Molecular Cell, Vol. 3, 413–422, April, 1999, Copyright ©1999 by Cell Press

# Phosphorylation and Inactivation of BAD by Mitochondria-Anchored Protein Kinase A

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## Summary

**Signaling pathways between cell surface receptors and the BCL-2 family of proteins regulate cell death. Survival factors induce the phosphorylation and inactivation of BAD, a proapoptotic member. Purification of BAD kinase(s) identified membrane-based cAMP-dependent protein kinase (PKA) as a BAD Ser-112 (S112) site-specific kinase. PKA-specific inhibitors blocked the IL-3-induced phosphorylation on S112 of endogenous BAD as well as mitochondria-based BAD S112 kinase activity. A blocking peptide that disrupts type II PKA holoenzyme association with A-kinase-anchoring proteins (AKAPs) also inhibited BAD phosphorylation and eliminated the BAD S112 kinase activity at mitochondria. Thus, the anchoring of PKA to mitochondria represents a focused subcellular kinase/substrate interaction that inactivates BAD at its target organelle in response to a survival factor.**

## Introduction

Programmed cell death is a distinct genetic pathway with evolving biochemical detail. This apoptotic pathway, apparently shared by all multicellular organisms, is critical for the successful development of multiple tissues and the maintenance of normal tissue homeostasis. In order for cells to avoid a suicidal fate, they must receive cues from their extracellular environment (Raff, 1992). A series of cytokines, such as interleukin-3 (IL-3), nerve growth factor (NGF), or insulin-like growth factor (IGF), function as survival factors and transduce signals through surface receptors to increase cellular resistance to apoptosis. Downstream of these proximal signals lie the BCL-2 family of proteins, which themselves reside upstream to the irreversible damage of cellular constituents. The pro- versus antiapoptotic BCL-2 members constitute a critical, decisional checkpoint that helps determine how cells respond to proximal death and survival signals (Oltvai et al., 1993). Activation of proapoptotic BCL-2 members such as BAX results in the activation of caspases as well as the onset of mitochondrial dysfunction (Xiang et al., 1996; Pastorino et al., 1998). Consequently, BCL-2 members represent an attractive target for posttranslational modification in response to proximal signal transduction events in order to alter susceptibility to apoptosis.

BAD represents a divergent BCL-2 family member belonging to a subset that shares substantial sequence homology only within the BH3 amphipathic $\alpha$-helical domain (Yang et al., 1995; Zha et al., 1997). Members of this "BH3 domain–only" subset include the mammalian BID, BIK, BIM, BLK, and HRK molecules, all of which are proapoptotic, lending support to the thesis that BH3 represents a minimal death domain (Boyd et al., 1995; Wang et al., 1996b; Inohara et al., 1997; Hegde et al., 1998; O'Connor et al., 1998). Moreover, the identification of a BH3 domain–only member in *Caenorhabditis elegans*, EGL-1, which maps upstream to CED-9 and regulates all developmental deaths, argues that these molecules are conserved components of the central death pathway (Conradt and Horvitz, 1998). Deletion mapping and site-directed mutagenesis indicated that the BH3 domain of BAD was critical for its heterodimerization with BCL-$X_L$ or BCL-2 as well as its death agonist activity (Kelekar et al., 1997; Ottilie et al., 1997; Zha et al., 1997). This is consistent with the NMR analysis of a BH3 amphipathic $\alpha$-helical peptide from BAK within the BCL-$X_L$ pocket (Sattler et al., 1997). BAD lacks the typical hydrophobic COOH-terminal sequence that in the case of BCL-2 is required for its targeting mitochondria (Nguyen et al., 1993). Consistent with this, BAD displays cytosolic as well as membrane-associated localizations and may represent a death ligand (Wang et al., 1996b) that translocates between cytosol and membrane-based BCL-2 or BCL-$X_L$.

In the presence of a requisite survival factor, IL-3, cells phosphorylated BAD on two serine residues (S112 and S136) embedded within 14-3-3 consensus binding sites (Zha et al., 1996). This has the dual impact of dictating BAD's location as well as its protein partners. Phosphorylated BAD appears to be the inactive moiety sequestered in the cytosol bound to 14-3-3, freeing BCL-$X_L$ or BCL-2 to promote survival. Only the active, nonphosphorylated BAD heterodimerized with BCL-$X_L$

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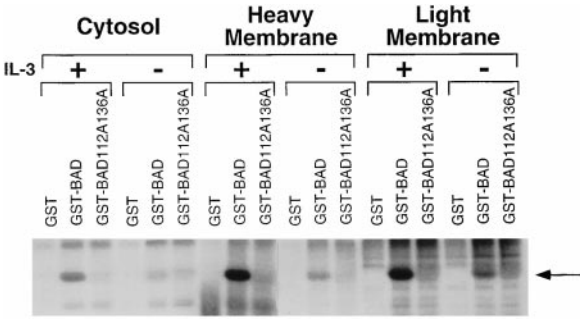


Figure 1. Localization of BAD Kinase(s)

IL-3 was withdrawn from FL5.12 BCL-$X_L$/BAD cells for 2 hr (−IL-3), followed by readdition of IL-3 for 15 min (+IL-3). Cells were fractionated into cytosol, heavy membrane, and light membrane fractions. An in vitro kinase assay was performed with GST, GST-BAD wild-type (GST-BAD), and GST-BAD whose S112 and S136 were substituted to alanine (GST-BAD112A136A) as substrates. The arrow indicates the position of phosphorylated GST-BAD.

