BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak

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The BH3-only proteins Bim and Bad bind to the anti-apoptotic Bcl-2 proteins and induce apoptosis in wild-type cells and cells from either bax−/− or bak−/− animals. In contrast, constitutively active forms of Bim and Bad failed to induce apoptosis in bax−/−bak−/− cells. Expression of Bax restored susceptibility of the cells to Bim and Bad. In addition, Bax but not Bim or Bad sensitized the bax−/−bak−/− cells to a wide variety of cell death stimuli including UV irradiation, chemotherapeutic agents, and ER stress. These results suggest that neither activation of BH3-only proteins nor suppression of pro-survival Bcl-2 proteins is sufficient to kill cells in the absence of both Bax and Bak. Furthermore, whereas mouse embryo fibroblasts (MEF) expressing only Bax or Bak displayed resistance to transformation, bax−/−bak−/− MEF were nearly as prone to oncogenic transformation as p53−/− MEF. Thus, the function of either Bax or Bak appears required to initiate most forms of apoptosis and to suppress oncogenic transformation.

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Bcl-2 family proteins play pivotal roles in controlling programmed cell death. Whereas some members prevent cell death, others exhibit a proapoptotic activity. Two major groups of Bcl-2 family proteins exist as follows: the pro-survival members including Bcl-2, Bcl-xL, Bcl-1/A1, Bcl-w, Mcl-1, Boo, NR-13, BHRF1, LMW5-HL, ORF16, KS-Bcl-2, E1B-19K, and Ced-9, and the proapoptotic members including Bax, Bak, and Bok [for review, see Adams and Cory 1998]. Three-dimensional imaging suggests that both proapoptotic and antiapoptotic Bcl-2 family members share a common structure. Bcl-2 proteins have been shown to interact and to be regulated by another group of proteins that share at least one common structural feature with Bcl-2 related proteins, an α-helical structure termed BH3-domain. BH3-only members include Bcl, Bim, Bid, Bik/Nbk, BNIIP3, Blk, Hrk, Noxa, and EGL-1. Some BH3-only proteins selectively interact with antiapoptotic Bcl-2 family members, whereas others also interact with proapoptotic family members. All BH3 proteins discovered to date are proapoptotic (for review, see Huang and Strasser 2000).

BH3-only proteins have been proposed to be allosteric regulators of the Bcl-2 family of proteins, and serve as central effectors of apoptotic signaling pathways. For example, Bid is normally localized in the cytosol in an inactive form. The engagement of Fas ligand with Fas results in the activation of caspase 8. Active caspase 8 in turn cleaves p22 Bid into the death-promoting p15 tBid, which is then targeted to the mitochondria and binds to either Bax or Bak and results in their oligomerization [Li et al. 1998; Luo et al. 1998; Wei et al. 2000].

Unlike Bid, activation of Bim and Bad does not require caspase cleavage. Three isoforms of Bim exist due to alternative mRNA splicing: BimEL, BimL, and BimS [O’Connor et al. 1998]. In healthy cells, BimEL and BimL are sequestered to the microtubule-associated dynein motor complex by binding to dynein light chain LC8, and are thereby unable to promote cell death. Certain death-inducing stimuli, such as serum withdrawal, induce the release of BimEL or BimL from the dynein motor complex. Once released, BimEL or BimL then translocates to mitochondria where they bind Bcl-2 and Bcl-xL and neutralizes their anti-apoptotic activity. BimS does not appear to interact with the microtubule complex, yet it is still capable of binding to Bcl-2 and Bcl-xL [Puthalakath et al. 1999]. Thus, BimS acts as a constitutive death inducer. Unlike tBid, Bim does not appear capable of binding the proapoptotic proteins Bax and Bak [O’Connor et al. 1998].

