Survival-factor-induced phosphorylation of Bad results in its dissociation from Bcl-x_L but not Bcl-2

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The pro-apoptotic Bcl-2-family protein Bad heterodimerizes with Bcl-2 and Bcl-x_L in the outer mitochondrial membranes, nullifying their anti-apoptotic activities and promoting cell death. We report that interleukin-3 (IL-3) stimulation induces Bad phosphorylation and triggers its translocation from mitochondria to cytoplasm in cells expressing Bcl-x_L but not Bcl-2. Overexpression of Bad sensitized Bcl-x_L-expressing FL5.12 cells to apoptosis induced by IL-3 deprivation, but had no effect on the viability of cells expressing Bcl-2. IL-3 stimulation induced Bad phosphorylation at Ser-112, impairing its binding to Bcl-x_L and resulting in its association with 14-3-3 proteins in the cytosol. However, Ser-112 phosphorylation could not trigger Bad dissociation from mitochondria in FL5.12 cells expressing Bcl-2. In 293T cells expressing Bcl-x_L, Bad was phosphorylated at three serines, 112, 136 and 155, and was largely localized in the cytosolic fraction. In contrast, overexpression of Bcl-2 prevented phosphorylation of Bad at Ser-136 and Ser-155, sequestering this protein in the mitochondrial membranes. When the N-terminal regions of Bcl-2 and Bcl-x_L were swapped with each other, the Bcl-x_L(N)–Bcl-2 chimaeric protein (containing the N-terminal region of Bcl-x_L) failed to prevent Bad phosphorylation in cells and was unable to block the cytosolic distribution of this pro-apoptotic protein. Additional experiments with the Bcl-2(N)–Bcl-x_L chimaeric protein (containing the N-terminal region of Bcl-2) indicated that, although the N-terminal region of Bcl-2 is necessary, it is not sufficient for sequestering Bad in the mitochondrial membranes. These observations suggest that growth-factor-mediated phosphorylation of Bad contributes to the cytoprotective function of Bcl-x_L but not Bcl-2.

Key words: apoptosis, IL-3, mitochondria, subcellular fractionation, translocation.

INTRODUCTION

Programmed cell death or apoptosis plays an important role in the development and homoeostasis of multicellular organisms by triggering an intrinsic cellular death programme when cells are damaged, infected or no longer receiving survival signals from their extracellular environment [1,2]. The Bcl-2 family of proteins consists of anti- and pro-apoptotic molecules that control a critical intracellular checkpoint within an evolutionarily conserved cell-death pathway [3]. The relative ratios between pro- and anti-apoptotic members of this family determine the ultimate sensitivity or resistance of cells to apoptotic signals [4]. Many of the Bcl-2-family proteins are capable of interacting with each other through a complex network of homo- and hetero-dimers. In order to avoid a suicidal fate, cells require continuous survival signals from their surface receptors. A number of survival factors, such as insulin-like growth factor, platelet-derived growth factor, nerve growth factor and interleukin-3 (IL-3), promote cell survival at least in part through a mechanism of post-translational modification for inactivating pro-apoptotic Bcl-2-family members and preventing their interaction with anti-apoptotic Bcl-2-family members [5].

The Bad protein is a pro-apoptotic member of the Bcl-2 family that can bind to anti-apoptotic proteins such as Bcl-2 and Bcl-x_L and promote cell death [6]. Recent data indicate that the ability of Bad to heterodimerize with Bcl-2 or Bcl-x_L and to promote apoptosis is modulated by mechanisms that control the state of phosphorylation of this pro-apoptotic protein [5,7,8]. As a large proportion of Bcl-2 and Bcl-x_L protein molecules are anchored in the outer membranes of mitochondria with orientation toward the cytosol, phosphorylation of Bad impairs its binding to Bcl-x_L and results in a translocation of Bad from the surface of mitochondria to the cytosol [9]. At least three sites on Bad (Ser-112, Ser-136 and Ser-155 in the murine protein) can become phosphorylated by growth factors through activating survival kinases such as Raf-1 when targeted to mitochondria [7,10–12], Akt/protein kinase B [13–15], protein kinase A [16–20], the p21-activated kinases [21] or the mitogen-activated protein kinase-activated kinase RSK [18,22]. Ser-112 and Ser-136 of the mouse Bad protein lie within the potential 14-3-3 binding sites [23]. Consistent with this, phsophorylation of Ser-112 and Ser-136 results in Bad sequestration by cytosolic 14-3-3-family proteins [9]. In contrast, Ser-155 is localized in the BH3 domain of Bad (where BH means Bcl-2 homology) that is embedded within the surface pocket created by the combination of BH1, BH2 and BH3 domains of dimerization partners Bcl-2 and Bcl-x_L [24], thus phosphorylating Ser-155 must require a conformational change in the Bad complexes. However, it is unclear how phosphorylation of Bad causes its dissociation from Bcl-2 and Bcl-x_L, though it has been proposed that Ser-136 phosphorylation of Bad recruits 14-3-3 proteins and weakens the association between Bad and Bcl-x_L, accessing Ser-155 kinases for complete disruption of the Bad–Bcl-x_L complex [17]. Here we provide evidence that phosphorylation of Bad by survival-factor-dependent kinases impairs its binding to Bcl-x_L but not Bcl-2 and that differences in the BH4 and loop regions of Bcl-2 and Bcl-x_L contribute to the phenotypes of these proteins.

