Interleukin-3–Induced Phosphorylation of BAD Through the Protein Kinase Akt

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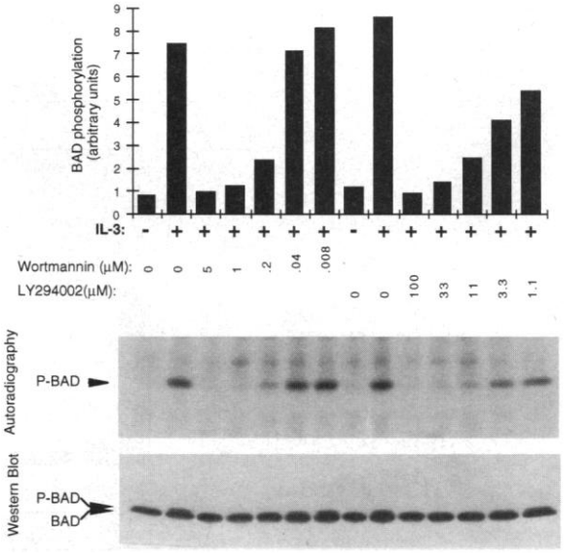
BAD is a distant member of the Bcl-2 family that promotes cell death. Phosphorylation of BAD prevents this. BAD phosphorylation induced by interleukin-3 (IL-3) was inhibited by specific inhibitors of phosphoinositide 3-kinase (PI 3-kinase). Akt, a survival-promoting serine-threonine protein kinase, was activated by IL-3 in a PI 3-kinase–dependent manner. Active, but not inactive, forms of Akt were found to phosphorylate BAD in vivo and in vitro at the same residues that are phosphorylated in response to IL-3. Thus, the proapoptotic function of BAD is regulated by the PI 3-kinase–Akt pathway.

Proteins of the Bcl-2 family are important regulators of cell death in mammalian cells (*1*). BAD, a distant member of the Bcl-2 family, promotes cell death at least in part through heterodimerization with the survival proteins Bcl-2 and Bcl-x_L (*2*). BAD resides in the cytosol and is phosphorylated on serine residues after cells are stimulated with IL-3 (*3*). Phosphorylation of BAD results in its cytosolic sequestration by the tau form of 14-3-3 proteins and its inactivation, as the phosphorylated form has reduced ability to bind to membrane Bcl-x_L (*3*).

The survival of cells in multicellular organisms requires continuous stimulation from the extracellular enviroment. Certain growth factors maintain cell survival during embryonal and postnatal development (*4*). The intracellular signaling pathways by which growth factors promote survival are poorly understood. PI 3-kinase is recruited and activated during the intracellular signal transduction of many receptors and has been implicated in the signaling of survival factors (*5*). PI 3-kinase phosphorylates inositol lipids that act as second messengers for several targets, including the serine-threonine Akt kinase (*6*, *7*, *8*). Akt was initially described as an oncogene (*9*) and is activated by a variety of growth factors through a PI 3-kinase–dependent pathway (*6*, *7*). Activation of Akt is known to deliver a survival signal that inhibits the apoptosis induced by growth factor withdrawal in neurons, fibroblasts, and lymphoid cells (*10*, *11*). Activation of Akt ultimately leads to inhibition of caspase activity and protection from apoptotic cell death (*11*). However, the precise mechanism by which the PI 3-kinase–Akt signaling pathway transduces a survival signal that inhibits apoptosis is unknown.

To determine whether BAD phosphorylation is regulated through the PI 3-kinase–Akt signaling pathway in vivo, IL-3–dependent FL5.12 lymphoid progenitor cells that coexpress Bcl-2 and BAD (*12*) were incubated with increasing concentrations of wortmannin and LY294002, two specific inhibitors of PI 3-kinase (*5*, *10*, *11*). Stimulation of the cells with IL-3 resulted in BAD phosphorylation that was inhibited by concentrations of wortmannin and LY294002 known to specifically inhibit PI 3-kinase activity (Fig. 1). Because Akt is a target of the PI 3-kinase, we determined next whether stimulation of FL5.12 cells with IL-3 could increase the activity of Akt through a PI 3-kinase–dependent mechanism. FL5.12 cells were stimulated with recombinant IL-3 in the presence or absence of PI 3-kinase inhibitors, and the kinase activity of endogenous Akt was assayed in Akt immunoprecipitates with the use of histone H2B as a substrate (*6*). Stimulation with IL-3 increased Akt kinase activity four times and was inhibited by wortmannin and LY294002 (Fig. 2). Thus, IL-3 activated the Akt kinase through a PI 3-kinase–dependent pathway in FL5.12 cells, which made it a candidate kinase for BAD.

