Bak BH3 Peptides Antagonize Bcl-xL Function and Induce Apoptosis through Cytochrome c-independent Activation of Caspases*

(Received for publication, August 12, 1998, and in revised form, December 8, 1998)

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The Bcl-2 homology (BH3) domain is crucial for the death-inducing and dimerization properties of pro-apoptotic members of the Bcl-2 protein family, including Bak, Bax, and Bad. Here we report that synthetic peptides corresponding to the BH3 domain of Bak bind to Bcl-xL, antagonize its anti-apoptotic function, and rapidly induce apoptosis when delivered into intact cells via fusion to the Antennapedia homeoprotein internalization domain. Treatment of HeLa cells with the Antennapedia-BH3 fusion peptide resulted in peptide internalization and induction of apoptosis within 2–3 h, as indicated by caspase activation and subsequent poly-(ADP-ribose) polymerase cleavage, as well as morphological characteristics of apoptosis. A point mutation within the BH3 peptide that blocks its ability to bind to Bcl-xL abolished its apoptotic activity, suggesting that interaction of the BH3 peptide with Bcl-2-related death suppressors, such as Bcl-xL, may be critical for its activity in cells. While overexpression of Bcl-xL can block BH3-induced apoptosis, treatment with BH3 peptides resensitized Bcl-xL-expressing cells to Fas-mediated apoptosis. BH3-induced apoptosis was blocked by caspase inhibitors, demonstrating a dependence on caspase activation, but was not accompanied by a dramatic early loss of mitochondrial membrane potential or detectable translocation of cytochrome c from mitochondria to cytosol. These findings demonstrate that the BH3 domain itself is capable of inducing apoptosis in whole cells, possibly by antagonizing the function of Bcl-2-related death suppressors.

The Bcl-2 protein family plays an important role in the regulation of apoptosis in evolutionarily diverse species. Certain family members, including Bcl-2 and Bcl-xL, act as potent suppressors of apoptosis, whereas other homologs (e.g. Bak and Bax) have opposing functions and promote cell death. Although the mechanistic details by which these proteins function to regulate apoptosis are still unclear, certain domains of homology between family members, termed Bcl-2 homology (BH) domains, are critical for various aspects of their activities, including the induction or suppression of cell death, and the ability to heterodimerize with other family members (2–6).

The involvement of the BH3 domain in both the death-promoting and protein binding functions of several of the pro-apoptotic family members (such as Bak, Bax, Bik, and Bad) is now well established (6–9). Expression of truncated derivatives of Bak containing the BH3 domain is sufficient to induce cell death in transfected cells (6) and synthetic peptides derived from the BH3 domains of Bak, Bax, and Bid can induce biochemical events in a cell-free system that are characteristic of cellular apoptosis, such as cytochrome c release, caspase activation and DNA fragmentation (10). BH3 peptides can bind directly to death suppressors such as Bcl-xL (3) and block their subsequent heterodimerization with death promoters in vitro (11, 12), suggesting BH3 domains may interfere with the function of death suppressors in vivo. In support of this possibility, Bad, a “BH3-only” member of the Bcl-2 family, appears to promote apoptosis by forming inactivating dimers with death suppressors through its BH3 domain (13).

In this study, we have examined whether synthetic BH3 peptides can function as antagonists of Bcl-xL, and have assessed their biological activity when introduced into intact cells. A Bak BH3 peptide was found to antagonize the protective effects of microinjected Bcl-xL in α-Fas-treated HeLa cells, whereas a mutant Bak BH3 peptide that no longer binds Bcl-xL was inactive. Delivery of synthetic BH3 peptides into the cytosol of intact HeLa cells via facilitated diffusion as a fusion with the internalization domain of the Antennapedia homeoprotein, resulted in the induction of a caspase-dependent apoptotic program, characterized by cleavage of poly(ADP-ribose) polymerase (PARP) and morphological changes such as cytoplasmic contraction, membrane blebbing, and the formation of apoptotic bodies. This BH3-mediated cell death was not, however, accompanied by an early loss of mitochondrial membrane potential or release of cytochrome c from the mitochondria, suggesting the engagement of a cytochrome c-independent pathway to caspase activation. In addition, the Antennapedia-BH3 fusion peptide (Ant-BH3) was able to overcome the protective effects of Bcl-xL in α-Fas-treated HeLa cells. Our results demonstrate that the Bak BH3 domain is sufficient to antagonize the function of Bcl-xL and to trigger the apoptotic program in intact cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were obtained from the American Type Culture Collection, and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 4 mM l-glutamine (complete DMEM) at 37 °C with 6% CO₂. Cells were maintained in constant logarithmic growth in plastic culture dishes (Costar) for less than 15 passages.