MATERIALS AND METHODS

Plasmid constructions

The pSFFV-Bcl-x_L plasmid was generated by subcloning the human Bcl-x_L cDNA into the pSFFV-Neo vector [25] using

Abbreviations used: TM, transmembrane; HM, heavy membrane; BH, Bcl-2 homology; FCS, fetal calf serum; IL-3, interleukin-3.

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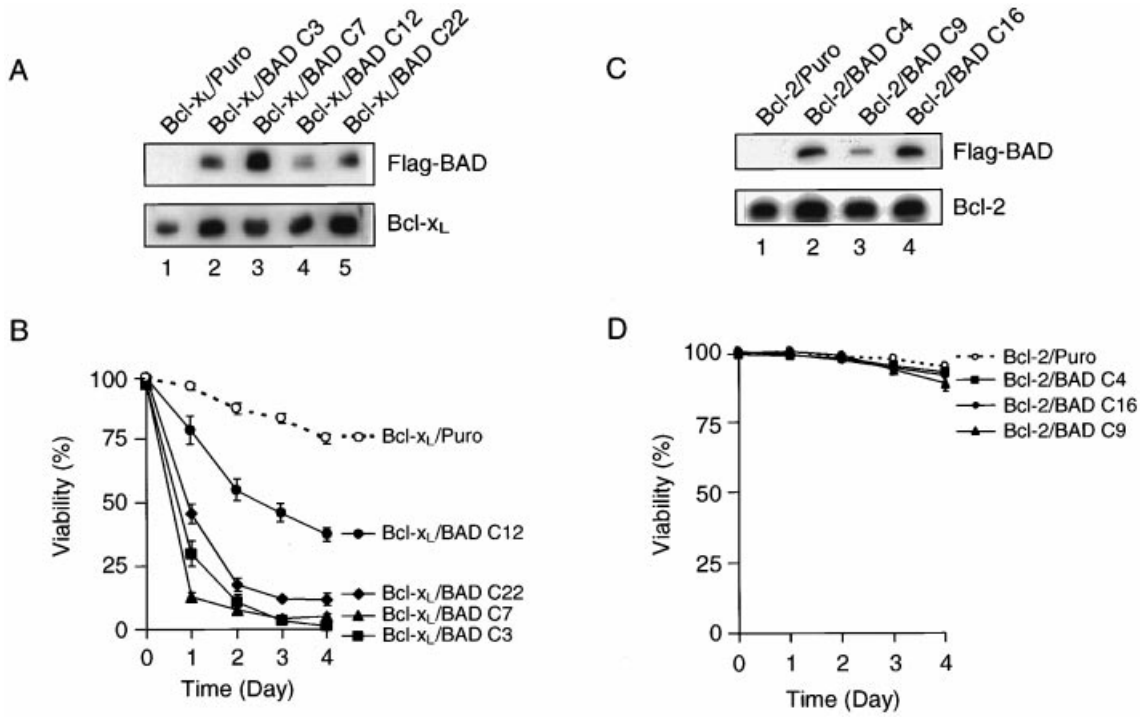

Figure 1 Bad counters the anti-apoptotic activity of Bcl-x_L but not that of Bcl-2 in FL5.12 cells following IL-3 withdrawal

FL5.12 cells stably expressing Bcl-x_L (**A**, **B**) or Bcl-2 (**C**, **D**) were transfected with FLAG-Bad-producing plasmid (BAD C3 etc., where Cx refers to the subclone number) or parental vector (Puro) DNA using electroporation. In (**A**) and (**C**), transfected FL5.12 cells were subcloned by limiting dilution and cell lysates were prepared for SDS/PAGE and immunoblot analysis using anti-FLAG M2 monoclonal antibody and polyclonal rabbit antisera specific for Bcl-x_L or Bcl-2. In (**B**) and (**D**), the same subclones were cultured for various times in the absence of IL-3 and the percentage of variable cells was determined by Trypan Blue dye exclusion (means $\pm$ S.D.; $n = 3$).

the *Eco*RI site. For construction of pcDNA3-Bcl-2(N)–Bcl-x_L chimaeric plasmid (containing the N-terminal region of Bcl-2), the *Hin*dIII–*Blp*I fragment of human Bcl-2 cDNA was ligated with the *Sma*I–*Eco*RI fragment of human Bcl-x_L cDNA, then subcloned into the *Hin*dIII/*Eco*RI-digested pcDNA3 vector (Invitrogen). The Bcl-x_L(N)–Bcl-2 chimaera (containing the N-terminal region of Bcl-x_L) was generated by subcloning the *Eco*RI–*Sma*I fragment of human Bcl-x_L cDNA and the *Blp*I–*Xba*I fragment of human Bcl-2 cDNA into the *Eco*RI-digested pSFFV-Neo vector after blunt-end treatment.

Cell culture and transfection

293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FCS, 50 units/ml penicillin G and 50 μg/ml streptomycin. FL5.12 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal-calf serum (FCS), 15 % (v/v) conditioned medium from WEHI 3B cells, as a source of IL-3, and antibiotics. 293T cells were transfected by calcium phosphate precipitation and FL5.12 cell lines were transfected by electroporation as described previously [26].