The activation of a third well-characterized BH3-only protein, Bad, is regulated by phosphorylation. Survival signals derived from growth factors lead to Bad phosphorylation and its retention in the cytosol in an inactive form bound to 14-3-3 scaffold proteins. BAD then binds to and inhibits Bcl-2 and Bcl-xL and thereby promotes cell death. Re-exposure of the cells to growth factors inactivates Bad via phosphorylation mediated by PI3-kinase [Zha et al. 1996; Dutta et al. 1997]. In support of the role of serine phosphorylation in regulating Bad function, the mutation of serines 122, 136, 155 to alanine, converts Bad into a constitutive death-inducing molecule Bad3A [Datta et al. 1997; Virdee et al. 2000].

Bax/Bak double-deficient cells are defective in apoptosis [Lindsten et al. 2000]. These cells are also defective in cell death initiated by tBid, demonstrating not only that tBid selectively interacts with Bax and Bak, but also that this interaction is required for the ability of tBid to initiate apoptosis [Wei et al. 2001]. However, although these studies suggested that Bax and Bak can function as death effectors, they do not resolve whether antiapoptotic proteins such as Bcl-2 and Bcl-xL have an apoptotic function regulated by BH3-only proteins that is independent of Bax and Bak. To address this issue, we have used the constitutively active, death-promoting forms of Bim

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and Bad to neutralize the pro-survival Bcl-2 proteins. We report that Bim and Bad are incapable of inducing apoptosis in \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF. Thus, regulation of the proapoptotic function of Bax and Bak appears to be the point at which the function of Bcl-2 related proteins is integrated.

**Results**

The BH3-only protein Bim does not induce apoptosis in \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells

Bim proteins induce apoptosis by interacting with Bcl-x\(_L\) and Bcl-2, and the short form Bim\(_S\) is constitutively proapoptotic [O'Connor et al. 1998]. It remains uncertain whether Bim induces apoptosis by directly suppressing the pro-survival functions of BcL-2 and BcL-x\(_L\), or by releasing the proapoptotic functions of Bax-like proteins from repression by Bcl-2 and Bcl-x\(_L\). To clarify this issue, we tested the ability of Bim\(_S\) to induce apoptosis in \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF.

MEF obtained from wild-type, \( \text{bax}^{-/-}, \text{bak}^{-/-}, \) or \( \text{bax}^{-/-}\text{bak}^{-/-} \) backgrounds were infected with retrovirus expressing either green fluorescence protein (GFP), or both murine Bim\(_S\) and GFP from the same promoter using an internal ribosomal entry site (IRES). MEF with early passage number (<6) were used to minimize the possibility of mutations acquired during culture. Whereas wild-type, \( \text{bax}^{-/-} \), and \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF infected with Bim\(_S\) showed massive cell death, \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells were resistant to the proapoptotic action of Bim\(_S\) (Fig. 1A). Quantitation by 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining showed that >70% of GFP-positive cells from wild-type and single deficient MEF were killed. Conversely, a marginal percentage of GFP-positive cell\(_S\)s were DAPI positive (Fig. 1B). Despite their resistance to cell death, \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF accumulated substantial amounts of Bim\(_S\) (Fig. 1C).

The constitutively active BH3-only protein Bad fails to induce apoptosis in \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells

Bad is another BH3-only protein that induces cell death by neutralizing the pro-survival Bcl-2 family members [Yang et al. 1995]. We tested whether suppression of pro-survival Bcl-2 family members by Bad\(_{3A}\), the constitutive death-inducing form of Bad, could also lead to cell death in \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF. As predicted, 24 h after retroviral infection, significant numbers of Bad\(_{3A}\) infected wild-type and Bax or Bak single knockout cells were dead, whereas the \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells survived (Fig. 2). Examination of the cells at later times failed to uncover any additional death in the \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells although additional GFP-positive apoptotic cells were observed in the wild-type, \( \text{bax}^{-/-} \), and \( \text{bax}^{-/-}\text{bak}^{-/-} \) cells (data not shown).

These findings show that the BH3-only proteins Bim and Bad could not kill cells in the absence of Bax and Bak, suggesting that it is the activation of Bax or Bak, rather than the suppression of pro-survival Bcl-2 members, that is required to effect apoptosis.