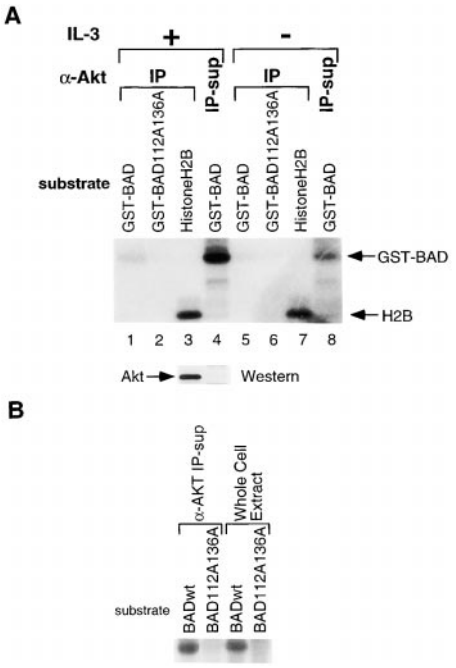


Figure 2. AKT Does Not Appear to Be the Principal BAD Kinase(s)

(A) IL-3 was withdrawn from FL5.12 BCL-$X_L$/BAD cells for 2 hr (−IL-3), followed by readdition of IL-3 for 15 min (+IL-3). Whole-cell extracts were then immunoprecipitated with an anti-AKT antibody (α-AKT). An AKT kinase assay was performed with the immunoprecipitates (IP), and histone H2B, GST-BAD, and GST-BAD112A136A as substrates (lanes 1–3, and 5–7). The BAD kinase assay was also performed using the supernatant following successful depletion of AKT by an anti-AKT antibody (IP-sup), with GST-BAD as a substrate (lanes 4 and 8). A Western blot of the α-AKT immunoprecipitate (IP) and the supernatant (IP-sup) was developed with anti-AKT Ab, confirming the effectiveness of the immunodepletion.

(B) Whole-cell extracts from IL-3-stimulated cells as in (A) were assayed for BAD kinase activity before and after immunodepletion of AKT (α-AKT IP-sup).

or BCL-2 at membrane sites to promote cell death. Moreover, BAD that was singly phosphorylated on S112 proved incapable of binding BCL-$X_L$, implying a conformational change that masks the BH3 domain (Zha et al., 1996). Functionally, the rapid phosphorylation of BAD that follows IL-3 connects proximal survival signaling with the BCL-2 family, resetting this checkpoint for apoptosis. Thus, identifying the signaling components between the surface receptor and BAD, including the identification of the BAD kinase(s), is a critical objective in the interconnection of signal transduction and distal death effector pathways. Recently, AKT/PKB/RAC, a Ser/Thr kinase downstream of phosphatidylinositol 3-kinase (PI-3K), was shown to be capable of phosphorylating S136 of BAD. Moreover, expression of AKT will block the death of cells induced by expression of BAD (Datta et al., 1997; del Peso et al., 1997; Blume-Jensen et al., 1998). In addition, BAD is also inactivated in vivo by phosphorylation on S112, prompting our purification of that responsible kinase.

The differential phosphorylation of highly selected protein substrates is a key element in the surprising specificity that follows engagement of surface receptors. Signaling cascades downstream of receptors often employ rather ubiquitous intermediates, raising important questions as to how activated kinases and phosphatases preferentially phosphorylate target substrates. An attractive "targeting hypothesis" holds that phosphorylation is controlled at least in part by localization of individual kinases and phosphatases to distinct subcellular compartments (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). A striking example of subcellular targeting of kinase activity is the type II cAMP-dependent kinase (PKA) holoenzyme (Dell'Acqua and Scott, 1997). The PKA holoenzyme complex is comprised of an inhibitory regulatory R subunit dimer that holds two catalytic C subunits in a dormant state. Another property of the RII dimer is its direction of the subcellular location of PKA holoenzyme through its association with a series of tethering proteins known as A-kinase-anchoring proteins (AKAPs). AKAPs represent a functionally related family of signaling proteins that contain a conserved anchoring domain that associates with RII and unique targeting sequences that direct the PKA holoenzyme complex to specific subcellular addresses including the mitochondria (Carr et al., 1992; Lin et al., 1995; Huang et al., 1997). Here we purify an IL-3-induced kinase responsible for the phosphorylation of S112 of BAD. It proves to be mitochondria-tethered PKA, thus focusing the inactivation of BAD to the site where it damages an organelle.

## Results

### Localization of BAD Kinase Activity

To determine whether a BAD-specific kinase is activated in response to the survival factor IL-3, we established an in vitro kinase assay. To insure specificity, GST-BAD wild-type and a GST-BAD mutant protein whose S112 and S136 residues were substituted to Ala were compared as substrates. We utilized an IL-3-dependent cell line, FL5.12, to examine BAD kinase(s) in that the differential phosphorylation of BAD following IL-3 addition or withdrawal dictates cell survival (Zha et al., 1996). Subcellular fractions of FL5.12 cells expressing BCL-$X_L$

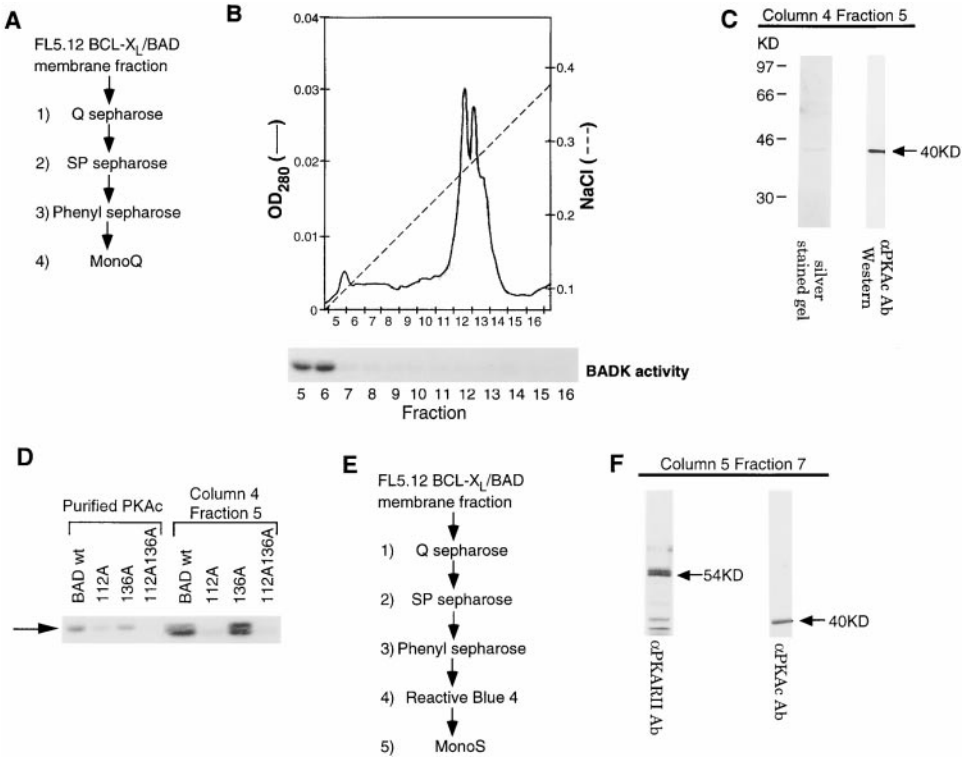


Figure 3. Purification of the BAD Ser-112-Specific Kinase

(A) The purification scheme used for a BAD S112–specific kinase.