We determined next whether expression of activated Akt leads to phosphorylation of BAD in vivo. In these experiments, 293T human kidney cells were transiently cotransfected with expression plasmids producing AU1 epitope-tagged BAD and HA-tagged active and inactive Akt (*6*, *11*). BAD phosphorylation was assayed by auto-



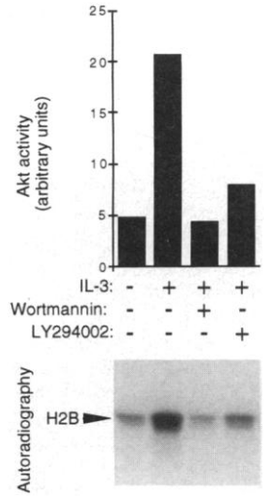


Fig. 1 (left). PI 3-kinase inhibitors block IL-3–induced BAD phosphorylation in FL5.12 cells. (Top) Quantitation of phosphorylated BAD. (Middle) ^{32}P-labeled BAD (P-BAD) from which quantitation was done (*18*). (Bottom) Immunoblot (Western blot) of the same membrane with polyclonal antibody to BAD (Santa Cruz), developed by enhanced chemiluminescence (Amersham). The results are representative of three independent experiments. **Fig. 2 (right).** IL-3 induces Akt activation in a PI 3-kinase–dependent manner in FL5.12 cells. Parental FL5.12 cells were starved of IL-3 for 2 hours, incubated with or without wortmannin (200 nM) or LY294002 (10 μM) for 30 min, and then stimulated with rIL-3 (150 ng/ml) for 10 min or left untreated. Cells were lysed, and cell lysates were precleared with normal rabbit serum and protein A–Sepharose. Endogenous Akt was immunoprecipitated with polyclonal antibody specific for Akt, and immunocomplexes were collected with protein A–Sepharose and used in an in vitro kinase reaction using [γ-^{32}P]ATP (10 μCi per reaction, 3000 Ci/mmol) and histone H2B (50 μg/ml; Boehringer) as a substrate (*6*). Reaction products were resolved by SDS-PAGE and transferred to nitrocellulose filters for quantification with a PhosphorImager system (Molecular Dynamics). The amount of ^{32}P incorporated into H2B is shown in the upper panel. Autoradiography of the filter is shown in the lower panel. The experiment is representative of two independent experiments.

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radiography. Expression of wild-type (WT) or an activated form of Akt with the Src myristoylation signal fused in-frame to the c-Akt coding sequence (MyrAkt) resulted in increased BAD phosphorylation (Fig. 3A). In contrast, expression of a kinase-defective Akt mutant (KDAkt) in which the lysine of the adenosine triphosphate (ATP)–binding site at position 179 was replaced by a methionine did not induce significant BAD phosphorylation (Fig. 3A). Protein immunoblot analysis revealed that the amount of BAD was equivalent in cells expressing active and inactive forms of Akt (Fig. 3A), which indicates that these results represented differences in BAD phosphorylation. Analysis of five independent experiments confirmed that expression of WT and active Akt, but not inactive Akt, led to significant phosphorylation of BAD in vivo (Fig. 3B). Similar results were obtained when active or inactive Akt constructs were expressed in COS cells (*13*). Consistent with the fact that Akt is dowstream of the PI 3-kinase, BAD phosphorylation induced by Akt was not inhibited by wortmannin (*13*).

