A Chimeric Protein Induces Tumor Cell Apoptosis by Delivering the Human Bcl-2 Family BH3-Only Protein Bad

Antonella Antignani and Richard J. Youle*

Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: Deregulation of PI3K/Akt and Raf/Mek/Erk signal transduction cascades is one of the principal causes of neoplastic transformation. The inactivation of the proapoptotic protein Bad, upon phosphorylation by different kinases of these two pathways, may play an important role in different human malignancies. Therefore, we have expressed and purified a new chimeric protein, hGM-CSF−Bad, linking the human granulocyte-macrophage colony-stimulating factor to the N-terminus of the proapoptotic protein human Bad, to deliver Bad into tumor cells and induce apoptosis. Indeed, the human GM-CSF receptor is a good target because it is overexpressed on many leukemias and solid tumors and is not detectable on stem cells. We found that the chimeric protein binds the human GM-CSF receptor, is endocytosed, and appears to reach the cytosol via retrograde ER transport. After entering cells, the protein is able to induce apoptosis of human leukemia cells and human colon and gastric carcinoma cell lines (IC_{50} values as low as 1 μM). We conclude that GM-CSF−Bad can overcome the inappropriate survival stimuli in transformed cells and restore the apoptotic pathway. The completely human sequence and the elevated selectivity for cancer cells could prevent immunogenicity and the nonspecific toxicity of targeted toxins in future clinical application of this fusion protein.

As apoptosis is a mechanism used by cells to regulate tissue homeostasis through the elimination of redundant or potentially deleterious cells (*1*, *2*), insufficient apoptosis underlies many diseases, including cancer. The Bcl-2 family of proteins, classified by sequence homology of four α-helix segments, called BH1−BH4, governs commitment to apoptosis in response to diverse physiological clues (*3*). The antiapoptotic members (Bcl-2, Bcl-XL, Mcl1, and A1) contain all four BH domains (*4*−*6*). The proapoptotic proteins can be subdivided into two groups. Bax, Bak, and Bok (*7*, *8*) are multidomain proteins with the BH1−BH3 domains, whereas the BH3-only members (Bid, Bad, Noxa, and Puma) contain only the BH3 minimal death domain and do not exhibit sequence conservation outside this domain. The NMR and the crystal structure of Bcl-XL bound to Bak, Bad, or a Bim peptide reveal that the pro-survival protein forms a pocket capable of binding the BH3 domains of the other family members (*9*−*12*).

Bax and Bak molecules constitute requisite gateways to the mitochondria, as well as the endoplasmic reticulum, for the control of apoptosis (*13*, *14*). BH3-only molecules operate upstream, connecting proximal death signals to the activation of Bax and Bak (*15*). That leads to a cascade of events, including a collapse of mitochondrial membrane potential, release of cytochrome *c* and SMAC, and activation of caspases (*16*−*21*).

* To whom correspondence should be addressed: Biochemistry Section, SNB, NINDS, NIH, Bldg 35, Room 2C917, 35 Convent Dr. 2C917, MSC3704, Bethesda, MD 20892-3704. Phone: (301) 496-6628. Fax: (301) 496-3444. E-mail: youler@ninds.nih.gov.

Results from three different systems, Bad knockout and knockin mice and Bad BH3 peptides (*22*−*24*), support a model where this proapoptotic BH3-only protein has been implicated in coordinating survival signals with the mitochondrial cell death machinery. In the presence of growth factors promoting cell survival, Bad is phosphorylated and sequestered in the cytosol by binding to 14-3-3 proteins, a large family of proteins that interact with phosphoserine- and phosphothreonine-containing ligands that are thought to act as molecular chaperones (*25*). A number of different protein Ser/Thr protein kinases are known to phosphorylate Bad at different serine residues. The most well characterized phosphorylation sites occur at serine 112, 136, and 155 in the mouse protein (*22*, *26*, *27*), which correspond to serine 75, 99, and 118, respectively, in human Bad (*28*). Phosphorylated Ser112 and -136 are responsible for binding 14-3-3, whereas the phosphorylation of Ser155 prevents the interaction of Bad with the antiapoptotic proteins. Apoptotic signals cause Bad dephosphorylation and release from 14-3-3. At this point, Bad binds Bcl-XL, triggering its translocation to mitochondria and resulting in mitochondrial dysfunction, cytochrome *c* release, caspase activation, and apoptosis (*22*−*29*).

Impaired apoptosis is a crucial step in tumorigenesis as a defective cell death program endows nascent neoplastic cells with multiple selective advantages. The cells can persist in hostile environments (for example, where cytokines or oxygen is limiting), escape from death imposed by tumor suppressive fail-safe mechanisms, ignore restraining signals from neighbors, and survive detachment from the extracellular matrix leading to metastasis. A high level of

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expression of death antagonists, such as Bcl-2 and Bcl-XL, is found in a wide variety of human cancers and mediates the resistance of cancers to a wide spectrum of chemotherapeutic drugs which act by inducing apoptosis in tumor cells (*30*).

Delivery of proapoptotic members of the Bcl-2 family to tumor cells could be a strategy for restoring the defective apoptotic process. The first prototype chimeric protein, IL2-Bax, specifically targeted cells expressing the IL-2 receptor (T-cell lymphoma) and induced cell specific apoptosis in the absence of any additional death stimulus (*31*). Bik, Bax, and Bak fused to the gonadotroph-releasing hormone (GnRH) target and kill adenocarcinoma cells via the apoptotic pathway (*32*). Bad is also a good candidate for killing tumor cells as it has been demonstrated to bind and antagonize pro-survival Bcl-XL (*33*).

Human granulocyte-macrophage colony-stimulating factor (GM-CSF)[1] is a 127-amino acid cytokine that triggers the growth, differentiation, and functional enhancement of both granulocytes and macrophages from myeloid progenitor cells (*34*). The aberrant expression of GM-CSF and its high-affinity receptor have been postulated to play a central role in drug resistance AML leukemogenesis, the most common type of leukemia. Autonomous growth related to GM-CSF autocrine or paracrine production and secretion has been identified in blast samples from a majority of patients with AML and has been associated with the therapy-refractory disease and inferior survival (*35*). The GM-CSF receptor is also expressed on solid tumors, including lung, breast, and gastrointestinal carcinomas (*36*). Because GM-CSF receptors cannot be detected on early normal hematopoietic stem cells, it is a useful ligand for delivering proteins able to induce cell death, as the proapoptotic members of the Bcl-2 protein family, to myelogenous tumor cells. Different groups have found that immunotoxins with the translocation and catalytic domains of the diphtheria toxin or *Pseudomonas aeruginosa* exotoxin linked to human GM-CSF were cytotoxic in vitro to chemotherapy-resistant cell lines and therapy-refractory AML patient progenitor cells but were nontoxic to normal human myeloid progenitors (*35*, *37*−*39*). Dramatic antileukemic effects were observed also in vivo in mice with human leukemia (*39*, *40*). The immunotoxin can produce complete and partial remissions in patients with relapsed or refractory AML, but liver injuries and the presence of antibodies against DT portions of the immunotoxin were observed (*41*).