(B) The phenyl-Sepharose fraction (column 3 in [A]) that shows a BAD kinase activity was chromatographed on a Mono Q column, and the bound proteins were eluted with a NaCl gradient (the region of the gradient from 80 mM to 380 mM NaCl is shown). Fractions 5–16 were assayed for BAD kinase activity with GST-BAD wild-type as a substrate.

(C) Left, a silver-stained gel of fraction 5 from the Mono Q column. Right, a Western blot of the same fraction using an anti-PKA catalytic subunit antibody.

(D) Purified BAD kinase (Mono Q, fraction 5) was tested for its ability to phosphorylate GST-BAD wild-type (BAD wt), and GST-BAD whose S112 and/or S136 were substituted to Ala (112A, 136A, and 112A136A). Purified PKA catalytic subunit (Sigma) was used to show Ser-112 specificity of BAD phosphorylation in vitro.

(E) An alternative purification scheme used for a BAD S112–specific kinase.

(F) A Western blot of the peak fraction of BAD kinase activity (fraction 7) from a Mono S column using an anti-PKA regulatory subunit type II (left) and an anti-PKA catalytic subunit (right) antibody.

and BAD (FL5.12 BCL-X$_L$/BAD) following the deprivation of IL-3 (2 hr) or its readdition (15 min) were examined. Only low-level BAD kinase activity was detected in the absence of IL-3. Exposure to IL-3 markedly induced kinase activity for wild-type BAD but not the BAD 112A136A mutant. Moreover, this IL-3-activated kinase was predominantly localized to the heavy membrane (enriched for mitochondria) and light membrane (enriched for endoplasmic reticulum) fractions (Figure 1). Therefore, a crude membrane fraction of FL5.12 BCL-X$_L$/BAD cells cultured in the presence of IL-3 was used as the source to purify the BAD kinase(s) (see Figures 3A and 3E).

### AKT Does Not Appear to Be the Principal BAD Kinase following IL-3 Stimulation

AKT (protein kinase B/RAC) is a Ser/Thr kinase downstream of PI-3 kinase and has been shown to suppress apoptosis following a variety of stimuli in multiple cell types (Franke et al., 1997). The pS112 and pS136 14-3-3 binding sites of BAD constitute attractive consensus sites for AKT phosphorylation. Recently, AKT has been shown to be capable of phosphorylating the S136 site of BAD and, when overexpressed, blocked BAD-induced death of primary neurons (Datta et al., 1997). Overexpression of AKT has also been reported to inhibit IL-3 deprivation death (del Peso et al., 1997; Songyang et al., 1997). However, to assess whether AKT was the endogenous kinase primarily responsible for the phosphorylation of BAD following IL-3 treatment, we pursued an immunodepletion approach. AKT was immunoprecipitated by an anti-AKT antibody (Franke et al., 1995) that recognizes AKT1 and AKT2, and in vitro kinase assays were performed. AKT phosphorylated its classic substrate H2B robustly (Figure 2A, lanes 3 and 7). Comparatively, BAD was only minimally phosphorylated by the immunoprecipitated AKT (Figure 2A, lanes 1 and 5). However, BAD kinase activity increased approximately 10-fold following IL-3 but resided principally in the AKT-depleted extract (Figure 2A, lanes 4 and 8; Figure 2B). These results argue that AKT is not the major kinase responsible for the site-specific phosphorylation of BAD following IL-3 receptor engagement.

Raf-1, a Ser/Thr kinase in the MAPK pathway, can

weakly phosphorylate BAD (Wang et al., 1996a; Zha et al., 1996). However, immunoprecipitates of Raf-1 in an in vitro kinase assay revealed that the mutant GST-BAD 112A136A was phosphorylated just as well as wild-type BAD (data not shown). This result, together with the results using kinase inhibitors (see Figure 5), indicates that Raf-1 is not the principal kinase.

### Purification of Protein Kinase A as a Ser-112-Specific BAD Kinase

A series of column chromatography purification steps were utilized to enrich the kinase activity from the membrane fraction of IL-3-stimulated cells that had specificity for wt BAD but not mutant BAD 112A136A (Figure 3A). The peak BAD kinase activity was present in fractions 5 and 6 from the final, Mono Q column (Figure 3B). A silver-stained SDS-PAGE gel of fraction 5 revealed a prominent 40 kDa band (Figure 3C). Nano-electrospray mass spectroscopy identified the protein as the $\alpha$ catalytic subunit of protein kinase A. Furthermore, Western blot analysis of fraction 5 confirmed that the 40 kDa protein was PKAc (Figure 3C). Furthermore, the purified BAD kinase of fraction 5 displayed S112 specificity typical of PKAc (Figure 3D). These results implicate PKAc as the candidate kinase for S112 phosphorylation in vivo. Of note, S136-specific BAD kinase activity was noted to separate from S112 kinase activity during the chromatographic purification steps (data not shown).