Cell viability assay

Stably transfected FL5.12 cells were washed three times with PBS and cultured in RPMI 1640 medium containing 10 % FCS without IL-3 for 4 days. The cell viability was determined by Trypan Blue dye exclusion at each time point.

Preparation of subcellular fractions

The subcellular fractions were prepared as described in [10] with some modifications. Namely, 2×10^8 cells were suspended in 2 ml of hypotonic buffer (3.3 mM Hepes, pH 7.5, 1.7 mM KCl, 0.25 mM $MgCl_2$, 0.17 mM EGTA and 0.17 mM EDTA) containing 3.3 μg/ml aprotinin, 6.6 μg/ml leupeptin, 3.3 μg/ml pepstatin, 0.3 mM PMSF, 16.7 mM NaF, 66.7 μM Na_3VO_4 and 8.3 mM sodium β-glycerophosphate. After incubation on ice for 30 min, cells were homogenized with a Dounce homogenizer and centrifuged at 1000 ***g*** for 5 min at 4 °C to discard unbroken cells and nuclei. The resulting supernatant was centrifuged at 10000 ***g*** for 10 min at 4 °C to obtain the heavy-membrane (HM) fraction (pellet). The resulting supernatant was then centrifuged for 30 min at 100000 ***g*** at 4 °C to obtain the cytoplasmic fraction (supernatant).

Immunoblotting and immunoprecipitation analysis

Cells were lysed in Triton X-100 lysis buffer (10 mM Hepes, pH 7.5, 143 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 0.5 % Triton X-100) containing 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM PMSF, 50 mM NaF, 100 μM Na_3VO_4 and 25 mM sodium β-glycerophosphate. Immunoblotting and immunoprecipitation were carried out as described previously [27].

RESULTS

IL-3-induced Ser-112 phosphorylation frees Bad from Bcl-x_L but not Bcl-2

To study the effects of Bad phosphorylation on its ability to bind to Bcl-2 and Bcl-x_L, we transfected IL-3-dependent FL5.12 cell lines stably expressing Bcl-2 or Bcl-x_L with plasmids encoding FLAG-tagged Bad. Several independent clones of FL5.12 transfectants with a wide range of FLAG-Bad protein levels were obtained and used to determine the association between expression of the Bad protein and promotion of apoptosis (Figure 1). Following IL-3 withdrawal, a general correlation between higher levels of FLAG-Bad protein and faster cell-death kinetics was observed in FL5.12 clones co-expressing Bcl-x_L (Figure 1B). In contrast, no significant differences in the viability of FL5.12 cells were observed among the Bcl-2-overexpressing clones, regardless of their relative levels of FLAG-Bad protein (Figure 1D). This result, together with the previous report [6], indicates that the pro-apoptotic protein Bad nullifies the anti-apoptotic activity of Bcl-x_L, but not that of Bcl-2.

Next, we performed subcellular fractionation experiments to examine the intracellular translocation of Bad in response to IL-3 stimulation. When cultured in the absence of IL-3, the FLAG-Bad protein was contained predominantly within the mitochondrion-enriched HM fraction, where Bcl-2 or Bcl-x_L resides (Figure 2). As reported previously [28], Bcl-2 was mainly associated with membranes (Figure 2B) whereas a small proportion of Bcl-x_L was localized in the cytosolic fraction (Cyt, Figure 2A). Immunoblot analysis with antibodies specific for the mitochondrial protein Cox IV was used as a control for the fractionation procedure. IL-3 stimulation resulted in a slower-migrating form of FLAG-Bad in gels as a doublet (Figure 2A, lane 1), indicating the presence of phosphorylated Bad protein. To map the phosphorylation sites in Bad protein, we employed the anti-phospho-Bad antibodies that have been shown to specifically recognize phosphorylated Ser-112, Ser-136 or Ser-155 residues in the murine Bad [17,18]. As shown in Figure 2, Bad was phosphorylated at Ser-112, but not Ser-136 and Ser-155, in response to IL-3 stimulation. Ser-112-phosphorylated Bad was detected in both the HM fraction and the cytosolic fraction of FL5.12 cells co-expressing FLAG-Bad and Bcl-x_L (Figure 2A), suggesting that phosphorylation of Bad by IL-3 disrupts its binding to Bcl-x_L. In contrast, surprising results were obtained for FL5.12 cells co-expressing FLAG-Bad and Bcl-2, in which Ser-112 phosphorylation of Bad was observed only in the HM fraction and IL-3 stimulation failed to induce the translocation of Bad from the mitochondria to the cytosol (Figure 2B).

Only cytosolic Bad binds to 14-3-3 protein

To determine whether phosphorylation-induced Bad dissociation for Bcl-x_L requires the recruitment of 14-3-3 proteins, we performed co-immunoprecipitation experiments using subcellular fractions prepared from FL5.12 transfectants. As shown in Figure 3(A), 14-3-3 was exclusively co-immunoprecipitated with FLAG-Bad in the cytosolic fraction prepared from IL-3-stimulated Bcl-x_L/FLAG-Bad FL5.12 cells, consistent with the previous report [9] that phosphorylated Bad translocates to