To investigate whether human GM-CSF can be used to deliver proapoptotic proteins to tumor cells, we fused human Bad to human GM-CSF (GM-CSF−Bad) and tested the cytotoxicity to leukemia cells and human gastrointestinal carcinomas. We also tested a mutant of hGM-CSF−Bad (GM-CSF−Bad AA) in which serine 75 and serine 99 (homologous to the phosphorylation sites in mouse Bad) were substituted with alanine, to prevent Bad inactivation by kinases and to enhance the ability to induce apoptosis.

EXPERIMENTAL PROCEDURES

Constructions of the Fusion Proteins. The polymerase chain reaction (PCR) was performed using the Platinum Pfx DNA polymerase kit (Invitrogen). The denaturation temperature was 94 °C for 1 min and 55 °C for 1 min, and polymerization was carried out at 68 °C for 1 min.

To assemble GM-CSF−Bad and GM-CSF−Bad AA, the cDNA of human GM-CSF was amplified from the pRKDT-GM plasmid, containing the cDNA of a recombinant fusion toxin with the first 388 residues of diphtheria toxin with an H−M linker fused to human GM-CSF, using primers that introduced a NdeI site 5′ of the cDNA of GM-CSF (5′-GAAATTCCATATGGCACCAGCACGATCGCC-3′) and a BamHI site 3′ (5′-CGCGGATCCTTCCTGTACTGGCTC-CCAGCA-3′). The cDNA of human Bad, cloned in the plasmid pCDNA3, was amplified by introducing a BamHI site at the 5′ end (5′-CGCGGATCCTTCCAGATCCCA-GAGTTTGAG-3′) and a HindIII site at the 3′ (5′-C-CCAAGCTTTCACTGGGAGGGGGCGGAGCT-3′).

The fragments were digested with the respective restriction enzymes (New England Biolabs), and purified from gels with the QIAquick Gel Extraction Kit (Qiagen). GM-CSF digested with NdeI and BamHI and Bad digested with BamHI and HindIII were incubated for 1 h a 25 °C in the presence of T4 ligase (Promega), and then ligated by T4 DNA ligase to the expression plasmid, pET22b(+) (Novagen), digested with NdeI and HindIII, by incubation for 1 h at 25 °C and overnight at 16 °C. The BamHI site in the recombinant cDNA introduces a glycine-serine linker between the two proteins.

For GM-CSF−Bad AA, pET22b(+)−GM-CSF−Bad was the template for introducing the substitutions of serine with alanine at serine 75 and 99 of human Bad in both expression plasmids, by the PCR-based overlap extension method. Mutagenic primers were oligonucleotides 5′-GGAGTCGC-CACAGCGCCTACCCCGCGGGG-3′ and 5′-CCCCGCGGG-GTAGGCGCTGTGGCGACTCC-3′ for the S75A mutation and 5′-CGGGGCCGCTCGCGCTCGCGCGCGGCGCCCC-CCAAC-3′ and 5′-GGTTGGGGGGCGCCGCGCGAGC-GCGGCCCCG-3′ for the S99A mutation. The outmost amplification primers were the primers used to amplify hBad introducing the BamHI and HindIII sites.

To construct Bad−GM-CSF, the cDNA for human Bad was amplified introducing a NdeI site at the 5′ end (5′-GGGATTCCATATGTTCCAGATCCCAGAGTT-3′) and a BamHI site at the 3′ end (5′-CGCGGATCCCTGGGAGG-GGGCGGAGCTTCC-3′). The cDNA of human GM-CSF was amplified introducing a BamHI site 5′ of the gene (5′-CGCGGATCCGCACCAGCACGATCGCCAAGC-3′) and a HindIII site 3′ (5′-CCCAAGCTTTCATTCCTGTACTG-GCTCCCA-3′). To insert the mutations in positions 75 and 99, the same mutagenic primers of GM-CSF−Bad were used; the outmost amplification primers were the primers used to amplify GM-CSF introducing the BamHI and HindIII sites.

The four expression gene constructions were verified by DNA sequencing (National Institute of Neurological Disorders and Stroke DNA Facility).

Protein Expression and Purification. Escherichia coli BL21 DE3 (Invitrogen) was used to express GM-CSF−Bad, GM-CSF−Bad AA, Bad−GM-CSF, and Bad AA−GM-CSF. Recombinant bacteria were grown in 1 L of Super Broth (KD Medical, Columbia, MD) containing 50 μg/mL ampicillin (Sigma Chemical Co., St. Louis, MO) in 2 L flasks at 37 °C. Protein expression was induced by addition of 1 mM IPTG (Sigma) when the OD_{600} reached 0.8−1. After

[1] Abbreviations: AML, acute myeloid leukemia; GM-CSF, granulocyte-macrophage colony-stimulating factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; ER, endoplasmic reticulum.

incubation for 3 h, cells were harvested by centrifugation at 5000*g*, and after resuspension in lysis buffer [0.1 M Tris-HCl (pH 8) and 10 mM EDTA], pellets were lysed with a French press. The inclusion bodies were collected by centrifugation at 20000*g* and washed four times with lysis buffer containing 2% Triton-100 and 2 mM urea and twice with lysis buffer alone. The inclusion bodies were dissolved in 0.1 M Tris-HCl (pH 8) containing 6 M guanidine, 10 mM EDTA, a protease inhibitor cocktail, and 25 mM DTT at 4 °C overnight. The proteins were refolded by dilution in a 100-fold volume of the refolding buffer [0.1 M Tris-HCl (pH 8), 0.5 M arginine, and 0.9 mM oxidized glutathione] followed by incubation at 25 °C for 48−72 h. Ion exchange chromatography was performed on an HiPrep 16/10 DEAE column and a subsequent gel filtration on a HiPrep 16/60 Sephacryl S-200 column (FPLC apparatus, Pharmacia). The fractions, eluted in PBS, were pooled and stored at 4 °C. The quality of purified proteins was analyzed by 10−20% SDS−PAGE stained with Brilliant Blue R, and Western blotting using primary antibodies against human Bad (Cell Signaling, Beverly, MA) and GM-CSF (Sigma).