**Bax restores susceptibility of \( \text{bax}^{-/-}\text{bak}^{-/-} \) MEF to Bim and Bad**

To confirm that both Bim and Bad-mediated cell deaths are dependent on the function of a proapoptotic Bcl-2
family member, Bax was re-introduced into bax<sup>−/−</sup>bak<sup>−/−</sup> MEF together with Bim<sup>S</sup> or Bad<sup>(3A)</sup> mutant. Coexpression of Bax and control GFP was not sufficient to kill bax<sup>−/−</sup>bak<sup>−/−</sup> cells. In contrast to the resistance to Bim<sup>S</sup> or Bad<sup>(3A)</sup> expression, the bax<sup>−/−</sup>bak<sup>−/−</sup> cells were killed by Bim<sup>S</sup> or Bad<sup>(3A)</sup> when Bax was co-expressed [Fig. 3A,B].

**Bax but not Bim or Bad sensitizes bax<sup>−/−</sup>bak<sup>−/−</sup> cells to cell death stimuli**

The above results demonstrated that either Bax or Bak is required for cell death mediated by Bim or Bad. These data suggest that the ability of Bim and Bad to bind pro-survival Bcl-2 proteins is insufficient to mediate cell death. However, it remained possible that Bim or Bad regulation of the function of the anti-apoptotic Bcl-2 proteins could be uncovered if bax<sup>−/−</sup>bak<sup>−/−</sup> cells were subjected to exogenous apoptotic stimuli. To address this issue, Bim<sup>S</sup>, Bad<sup>(3A)</sup>, or Bax was expressed in bax<sup>−/−</sup>bak<sup>−/−</sup> MEF, and the cells were then challenged with a spectrum of cell death-inducing stimuli including DNA-damaging agents, chemotherapeutic agents, and ER stress stimuli. Neither Bim<sup>S</sup> nor Bad<sup>(3A)</sup> could sensitize the bax<sup>−/−</sup>bak<sup>−/−</sup> cells to these treatments in the absence of Bax and Bak [Fig. 3C]. In sharp contrast, expression of Bax in bax<sup>−/−</sup>bak<sup>−/−</sup> cells restored the susceptibility of these cells to UV, etoposide, staurosporine, or brefeldin A [Fig. 3C]. Furthermore, cells from mice having a single functional allele of either Bax or Bak had a pattern of cell death susceptibility to these agonists that was comparable with that of cells from wild-type mice (data not shown).

**Absence of Bax and Bak promotes oncogenic transformation**

It has been shown that Bax may contribute to p53-dependent suppression of oncogenic transformation [McCurrah et al. 1997] and the cell death induced by certain chemotherapeutic agents [Zhang et al. 2000]. However, loss of bax results in only partially increased oncogenic transformation as compared with the loss of p53 [McCurrah et al. 1997]. This has been argued to result from the ability of BH3-only proteins to promote apoptosis by directly inhibiting the antiapoptotic function of the pro-survival Bcl-2 family members [Huang and Strasser 2000]. Recently, p53 has been shown to directly induce two proapoptotic BH3 containing proteins Noxa [Oda et al. 2000] and Puma [Nakano and Vousden 2001; Yu et al. 2001]. In addition, Bim has been reported to be the apoptotic mediator of cell death in response to cytoskeleton disruption [Puthalakath et al. 1999], whereas Bad has been reported to mediate growth factor withdrawal-induced death. However, our findings suggest that either Bax or Bak is required to induce cell death in response to loss of attachment (anoikis) or serum deprivation [Fig. 4A]. Both growth factor and anchorage independence have been associated with tumor development. This suggests that Bax and Bak might also share a redundant function that suppresses tumorigenicity through the independent ability of either of these proteins to initiate apoptosis in response to oncogenic transformation. To address this issue, we performed a soft agar focus-formation assay using primary MEF of different genotypes (wild-type, bax<sup>−/−</sup>, bak<sup>−/−</sup>, bax<sup>−/−</sup>bak<sup>−/−</sup>, and p53<sup>−/−</sup>). As anticipated, oncoprotein Ras and the adenovirus-5 E1A induced focus formation when p53 was inactivated [Fig. 4B,C,D]. Both bax<sup>−/−</sup> and bak<sup>−/−</sup> single knockout cells produced a modest number of foci that was significantly less than that obtained from p53<sup>−/−</sup> cells. Significantly, bax<sup>−/−</sup>bak<sup>−/−</sup> MEF transformed with Ras and E1A developed comparable numbers of foci, with that observed following transformation of p53<sup>−/−</sup> MEF [Fig. 4B, C, D].