An alternative column chromatography purification scheme revealed a peak of S112-specific kinase activity in fraction 7 from the final, Mono S column (Figure 3E). Of note, immunoblot analysis revealed the presence of the 54 kDa PKA regulatory subunit type II (PKARII) doublet copurifying with PKAc in this fraction (Figure 3F). It has been established that the type II PKA holoenzyme is targeted to select intracellular sites by association of the regulatory RII subunit with a series of A-kinase-anchoring proteins (AKAPs) (Dell'Acqua and Scott, 1997). Thus, the above results suggested a thesis for further testing: that PKA tethered to membranes by RII/AKAP interaction could provide a localized pool of anchored PKA responsible for the phosphorylation on S112 of BAD.

### Mitochondrial Membrane-Based S112 BAD Kinase Is Activated by IL-3 and Blocked by PKA Inhibitors

We next examined site-specific BAD kinase activity in the mitochondrial heavy membrane fraction from cells maintained in or deprived of IL-3. S112-specific, as well as S136-specific activities were consistently increased in the mitochondrial fraction of IL-3-treated cells (Figure 4A). The S112-specific kinase activity was strongly diminished to nearly background levels following the addition of a PKA-specific inhibitory peptide (PKI) to the heavy membrane fraction while the S136-specific kinase activity was unaffected (Figure 4B). Moreover, the addition of 10 $\mu$M H-89, an ATP analog that functions as a PKA inhibitor, to the heavy membrane preparation reduced the IL-3-induced S112 kinase activity (Figure 4C). Collectively, these results implicate PKA as the principal mitochondria-based S112 kinase.

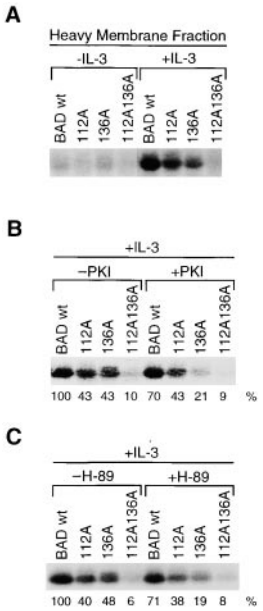


Figure 4. Ser-112-Specific BAD Kinase Induced by IL-3 in the Heavy Membrane Fraction Is Blocked by a PKA-Specific Inhibitor

(A) S112 and S136 BAD kinase activities in the heavy membrane fraction are induced by IL-3. IL-3 was withdrawn from FL5.12 BCL-$X_L$/BAD cells for 2 hr (−IL-3), followed by readdition of IL-3 for 15 min (+IL-3). The heavy membrane fractions were used for an in vitro kinase assay with BAD wt, 112A, 136A, and 112A136A as substrates.

(B) PKA-specific inhibitor peptide, PKI, blocks S112-specific BAD kinase activity induced by IL-3. The heavy membrane fraction of FL5.12 BCL-$X_L$/BAD cells induced by IL-3 was used for an in vitro kinase assay with (100 nM) or without PKI. Lower numbers indicate the relative phosphorylation level of each substrate when the level of GST-BAD wild-type phosphorylation without PKI is considered as 100% measured by phosphorimage analyzer. Data shown are representative of four independent experiments.

(C) A PKA inhibitor, H-89, inhibits the IL-3-induced S112 kinase activity. The heavy membrane fractions of the IL-3-induced FL5.12 BCL-$X_L$/BAD cells treated with (10 $\mu$M) or without H-89 were used for an in vitro kinase assay. Lower numbers indicate the relative phosphorylation level of each substrate when the level of GST-BAD wild-type phosphorylation without H-89 is considered as 100% measured by phosphorimage analyzer. Data shown are representative of three independent experiments.

### PKA Inhibitors Block IL-3-Induced Phosphorylation of Endogenous BAD

To examine the phosphorylation of BAD in vivo, FL5.12 BCL-$X_L$/BAD cells were labeled with $^{32}$P-orthophosphate and immunoprecipitated with an anti-BAD antibody (Zha et al., 1996). There was very little residual phosphorylation of BAD after 4 hr of IL-3 deprivation (Figure 5A). However, BAD phosphorylation was induced approximately 12-fold following 15 min of IL-3 exposure. IL-3-induced phosphorylation of endogenous BAD was inhibited by approximately 50% when cells were pretreated with the PKA inhibitors Rp-cAMP or H-89, consistent with the inhibition of S112 but not S136 phosphorylation (Figures 5A and 5B). In further support of this conclusion, inclusion of a myristoylated PKI peptide that gains access into cells also reduced the IL-3-induced phosphorylation of endogenous BAD (Figure 5B). These data confirm that PKA phosphorylates endogenous BAD in response to IL-3.

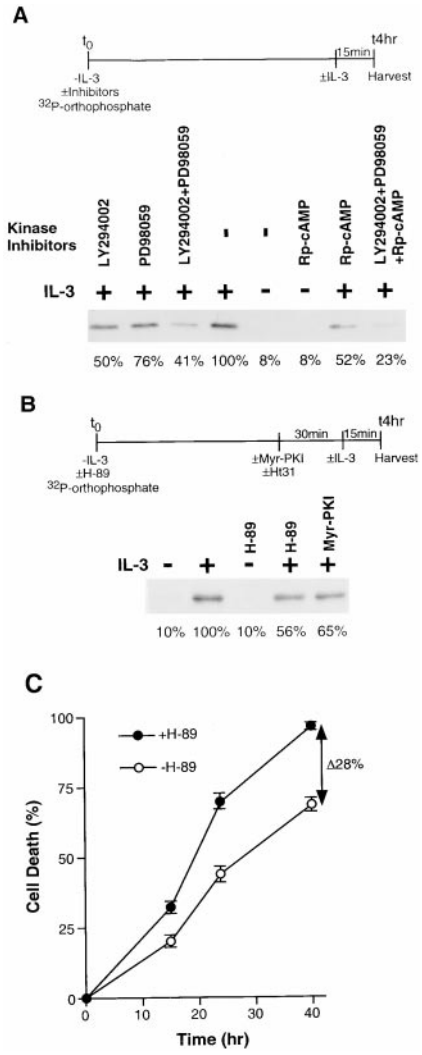


Figure 5. PKA Inhibitors Block IL-3-Induced BAD Phosphorylation In Vivo

(A) BAD immunoprecipitates from $^{32}$P-labeled FL5.12 BCL-$X_L$/BAD cells were prepared after 4 hr of IL-3 deprivation (−IL-3) or 15 min following IL-3 readdition (+IL-3). PI-3 kinase inhibitor (LY294002, 10 μM), MEK inhibitor (PD98059, 50 μM), and PKA inhibitor (Rp-cAMP, 100 μM) were added together with $^{32}$P-orthophosphate at time 0. Cellular extracts were immunoprecipitated with an anti-BAD antibody (2G11). The experimental time course is shown at the top. Percentage values indicate the relative BAD phosphorylation level for each treatment when the level of BAD phosphorylation induced by IL-3 is set at 100% as measured by phosphorimage analyzer. Data shown are representative of three independent experiments.