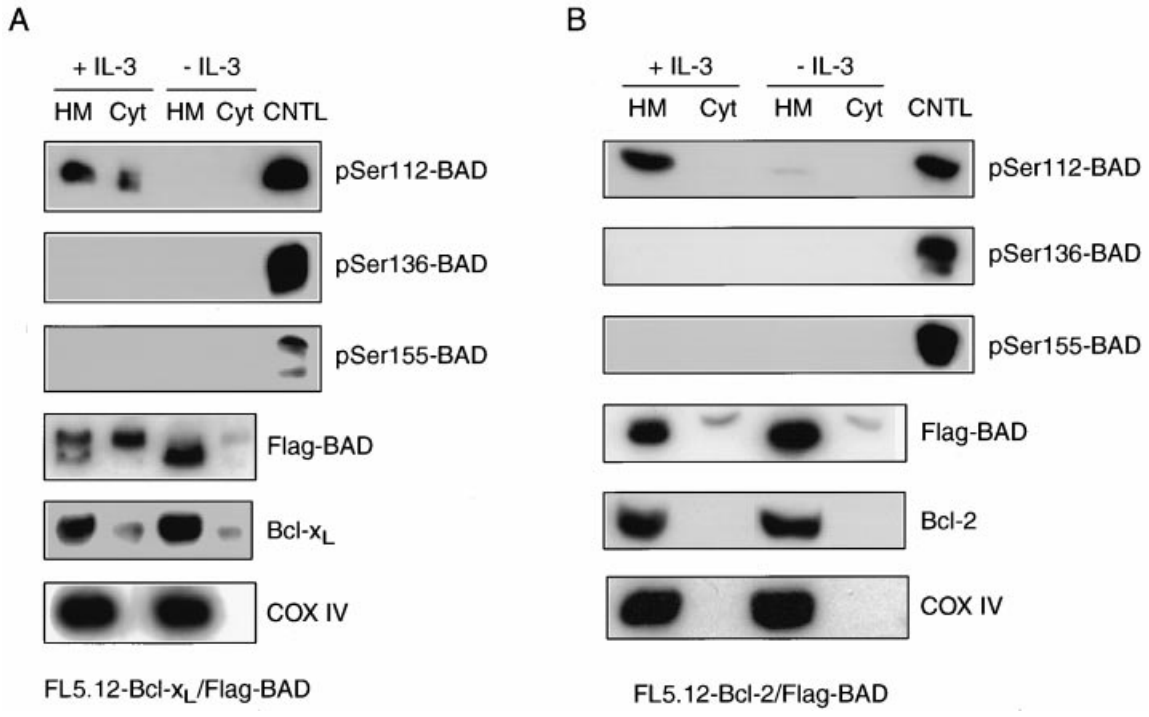

Figure 2 IL-3-induced Ser-112 phosphorylation of Bad triggers Bad dissociation from Bcl-x_L but not Bcl-2

FL5.12-Bcl-x_L/FLAG-Bad (**A**) and FL5.12-Bcl-2/FLAG-Bad (**B**) transfectants were cultured in the absence of IL-3 for 8 h and 12 h, respectively, prior to stimulation with or without 100 ng/ml recombinant mouse IL-3 (BioSource International) for 30 min. Then, cells were fractionated into cytosolic (Cyt) and HM fractions, which were normalized for protein content and subjected to SDS/PAGE and immunoblot analysis (30 μg of protein/lane) with antibodies specific for FLAG-epitope, pSer112-Bad (BioSource International), pSer136-Bad (New England BioLabs), pSer155-Bad (New England BioLabs), Bcl-x_L, Bcl-2 or COX IV (cytochrome *c* oxidase complex IV; Molecular Probes). In addition, FLAG-Bad-transfected 293T cell lysates were used in lanes 5 (CNTL) as positive controls for the anti-phospho-Bad antibodies.