**Discussion**

The pro-survival Bcl-2 proteins such as Bcl-2/Bcl-x<sub>L</sub> block apoptosis, whereas the Bax-like proteins Bax/Bak induce apoptosis. However, because these two groups of proteins antagonize each other’s functions, it has been difficult to determine which molecules are the primary effectors of cell survival/death. Using bax<sup>−/−</sup>bak<sup>−/−</sup> genetic background, we were able to address this issue in fibroblasts by manipulating the level of the pro-survival Bcl-2 proteins using the BH3-only agonists. We showed that neutralizing pro-survival Bcl-2 family members by the BH3-only proteins Bim and Bad was not sufficient to induce apoptosis in the absence of Bax and Bak. Introducing Bax back into the bax<sup>−/−</sup>bak<sup>−/−</sup> background enabled Bim or Bad to kill these cells. Moreover, enforced expression of Bax, but not Bim or Bad, sensitized the bax<sup>−/−</sup>bak<sup>−/−</sup> MEF to physiological death stimuli. These
findings suggest that the proapoptotic proteins Bax and Bak are the effectors that execute cell death, and the antiapoptotic proteins Bcl-xL and Bcl-2 function to antagonize the Bax-like proteins. Whereas Bax and Bak appear to be the only proapoptotic Bcl-2 proteins that act as death effectors in fibroblasts, other proapoptotic family members may also act as death effectors in other tissues. For example, the third well-characterized proapoptotic family member, Bok, has a more restricted expression pattern than Bax or Bak and is expressed primarily in reproductive tissues (Hsu et al. 1997a).

The data support a model in which the BH3-only proteins are activated in response to death-inducing stimuli, and function to neutralize pro-survival Bcl-2 proteins and free the Bax-like proteins to execute cell death (Fig. 5). According to this model, the default status of a cell in the absence of both pro- and anti-apoptotic Bcl-2 family members is survival. MEF cell death requires the effector function of either Bax or Bak. In a healthy cell, this effect is antagonized by pro-survival Bcl-2 proteins. The balance between these two groups can be disrupted by the BH3-only proteins. In response to cell death signals, the Bcl-2/Bcl-xL selective, BH3-only proteins are activated through different mechanisms to bind to pro-survival Bcl-2 members. In the absence of the pro-survival Bcl-2 members, Bax-like proteins function unopposed to initiate apoptotic cell death. This suggests that there is an additional level of complexity in the central apoptotic pathway of vertebrates not observed in genetic studies of Caenorhabditis elegans. In the worm, Ced-9 functions as a genetic inhibitor of Ced-4. In contrast, our studies in the mouse suggest that the Ced-9 homologs Bcl-2 and Bcl-xL serve as inhibitors of Bax and Bak. No proapoptotic Bcl-2 family members have been uncovered so far in the C. elegans genome. However, given the low level of sequence conservation between Bcl-2 family members, such proteins could easily be missed. If two such proapoptotic family members exist in C. elegans and they were functionally redundant in a manner similar to Bax and Bak, it is unlikely that the reported cell death screens in C. elegans would have identified them. Because the apoptotic steps upstream and downstream of Bax/Bak appear to be evolutionarily conserved, the present data suggest that additional screening may be necessary to identify whether the C. elegans cell death pathway contains homologs of the apoptotic mediators Bax and Bak.