(B) BAD immunoprecipitates from $^{32}$P-labeled FL5.12 BCL-$X_L$/BAD cells were prepared after 4 hr of IL-3 deprivation (−IL-3) or 15 min following IL-3 readdition (+IL-3). The prepared PKA inhibitor, H-89 (10 μM), was added together with $^{32}$P-orthophosphate. The myristoylated PKI peptide (100 μM) was added to the culture medium 30 min prior to IL-3 readdition. Cellular extracts were immunoprecipitated with an anti-BAD antibody (2G11). The experimental time course is shown in the upper panel. Percentage values indicate the relative BAD phosphorylation level for each treatment when the level of BAD phosphorylation induced by IL-3 is set at 100% as measured by phosphorimage analyzer. The effectiveness of PKA inhibition was confirmed by assessing PKA activity within cellular lysates to the Kemptide (LRRASLG) substrate. For example, when background PKA activity in IL-3-deprived cells is set as 1.0, the inclusion of 10 μM H-89 decreased the IL-3-induced PKA activity from 2.7-fold to 0.4-fold. Data shown are representative of two independent experiments.

(C) FL5.12 BCL-$X_L$/BAD 136A cells in the presence of IL-3 were pretreated (+) or not (−) with the PKA inhibitor H-89 (10 μM). Following IL-3 deprivation, viability was assessed by trypan blue exclusion.

Two distinct regions within the cytoplasmic domain of the IL-3 receptor βc chain are recognized to result in separate signal transduction pathways. A membrane proximal region is essential for JAK-STAT activation, while a distal region is required for activation of the RAS-RAF-1-MEK-MAP kinase (MAPK) pathway and the PI3K-p70 S6 kinase (p70S6K) pathway (Miyajima et al., 1993; Ihle, 1995). To assess whether these pathways contribute to BAD phosphorylation, a panel of kinase-specific inhibitors was used. The PI3K inhibitor LY294002 (Vlahos et al., 1994) or Wortmannin (data not shown) also inhibited the phosphorylation of BAD by approximately 50% (Figure 5A). In addition, the MEK inhibitor PD98059 (Alessi et al., 1995) also partially inhibited (∼25%) the phosphorylation of BAD (Figure 5A). The MEK and PI-3K inhibitors proved additive in their effect (Figure 5A), suggesting that both of these pathways, in addition to the PKA pathway, can contribute to IL-3-induced BAD phosphorylation. However, the protein tyrosine kinase inhibitor Genestein and the p70S6K inhibitor Rapamycin had no effect on the phosphorylation (data not shown). Furthermore, when inhibitors of PI3K (LY294002), MEK (PD98059), and PKA (Rp-cAMP) were added simultaneously, a consistent, further reduction in phosphorylation was observed (>75%) (Figure 5A).

To determine whether PKA activity has a biologic role in survival, we assessed the effects of the PKA inhibitor H-89 upon FL5.12 BCL-$X_L$/BAD 136A cells, which possess an intact S112 site. Cells were pretreated with H-89 to inhibit PKA and deprived of IL-3 as a death stimulus. Approximately 28% more death was observed with the inhibition of PKA (Figure 5C). A more stringent test of PKA's singular importance to cell survival asked whether the inhibition of PKA in the continued presence of IL-3 was sufficient to cause death. Cells were deprived of IL-3 to initiate a death signal, but IL-3 with or without H-89 was added back at 8 hr before irreversible damage occurred. Inhibition of PKA was sufficient to kill ∼20% of cells even in the presence of IL-3 providing its other survival signaling pathways. The data in Figure 5 suggest that PKA as well as PI-3K and MAPK pathways impact BAD phosphorylation and cell survival.

### Anchoring Inhibitor Peptide Inhibits BAD Ser-112 Phosphorylation In Vivo

The observation that PKA inhibitors would block the mitochondria-based S112 BAD kinase activity (Figure 4) suggests the involvement of a mitochondria-based AKAP that would localize the PKA holoenzyme to that membrane. To examine this, we performed an RII overlay experiment. Radiolabeled $^{32}$P-RIIα revealed a prominent binding protein in lysates of enriched mitochondria (Figure 6A). Pretreatment with a peptide (Ht31) of the amphipathic α helix representing the site of RII/AKAP interaction (Carr et al., 1992) specifically blocked the interaction of $^{32}$P-RIIα with this band as has been shown

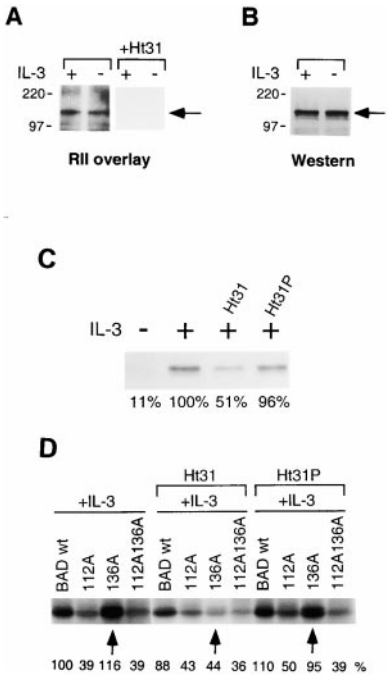


Figure 6. Anchoring Inhibitor Peptide Inhibits BAD Ser-112 Phosphorylation In Vivo

(A) The mitochondria-enriched heavy membrane fraction prepared from FL5.12 BCL-$X_L$/BAD cells in the presence of IL-3 (+) or after IL-3 withdrawal (−IL-3) were examined using an RII overlay method. Mitochondrial extracts were size fractionated by SDS-PAGE and transferred to nitrocellulose. A matched filter was pretreated with the Ht31 peptide that disrupts RII/AKAP interactions (+Ht31), and both filters were hybridized with $^{32}$P-labeled RII$\alpha$. The arrow indicates a specific RII-binding protein consistent in size with the 132 kDa isoform of the mitochondrial AKAP (D-AKAP 1/S-AKAP84).

