Overexpression of BAD Potentiates Sensitivity to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Treatment in the Prostatic Carcinoma Cell Line LNCaP

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Abstract

Here we show that LNCaP, which is resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, becomes sensitive to TRAIL after overexpression of full-length, wild-type BAD (BAD WT). TRAIL induces caspase-dependent cleavage of BAD WT that results in generation of a Mr 15,000 protein. LNCaP stably expressing truncated BAD (tBAD) and cells expressing mutated BAD at the caspase cleavage site were less sensitive to TRAIL treatment when compared to LNCaP expressing BAD WT. Cytochrome c and Smac/DIABLO release from mitochondria into cytosol was found after TRAIL treatment only in cells overexpressing BAD WT. Furthermore, differences in phosphorylation of serine residues for BAD WT and tBAD were identified. BAD WT was phosphorylated at positions S136 and S155, whereas tBAD was phosphorylated at positions S112, S136, and S155. LNCaP stably expressing BAD mutated at serine 112 to alanine was less sensitive to TRAIL treatment when compared to LNCaP expressing BAD WT. Lastly, recombinant BAD cleaved by caspase-3 is a more potent inducer of cytochrome c and Smac/DIABLO release than BAD WT. In summary, BAD-mediated sensitivity of LNCaP to TRAIL depends on the phosphorylation status of BAD WT and tBAD.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), or Apo2L, is a type II transmembrane protein that was initially identified based on its homology with FasL and TNF (1, 2). TRAIL can induce apoptosis by interaction with two receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR4, TRICK2, KILLER) (3–8). Analysis of the TRAIL death-inducing signaling complex (DISC) revealed ligand-dependent recruitment of FADD and caspase-8 (9, 10).

TRAIL is capable of inducing apoptosis in a wide variety of cancer cells in culture and in tumor implants in mice, including cancers of the colon, breast, lung, kidney, central nervous system, blood, and skin (1, 11–14). At the same time, unlike TNF-α and Fas ligand (15, 16), TRAIL has no toxic effects when systemically administered in rodents (14) and nonhuman primates (13).

The pathways that are activated in response to TRAIL in prostate cells have recently been investigated (17–25). We and others have reported that LNCaP is resistant to TRAIL-induced apoptosis but inhibition of the phosphatidylinositol 3′-kinase (PI3K) pathway converted the phenotype of LNCaP from TRAIL-resistant to -sensitive (20, 21, 24). LNCaP lacks the active lipid phosphatase PTEN, a negative regulator of the PI3K/Akt pathway (26). This pathway is required for cell survival in a wide variety of different tumors and this pathway has been reported to block apoptosis induced by a number of death stimuli (27–29). Compared to PC3 and DU145, LNCaP cells express the highest level of constitutively active Akt, which is directly correlated with TRAIL resistance. Downregulation of Akt by dominant negative Akt or PTEN renders LNCaP cells sensitive to TRAIL. Additional data show that inhibition of TRAIL-induced apoptotic signals occurs at the level of mitochondria (20, 21, 24, 30).

BAD is a death-promoting BH3-only member of the Bcl-2 family of proteins and its proapoptotic activity is regulated primarily by phosphorylation at several sites in response to survival factors (31). Ser-112 and Ser-136 have been identified as two major sites that when phosphorylated, in response to IL-3, blocked the proapoptotic function of BAD (32). Recently, two other sites, Ser-155 and Ser-170, have been shown to be phosphorylated in response to growth factors and prevented cytotoxic effects of BAD (33–38).