Figure 3. Bax restores the susceptibility of bax−/−bak−/− MEF to undergo apoptosis. [A] Bax restores apoptosis sensitivity to BimS and Bad3A. bax−/−bak−/− MEF were infected with Bax–IRES–GFP together with control (GFP), Bad3A–IRES–GFP, or BimS–IRES–GFP. Twenty-four hours after infection, cells were stained with DAPI and photographed by use of a FITC or DAPI filter. [B] bax−/−bak−/− MEF were infected with BimS or Bad3A together with control or Bax vectors. The percentage of dead cells was determined 24 h later by the number of DAPI-positive cells over that of the GFP-positive cells. [C] Bax, but not Bim or Bad, sensitizes bax−/−bak−/− cells to different death-inducing agents. bax−/−bak−/− MEF were infected with control (GFP), BimS–IRES–GFP, Bad3A–IRES–GFP, or Bax–IRES–GFP. Twenty-four hours later, cells were treated with etoposide (Eto, 100 µM), staurosporine (STS, 4 µM), brefeldin A (BFA, 10 µg/mL), or UV-irradiation (200 J/m²). Cell death was determined 24 h later by the number of DAPI-positive cells over that of GFP-positive cells.

Figure 4. Loss of both Bax and Bak facilitates oncogenic transformation. [A] bax−/−bak−/− MEF are resistant to death induced by loss of attachment and serum deprivation. MEF of different genotypes were cultured in uncoated bacterial culture dishes or in the absence of FBS for 48 h. The percentage of cell death was determined by PI-exclusion. [B–D] MEF of different genotypes were infected with viral constructs expressing adenovirus-5 E1A and an activated ras oncogene, and cultured in soft agar. Two weeks after infection, plates were photographed and the number of foci scored. [B] Photographs of representative plates. [C] Photographs of foci. [D] Graph quantifying the number of foci from three experiments.
It has been shown that Bax and Bak are activated by subcellular redistribution and/or conformational change after apoptosis is initiated. This cell death process can be blocked by overexpression of Bcl-2, Bcl-xL, or E1B-19K [Hsu et al. 1997b; Perez and White 2000; Wei et al. 2000]. Addition of recombinant Bax or Bak to isolated mitochondria induces cytochrome c release and the loss of mitochondrial potential [Jurgensmeier et al. 1998; Narita et al. 1997]. Bax mutants that do not translocate into mitochondria are no longer able to induce apoptosis [Nechushtan et al. 1999]. In contrast, Bcl-2 mutants that lack the membrane-anchoring domain but retain the ability to bind Bax can still block cell death [Borner et al. 1994].

One important question concerning antiapoptotic Bcl-2 proteins is whether they have a function in addition to antagonizing Bax-like proteins. All death stimuli we have tested have been reported to be suppressed by overexpression of Bcl-2/Bcl-xL. However, blocking Bcl-2/Bcl-xL function by use of Bim-S or Bad[3A] did not sensitize the cells to etoposide, staurosporine, brefeldin A, and UV-irradiation (Fig. 3C). This suggests that the primary function of Bcl-2 and Bcl-xL is to suppress the pro-apoptotic function of Bax and Bak. Thymocytes from bax−/−bak−/− mice are susceptible to Fas-mediated cell death [Lindsten et al. 2000], demonstrating that not all forms of apoptosis are dependent on Bax/Bak function. Interestingly, overexpression of Bcl-2 also fails to protect thymocytes from Fas-induced death [Strasser et al. 1995]. Thus, it appears that expression of endogenous Bcl-2 members does not provide any anti-apoptotic resistance beyond that observed in bax−/−bak−/− cells.

Deregulation of apoptosis plays an important role in tumorigenesis. The pro-survival Bcl-2 members can be oncogenic. The connection between the pro-survival Bcl-2 members and tumorigenesis has been found in both clinical cases and transgenic models [for review, see Evan and Littlewood 1998]. To a lesser extent, Bax muta-
tion has been implicated in human leukemias, gastrointestinal cancer, and colorectal cancer (Rampino et al. 1997; Zhang et al. 2000). Abrogation of Bak expression has been observed in skin cancer and gastric and colorectal cancers [Rosen et al. 1998; Jackson et al. 2000]. Expression of Bax may be regulated by p53 in some cells [Miyashita and Reed 1995], although there is no consensus p53 binding site in the mouse bax gene. A previous study has found that loss of Bax could reduce apoptosis and induce tumori-
genicity in a p53-dependent manner in MEF, although to a lesser extent than loss of p53 itself [McCurra et al. 1997]. Our finding that loss of both Bax and Bak resulted in a significant increase of transformation in comparison with the single deficiency of Bax, indicates that these proapop-
totic proteins are capable of compensating for each other in preventing transformation. Thus, the apparent redundancy of Bax and Bak may have been selected as a safeguard against oncogenic transformation as well as to assure the effective elimination of damaged or excess cells.