(B) Western blot of the same mitochondria-enriched heavy membrane fraction (as in [A]) was developed with an anti-D-AKAP 1/S-AKAP84 Ab (Huang et al., 1997). The prominent immunoreactive band (arrow) confirms that the comparably sized RII-binding protein (as in [A]) is the ~132 kDa isoform of the mitochondrial AKAP.

(C) BAD immunoprecipitates from $^{32}$P-labeled FL5.12 BCL-$X_L$/BAD cells were prepared after 4 hr of IL-3 deprivation (−IL-3) or 15 min following IL-3 readdition (+IL-3). Myristoylated peptides (200 $\mu$M) were added to the culture medium 30 min prior to IL-3 readdition as diagrammed in Figure 5B. Cellular extracts were immunoprecipitated with an anti-BAD antibody (2G11). Percentage values indicate the relative BAD phosphorylation level for each treatment when the level of BAD phosphorylation induced by IL-3 is set at 100% as measured by phosphorimage analyzer. Data shown are representative of two independent experiments.

(D) The heavy membrane fraction of FL5.12 BCL-$X_L$/BAD cells induced by IL-3 was used in an in vitro kinase assay to examine specificity for BAD site-specific substrates. The myristoylated Ht31 and Ht31P peptides were added (as in [C]), except the final concentration was 100 $\mu$M. Data shown are representative of two independent experiments.

previously for RII/AKAP interactions in vitro (Rosenmund et al., 1994). An immunoblot of this same mitochondrial fraction was developed with an antibody to D-AKAP 1/S-AKAP84, confirming that this RII-binding protein represents the ~132 kDa isoform of this mitochondrial AKAP (Figure 6B) (Lin et al., 1995; Huang et al., 1997).

Only the nonphosphorylated BAD appears to be an active death agonist found at membrane sites where it can heterodimerize with BCL-$X_L$/BCL-2 (Zha et al., 1996).

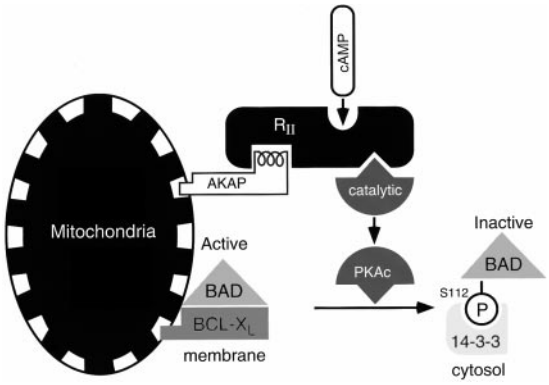


Figure 7. Schematic Representation in which the IL-3-Induced Phosphorylation of BAD on S112 Is Regulated by Mitochondria-Anchored cAMP-Dependent Protein Kinase

The observation that the PKA regulatory II subunit (RII), along with the catalytic subunit (PKAc), copurified during the identification of the S112 kinase (Figure 3F) suggested that the anchoring of PKA to the mitochondrial membrane may be critical for the regulation of BAD S112 phosphorylation. To test this thesis, we introduced a myristoylated Ht31-blocking peptide that serves as a competitive inhibitor of RII/AKAP interaction in vivo in order to globally disrupt anchoring of PKA (Lester et al., 1997). The myristoylated Ht31 peptide inhibited IL-3-induced phosphorylation of endogenous BAD (~50%), consistent with loss of the S112 portion of phosphorylation (Figure 6C). The control peptide, Ht31P, which contains a proline residue that disrupts the $\alpha$ helix, did not alter phosphorylation (Figure 6C). Furthermore, at the level of the mitochondrial membrane, the IL-3-induced S112-specific kinase activity was selectively reduced by treating cells with Ht31 but not Ht31P peptide (Figure 6D). Thus, the displacement of RII-anchored PKA appears to have essentially eliminated the S112 kinase activity at the mitochondrial membrane.

## Discussion

The phosphorylation of BAD following exposure to IL-3 provides a link between an extracellular survival factor and altered susceptibility to apoptosis by a posttranslational modification at the BCL-2 checkpoint. Accumulating evidence suggests a model in which protein kinases and phosphatases are spatially and temporally activated in a cell (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). Here we show that PKA is responsible for site-specific, phosphorylated inactivation of BAD upon S112 both in vitro and in vivo. Compartmentalization of the PKA holoenzyme is conferred by the association of RII subunits with a family of AKAPs. These AKAPs bear subcellular addresses that focus the PKA holoenzyme in close proximity to preferred substrates and potentially position the kinase where it can optimally respond to alterations in cAMP. On the basis of our evidence that the type II PKA holoenzyme is an IL-3-induced kinase responsible for the S112 phosphorylation of BAD and that a blocking peptide that disrupts AKAP/RII interactions eliminated the S112-specific BAD

phosphorylation in vivo at the mitochondria, we propose that an anchored pool of PKA performs this function. Consistent with this involvement of PKA, we found a 5- to 6-fold increment in the level of cAMP and the induced phosphorylation of CREB in FL5.12 cells following IL-3 exposure (data not shown). Of note, it is the nonphosphorylated form of BAD that complexes with BCL-$X_L$/BCL-2 at membrane sites, especially mitochondria (Zha et al., 1996). This constellation of findings indicates that IL-3 survival signaling triggers the activation of mitochondrial membrane-targeted PKA where active BAD is localized and then inactivated (Figure 7).