Stock concentrations of GM-CSF−Bad and GM-CSF−Bad AA were determined by a colorimetric assay (BCA kit, Pierce). Final yields of GM-CSF−Bad and GM-CSF−Bad AA were between 2 and 6 mg/L of culture.

Cytotoxicity Assay. Cell lines HL-60, U937, LS-174, HTB103, HeLa, and A-431 were purchased from the American Type Culture Collection (ATCC). To access the cytotoxicity of the recombinant proteins, two kinds of assays were performed: cellular protein synthesis inhibition and apoptotic cell counting. Cellular protein synthesis inhibition was assessed as described previously (*33*) with slight modifications. Briefly, cells in 100 μL were incubated at concentrations of 1×10^5 cells/mL in 96-well microtiter plates overnight and treated with various concentrations of purified proteins for 48 h in leucine-free RPMI 1640 followed by a 1 h pulse with 0.1 mCi of [^{14}C]leucine. Then, cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Watertown, MA), and the radioactivity was counted by liquid scintillation counting. The results were expressed as a percentage of radiolabeled leucine incorporation in PBS-treated control cells. Cell viability was also determined with the Celltiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Values represent the mean of triplicate samples with a <10% standard error of the mean.

Calculation of the levels of apoptotic cells was performed as previously described (*42*), and the caspase 3/7 protease activity was measured using the ApoOne Homogeneous Caspase 3/7 Assay kit (Promega).

RESULTS

Construction and Expression of GM-CSF−Bad. To target Bad selectively to leukemia cells, the gene for GM-CSF was fused to the 5′ end of the Bad gene using cDNAs as templates for the construction of two chimeric proteins (Figure 1a). To prevent Bad inactivation by kinases and thereby potentially enhance the ability to induce apoptosis, we created a second construct with the same domain orientation, GM-CSF−Bad AA, with mutations to alanine in the sequence of human Bad at serine 75 and serine 99. These serines are homologous to the principal phosphorylation sites, Ser112 and Ser136, in mouse Bad (Figure 1a).

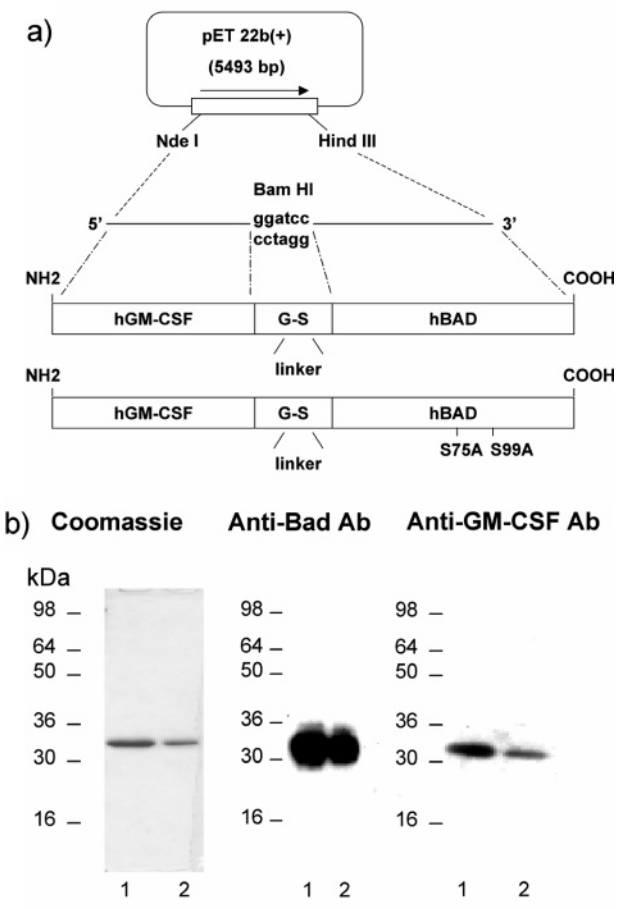

FIGURE 1: Construction and expression of the Bad and GM-CSF fusion proteins. (a) Schematic diagram of the GM-CSF fusion proteins. The cDNA for the human GM-CSF, digested with NdeI and BamHI, was fused with the cDNA of human Bad (wild-type or mutant), digested with BamHI and HindIII. The fused genes, GM-CSF−Bad and GM-CSF−Bad AA, were cloned into vector pET22b(+). The presence of the BamHI restriction site, required for the ligation of the two cDNAs, introduces a glycine and a serine as a linker between the two proteins. (b) Coomassie and Western blot analysis of GM-CSF−Bad (lane 1) and GM-CSF−Bad AA (lane 2). Purified proteins were subjected to SDS−PAGE (10−20%) and visualized by Coomassie brilliant blue staining. Western blot analysis was conducted using either anti-Bad or anti-GM-CSF antibodies.

To explore different possibilities for refolding the recombinant fusion protein expressed in *E. coli*, we assembled two additional constructs, Bad−GM-CSF and Bad AA−GM-CSF, with the GM-CSF domain C-terminal to the Bad domain. All four constructs were cloned into the pET22b-(+) expression plasmid. DNA sequencing confirmed that no errors had been introduced during the cloning and, in addition, that the recombinant proteins were inserted with the correct reading frame in the vector.

The recombinant proteins were expressed in *E. coli*, and although after induction with IPTG more than 90% of the expressed proteins were found in inclusion bodies, we were not able to purify the two recombinant proteins with Bad at the N-terminus because they were completely degraded proteolytically during purification. After denaturation and