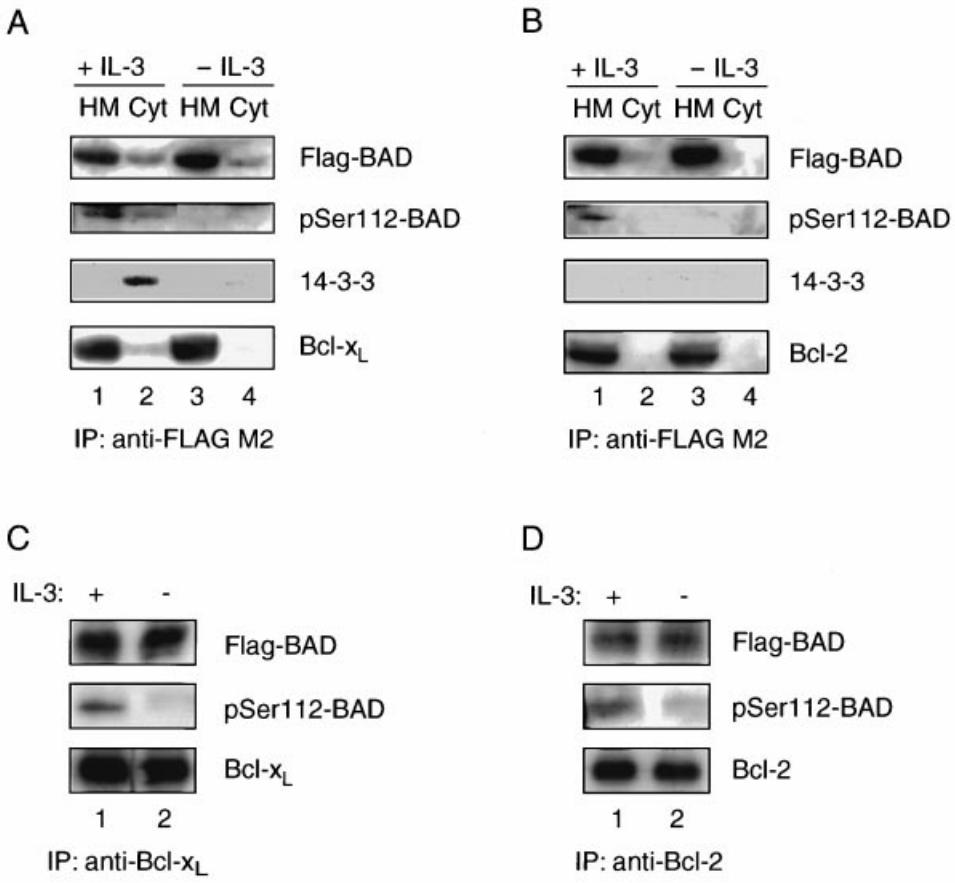

Figure 3 IL-3-stimulation-induced Bad dissociation from mitochondria does not require the recruitment of 14-3-3

Each subcellular fraction (100 μg) prepared from FL5.12-Bcl-x$_L$/FLAG-Bad (**A**) or FL5.12-Bcl-2/FLAG-Bad (**B**) transfectants was subjected to immunoprecipitation (IP) with anti-FLAG M2 monoclonal antibody, followed by immunoblot analysis with the indicated polyclonal antibodies. In (**C**) and (**D**), stably transfected FL5.12 cells were cultured in the presence or absence of IL-3 and lysed in Triton X-100 lysis buffer. Whole-cell lysates (500 μg) were used for immunoprecipitation with monoclonal antibodies specific for Bcl-x$_L$ or Bcl-2 and analysed by immunoblotting with the indicated polyclonal rabbit antisera. Cyt, cytosolic fraction.

the cytosol where it binds to 14-3-3 molecules. IL-3 simulation induced phosphorylation of Bad Ser-112 on mitochondria, but did not recruit 14-3-3 to the mitochondrial Bad complexes (Figures 3A and 3B, lanes 1). Moreover, Ser-112-phosphorylated Bad could associate with both Bcl-x$_L$ and Bcl-2, although less Ser-112 phospho-Bad was detected in Bcl-x$_L$ immune complexes compared with that found in Bcl-2 immunoprecipitates (Figures 3C and 3D). These results suggest that phosphorylation of Ser-112 weakens the association of Bad with Bcl-x$_L$ but not Bcl-2 and that 14-3-3 molecules may not be involved in this process.

Bcl-2 inhibits Bad phosphorylation at Ser-136 and Ser-155 in 293T cells

To further confirm the differences in Bcl-2 and Bcl-x$_L$ for controlling Bad phosphorylation in cells, 293T cells were transiently co-transfected with plasmids encoding FLAG-Bad and Bcl-2 or Bcl-x$_L$. Immunoblot analysis showed that phosphorylation of Bad at Ser-136 and Ser-155 was dramatically reduced in 293T cells co-expressing Bcl-2 (Figure 4, lane 2). In contrast, expression of Bcl-x$_L$ had no effect on Bad phosphorylation in 293T cells compared with the control transfection (Figure 4, lanes 1 and 3).

The N-terminal region of Bcl-2 is critical for sequestering Bad in mitochondria

Both Bcl-2 and Bcl-x$_L$ contain four conserved domains (BH1, BH2, BH3 and BH4) as well as a loop region and a transmembrane (TM) domain [3]. To gain further insights into these molecules, we calculated theoretical isoelectric point (pI) values for each of the conserved domains in the human Bcl-2 and Bcl-x$_L$ proteins. As shown in Table 1, the pI values between the N-terminal regions of Bcl-2 and Bcl-x$_L$, in which the BH4 and loop domains reside, are apparently different, with 8.17 for Bcl-2 and 4.41 for Bcl-x$_L$. In contrast, there is relatively no difference in pI values between the remaining C-terminal parts of the proteins from BH3 through to the TM domain.

We hypothesized that differences in pI values between the N-terminal regions of Bcl-2 and Bcl-x$_L$ reflect their characters in controlling the subcellular localization of Bad in cells. To test this hypothesis, we made chimaeric Bcl proteins by exchanging their N-terminal regions with each other. The pI values for these chimaeric molecules, Bcl-2(N)–Bcl-x$_L$ and Bcl-x$_L$(N)–Bcl-2, were very similar to those of Bcl-2 and Bcl-x$_L$, respectively (see Table 1 for details).