The inhibition of apoptosis by cAMP has been reported in several systems including the the ability of dibutyryl cAMP to retard the apoptosis of IL-3-deprived cells (Berridge et al., 1993). Derivatives of cAMP also protected cells from chemotherapy-induced death (von Knethen, 1998). In addition, elevation of cAMP has been shown to enhance the responsiveness of retinal ganglion cells to many different trophic factors (Meyer-Franke et al., 1995). The data here provide one target for cAMP effects, the inactivation of the proapoptotic molecule BAD.

IL-3 receptor signals are transduced via the associated βc chain (Miyajima et al., 1993; Ihle, 1995) also shared by IL-5 and GM-CSF receptors. Three downstream pathways are well documented following IL-3 receptor stimulation and include the Jak-Stat, Ras-Raf1-MEK-MAPK, and PI-3K pathways, which emanate from defined regions of the βc chain. While less explored, elevation of cAMP levels has been reported in IL-3-treated 32D cells (Berridge et al., 1993) and GM-CSF-stimulated cells (Coleman et al., 1989). Our initial examination of the βc chain indicates that the cAMP response resides in a distinct region of that receptor. Alternatively, the IL-3 activation of mitochondria-based PKA could reflect a more tightly compartmentalized mechanism that enables catalytic activity.

Inhibitors of PKA, PI-3K, and MEK kinases all diminished BAD phosphorylation in vivo, arguing that a combination of pathways controls BAD activity in IL-3-treated cells. AKT has been implicated in the phosphorylated inactivation of BAD in several systems that overexpress both AKT and BAD (Datta et al., 1997; del Peso et al., 1997; Blume-Jensen et al., 1998). In one of these, transiently expressed BAD and AKT were colocalized to the perinuclear region providing an explanation (Blume-Jensen et al., 1998). The immunodepletion of AKT performed here did not substantially alter the IL-3-induced BAD kinase activity. In that context, AKT is reported to be principally located at the plasma membrane (Andjelkovic et al., 1997), while nonphosphorylated BAD resides at the mitochondria and endoplasmic reticulum. This raises an issue as to whether native levels of AKT are solely responsible for the phosphorylation of endogenous BAD.

Another study provided evidence that a MEK-dependent pathway may contribute to the phosphorylation of BAD (Scheid and Duronio, 1998). The separation of S136 from S112 kinase activity after several chromatographic steps provides an opportunity to define the S136-specific kinase in the IL-3 pathway.

An expanding body of data supports a prominent role for mitochondria in many apoptotic deaths. In addition to the localization of BCL-2 members to mitochondria (de Jong et al., 1994; Hockenbery et al., 1990), mitochondrial dysfunction frequently occurs in apoptosis. This includes alterations of $\Delta\psi M$, production of reactive oxygen species, mitochondrial swelling, and release of cytochrome c (Xiang et al., 1996; Kroemer et al., 1997; Li et al., 1997; Vander Heiden et al., 1997). Expression of each proapoptotic member we have examined, including BAX, BAD, and BID, all induce mitochondrial dysfunction. The AKAP-mediated tethering of PKA to mitochondria is an attractive strategy to insure that damage to this organelle does not occur in the presence of adequate survival factor (IL-3). This also raises the possibility that other mitochondrial substrates beyond BAD may exist and influence cell death. AKAPs provide a clear example of subcellular signaling specificity that focuses kinase/substrate interactions at the principal sites of action. The distinct AKAP (D-AKAP1/S-AKAP84) that targets the outer mitochondrial membrane (Lin et al., 1995; Huang et al., 1997) would provide PKA the proper localization to phosphorylate BAD. This does not eliminate the possibility of additional subcellularly focused BAD kinase(s).

Evolving evidence suggests that all proapoptotic BCL-2 members may exist in inactive and active conformations (McDonnell et al., 1999). Activation represents posttranslational modifications in response to death signals that also result in translocation between the cytosol and mitochondria. The withdrawal of survival factor or treatment with staurosporine results in the translocation of monomeric BAX from the cytosol to the mitochondria, where it is a homodimerized, integral membrane protein (Wolter et al., 1997; Gross et al., 1998). The BH3 domain–only BID provides another example in which Caspase-8 activation by Fas or TNF results in proteolytic cleavage to an active p15 form of BID that targets mitochondria and releases cytochrome c (Luo et al., 1998; Li et al., 1998; Gross et al., 1999). The phosphorylation of BAD provides yet another example of how distinct signal pathways alter death effectors at intracellular sights. The differential phosphorylation of BAD indicates the complexity of death and survival pathways (Figure 7). This single proapoptotic molecule has two nearly identical serine phosphorylation sites, both of which bind 14-3-3. However, each site has a distinctly different kinase responsible downstream from a single receptor, IL-3R. This argues that the effects of posttranslational modification on the central apoptotic pathway will be substantial.

## Experimental Procedures

### Plasmid Construction

Standard polymerase chain reactions (PCRs) were used to construct the GST-BAD fusion plasmids. The *BAD* cDNAs encompassing amino acids from 104 to 141 were subcloned between BamHI and EcoRI sites of pGEX4T-1. The plasmids pSFFV-BADwt, -BAD112A, -BAD136A, and -BAD112A136A (Zha et al., 1996) were used as templates for amplifying cDNAs with a forward primer of sequence ATTGGATCCATGGAGACTCGGAGTCGC and a reverse primer of sequence TGCGAATTCTCAGAGATTGGGGGGAGC.

### Antibodies

Anti-BAD antibody (Ab) is a hamster monoclonal Ab (2G11) (Zha et al., 1996). Anti-AKT Ab is a rabbit polyclonal Ab provided by Dr.

Philip Tsichlis, Fox Chase Cancer Center. Anti-PKAc and anti-D-AKAP1/S-AKAP84 Abs are rabbit polyclonal Abs provided by Dr. Susan Taylor, UCSD. Anti-PKARII Ab is a rabbit polyclonal Ab purchased from Santa Cruz.

**Western Blot Analysis**

The analysis was carried out as described previously (Zha et al., 1996). Anti-AKT Ab was used at 1:1000 dilution, anti-PKAc Ab was used at 1:10000, anti-PKARII Ab was used 1:300, anti-D-AKAP 1 Ab was used 1:5000, and the secondary Abs were used at 1:2000.