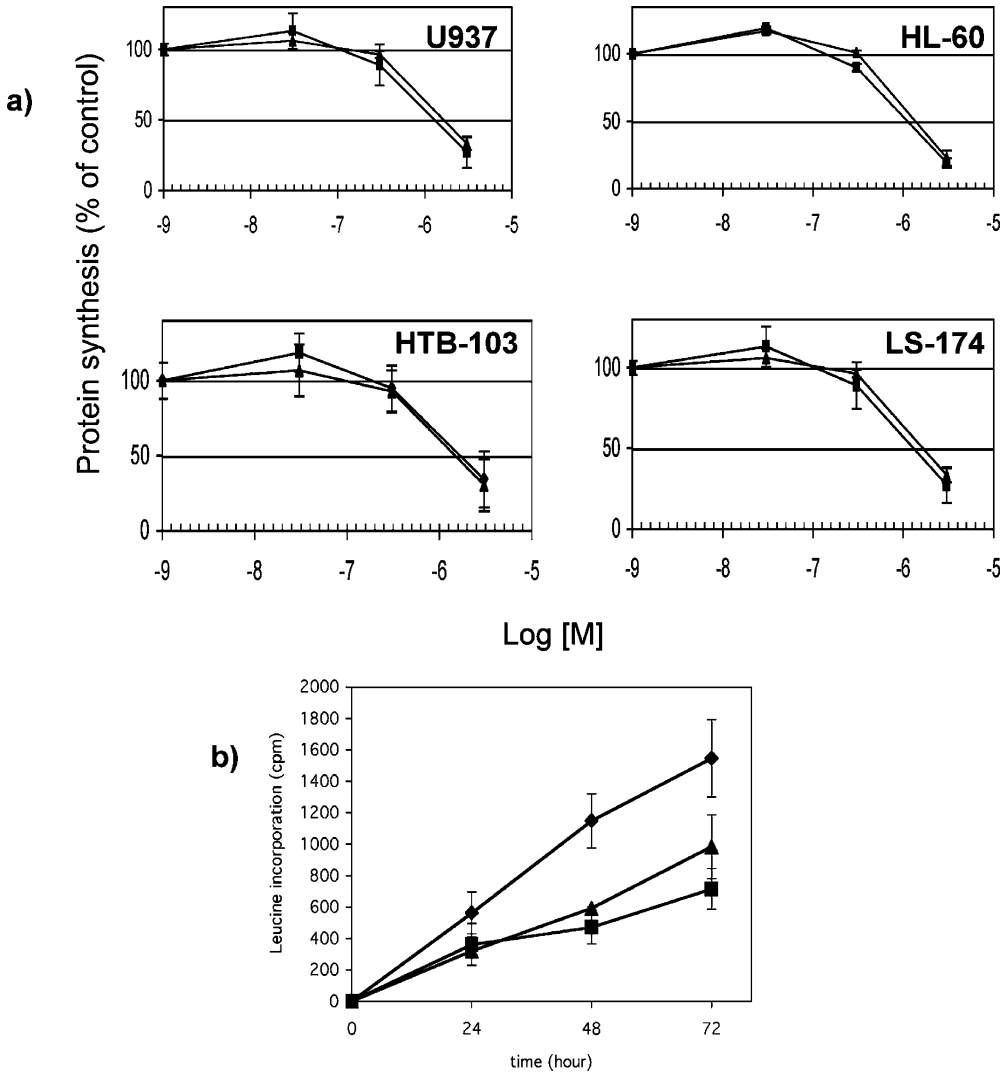

FIGURE 2: Toxicity of GM-CSF chimeric proteins to target cells. (a) The human leukemia lines, U937 and HL-60, the human gastric carcinoma line, HTB-103, and the human colon carcinoma cell line, LS174T, were incubated in the presence of GM-CSF−Bad (■) and GM-CSF−Bad AA (▲), at different concentrations, for 48 h. The cells were pulsed with [^{14}C]leucine for 1 h and harvested. The extent of leucine incorporation was measured and presented as a percentage relative to PBS-treated cells. The mean values determined from triplicate measurements are plotted vs the concentration of fusion proteins. The error bars represent the standard error of the mean. (b) Time course of GM-CSF−Bad (1 μM) and GM-CSF−Bad AA (1 μM) toxicity to human leukemia HL60, measured by the incorporation of radioactivity, after a pulse with [^{14}C]leucine, by HL-60 cells. The extent of leucine incorporation was measured and presented as a percentage relative to PBS-treated cells (◆). The error bars represent the standard error of the mean.

refolding of the GM-CSF−Bad proteins, two consecutive steps of purification, ion exchange and gel filtration chromatography, were necessary to obtain homogeneous proteins. The purified products migrated on SDS gels at the expected size of ~33 kDa (Figure 1b), and the theoretical M_r of the wild-type Bad fusion protein of 32 983.49 was verified by mass spectrometry. Western blot analysis, performed with the anti-hGM-CSF and anti-hBad antibodies, confirmed the cloning and production of the in-frame full-length chimeric protein and also the absence of proteolytic degradation products (Figure 1b).

Cytotoxic Activity of the GM-CSF−Bad Fusion Proteins on Different Tumor Cell Lines. We evaluated the effect of GM-CSF−Bad on four tumor cell lines expressing the human GM-CSF receptor: two leukemia (HL-60 and U937) and two gastrointestinal carcinoma (LS-174T and HTB-103) cell lines. The cells were incubated for 48 h in the presence of different concentrations of fusion proteins, and after a pulse with [^{14}C]leucine, the percent of protein synthesis was calculated (Figure 2a). GM-CSF−Bad and GM-CSF−Bad AA inhibited protein synthesis in a dose-dependent manner (Figure 2), with an IC_{50} value between 1 and 2 μM (Table 1). Surprisingly, GM-CSF−Bad AA was not substantially different in cytostatic activity than the wild-type human Bad when fused to GM-CSF (Figure 2b). This contrasts with results reported with mouse Bad fusion proteins (*33*).

We confirmed that, despite the different numbers of GM-CSF receptors on their plasma membranes, the leukemia and gastric intestinal cell lines were not differentially susceptible to the GM-CSF proteins (Table 1).

There was a slight activation of protein synthesis at low concentrations of the fusion proteins (Figure 2a) that may result from the GM-CSF portion of the protein binding the GM-CSF receptor and activating the Jak-Stat pathway. However, an increasing concentration allows the proapoptotic Bad portion of the chimera to effect cell death.

These results show that GM-CSF−Bad and GM-CSF−Bad AA are cytotoxic for GM-CSF receptor-expressing cells

Table 1: Cytotoxicity of the GM-CSF Fusion Proteins

cell type	no. of sites per cell[b]	tumor type	IC_{50}[a] (μM) GM-CSF−Bad	IC_{50}[a] (μM) GM-CSF−Bad AA
HL-60	540 ± 50	promyelocytic	1.12 ± 0.79	1.37 ± 0.15
U937	3500 ± 350	monocytic	1.28 ± 0.25	1.77 ± 0.35
HTB-103	500 ± 200	gastric	1.47 ± 0.16	1.62 ± 0.19
LS174T	900 ± 700	colon	1.9 ± 0.31	1.99 ± 0.56

[a] Values are the mean ± standard error of the mean. [b] From ref *38*.

and suggest that the GM-CSF receptor binding mediates the internalization of Bad into cells.

