimEL phosphorylation include not only ERK, which has previously been documented, but also the Akt kinase. We provide evidence that Akt phosphorylation of BimEL, and not ERK, acts as a signal for BimEL binding to, and likely sequestration by, 14-3-3 proteins in that 14-3-3 binding correlates with Akt activation and not that of ERK (Fig. 5B). Finally, we demonstrate that mutation of the Akt phosphorylation site in BimEL, Ser87, to alanine dramatically increases the apoptotic potency of BimEL.

Our results demonstrate that IL-3 stimulation of Ba/F3 cells induced phosphorylation of BimEL at multiple sites, as evidenced by the formation of at least three to four bands by Western blotting that were sensitive to phosphatase digestion. This finding is in agreement with the results of two previous studies in which the number of BimEL species was examined by two-dimensional gel electrophoresis (25, 27). For example, it was shown that BimEL immunoprecipitated from [32P] metabolically labeled murine HT-2 or Bal17 lymphocytes, separated into at least four [32P]-labeled forms (27). Furthermore, some spots appeared to contain more than one form of [32P]-labeled BimEL. Phospho-amino acid analysis revealed only [32P]-labeled serine and not threonine or tyrosine residues. After IL-2 deprivation, at least two of the [32P]-labeled forms of BimEL were decreased in HT-2 cells (27). Similar results were obtained in CCL39 fibroblasts, where addition of fetal bovine serum to serum-starved cells increased the appearance of three, presumably phosphorylated, forms of immunoreactive BimEL (25). The results of both of these studies provide strong evidence that BimEL is highly phosphorylated and that this phosphorylation is tightly regulated by cytokines.

The role of the ERK pathway in Bim-mediated phosphorylation has attracted the most investigation. The fetal bovine serum-induced appearance of multiple forms of BimEL in CCL39 cells was shown to be sensitive to the ERK1/2 pathway inhibitor, PD184352, suggesting that multiple ERK phosphorylation sites exist in BimEL (25). A more recent study, in the IL-3-dependent murine pro-B cell line, FL5.12, demonstrates that when IL-3 is added back to cytokine-deprived cells there is a rapid shift in the mobility of BimEL to an apparently higher molecular weight form, consistent with its phosphorylation (45). Further, six BimEL mutants were generated by alanine substitution of individual, putative ERK phosphorylation sites and tested for their ability to be phosphorylated when transfected into IL-3-stimulated FL5.12 cells. Three mutants, S55A, S65A, and S100A, failed to undergo a mobility shift, indicating that ERK could potentially phosphorylate BimEL at these three sites. Western blotting with anti-BimEL antibodies that recognize only the phosphorylated Ser55, Ser65, and Ser100 sites confirmed that all of these sites were phosphorylated in cells overexpressing wild-type BimEL after IL-3 stimulation and were sensitive to the MEK/ERK pathway inhibitor, U0126.

Despite the above evidence that ERK phosphorylates BimEL at multiple sites, two studies have demonstrated that a single mutation in GST-BimEL fusion proteins at Ser65 of murine BimEL (25), or the equivalent Ser66 of human BimEL (26), is sufficient to completely abrogate phosphorylation by recombinant ERK in an in vitro kinase assay. ERK was shown not to phosphorylate the BimL or BimS isoforms of Bim in vitro (25). Thus, it is unlikely that the multiple phosphorylated forms of BimEL observed by us, as well as others, can be accounted for by only ERK phosphorylation. In this regard, Bim has also been reported to be a substrate for JNK-mediated phosphorylation (34). However, all of the three potential INK phosphorylation sites identified were found using the BimL isoform as substrate and are not present in the “EL-specific” domain of Bim. In our study, we did not observe any IL-3-induced mobility shift in BimEL (Fig. 1D), and a specific INK inhibitor, SP600125, had no effect on IL-3-induced BimEL phosphorylation (Fig. 2E). Furthermore, the p38 inhibitor SB202190 also had no effect on IL-3-induced BimEL phosphorylation, whereas the ERK pathway inhibitors PD98059 and U0126 inhibited phosphorylation, as expected (Fig. 2E). Therefore, it is likely that only the ERK MAPK pathway is involved in IL-3 stimulated BimEL phosphorylation in Ba/F3 cells.