BAD can be phosphorylated by different kinases, in particular, Akt/PKB at Ser-136 (39, 40). BAD at Ser-112 can be phosphorylated by RSK2, PKA, and PAK (41–43). PKA and RSK1 can phosphorylate BAD at Ser-155 (33–37). Phosphorylation is necessary for the association of BAD with 14-3-3 proteins that prevent BAD translocation to the mitochondria and interaction with Bcl-XL or Bcl-2 (32, 44), thus allowing the latter proteins to promote cell survival. BAD is cleaved by caspase(s) at its NH2 terminus to generate a Mr 15,000 truncated protein that is a more potent inducer of apoptosis than the wild-type (WT) protein. Mutant BAD at position 36/61 is resistant to caspase-3 cleavage and is a weaker inducer of apoptosis (45–47).
Here we show that overexpression of wild-type BAD (BAD WT) renders LNCaP sensitive to TRAIL and is able to overcome a block in apoptosis signaling. Our data show that full-length BAD and truncated BAD (tBAD) can act together to promote apoptosis. These results suggest that induction of apoptosis can be regulated not only by phosphorylation of BAD, but also by phosphorylation of tBAD. We also show here for the first time that BAD is able to induce Smac/DIABLO release from isolated mitochondria.

**Results**

**LNCaP Cells Transfected With BAD WT Are Sensitive to TRAIL Treatment**

In the presence of wortmannin, an inhibitor of the PI3K/Akt pathway, LNCaP became sensitive to TRAIL treatment. BAD is one of the downstream phosphorylation targets of PKB/Akt. To investigate the role of BAD, we overexpressed BAD WT in LNCaP (LNCaP-BAD WT) (Fig. 1A) and compared the response of LNCaP-Hygro and LNCaP-BAD WT to TRAIL treatment. As can be seen from Fig. 1B, overexpression of BAD WT converts LNCaP from TRAIL-resistant to -sensitive.

**Overexpression of BAD Enhances Caspase Activity After TRAIL Treatment**

We found that TRAIL induced activation caspases-3, -7, and -9 after 1 h of treatment, and caspase-2 after 2 h of treatment in LNCaP-BAD WT but not in LNCaP-Hygro (Fig. 2A). We did not find any difference in caspase-8 activation between LNCaP-Hygro and LNCaP-BAD WT. DEVDase and VEIDase activities were increased in LNCaP-BAD WT compared to LNCaP-Hygro. As can be seen from Fig. 2B, caspase activity of LNCaP-BAD WT increased after 2 h, increased further after 4 and 8 h of TRAIL treatment and decreased after 16 h of treatment. In contrast, caspase activity in LNCaP-Hygro was low and did not change significantly over the time. We also examined the proteolysis of the nuclear enzyme PARP into its Mr 85,000 COOH-terminal fragment (Fig. 2C), which has been widely used as a biochemical hallmark for the executioner phase of apoptosis (48). TRAIL induced partial proteolysis of PARP in LNCaP-BAD WT but not in LNCaP-Hygro. As can also be seen from Fig. 2C, pretreatment of LNCaP-BAD WT with the pan-caspase inhibitor Z-VAD-fmk before TRAIL treatment prevented PARP cleavage. Taken together, these data indicate that TRAIL-induced apoptosis in LNCaP-BAD WT cells is caspase dependent and occurs downstream of caspase-8.

**TRAIL-Mediated Apoptosis Is Accompanied by Proteolysis of BAD in LNCaP-BAD WT**

Because BAD WT plays a major role in sensitivity of LNCaP-BAD WT to TRAIL-mediated apoptosis, we investigated the level of BAD proteolysis. As can be seen from Fig. 3, treatment of LNCaP-BAD WT with TRAIL for 4 h resulted in the appearance of Mr 15,000 tBAD. This was prevented by Z-VAD-fmk pretreatment. These data indicate that proteolysis of BAD is caspase dependent and suggest that tBAD may play a role in TRAIL-mediated apoptosis.