Materials and methods

Retroviral constructs

Murine Bim-S cDNA was obtained by RT-PCR by use of oligos 5′-AG CCAGGATCCCTCGAGCTATGGCAACCTTC-3′ and 5′-AGGC AAGATTGTCGACCATGCTTTCTCCATAC-3′. Bim-S mutant cDNA was a gift from Dr. Michael Greenberg (MIT, Cambridge, MA). cDNAs of Bim-S, Bad[3A], and Bak were cloned into retroviral expression vector pBabeMN-IRES-GFP. Retroviral expression vectors for control (LPC), E1A (LPC-12S), and Ras (pBabe-Ras) were gifts of Dr. Scott W. Lowe (Cold Spring Harbor, NY).

Cell culture

Murine embryonic fibroblasts were generated from wild type, bax−/−, bak−/−, or p53−/− embryos. MEF were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin.

Retroviral infection

Phoenix cells (5 × 10^6) were plated in a 10-cm culture dish the day before transfection. Cells were transfected with retroviral vectors expressing GFP [control], GFP-IRES-Bim-S (Bim-S), GFP-IRES-Bad[3A] (Bad[3A]), or GFP-IRES-Bax (Bax). Viral supernatants were collected during the 48–96-h period post-transfection, and centrifuged at 2000 rpm for 10 min to get rid of contaminating packaging cells. MEF were infected with the viral supernatant in the presence of 10 µg/mL of polybrene [Sigma].

Death assays

MEF were left untreated or treated with staurosporine (4 µM, Sigma), UV-irradiation (300 Jules/m², Stratalinker, Stratagene), etoposide (100 µM, Clontech), or brefeldin A (10 µg/mL, Sigma) for 24 h, or cultured in uncoated bacterial culture dishes or in the absence of FBS for 48 h. A total of 1 µg/mL DAPI or propidium iodide was added to cell culture. Cells were observed and photographed with the Nikon Eclipse TE300 microscope, and subjected to flow cytometry to determine the number of DAPI-positive cells over that of GFP-positive cells. Cell death was also determined by PI-exclusion.
**Immunoprecipitation and immunoblotting**

One-million MEF were infected with control or BimS. Forty-eight hours after infection, cells were lysed in 500 µl of 60 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM MgCl2, 10% Glycerol, and 0.5% NP-40 in the presence of a protease inhibitor cocktail (Roche Inc.). The lysates were pre-cleaned with Protein G sepharose for 30 min at 4°C, incubated with a polyclonal anti-Bim antibody (M-20, Santa Cruz) for 2 h, followed by Protein G agarose beads (Life Technologies) for 1 h at 4°C. The proteins were washed in the lysis buffer three times, and loaded on pre-cast SDS-PAGE gel (Bio- Rad). For immunoblotting, 20 µg of protein was loaded on pre-cast gels in some experiments. Immunoblotting was performed by use of polyclonal antibodies against Bim (BD-Pharmingen), Bcl-xL (13.6), Bcl-2 (BD-Pharmingen), and actin (Santa Cruz).

**Soft agar transformation assay**

MEF with different genotypes (wild type, bak−/−, bak−/−, bak−/−bak−/−, or p53−/−) were infected with GFP or E1A together with Ras retrovirus. Twenty-four hours later, the infection efficiency was determined by the number of GFP-positive MEF present, as analyzed by FACS. Equal amounts of infected cells were plated in soft agar. Transformed foci were scored 14 d later, and photographed.

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