Based on these previous studies, we reasoned that kinases other than ERK and JNK may mediate the IL-3-induced phosphorylation of BimEL observed in our studies of Ba/F3 cells. We decided to explore a potential role for Akt in BimEL phosphorylation because of the well recognized role of the Akt pathway in cell survival in general (36–38), and Ba/F3 cells in particular (15, 17, 46), although there are contradictory reports.
that the PI3K-Akt pathway plays no role in Ba/F3 apoptosis (47, 48). Our results demonstrate that higher doses of IL-3 stimulate activation of the Akt pathway, in addition to the ERK pathway, which correlate with BimEL phosphorylation at multiple sites. Furthermore, the PI3K/Akt pathway inhibitor LY294002 consistently blocked both IL-3-stimulated Akt activation and BimEL phosphorylation, supporting a role for Akt in BimEL phosphorylation. The blockade of BimEL phosphorylation by LY294002 was of note, because it has been reported that this inhibitor does not block acute (<1 h) Bim phosphorylation induced by nerve growth factor in PC12 cells (21), Raf overexpression or phorbol 12-myristate 13-acetate treatment in 293 cells (26), or IL-3 stimulation in FL5.12 cells (45). LY294002 also had no effect in more long-term studies (6–18 h) of BimEL phosphorylation induced by fetal bovine serum in CCL39 cells (29) or Raf overexpression in MCF-10A cells (30). The reason for these apparent contradictory results with the LY294002 inhibitor may be related to several factors, including cell type, culture conditions, or strength of stimulus. In addition, in this study we routinely observed more phosphorylated BimEL forms by Western blot analysis than that found in these other studies, and thus effects on phosphorylated species may be more easily detected. We also obtained identical inhibition of IL-3-stimulated BimEL phosphorylation with wortmannin, another PI3K inhibitor structurally unrelated to LY294002, further supporting a role of the PI3K/Akt pathway.

Our data demonstrating that both recombinant Akt and Akt immunoprecipitated from IL-3-stimulated Ba/F3 cells could phosphorylate a GST-BimEL fusion protein supports a role for Akt in IL-3-stimulated BimEL phosphorylation in vivo. The Akt phosphorylation site we identified in GST-BimEL by mutagenesis, Arg-Arg-Ser-Ser^87, is conserved in both human and mouse species, suggesting it plays an important biological role. Although this sequence does not constitute the full Akt consensus phosphorylation site often cited, RXRXX(S/T) (35–38), other Akt substrates containing only an RXRXX(S/T) phosphorylation site have been reported, such as cAMP-response element-binding protein (37). In addition, the Akt phosphorylation site in BimEL, Ser^87, is immediately followed by the amino acid leucine in both the human and mouse protein sequence. It is thought that the presence of a bulky, hydrophobic

FIGURE 6. The S87A mutant of BimEL is a more potent inducer of cell death compared with wild-type BimEL. A, duplicate plates of FAO cells were transiently transfected with either 5 or 15 μg of wild-type BimEL vector or 15 μg of GFP vector using Lipofectamine Plus. After 24 h, the cells were harvested, and lysates were prepared for analysis of either DNA ladder formation (left panel) or BimEL expression by Western blotting (right panel). In the left panel, lane 1 contains a standard 100-bp DNA ladder as a size marker. B, FAO cells were co-transfected with TK-Renilla vector and increasing concentrations of wild-type BimEL or GFP vector, as indicated. After 24 h, the cells were lysed and assayed for luciferase activity. The values shown represent the average of quadruplicate wells ± S.D. of a representative experiment. C, similar to panel A except that four different BimEL vector constructs were used in transfections: wild-type and S69A, S87A, and S94A mutants. Only 5 μg of vector was used in transfections for DNA ladder experiments (top panel). Western blotting results are shown in the bottom panels. D, similar to panel B except that the four different BimEL vector constructs mentioned above were tested.
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residue, such as phenylalanine or leucine, C-terminal to serine is favorable for Akt phosphorylation (35, 36). It is interesting to note that there are no other potential Akt phosphorylation sites in the Bim protein sequence besides the two RXXS sites located in the C terminus of the Bim EL domain. Thus, only Bim isoforms containing this unique domain will be subject to Akt phosphorylation and regulation. This may be another reason why BimEL is the weakest inducer of apoptosis compared with BimL and BimS when overexpressed (1, 6, 21).