**LNCaP-tBAD68 and LNCaP-BADD61E Were Less Sensitive to TRAIL Than LNCaP-BAD WT**

To understand the role of the p15 band of BAD in apoptosis, we stably transfected LNCaP with cDNAs encoding tBAD starting from amino acid 68 (LNCaP-tBAD68) and BAD mutated at the site of caspase cleavage (LNCaP BAD D61E) (45). As shown in Fig. 4, both LNCaP-tBAD68 and LNCaP-BADD61E were relatively sensitive to TRAIL compared to LNCaP-Hygro, but were less sensitive than LNCaP-BAD WT. The sensitivity of LNCaP-tBAD68 and LNCaP-BADD61E at the highest concentration (1 µg/ml) of TRAIL was the same after 48 h of treatment. Because tBAD68 and BADD61E are expressed at the same level (data not shown) and sensitizes LNCaP to the same extent to TRAIL, the data suggest that full-length BAD and tBAD are both important in mediating TRAIL-induced apoptosis in LNCaP-BAD WT.

**Overexpression of BAD Potentiates TRAIL-Induced Cytochrome c and Smac/DIABLO Release From Mitochondria**

Release of cytochrome c, Smac/DIABLO, AIF, and HtrA2/Omi from mitochondria into the cytosol are important events of apoptosis signaling (49–59). To address the mechanism of
TRAIL-induced apoptosis, cytochrome c, Smac/DIABLO, AIF, and HtrA2/Omi release were assessed in different LNCaP transfectants after 2 and 4 h of TRAIL treatment. We found that TRAIL induced cytochrome c release to a high level in LNCaP-BAD WT, compared to LNCaP-Hygro and to other transfectants. LNCaP-tBAD68 also released cytochrome c, but at a lower level compared to LNCaP-BAD WT. Smac/DIABLO release was detected at a higher level in LNCaP-BAD WT compared to LNCaP-tBAD68 and LNCaP-BADD61E (Fig. 5). These data correlate with our results obtained from a cell viability assay (Fig. 4) and suggest that tBAD and full-length BAD may function differently in LNCaP under TRAIL treatment. Release of AIF and HtrA2/Omi from mitochondria into cytosol was also detected after TRAIL treatment. However, we did not reveal any difference between cell lines (data not shown).

**Phosphorylation Status of BAD WT and tBAD68 in LNCaP**

It has recently been reported that Mr 15,000 tBAD is a more potent inducer of apoptosis than the WT protein (45). At the same time, it is well known that the proapoptotic activity of BAD is regulated primarily by phosphorylation at several serine residues (31).

BAD WT and tBAD were found at the same level in cytosolic and mitochondrial fractions but their phosphorylation status was different.
different. BAD WT was phosphorylated at serine residues S136 and S155, but not at S112. tBAD68 was phosphorylated at all serine residues in total cell lysates and in the cytosolic fraction, but not at S112 in the mitochondrial fraction (Fig. 6). The level of tBAD phosphorylation at S136 and S155 in the mitochondrial fraction was lower compared to BAD WT. Taken together, these results show that BAD WT and tBAD 68 have a different phosphorylation status in LNCaP.

Non-Phosphorylated tBAD Is a More Potent Inducer of Cytochrome c and Smac Release From LNCaP Mitochondria Than Non-Phosphorylated BAD WT

Our results show that cytochrome c and Smac/DIABLO release after TRAIL treatment occurred in LNCaP-BAD WT earlier and at a higher level than in LNCaP-tBAD68 (Fig. 5). To prove that the ability of tBAD to induce release of cytochrome c and Smac/DIABLO depends on phosphorylation, we incubated isolated mitochondria from LNCap with different concentrations of recombinant, non-phosphorylated BAD WT protein in the presence or absence of active recombinant caspase-3 (45). The appearance of tBAD in samples incubated together with caspase-3 was confirmed by Western blot analysis (data not shown). We noted that 200 and 500 nm BAD did not induce cytochrome c but did induce Smac/DIABLO release (Fig. 7). However, 500 nm BAD incubated with 20 and 30 ng of recombinant active caspase-3 induced cytochrome c release in a dose-dependent manner. Smac/DIABLO release occurred at a higher level when compared to samples incubated without caspase-3. These data indicate that non-phosphorylated tBAD is a more potent inducer of cytochrome c and Smac/DIABLO release than non-phosphorylated BAD WT.