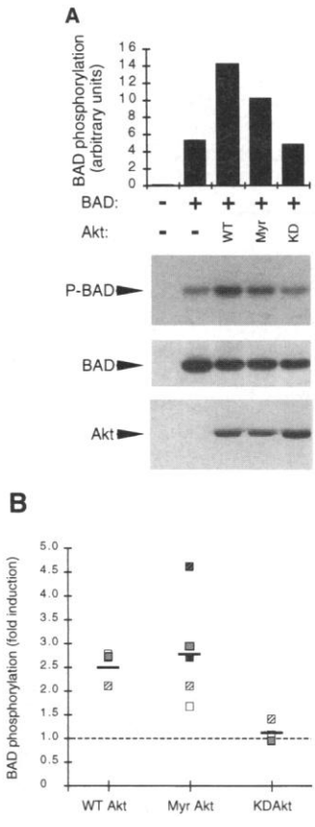

Fig. 3. Induction of BAD phosphorylation by Akt in vivo. (**A**) 293T cells were transfected with empty vector (–) or pcDNA3-AU1-BAD (+) alone or in combination with pCMV6-Akt-HA (WT), pCMV6-Myr-Akt-HA (Myr), or pCMV6-Akt-HA K179M (KD). Twenty-four hours after transfection, cells were labeled with ^{32}P-orthophosphate (100 μCi/ml; NEN) in phosphate-free DMEM for 1 hour. After labeling, cells were harvested and lysed, and BAD was immunoprecipitated as described in Fig. 1. Quantification of the amount of ^{32}P incorporated into BAD is shown in the upper panel. Autoradiography of the original membrane is shown in the panel below. Immunoblots of the same membrane incubated with polyclonal antibody to BAD or to Akt are shown in the lower panels. (**B**) Fold induction of BAD phosphorylation induced by WT Akt (n = 3), Myr-Akt (n = 5), or KDAkt (n = 3). We assigned a value of 1 to the amount of BAD phosphorylation obtained in cells transfected with BAD alone (dotted horizontal line). BAD phosphorylations shown were normalized to represent equal amounts of immunoprecipitated BAD. The average fold increase in phosphorylation is indicated (solid horizontal lines). Differences in the levels of BAD phosphorylation induced by WT Akt or Myr-Akt and the KDAkt mutant were statistically significant ($P < 0.05$, Student's t test). Each set of patterned boxes represents an independent experiment.

To determine whether Akt could phosphorylate BAD directly, recombinant BAD was purified (*15*) and incubated with Akt or control immunoprecipitates from 293T cells expressing active and inactive Akt forms. Active Akt induced a significant increase in BAD phosphorylation, whereas inactive Akt failed to phosphorylate BAD even when the expression of the inactive Akt kinase was greater than that of active Akt (Fig. 4B). Because BAD is known to be phosphorylated at Ser^{112} and Ser^{136} residues in vivo after IL-3 stimulation (*3*), we engineered a BAD mutant in which these two amino acids were mutated to alanine residues (*15*). In contrast to the WT protein, mutant BAD with alanine substitutions at Ser^{112} and Ser^{136} was not phosphorylated by active Akt (Fig. 4B), which indicates that Akt phosphorylated BAD at serine residues known to be phosphorylated under physiological conditions (*3*).

Stimulation of the PI 3-kinase–Akt signaling pathway through several growth factor receptors, including the IL-3 receptor, delivers a survival signal that ultimately leads to inhibition of apoptosis. The results presented herein identify the death agonist BAD as a substrate of Akt. The phosphorylation of BAD results in its cytosolic sequestration by the tau form of 14-3-3 proteins and prevents its binding to the survival factor Bcl-x_L at intracellular membrane sites (*3*). Because BAD exerts its death-promoting effects by heterodimerizing with and inhibiting the death antagonist Bcl-x_L, phos-