Next, we determined the effects of these Bcl chimaeric proteins on the intracellular distribution and phosphorylation of the Bad

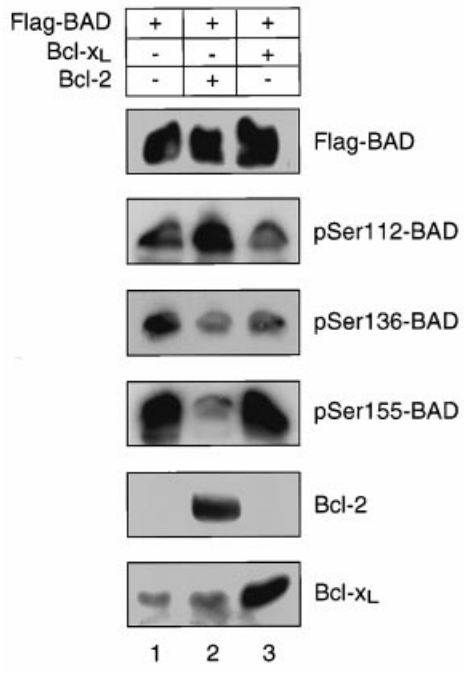

Figure 4 Bcl-2 prevents Bad phosphorylation in 293T cells

293T cells were transiently transfected with the indicated plasmids encoding FLAG-Bad, Bcl-2 or Bcl-x_L. Cells were lysed 24 h later and subjected to SDS/PAGE and immunoblot assay with the indicated specific antibodies.

Table 1 Bcl proteins and their theoretical pI values

Protein	Theoretical pI
Bcl-2	6.49
Met-1–Arg-6	9.52
Thr-7–Tyr-28 (BH4)	9.3
Glu-29–Pro-88 (loop domain)	5.26
Met-1–Pro-88 (BH4, loop domain)	8.17
Val-89–Lys-239 (BH3, BH1, BH2)	5.92
Bcl-x_L	4.86
Met-1–Tyr-22 (BH4)	8.19
Ser-23–Val-80 (loop domain)	4.12
Met-1–Val-80 (BH4, loop domain)	4.41
Ile-81–Lys-233 (BH3, BH1, BH2)	5.41
Bcl-2(N)–Bcl-x_L	6.31
Bcl-x_L(N)–Bcl-2	5.01

protein in 293T cells. As shown in Figure 5(B), FLAG-Bad was mainly distributed in the cytosolic fraction and phosphorylated at serines 112, 136 and 155 in 293T cells expressing FLAG-Bad alone. When co-transfected with Bcl-x_L, FLAG-Bad was apparently detected in both cytosolic and HM fractions. In this case, the cytosolic FLAG-Bad protein was also phosphorylated at all of these three serine residues, whereas no or very little phosphorylated Bad was detected in the HM fraction. Strikingly, FLAG-Bad was predominantly detected in the HM fraction of 293T cells overexpressing Bcl-2, even in the presence of growth factors. However, when co-transfected with Bcl-x_L(N)–Bcl-2, FLAG-Bad was distributed and phosphorylated essentially the same as that in 293T cells co-expressing Bcl-x_L, suggesting that Bcl-2 is converted into Bcl-x_L by swapping its N-terminal regions with Bcl-x_L. In the case of co-expression of Bcl-2(N)–Bcl-x_L, FLAG-Bad was also phosphorylated and distributed in both fractions, although a large proportion of FLAG-Bad was detected in the HM fraction compared with the cytosolic fraction. These results indicate that the N-terminal region of Bcl-2 is necessary but not sufficient for inhibiting Bad phosphorylation and its subsequent translocation to the cytosol in response to survival signals. These data, together with the results from FL5.12 transfectants, suggest that growth factor-induced phosphorylation of Bad impairs its binding to Bcl-x_L, whereas Bcl-2 prevents Bad phosphorylation, especially at Ser-136 and Ser-155, sequestering it in the outer membranes of mitochondria.

DISCUSSION

The BH3-only death promoter Bad interacts with anti-apoptotic molecules Bcl-2 and Bcl-x_L and promotes apoptosis [6]. Phosphorylation of Bad is induced by growth factors such as IL-3, insulin-like growth factor 1 and platelet-derived growth factor, disrupting its interaction with Bcl-x_L and abrogating its pro-apoptotic effects in cells [9,13]. Unlike the Bad–Bcl-x_L association, we show here that the binding of Bad to Bcl-2 is not mediated by growth-factor-dependent kinases. In response to IL-3 stimulation, Bad was phosphorylated at Ser-112 but not Ser-136 and Ser-155, as demonstrated by immunoblotting with anti-phospho-Bad antibodies. In the absence of IL-3, Bad was predominantly detected in the mitochondrion-enriched HM fraction in which Bcl-2 and Bcl-x_L reside. IL-3 stimulation resulted in the redistribution of Bad to the cytoplasm where it was captured by 14-3-3 proteins in FL5.12 cells co-expressing FLAG-Bad and Bcl-x_L, consistent with the results of Zha et al. [9]. It has been proposed that phosphorylation of Bad Ser-136 is required for the recruitment of 14-3-3 proteins that weaken the interaction of Bad with Bcl-x_L and increase the access of Ser-155 kinases to the Bad protein [17]. Thus Ser-155 phosphorylation of Bad is essential for completing its dissociation from the Bad–Bcl-x_L complexes and subsequent translocation to the cytosol [17–20]. However, at least in our FL5.12 cell lines, Ser-112 phosphorylation seems to be sufficient to induce the dissociation of Bad from Bcl-x_L in response to IL-3 stimulation, but we cannot exclude the possibility that the anti-phospho-Bad antibodies used for this study are less sensitive for Ser-136 and Ser-155 detection. It should be noted, however, that all of these three serine residues in FLAG-Bad were strongly phosphorylated in 293T human embryonic kidney cells. Moreover, the data showing that 14-3-3 association with Bad occurred only in the cytosolic fraction argue that the recruitment of 14-3-3 to the Bad–Bcl-x_L complex by Ser-136 phosphorylation may not be the primary mechanism used by IL-3 to disrupt the interaction between Bcl-x_L and Bad. In contrast with Bcl-x_L-expressing cells, Ser-112 phosphorylation of Bad was only detected in the HM fraction of FL5.12 cells overexpressing Bcl-2, and no Bad translocation occurred following IL-3 stimulation. This phosphorylation did not result in Bad association with 14-3-3 proteins when overexpressed with Bcl-2. In addition, co-immunoprecipitation experiments using anti-Bcl-2 and anti-Bcl-x_L monoclonal antibodies showed that the association between Ser-112-phosphorylated Bad and Bcl-2 is more stable compared with that with Bcl-x_L. Thus these results, together with the distinct distribution of Bad in 293T cells expressing Bcl-2 versus Bcl-x_L, suggest that the protein complexes of Bad with Bcl-2 and Bcl-x_L are structurally different.