LNCaP-BAD S112A Is Less Sensitive to TRAIL Treatment Than LNCaP-BAD WT

To understand the role of the phosphorylation tBAD at position S112, we stably transfected LNCaP with a cDNA encoding mutated BAD at serine 112 to alanine (BAD S112A). As shown in Fig. 8A, LNCaP-BAD S112A was relatively less sensitive than LNCaP-BAD WT. Treatment of LNCaP-BAD WT and LNCaP-BAD S112A with TRAIL for 4 and 6 h resulted in the appearance of Mr 15,000 tBAD. This was prevented by Z-VAD-fmk pretreatment (Fig. 8B). These data suggest that unphosphorylated full-length BAD and phosphorylated tBAD at S112 are both important in mediating TRAIL-induced apoptosis in LNCaP-BAD WT.

**Discussion**

In this report, we demonstrate for the first time that LNCaP stably overexpressing BAD WT became sensitive to TRAIL treatment. We also show that TRAIL treatment induced activation caspases-2, -3, -7, and -9, even after 1 h of treatment in LNCaP-BAD WT but not in LNCaP-Hygro (Fig. 2A). However, we did not find any differences in caspase-8 activation and Bid cleavage (data not shown) between LNCaP-Hygro and LNCaP-BAD WT. This result supports the conclusion that TRAIL-mediated apoptosis in LNCaP is
different phosphorylation pattern. tBAD was phosphorylated at WT. Our results indicate that tBAD and BAD WT have a developing an apoptotic response to TRAIL in LNCaP-BAD. LNCaP-BAD WT had readily detectable release of cytochrome c

Methods.

Cell Culture and Transfection

The human prostatic carcinoma cell line LNCaP was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Effectene Transfection reagent kit (Qiagen, Valencia, CA) blocked downstream of caspase-8 and at the level of Bid (21, 24). Absence of Bid cleavage in BAD WT-overexpressing cells also indicates that these BCL-2 family members work independently of each other.

We also detected TRAIL-induced cleavage of BAD WT. Because Z-VAD-fmk pretreatment totally abrogated the appearance $M_t$ 15,000 of tBAD, we concluded that cleavage of BAD WT after TRAIL treatment in LNCaP-BAD WT is caspase dependent. The appearance of tBAD was first found after interleukin-3 deprivation in 32Dcl3 murine myeloid precursor cells by Condorelli et al. (45). In the same paper, and later by others (46, 47), it was shown that tBAD is a more potent inducer of apoptosis than the WT protein. To assess the potential role of tBAD in developing an apoptotic signal after TRAIL treatment in LNCaP, we stably expressed tBAD (tBAD68) and BAD mutated at the caspase cleavage site (BAD61E). Surprisingly, LNCaP-
tBAD68 and LNCaP-BAD61E were equally sensitive to TRAIL treatment but less sensitive than LNCaP-BAD WT. TRAIL treatment of different transfectants also showed that only LNCaP-BAD WT had readily detectable release of cytochrome c and Smac/DIABLO.

Thus, full-length BAD and tBAD are equally important in developing an apoptotic response to TRAIL in LNCaP-BAD WT. Our results indicate that tBAD and BAD WT have a different phosphorylation pattern. tBAD was phosphorylated at all examined sites but phosphorylation was not detected at S112 in the mitochondrial fraction. On the other hand, BAD WT was phosphorylated at higher levels at S136 and at S155 in the mitochondrial fraction but was not phosphorylated at S112 in any extracts. LNCaP stably expressing BAD mutated at S112 to alanine was less sensitive to TRAIL treatment despite the appearance of tBAD. These data suggest that unphosphorylated full-length BAD and phosphorylated tBAD at S112 are both important in mediating TRAIL-induced apoptosis in LNCaP-BAD WT. It has been shown that IL-3 stimulation induces BAD phosphorylation at S112, impairing its binding to Bcl-xL (60). MAP kinase pathway-dependent S112 phosphorylation has been shown to be required for dissociation of BAD from Bcl-xL (61).