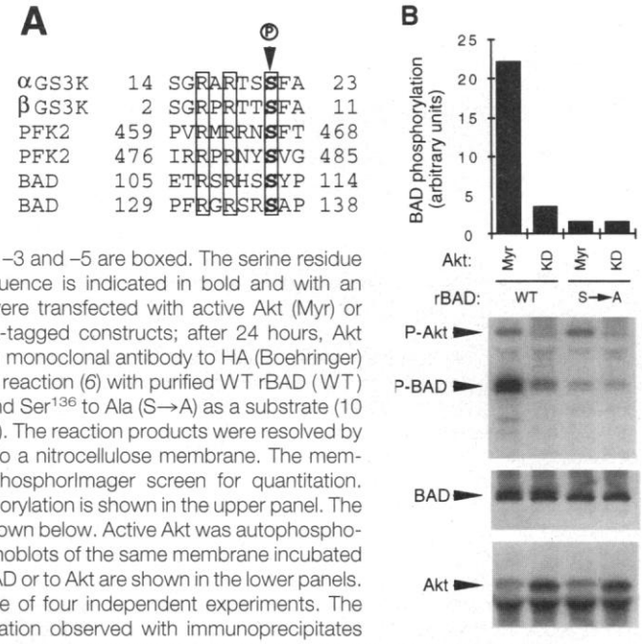

Fig. 4. Akt phosphorylated rBAD in vitro at the same residues that are phosphorylated in response to IL-3. (**A**) Alignment of protein sequences phosphorylated in α and β GSK3 and in PFK-2 (heart isoenzyme) by Akt and the regions phosphorylated in BAD (*19*). Conserved arginine residues at positions –3 and –5 are boxed. The serine residue phosphorylated in each sequence is indicated in bold and with an arrowhead. (**B**) 293T cells were transfected with active Akt (Myr) or kinase-deficient Akt (KD) HA-tagged constructs; after 24 hours, Akt was immunoprecipitated with monoclonal antibody to HA (Boehringer) and used in an in vitro kinase reaction (*6*) with purified WT rBAD (WT) or mutant BAD with Ser^{112} and Ser^{136} to Ala (S→A) as a substrate (10 μg/ml, approximately 0.3 μM). The reaction products were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was exposed to a PhosphorImager screen for quantitation. Quantification of BAD phosphorylation is shown in the upper panel. The original autoradiography is shown below. Active Akt was autophosphorylated as reported (*6*). Immunoblots of the same membrane incubated with polyclonal antibody to BAD or to Akt are shown in the lower panels. The results are representative of four independent experiments. The amount of rBAD phosphorylation observed with immunoprecipitates from mock-transfected cells was the same as that obtained with immunoprecipitates from cells transfected with the inactive Akt construct (*13*).

phorylation of BAD by Akt will preclude its binding to membrane-anchored Bcl-x_L, leading to increased cell survival. Thus, BAD phosphorylation by Akt is a mechanism by which growth factor receptors could deliver a survival signal that leads to the inhibition of apoptosis. However, these results do not rule out the possibility that Akt promotes cell survival by other mechanisms in addition to that mediated by phosphorylation of BAD. In this respect, it has been shown that Akt promotes expression of Bcl-2 in certain cell lines but not in others (*11*), which suggests that Akt mediates cell survival by at least one other mechanism. Previous studies have indicated that another kinase, Raf-1, could phosphorylate BAD in vitro (*16*). Unlike Akt, however, Raf-1 and another kinase, PKC, phosphorylated BAD in vitro at serine residues other than Ser^{112} and Ser^{136}, which suggests that BAD is not a physiological target of Raf-1 in vivo (*3*). Akt was originally identified as an oncogene in mice and is overexpressed in some human tumors (*17*). Because Bcl-2 and Bcl-x_L are known to deliver oncogenic signals that result in tumor development, these results suggest that active Akt promotes tumor development, at least in part, by acting on Bcl-2–related survival factors through phosphorylation and inactivation of BAD.