Specificity of the Fusion Protein. To determine if the internalization of the two fusion proteins required binding to the GM-CSF receptor, we examined the inhibition of protein synthesis in the presence of a concentration of GM-CSF−Bad and GM-CSF−Bad AA that causes the maximum cytotoxic effect on the cells in the presence or absence of an excess of free GM-CSF (5 μg/mL). Excess hGM-CSF largely blocked the cytotoxicity of both GM-CSF−Bad and GM-CSF−Bad AA (Figure 3a), indicating that the binding to the GM-CSF receptor is required for cytotoxicity.

To confirm the specificity of the proteins for the human GM-CSF receptor, we tested the biological activity also on two tumor cell lines that do not express the GM-CSF receptor. When Bad protein fused to hGM-CSF was added to the media of cells, no effect of GM-CSF−Bad was detected on proliferation of the human cervical adenocarcinoma cell line (HeLa) and epidermoid carcinoma cell line (A431) in contrast to the human monocytic cell line, HL-60 (Figure 3b).

GM-CSF−Bad Induces Apoptosis in the HL-60 Leukemia Cell Line. We examined if GM-CSF−Bad induced target cell apoptosis. Using Hoechst 333242 to assess apoptotic cells and propidium iodide to assess necrotic cells, we found that GM-CSF−Bad induced nuclear fragmentation (early apoptotic cells) during the first 24 h of incubation. After 48 h, the percent of apoptosis was ~65% (Figure 4a). The apparent necrosis that was detected may only represent the final stage of apoptosis, and does not imply that there are two death mechanisms involved.

Induction of apoptosis was confirmed by assessing the caspase activation in GM-CSF−Bad-treated cells. Caspase 3 and 7 are effector caspases, and their actions lead to the characteristic apoptotic morphological changes such as membrane blebbing, nuclear condensation, and DNA fragmentation. After 48 h, GM-CSF−Bad exposure induces an increase in caspase activity in HL-60 cells with no detectable effect on the A431 or HeLa cells (Figure 4b). These results demonstrate that the chimera induces apoptosis, consistent with the known bioactivity of Bad, and validates the specificity of this effect for GM-CSF receptor-expressing cells.

Intracellular Transport of GM-CSF−Bad. To investigate the role of internalization of the fusion proteins after binding of the GM-CSF receptor, we examined the effect of monensin, NH_4Cl, and brefeldin A on the chimera cytotoxic activity. NH_4Cl and monensin increase the endosomal pH in cells, reducing the proton gradient across the membranes and causing a block in the action of toxins such as diphtheria toxin that translocate, directly, from the endosomes to the cytosol. Brefeldin A blocks the vesicular transport from the ER to the cis Golgi apparatus and causes a termination of the retrograde vesicular transport from the cis Golgi to the ER.

When GM-CSF−Bad was tested in the presence of these three compounds, only brefeldin A dramatically reduced the cytotoxic activity (Figure 5). These data show that GM-CSF−Bad enters through the GM-CSF receptor and suggest that it does not translocate from the endosomes in the cytosol but that retrograde transport to the ER may be the intracellular route of the protein to the cytosol. This would represent a pathway similar to that of ricin, cholera, and pseudomonas toxin and in contrast to that of diphtheria toxin.

DISCUSSION

The PI3K/Akt signal transduction cascade that controls Bad phosphorylation is deregulated in cancer, and mutations in this pathway have been linked to a variety of human tumors, including breast cancer, lung cancer, and leukemia (*43*). A multitude of extracellular growth factors bind to receptor tyrosine kinases and activate the phosphatidylinositol 3-kinase (PI3K) signaling pathway. PI3K is a heterodimeric kinase that phosphorylates the lipid phosphatidylinositol 4,5-biphosphate (PIP2) at the 3′ position to generate phosphatidylinositol 3,4,5-triphosphate (PIP3). Akt and phosphoinositide-dependent kinase 1 (PDK1) are serine/threonine kinases that are recruited to the membrane through binding of their pleckstrin homology domain to PIP3. Following recruitment to the membrane, AKT is activated by phosphorylation and thereupon phosphorylates several targets to promote cell survival and progression that may contribute to malignant transformation. The activity of the PI3K/Akt pathway is negatively regulated by the phosphatase PTEN that dephosphorylates PIP3 and thereby directly counteracts PI3K signals (*44*). Mutations in PTEN that eliminate its phosphatase activity can lead to tumor progression (*45*). One of the targets of Akt identified with direct implications for regulating cell survival was the proapoptotic protein Bad, and several others kinases that have been implicated in survival signaling have been proposed to mediate Bad phosphorylation, including Rsk, PAK, and PKA (*46*−*50*). Bad represents an important stage at which activation of the Raf/MEK/ERK and the PI3K/Akt pathways can synergize to induce cell survival and cellular transformation.

We have produced a chimeric protein to deliver human Bad to tumor cells via the GM-CSF receptor to prevent uncontrolled cellular proliferation. The purified recombinant protein is cytotoxic to different types of tumor cells by directly inducing apoptosis, in the absence of other apoptotic stimuli.

We compared the toxicity of GM-CSF−Bad with that of GM-CSF−Bad AA (with the Ser75A and Ser99A mutations