**RII Overlay**

The presence of AKAPs in mitochondrial heavy membrane preparations was deleted by a solid phase RII overlay method (Carr et al., 1992). Lysates of mitochondria were separated by SDS-PAGE and transferred to nitrocellulose. Filters with or without pretreatment with 3 μM Ht31 peptide were incubated with $^{32}$P-labeled RIIα overnight at room temperature. RII-binding proteins were detected by autoradiography.

**In Vitro Kinase Assays**

The BAD kinase assays were carried out in the presence of 5 μCi of [γ-$^{32}$P]ATP and 50 mM cold ATP in a buffer containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM ditiothreitol (DTT), 10 mM β-glycerophosphate. Each of the GST-BAD fusion proteins was added at 10 μg. The reaction mixtures were incubated at 30°C for 20 min and stopped by the addition of SDS-PAGE loading buffer. Proteins were resolved on an SDS-polyacrylamide gel and were visualized by autoradiography. The AKT immunocomplex protein kinase assay was performed as described in Franke et al. (1995).

**Cellular Fractionation**

FL5.12 BCL-$X_L$/BAD cells were washed with phosphate-buffered saline twice, resuspended in a buffer (20 mM Tris [pH 7.5], 25 mM NaF, 1 mM $Na_3VO_4$, 10 mM β-glycerophosphate, 1 mM DTT, 0.15 U/ml aprotinin, 20 mM leupeptin, and 1 mM phenylmethylsulfonylfluoride [PMSF]), incubated on ice for 15 min, and homogenized in a Dounce homogenizer. Nuclei were removed by centrifugation at 600 × g for 10 min. The supernatant was centrifuged at 10,000 × g for 20 min, and the pellet was used for the heavy membrane fraction. The supernatant was further centrifuged at 100,000 × g for 30 min to separate cytosol from the light membrane fraction. The membrane fractions were solubilized with an equal volume of the cytosol fraction in buffer A (the above buffer plus 100 mM NaCl and 0.1% Nonidet P-40) at 4°C for 30 min. Solubilized proteins were used for the kinase assays or the kinase purification.

For preparing the heavy membrane fractions in Figure 4 and Figure 6D, the cells were resuspended in mito buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES [pH 7.4], 1 mM $Na_3VO_4$, 10 mM β-glycerophosphate, 0.15 U/ml aprotinin, 20 mM leupeptin, and 1 mM PMSF), homogenized by polytron, added another 4× volume of mito buffer, and centrifuged to remove nuclei. The supernatant was then centrifuged 10,000 × g for 20 min, and the pellet was resuspended in buffer A.

**Purification of Ser-112-Specific BAD Kinase**

The entire purification was done at 4°C or on ice. The crude membrane fraction (180 mg of protein) from 5 liters of FL5.12 BCL-$X_L$/BAD cells (about $10^{10}$ cells) was applied to a 10 ml HiTrap Q column (Pharmacia) equilibrated with buffer A. After washing with buffer A, bound proteins were eluted with a 200 ml linear gradient of 0.1–0.5 M NaCl in buffer A. Fractions of 10 ml were collected and assayed for BAD kinase activity. The active fractions (8.5 mg of protein) were pooled and buffer exchanged into buffer B (50 mM sodium phosphate [pH 7.0], 1 mM EDTA, 1 mM DTT, 0.05% Brij-35) using a Sephadex G-25 column (Pharmacia). Appropriate fractions were loaded onto a 2 ml HiTrap SP column (Pharmacia) equilibrated with buffer B. Ammonium sulfate (1 M) was added to the flowthrough fraction of HiTrap SP column (4.2 mg of protein) and loaded onto a 1 ml phenyl-Sepharose column (Pharmacia) equilibrated with buffer B containing 1 M ammonium sulfate. The column was eluted with a 15 ml linear gradient of 1.0–0 M ammonium sulfate in buffer B. BAD kinase activity was monitored after removal of salt by Centricon-10 (Amicon). Active fractions (0.8 mg of protein) were pooled and buffer exchanged into buffer C (20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM DTT, 0.05% Brij-35) using a Sephadex G-25 column. Appropriate fractions were loaded onto a Mono Q 5/5 column (Pharmacia) equilibrated with buffer C. The column was eluted with a 20 ml linear gradient of 0–0.5 M NaCl in buffer C.

On the purification described in Figure 3E, after the loading onto a 5 ml HiTrap SP column (46.2 mg of protein), bound proteins were eluted with a 100 ml linear gradient of 0–0.5 M NaCl in buffer B. Then ammonium sulfate (1 M) was added to the active fractions (11.6 mg of protein) and loaded onto a 1 ml phenyl-Sepharose column, and bound proteins were eluted at the same condition described above. The salt-removed, active fractions (2.8 mg of protein) were loaded onto a 1 ml Reactive Blue 4 column (Sigma) equilibrated with buffer B. The column was eluted with a 20 ml linear gradient of 0–1.0 M NaCl in buffer B. The active fractions (0.432 mg of protein) were pooled, diluted, and loaded onto a Mono S 5/5 (Pharmacia) equilibrated with buffer B. The column was eluted with a 20 ml linear gradient of 0–0.5 M NaCl in buffer B.

**Metabolic Labeling and Immunoprecipitation**

FL5.12 BCL-$X_L$/BAD cells were labeled in phosphate-free RPMI1640 medium with [$^{32}$P] orthophosphate (1 mCi/$10^7$ cells) for 4 hr. Immunoprecipitation using an anti-BAD antibody (2G11) was carried out as previously described (Zha et al., 1996).

**Acknowledgments**

We are grateful to Dr. Philip Tsichlis for providing the anti-AKT Ab. We thank Drs. Andrey Shaw and Helen Piwnica-Worms for their stimulating discussion and technical comments. We thank Ms. Deborah S. Maher for secretarial assistance. Hisashi Harada is a special fellow of the Leukemia Society of America. This work was sponsored by 5R01 CA 50239-10.

Received December 14, 1998; revised February 9, 1999.

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