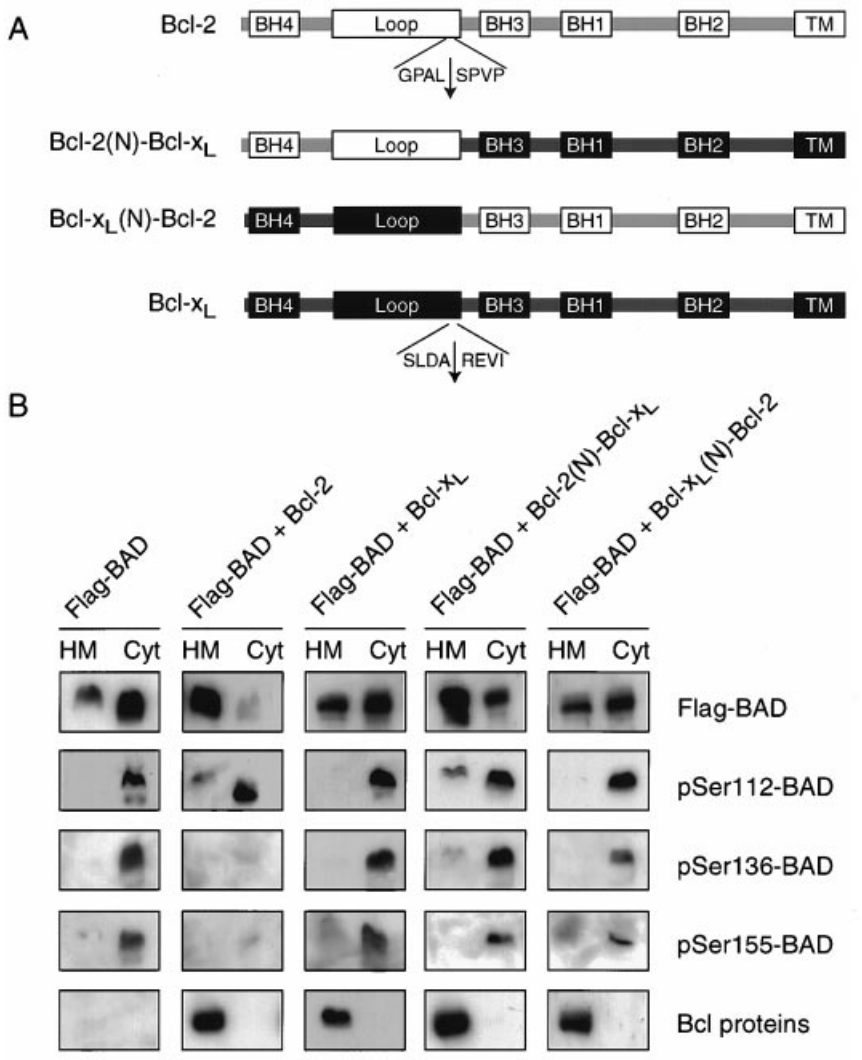

Figure 5 Importance of the N-terminal loop region of Bcl-2 and Bcl-x_L for regulating the intracellular distribution of Bad

(**A**) The predicted positions of the conserved domains within the human Bcl-2 and Bcl-x_L proteins are depicted and the chimaeras of Bcl-2 and Bcl-x_L are illustrated. Arrows indicate amino acid positions where Bcl-2 and Bcl-x_L were swapped with each other. (**B**) 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and transiently transfected with FLAG-Bad alone or together with plasmids encoding Bcl-2, Bcl-x_L or chimaeric Bcl proteins as indicated. After 24 h of transfection, cells were subjected to subcellular fractionation and 30 μg of protein from each fraction was applied to SDS/PAGE and immunoblot analysis with the indicated antibodies. Cyt, cytosolic fraction.

Bcl-2-family proteins contain up to four evolutionarily conserved domains: BH1, BH2, BH3 and BH4. Both Bcl-2 and Bcl-x_L have all four BH domains, which are quite similar between these proteins, and they also have a TM domain near their C-termini (Figure 5A) [3]. A region that lies between the N-terminal BH4 domain and the BH3 domain is designated as the loop domain or loop region, which is totally different between Bcl-2-family proteins; it has been reported that modification of the loop domain could affect their anti-apoptotic activities [29–32]. Furthermore, caspase cleavage of the loop region can convert anti-apoptotic Bcl-2 and Bcl-x_L into Bax-like pro-apoptotic proteins [33,34]. Although the X-ray and NMR structures of Bcl-x_L have been reported [24,35], the structure of the loop region of this protein is not clear due to its flexibility.