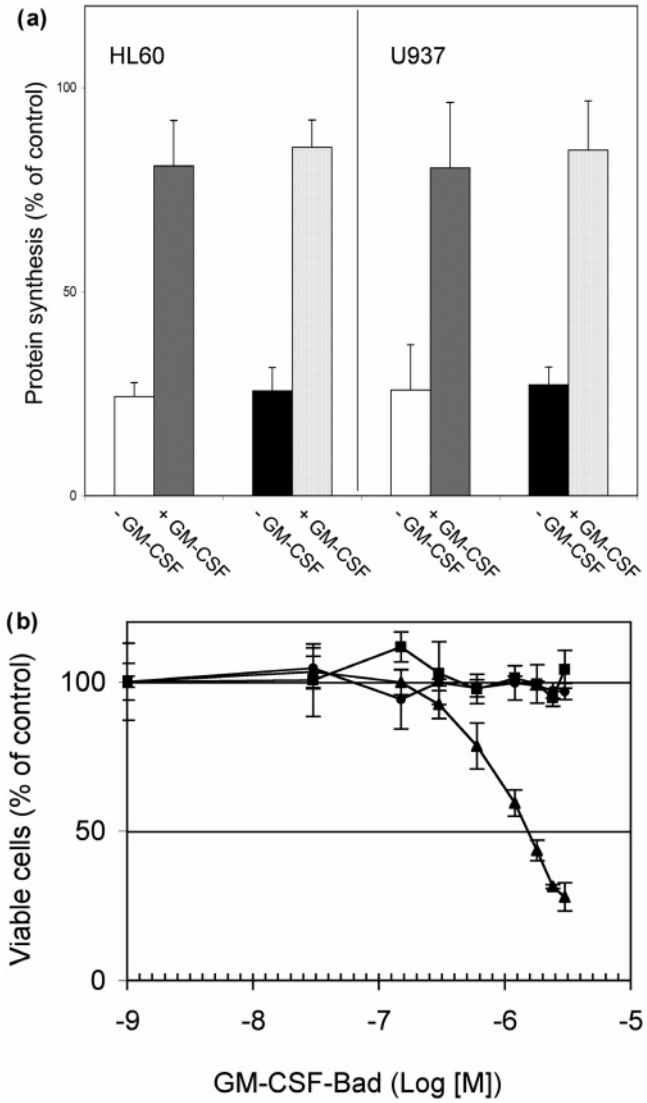

FIGURE 3: Specificity of GM-CSF fusion proteins for the human GM-CSF receptor. (a) Leukemia cell lines were incubated with GM-CSF−Bad (3 μM) (white and dark gray columns) and GM-CSF−Bad AA (3 μM) (black and light gray columns) in the presence or absence of 5 μg/mL human GM-CSF for 48 h. The cells were pulsed with [^{14}C]leucine for 1 h and harvested. The extent of leucine incorporation was measured and presented as a percentage relative to PBS-treated cells. The mean values determined from triplicate measurements are plotted vs the concentration of fusion proteins. The error bars represent the standard error of the mean. (b) Proliferation activity was measured on a human cervical adenocarcinoma (HeLa) (■), on a skin epidermoid carcinoma (A-431) (●), and on a human leukemia cell line (HL-60) (▲). The cells were incubated in the presence of GM-CSF−Bad at different concentrations, for 48 h. MTS was added to each well, and the plate was incubated for 1 h at 37 °C. The absorbance at 490 nm was measured using an EIA Multiwell Reader (Sigma Diagnostics) and presented as a percentage relative to PBS-treated cells. The mean values determined from triplicate measurements are plotted vs the concentration of the fusion protein. The error bars represent the standard error of the mean.

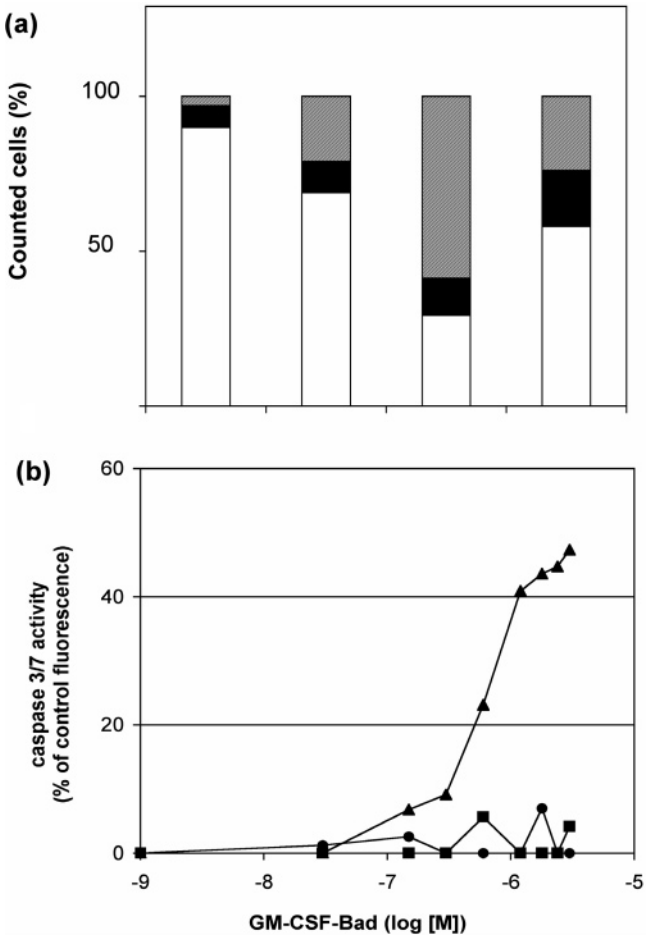

FIGURE 4: Quantitation of the type of GM-CSF−Bad cell death. HL-60 cells were treated for 24 or 48 h in the presence of 3 μM GM-CSF−Bad or for 6 h with 100 μM etoposide. The data represent the results of three separate experiments. The calculation of the different cell death types was performed as described in Experimental Procedures. Viable cell percent is indicated in white, necrotic cell percent in black, and the apoptotic percent in gray. (b) Caspase 3/7 activity was measured on a human cervical adenocarcinoma (HeLa) (■), on a skin epidermoid carcinoma (A-431) (●), and on a human leukemia cell line (HL-60) (▲). The cells were incubated in the presence of GM-CSF−Bad at different concentrations, for 48 h. Z-DEVD-R110 (1×) was added to each well, and the plate was incubated for 1 h at room temperature. The fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Wallack Victor^2 1420 Multilabel Counter.

in the sequence of human Bad). Both recombinant proteins have the same cytotoxic activity. As shown by Datta et al. (*51*), the phosphorylation of serine 112 and 136 enhances the binding of 14-3-3 protein to mouse Bad that weakens the effective interaction of Bad and Bcl-XL and improves the access of Ser 155 kinases to Bad Ser 155. The folding of GM-CSF−Bad could hide phosphorylation sites Ser 75 and Ser 99 on Bad without altering the conformation of the BH3 domain. In this way, the protein could preserve the functionality (binding of pro-survival members of the Bcl-2 protein family) but become insensitive to phosphorylation. An alternative hypothesis is that steric hindrance by the GM-CSF moiety may inhibit the sequestration of the fusion protein to 14-3-3 so that even if the Bad is phosphorylated it cannot be inactivated. As demonstrated by Ichinose et al. (*33*), it was necessary to mutate Ser112 and Ser136 in murine Bad to enhance the feable toxicity of Bad. In contrast with GM-CSF−Bad, the "wild-type" fusion protein has considerably more cytotoxicity than the murine Bad fusion protein prior to mutation, consistent with the above hyphotheses.

Excess GM-CSF competed for cytotoxicity, and cell lines that do not express the GM-CSF receptor were insensitive to the chimera, showing specificity for the GM-CSF receptor.