Further 14-3-3/BAD interactions are dependent on the presence of phosphorylated S136. However, mutation of S112 did not affect 14-3-3 binding (62). BAD phosphorylated at three serines, 112, 136, and 155, was localized in the cytosolic fraction (34, 40, 42). The phosphorylation of BAD at S155 within the BH3 domain inhibits death-promoting activity of BAD (33–37) and promotes its interaction with 14-3-3 proteins (36). It was proposed that phosphorylation of S136 changes the conformation of BAD that allows S155 to be phosphorylated (35). Other reports have suggested that S155 phosphorylation is independent of S136 and 14-3-3 (33, 34). Taken together, these data show that tBAD is phosphorylated at all serines, localized in the cytosol and can be blocked by 14-3-3. However, BAD WT can promote apoptosis even if unphosphorylated at S112 and phosphorylated at S136 and S155. We speculate that tBAD is more amenable to phosphorylation at S112 than BAD WT.

The in vitro experiments with recombinant BAD showed that cytochrome c and Smac/DIABLO release occurred at higher levels from isolated LNCaP mitochondria if they are incubated with tBAD rather than with full-length BAD. This indicates that non-phosphorylated tBAD is a more potent inducer of cytochrome c and Smac/DIABLO release than non-phosphorylated full-length BAD, which is consistent with the results of Condorelli et al. (45).

In conclusion, we have demonstrated that overexpression of BAD WT renders LNCaP cells sensitive to TRAIL and allows them to overcome a block at the mitochondrial level and undergo apoptosis. Our data also show that BAD WT and tBAD can act together to promote apoptosis. These results suggest that promotion of apoptosis can be regulated not only by phosphorylation of BAD WT, but also by phosphorylation of tBAD. In addition, isolated LNCaP mitochondria co-incubated with non-phosphorylated recombinant BAD and active caspase-3 released cytochrome c and Smac/DIABLO at higher levels than mitochondria incubated with BAD alone. We also show, for the first time, that recombinant BAD is able to induce Smac/DIABLO release from isolated mitochondria. Lastly, our results indicate that overexpression of BAD can override Akt-mediated resistance to TRAIL in cancer cells.

Materials and Methods

Cell Culture and Transfection

The human prostatic carcinoma cell line LNCaP was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Effectene Transfection reagent kit (Qiagen, Valencia, CA)

FIGURE 8. Assessment of cell death of LNCaP-BAD S112A in comparison to LNCaP-Hygro and LNCaP-BAD WT after TRAIL treatment and BAD cleavage of LNCaP-BAD WT and LNCaP-BAD S112A. A. Cells were treated for 48 h with different concentrations of TRAIL and cell death was estimated by calcein AM assay as described in “Materials and Methods.” Points, mean values of four separate experiments. B. BAD cleavage was examined in LNCaP-BAD WT and LNCaP-BAD S112A. Cells were treated with TRAIL (100 ng/ml) for indicated times. The effect of the pan-caspase inhibitor Z-VAD-fmk (50 μM) on the cleavage of BAD was also examined.
was used for transfection. LNCaP was transfected with the plasmid pSFFV/Hygro with or without a cDNA encoding WT mouse BAD or BAD S112A (32), and pcDNA 3/Neo with or without I-BAD68 and pcDNA 3/H-A-BADD61E (45), followed by hygromycin and neomycin selection, respectively. LNCaP-Hygro and LNCaP-Neo control cell lines showed the same sensitivity to TRAIL and further in experiments we used only LNCaP-Hygro. pcDNA3/I-BAD68 and pcDNA3/H-A-BADD61E constructs were a kind gift of Dr. B. Calabretta.

Estimation of Cell Viability
To measure cell viability, we used the calcine AM assay (Molecular Probes; Eugene, OR) as previously described (24). Briefly, cells were plated at a density of 7000 cells/well in 96-well flat-bottomed plates. TRAIL was added at the time of plating for 48 h. After incubation, medium was removed, the plates were washed with PBS, and incubated with 100 μl of 2 μg/ml calcine AM solution for 30 min at room temperature. Fluorescence, which is proportional to cell viability, was then measured with a FL600 fluorimeter (Bio-Tek Instruments, Inc., Burlington, VT).