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12. The mouse BAD cDNA containing the coding sequence was amplified by reverse transcription polymerase chain reaction (PCR) with specific primers (5′-AAAGATCTAGAATGGGAACCCCAAAGCAGCCCTCGCTG-3′ and 5′-TTGAATTCACTGGGAGGGGGTGGAGCCTCCTTTG-3′) and ligated in frame into the pcDNA3 vector (Invitrogen) containing an AU1-tag epitope. The authenticity of all constructs was confirmed by dideoxy sequencing. FL5.12 cells were transfected by electroporation (960 μF, 250 V) with the pcDNA3-AU1-BAD construct alone or in combination with a human Bcl-2–expressing plasmid (pSFFV–Flag–Bcl-2) and selected with G418 (1 mg/ml). After selection, expression was assessed in the bulk population as well as in independent clones by flow cytometry.
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15. To produce recombinant BAD protein (rBAD), the BAD coding sequence was cloned into the pET-30a(+) plasmid (Novagen). rBAD was purified as a histidine-tag fusion protein from BL21 (E30) Lys S bacteria by means of an Ni^{+2} affinity column according to the manufacturer's instructions (Novagen). Mutant BAD containing Ser^{112} to Ala and Ser^{136} to Ala mutations was generated by site-directed mutagenesis with the use of PCR (5′-CAGTGCGTACCCAGCGGGGACCGAGGAGGATGAAGGGTAGGAGGAGGAGTCTAGCCCTTTTCGAGGACGCTCGCGTGCGGCTCCC-3′ and 5′-GGGAGCCGCACGCGAGCGTCCTCGAAAAGGGCTAAGCTCCTCCTCCATCCCTTCATCCTCCTCGGTCCCCGCTGGGTAGCGACTG-3′), then subcloned into the pET-30a(+) plasmid and purified as WT rBAD. The authenticity of all constructs was confirmed by dideoxy sequencing.
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18. FL5.12 cells expressing AU1-tagged BAD and Flag-tagged Bcl-2 were starved of IL-3 for 2 hours and then labeled with [^{32}P] orthophosphate (100 μCi/ml) (NEN) in phosphate-free Dulbecco's modified Eagle's medium (DMEM) for 1 hour. After labeling, cells were incubated for 30 min with the indicated concentrations of wortmannin or LY294002 (Calbiochem) and then either incubated with mouse recombinant IL-3 (rIL-3) (150 ng/ml; Genzyme) (+) for 10 min or left untreated (–). After stimulation, cells were harvested by centrifugation at 4°C and lysed with 0.2% Nonidet P-40 (NP-40) lysis buffer [0.2% NP-40, 10 mM Hepes (pH 7.2), 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, aprotinin (2 μg/ml), leupeptin (2 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaF]. BAD was immunoprecipitated with an antibody to AU1 (Babco); immunocomplexes were recovered with protein G–Sepharose and resolved by SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a nitrocellulose membrane and exposed to a PhosphorImager screen (Molecular Dynamics) to quantitate the radioactivity incorporated into the BAD protein.
19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, Phe; G, Gly; H, His; I, Ile; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

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Population Diversity: Its Extent and Extinction

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Genetically distinct populations are an important component of biodiversity. This work estimates the number of populations per area of a sample of species from literature on population differentiation and the average range area of a species from a sample of distribution maps. This yields an estimate of about 220 populations per species, or 1.1 to 6.6 billion populations globally. Assuming that population extinction is a linear function of habitat loss, approximately 1800 populations per hour (16 million annually) are being destroyed in tropical forests alone.

Much of the current scientific and public concern over the extinction crisis centers on the loss of species globally (*1*). Most of the benefits biodiversity confers on humanity, however, are dependent on large numbers of populations of species, because each population ordinarily provides an incremental amount of an ecosystem good or service. Examples of these goods and services are seafood, timber, water purification, generation of soil fertility, pest control, mitigation of floods and droughts, and regulation of biogeochemical cycles (*2*). Populations also supply the genetic diversity that is crucial for the development and improvement of pharmaceuticals and agricultural crops (*3*).

Here we make a crude first approximation of population diversity (defined as the number of populations on the planet) and then estimate the extinction rate at this level of biodiversity. We reviewed the literature on population differentiation from a variety of taxa and estimated the average number of mendelian populations per unit area for a species. We then estimated the average range size of a species from a sample of distribution maps. The product of these two numbers is an approximation of the average number of populations per species, which, multiplied by the total number of species, yields an estimate of the number of populations on Earth (*4*).

Populations are normally defined as geographical entities within a species, distinguished either ecologically or genetically (*5*). We adopted the genetically based definition, or mendelian population (*6*), defined here as a group of individuals evolving independently of other groups because of limited gene flow and genetically distinguishable from other populations.

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