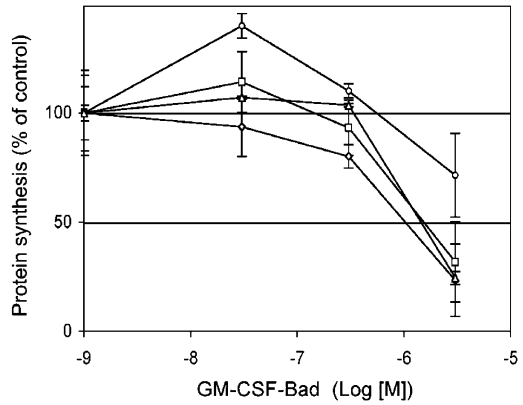

FIGURE 5: Effect of monensin, brefeldin A, and NH_4Cl on the toxicity of GM-CSF−Bad. HL-60 cells were plated for 24 h at 37 °C and were treated for 32 h in the presence of 3 μM GM-CSF−Bad. Brefeldin A [10 nM (○)], monensin [100 nM (□)], or NH_4Cl [25 μM (△)] was added, and the cells were incubated for an additional 14 h. The cells were pulsed with [^{14}C]leucine and harvested. The extent of leucine incorporation was measured and presented as a percentage relative to PBS-treated cells (◇). The mean values determined from triplicate measurements are plotted vs the concentration of fusion proteins. The error bars represent the standard error of the mean.

All the tumor cell lines positive for the GM-CSF receptor have the same sensitivity to GM-CSF−Bad despite the different numbers of receptors. As shown by Kreitman et al. (*38*), in addition to the number and affinity of receptors present on the plasma membrane, how the various cells route the protein intracellularly also appears to be important. GM-CSF−Bad appears to be internalized through a retrograde transport to the ER that may vary among tumor cells. This parameter could explain the discrepancy in the correlation between the calculated IC_{50} and the number of GM-CSF receptors on the different tumor cells and represent interesting aspects for future investigation.

Over the past 15 years, many different chimeric proteins have been produced to bind selectively to cancer cells, internalize, and kill the cells (*52*), in particular, for the treatment of hematological malignancies (*53*). These molecules are usually molecules which contain a plant or bacterial toxin connected to a targeting antibody or growth factor. The toxin part of the immunotoxin is extremely potent in killing cells since it acts enzymatically within the cytoplasm, and the resulting immunotoxins are much more potently toxic than GM-CSF−Bad. For example, the IC_{50} of immunotoxins containing GM-CSF and the diphtheria toxin as the catalytic domain, calculated on leukemia cells, is in the picomolar range (*37*, *38*) as well as the IC_{50} of the immutoxin containing GM-CSF and the catalytic domain of *P. aeruginosa*, measured on the gastric carcinoma cell lines (*38*). A major barrier to the clinical utility of classical immunotoxins is the immunogenicity associated mainly toward the bacterial or plant portion of the protein. GM-CSF−Bad is a completely human recombinant protein so it is expected to elicit a minimal immunogenic response. GM-CSF−Bad is one of few human chimeric proteins, compared to the large number of immunotoxins, produced thus far and offers another potential tool against tumor cells (*31*, *32*, *54*−*56*). The presence of GM-CSF receptors on both leukemia and solid tumors extends the opportunity to use this protein in vivo in comparison to other human chimeric proteins. In addition, the strategy described here introduces Bad, the actual protein that may be inactivated in cancer, into tumor cells to reconstitute the apoptosis pathway inducing perhaps less nonspecific toxicity that foreign and even human cytotoxic proteins.

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REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer 26*, 239−257.
2. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell death in development, *Cell 96*, 245−254.
3. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) Bcl-2 family members and the mitochondria in apoptosis, *Genes Dev. 13*, 1899−1911.
4. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) bcl-xl, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death, *Cell 74*, 597−608.
5. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993) MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2, *Proc. Natl. Acad. Sci. U.S.A. 90*, 3516−3520.
6. Choi, S. S., Park, I. C., Yun, J. W., Sung, Y. C., Hong, S. I., and Shin, H. S. (1995) A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow, *Oncogene 11*, 1693−1698.
7. Suzuki, M., Youle, R. J., and Tjandra, N. (2000) Structure of Bax: Coregulation of dimer formation and intracellular localization, *Cell 103*, 645−654.
8. Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions, *EMBO J. 14*, 5589−5596.
9. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death, *Nature 381*, 335−341.
10. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) Structure of Bcl-xL-Bak peptide complex: Recognition between regulators of apoptosis, *Science 275*, 983−986.
11. Liu, X., Dai, S., Zhu, Y., Marrack, P., and Kappler, J. W. (2003) The structure of a Bcl-XL/Bim fragment complex: Implication for Bim function, *Immunity 19*, 341−352.
12. Tan, Y., Demeter, M. R., Ruan, H., and Comb, M. J. (2000) BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival, *J. Biol. Chem. 275*, 25865−25869.
13. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death, *Science 292*, 727−730.
14. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: A control point for apoptosis, *Science 300*, 135−139.
15. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis, *Mol. Cell 8*, 705−711.
16. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked, *Science 275*, 1129−1132.

17. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) The release of cytochrome *c* from mitochondria: A primary site for Bcl-2 regulation of apoptosis, *Science 275*, 1132−1136.
18. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*, *Cell 86*, 147−157.
19. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition, *Cell 102*, 33−42.
20. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors, *Proc. Natl. Acad. Sci. U.S.A. 93*, 4974−4978.
21. Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death, *Nature 410*, 549−554.
22. Datta, S. R., Ranger, A. M., Lin, M. Z., Sturgill, J. F., Ma, Y. C., Cowan, C. W., Dikkes, P., Korsmeyer, S. J., and Greenberg, M. E. (2002) Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis, *Dev. Cell 3*, 631−643.
23. Ranger, A. M., Zha, J., Harada, H., Datta, S. R., Danial, N. N., Gilmore, A. P., Kutok, J. L., Le Beau, M. M., Greenberg, M. E., and Korsmeyer, S. J. (2003) Bad-deficient mice develop diffuse large B cell lymphoma, *Proc. Natl. Acad. Sci. U.S.A. 100*, 9324−9329.
24. Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics, *Cancer Cell 2*, 183−192.
25. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine phosphorilation of death agonist Bad in response to survival factors results in binding to 14-3-3 not Bcl-XL, *Cell 87*, 619−628.
26. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A, *Mol. Cell 3*, 413−422.
27. Lizcano, J. M., Morrice, N., and Cohen, P. (2000) Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155, *Biochem. J. 349*, 547−557.
28. Ottilie, S., Diaz, J. L., Horne, W., Chang, J., Wang, Y., Wilson, G., Chang, S., Weeks, S., Fritz, L. C., and Oltersdorf, T. (1997) Dimerization properties of human BAD. Identification of a BH-3 domain and analysis of its binding to mutant BCL-2 and BCL-XL proteins, *J. Biol. Chem. 272*, 30866−30872.
29. Jeong, S. Y., Gaume, B., Lee, Y. J., Hsu, Y. T., Ryu, S. W., Yoon, S. H., and Youle, R. J. (2004) Bcl-x(L) sequesters its C-terminal membrane anchor in soluble, cytosolic homodimers, *EMBO J. 23*, 2146−2155.
30. Shangary, S., and Johnson, D. E. (2003) Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family, *Leukemia 17*, 1470.
31. Aqeilan, R., Yarkoni, S., and Lorberboum-Galski, H. (1999) Interleukin 2-Bax: A novel prototype of human chimeric proteins for targeted therapy, *FEBS Lett. 457*, 271−276.
32. Azar, Y., and Lorberboum-Galski, H. (2000) GnRH−Bik/Bax/Bak chimeric proteins target and kill adenocarcinoma cells; the general use of pro-apoptotic proteins of the Bcl-2 family as novel killing components of targeting chimeric proteins, *Apoptosis 5*, 531−542.
33. Ichinose, M., Liu, X.-H., Hagihara, N., and Youle, R. J. (2002) Extracellular Bad fused to toxin transport domains induces apoptosis, *Cancer Res. 62*, 1433−1438.
34. Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., and Clark, S. C. (1985) Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins, *Science 228*, 810−815.
35. Shao, Y., Warman, B. E., and Perentesis, J. P. (2001) Recombinant fusion toxins directed against the human granulocyte-macrophage colony stimulating factor (GM-CSF) receptor, *Methods Mol. Biol. 166*, 31−53.
36. Barreda, D. R., Hanington, P. C., and Belosevic, M. (2004) Regulation of myeloid development and function by colony stimulating factors, *Dev. Comp. Immunol. 28*, 509−554.
37. Perentesis, J. P., Waddick, K. G., Bendel, A. E., Shao, Y., Warman, B. E., Chandan-Langlie, M., and Uckun, F. M. (1997) Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia cells by a recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor, *Clin. Cancer Res. 3*, 347−355.
38. Kreitman, R. J., and Pastan, I. (1997) Recombinant toxins containing human granulocyte-macrophage colony-stimulating factor and either *Pseudomonas* exotoxin or diphtheria toxin kill gastrointestinal cancer and leukemia cells, *Blood 90*, 252−259.
39. Perentesis, J. P., Gunther, R., Waurzyniak, B., Yanishevski, Y., Myers, D. E., Ek, O., Messinger, Y., Shao, Y., Chelstrom, L. M., Schneider, E., Evans, W. E., and Uckun, F. M. (1997) In vivo biotherapy of HL-60 myeloid leukemia with a genetically engineered recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor, *Clin. Cancer Res. 3*, 2217−2227.
40. Hall, P. D., Willingham, M. C., Kreitman, R. J., and Frankel, A. E. (1999) DT388-GM-CSF, a novel fusion toxin consisting of a truncated diphtheria toxin fused to human granulocyte-macrophage colony-stimulating factor, prolongs host survival in a SCID mouse model of acute myeloid leukemia, *Leukemia 13*, 629−633.
41. Frankel, A. E., Powell, B. L., Hall, P. D., Case, L. D., and Kreitman, R. J. (2002) Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia, *Clin. Cancer Res. 8*, 1004−1013.
42. Lee, J., and Shacter, E. (1997) Bcl-2 does not protect Burkitt's lymphoma cells from oxidant-induced cell death, *Blood 89*, 4480−4492.
43. Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., Franklin, R. A., and McCubrey, J. A. (2003) Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy, *Leukemia 17*, 590−603.
44. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. (1998) The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway, *Proc. Natl. Acad. Sci. U.S.A. 95*, 15587−15591.
45. Ali, I. U., Schriml, L. M., and Dean, M. (1999) Mutational spectra of PTEN/MMAC1 gene: A tumor suppressor with lipid phosphatase activity, *J. Natl. Cancer Inst. 91*, 1922−1932.
46. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms, *Science 286*, 1358−1362.
47. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cellular survival: A play in three Akts, *Genes Dev. 13*, 2905−2927.
48. Harada, H., Andersen, J. S., Mann, M., Terada, N., and Korsmeyer, S. J. (2001) p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD, *Proc. Natl. Acad. Sci. U.S.A. 98*, 9666−9670.
49. Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C., and Bokoch, G. M. (2000) p21-activated kinase 1 phosphorylates the death agonist Bad and protects cells from apoptosis, *Mol. Cell. Biol. 20*, 453−461.
50. Tan, Y., Ruan, H., Demeter, M. R., and Comb, M. J. (1999) p90(RSK) blocks Bad-mediated cell death via a protein kinase C-dependent pathway, *J. Biol. Chem. 274*, 34859−34867.
51. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation, *Mol. Cell 6*, 41−51.
52. Kreitman, R. J. (2000) Immunotoxins, *Expert Opin. Pharmacother. 1* (6), 1117−1129.
53. Kreitman, R. J. (2004) Recombinant immunotoxins for the treatment of haematological malignancies, *Expert Opin. Biol. Ther. 4*, 1115−1128.
54. De Lorenzo, C., Arciello, A., Cozzolino, R., Palmer, D. B., Laccetti, P., Piccoli, R., and D'Alessio, G. (2004) A fully human immunoRNAse selective for ErbB-2-positive carcinomas, *Cancer Res. 64*, 4870−4874.

55. Newton, D. L., Pollock, D., DiTullio, P., Echelard, Y., Harvey, M., Wilburn, B., Williams, J., Hoogenboom, H. R., Raus, J. C., Meade, H. M., and Rybak, S. M. (1999) Antitransferrin receptor antibody-RNase fusion protein expressedin the mammary gland of transgenic mice, *J. Immunol. Methods 231*, 159−167.

56. Newton, D. L., Nicholls, P. J., Rybak, S. M., and Youle, R. J. (1994) Expression and characterization of recombinant human eosinophil-derived neurotoxin-anti-transferrin receptor sFv, *J. Biol. Chem. 269*, 26739−26745.

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