Preparation of Total Cell Lysate and Western Blot Analysis
Preparation of total cell lysate and Western blot analysis was performed as previously described (24). The following primary antibodies were used: anti-PARP, anti-cytchrome c (Pharmingen, San Diego, CA); anti-BAD, anti-phospho-BAD (S112), anti-phospho-BAD (S136), anti-phospho-BAD (S155) (Cell Signaling Technology, Beverly, MA); anti-caspase-8 (Upstate Biotechnology, Inc., Lake Placid, NY); anti-caspase-3 (Transduction Laboratories, San Diego, CA); anti-caspase-2 (R&D Systems, Inc., Minneapolis, MN); anti-caspase-7, caspase-9 (Oncogene Science, Uniondale, NY); anti-Smac/ DIABLO (Zymed, San Francisco, CA); anti-AIF (Upstate Biotechnology); and anti-HtrA2/Omi (Apotech Corporation, Epalinges, Switzerland).

Assessments of Caspase Activity With Fluorogenic Substrates
To measure caspase activity with fluorogenic substrates, 25–40 μg of cell lysate were incubated at 37°C for 1 h in assay buffer [20 mM PIPES (pH 7.2), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose] with 20 μM of fluorescent substrates—Ac-DEVD-AMC, Ac-LEHD-AMC, and Ac-VEID-AMC (BIOMOL, Plymouth Meeting, PA), as previously described (24). DEVD is a substrate that is cleaved by several different caspases, including caspase-3, -7, and -8; VEID is known as a substrate of caspase-6 (63). Fluorescence at 360/460 nm was measured using a FL600 fluorimeter. Measurements were calibrated against a standard curve of 7-amino-4- methylcoumarin (AMC) (Sigma Chemical Co., St. Louis, MO) and data were expressed in nanomolars of released AMC per microgram of cytoplasmic extract proteins.

Preparation of Cytosolic and Mitochondrial Fractions of Cells
Mitochondria-free cytosol was prepared as described (61). Briefly, cells were lysed in ice-cold Mito-buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml pepstatin, leupeptin, and aprotinin] by homogenization in a small glass homogenizer with a teflon pestle (5–8 strokes on ice). The homogenates were first spun at 800 × g to remove nuclei and cell debris, then spun at 16,000 × g for 20 min at 4°C two times to pellet the mitochondria, and the supernatants were used for Western blot analysis. Pelleted mitochondria were washed twice with Mito-buffer and lysed in TNC buffer [10 mM Tris-acetate (pH 8.0), 0.5% NP40, 5 mM CaCl2, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml pepstatin, leupeptin, and aprotinin] on ice for 15 min and then used for Western blot analysis. Twenty micrograms of proteins from total cell lysate and cytosolic fraction, and 5 μg from mitochondrial fraction were analyzed by Western blotting.

Cytochrome c and Smac/DIABLO Release Assay in Vitro
Mitochondria were isolated from LNCaPs as described above. The mitochondrial pellet was washed once with ice-cold Mito-buffer-2 [10 mM HEPES (pH 7.5), 2 mM KH2PO4, 5 mM sodium succinate, 25 μM EGTA, 250 mM sucrose, 0.1 mM PMSF]. The reaction mixture contained 30–50 μg of mitochondria with 200 or 500 nm recombinant soluble BAD (Upstate Biotechnology) with or without 20 or 30 ng recombinant active caspase-3 (Upstate Biotechnology) in a final volume of 30 μl in mito-buffer-2. The reaction mixture was incubated 1 h at 30°C and spun down at 10,000 × g to pellet mitochondria. The supernatants were used to assay cytochrome c and Smac/DIABLO release by Western blotting.

References


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