Recently it has been shown that the ERK docking domain of Bim maps between amino acids Ala70 and Thr97 using GST pull-down experiments (25). More recently, this domain has been identified as a DEF (docking site for ERK, EXFP) domain containing the amino acid sequence FSF (49). The DEF domain is located at the extreme C-terminal end of the EL domain of BimEL, after the ERK phosphorylation site. Interestingly, the Akt phosphorylation site that we have identified, Ser87, maps between the ERK phosphorylation and docking sites. It is unclear, however, whether or not Akt phosphorylation of BimEL would affect the ability of ERK to dock with and phosphorylate BimEL, because it was also demonstrated that GST-BimEL fusion proteins that contained the ERK phosphorylation site, but either lacked the ERK docking site or from which the DEF domain was deleted, could still be phosphorylated by ERK (25, 49).

In the present study we also explored the effect of BimEL phosphorylation on 14-3-3 binding. Our finding that Akt stimulation in Ba/F3 and Hep3B cells promoted increased BimEL binding to 14-3-3 proteins is similar to results of previous studies demonstrating that Akt phosphorylation of another BH3-only protein, Bad, also results in 14-3-3 binding (41). It is postulated that one effect of binding to 14-3-3 proteins is to sequester a protein away from its normal binding partners, thereby causing a biological effect. In the case of BimEL, 14-3-3 sequestration may prevent its dimerization with and neutralization of the pro-survival proteins Bcl-2 and Bcl-X, leading to increased cell survival. Therefore, BimEL sequestration by 14-3-3 proteins may represent a secondary mechanism to rapidly neutralize excess, toxic BimEL proteins that escape ERK-induced phosphorylation and degradation. This idea is supported by our finding that hyperphosphorylated forms of BimEL are always observed, even after prolonged incubation with high doses of IL-3 (Fig. 1, A and B, using 1.0 ng/ml IL-3). This pool appears to represent only a small percentage of the total immunoreactive BimEL detected prior to IL-3 treatment (−CM); therefore, it is likely that ERK-induced degradation of BimEL represents the primary cellular mechanism to attenuate the apoptotic effects of BimEL. In this regard, we also consistently noticed that the amount of immunoreactive BimEL observed after LY294002 treatment was always greater than that detected in corresponding controls, even though the level of ERK activation, as measured by p-ERK Western blotting, was always greater in LY294002-treated samples (Fig. 2C). This suggests that Akt phosphorylation of BimEL may contribute to BimEL degradation through an as yet undescribed mechanism, which we are currently investigating.

Several studies have shown that LY294002 increases Bim protein levels in Ba/F3 cells (15), as well as other cells (14, 31). It is now clear that this effect of LY294002 is due to its downstream inhibition of Akt activity. Akt phosphorylates forkhead transcription factors, causing them to be retained in the cytoplasm via binding to 14-3-3 proteins (23). Inhibition of Akt activity by LY294002 allows forkhead proteins to translocate into the nucleus, where they are known to be direct activators of Bim gene expression (50–52). Therefore, Akt activation can down-regulate Bim transcription via its phosphorylation of forkhead proteins. The direct phosphorylation and sequestration of BimEL by Akt described herein would appear to complement this long term effect of Akt on Bim transcription by immediately neutralizing pre-existing Bim within a cell.

The biological importance of the Akt phosphorylation site, Ser87, was clearly demonstrated by the increased apoptotic potency in transient transfection studies of the S87A BimEL mutant compared with either wild-type BimEL or, unexpectedly, the S69A ERK phosphorylation site BimEL mutant (Fig. 6, C and D). Although many studies have made Ser to Ala mutations of Bim to study their effect on either Bim phosphorylation or apoptotic function (21, 24–26, 30, 34, 46), this is the first study to examine the biological importance of the Ser87 site. The most striking feature of this mutant was our inability to overexpress it, even at earlier time points (data not shown). Although weakly expressed, the S87A BimEL mutant demonstrated two bands by Western blotting, suggesting that it was still phosphorylated, presumably by ERK. The apoptotic potency of the Ser87 BimEL mutant indicates that this site may regulate more than just 14-3-3 binding and we are exploring this idea.

The existence of multiple signaling pathways regulating apoptosis is likely to be critical for cell survival. In the present study, we have provided evidence that the apoptotic function of Bim is regulated by Akt, in addition to ERK. Thus, Bim would represent another Bcl-2 protein, in addition to MCL-1 (53) and Bad (54–56), that is targeted by multiple kinase signaling pathways to regulate its function. It will be of interest to determine in future studies whether or not pro-apoptotic cytokines such as TGFβ, which we have previously shown to increase Bim expression levels (22), can enhance the apoptotic function of Bim by down-regulating ERK and Akt activity.

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