We hypothesized that this loop region might be important for Bcl-2 and Bcl-x_L to regulate Bad phosphorylation and intracellular translocation between cytoplasm and mitochondria. To demonstrate this hypothesis, we calculated the pI values for each domain in Bcl-2 and Bcl-x_L. The pI value is one of the calculable indices from primary protein sequence, which indicates acidity or alkalinity of a protein molecule in solution. Protein acidity or alkalinity affects protein–protein interactions. As we expected, there is no significant difference between the pI values of the well-conserved C-terminal half domains of Bcl-2 and Bcl-x_L. However, the pI value of the loop region is quite different between Bcl-2 and Bcl-x_L, indicating that the loop region dominates the pI values in these molecules. Indeed, the pI values were switched by swapping the N-terminal regions in which the loop domain resides. At least one of three serines, 112, 136 and 155, on mouse Bad can be phosphorylated in cells by survival-factor-dependent kinases, making this protein slightly acidic. It is possible that once Bad is phosphorylated the resulting acidic residue(s) facili-

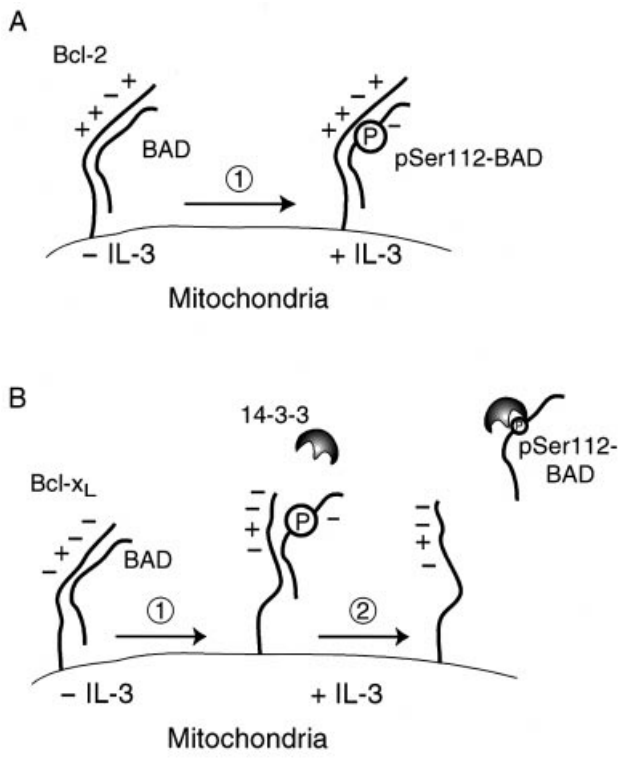

Figure 6 A model for Bad dissociation

(**A**) IL-3 stimulation induces Bad phosphorylation at Ser-112, which is insufficient to dissociate Bad from the Bcl-2 protein containing an alkaline N-terminal region. (**B**) IL-3-induced phosphorylation of Bad generates an acidic residue that weakens the interaction between Bad and the acidic Bcl-x_L molecule and results in Bad association with 14-3-3 in the cytosol.

tates dissociation of Bad from Bcl-x_L (pI = 4.86) but not Bcl-2 (pI = 6.49), as shown in Figure 6. This model is supported by our findings that when the N-terminal regions were swapped with each other, the Bcl-x_L(N)–Bcl-2 chimaeric protein (pI = 5.01) failed to inhibit Bad phosphorylation and was unable to sequester Bad in the outer membranes of mitochondria (Figure 5). In addition, substitution of the N-terminal region of Bcl-2 partially converted Bcl-x_L into a Bcl-2-like protein, suggesting that the N-terminal region of Bcl-2 is necessary but not sufficient to sequester Bad on the surface of mitochondria in the presence of growth factors.

Taken together, the observations described in this study indicate that Bcl-2 and Bcl-x_L represent two distinct binding partners for the pro-apoptotic Bad protein and that the N-terminal region of Bcl-2 and Bcl-x_L appears to be critical for controlling their interaction with Bad. Phosphorylation of Bad by growth factors induces Bad dissociation from Bcl-x_L but has no effect on Bad–Bcl-2 complex formation. This may reflect the bioactivities of Bcl-2 and Bcl-x_L, in that Bad counters the anti-apoptotic function of Bcl-x_L but not that of Bcl-2 in cells upon growth-factor deprivation. In other words, phosphorylation of Bad allows Bcl-x_L but not Bcl-2 to gain anti-apoptotic function in cells, because Bad constitutively binds to and inhibits Bcl-2 regardless of the presence or absence of growth factors. Thus alterations in the ratios of Bad and Bcl-2 may modulate Bcl-2 protein activity and put tumour cells into a more vulnerable state for therapeutic benefit.

We thank Hirohito Yamaguchi and Michael W. Lee for helpful discussion, the Molecular Biology core facility at the Moffitt Cancer Center and Research Institute for support, and BioSource International (Camarillo, CA, U.S.A.) for providing the anti-pSer112-Bad antibody. This work was supported by National Cancer Institute grant CA82197.

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Received 30 April 2001/13 June 2001; accepted 10 August 2001