Peptides—Peptides were synthesized by a commercial vendor utilizing diisopropylcarbodiimide/1-hydroxy-benzotriazole-activated Fmoc (N-(9-fluorenyl)methoxycarbonyl)-protected amino acids (Genzyme-Sygena, Cambridge, MA) on a model 396 Multiple Peptide Synthesizer.
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(Advanced Chemtech, Louisville, KY) and purified on Poros R2/2M 16 × 100-mm prep columns (PerSeptive Biosystems, Framingham, MA) with a gradient of 0–80% solvent B over 40 column volumes (solvent A = H2O/0.1% trifluoroacetic acid; solvent B = CH3CN/0.1% trifluoroacetic acid). Confirmation of molecular weight was determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Peptides were resuspended in Me2SO at 5 or 10 μM, and stored at −20 °C.

Microinjection—HeLa cells were plated in complete DMEM in 60-mm dishes at 2.4 × 10^6 cells/dish. Mixtures containing peptides and proteins as indicated in 25 mM HEPES buffer, pH 7.2, containing 3.3 mM NaCl and 1 mg/ml FITC-dextran as a marker were filter-sterilized and injected into the cytoplasm of cells using an Eppendorf micromanipulator and microinjector with femtotip capillary microtips. Following injection, the cells were returned to the incubator, and after 1 h the number of injected cells was determined by fluorescence microscopy. Cells were then treated with α-Fas mAb (7C11) and cycloheximide (10 μg/ml), and the recovery of injected cells remaining after 18 h was determined.

Internalization of Antennapedia Peptides—Biotinylated control or fusion peptides were added to cells grown on glass coverslips in serum-free DMEM supplemented with 4 mM l-glutamine (SF-DMEM). After a 30-min incubation with the peptides, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. After two additional washes with PBS, cells were incubated in blocking buffer (3% bovine serum albumin in PBS) for 1 h at room temperature, followed by incubation with streptavidin-FITC at 2 μg/ml in blocking buffer. Coverslips were washed three times and mounted in aqueous mounting medium (polyvinyl alcohol) on glass microscope slides for fluorescence microscopy using a Nikon Microphot-FXA upright fluorescence microscope.

Induction of Apoptosis by BH3 Peptides and Assessment of Cell Viability—Cells were plated in complete DMEM in 96-well tissue culture plates (Costar) at 4 × 10^3/well. After 24 h, cells were washed with PBS and treated with peptides (50 μM) in SF-DMEM. Cell viability was determined by staining unfixed cells with calcein AM/ethidium homodimer (Molecular Probes), followed by microscopic analysis of cell staining and cellular morphology on a Nikon Diaphot 300 inverted microscope equipped with a fluorescence module.

Transient Transfections—HeLa cells were plated in 24-well plates at 2.5 × 10^3/well 24 h prior to transfection. Cells were transfected using the Superfect transfection reagent (Qiagen) with a plasmid encoding the green fluorescent protein (GFP) as a marker. Each well was transfected with 0.1 μg of pCDNA3-GFP plasmid and 1.0 μg of either pCDNA-3 or pCDNA3-Bcl-xL plasmids. Following transfection, cells were incubated overnight in complete DMEM and subjected to peptide or α-Fas mAB treatment as described above.

Analysis of Poly(ADP-ribose) Polymerase (PARP) Cleavage—Cells were treated with Antennapedia fusion peptides in SF-DMEM or with α-Fas mAb (7C11) in complete DMEM for various times and stained directly into 1% SDS-polyacrylamide gel electrophoresis sample buffer (with 6 M urea added). Lysates were sonicated briefly (20 s, 40% duty..

RESULTS

BH3 Peptides Antagonize Bcl-xL Function—Synthetic BH3 peptides can bind to Bcl-2-like death suppressors and block heterodimerization with pro-apoptotic Bcl-2 homologues in vitro (3, 11, 14). To test the ability of a BH3 peptide to antagonize the protective function of a death suppressor, Bcl-xL was delivered into the cytosol of cells by microinjection either in the presence or absence of a Bcl-3 peptide. Microinjection of a bacterially expressed GST-Bcl-xL fusion protein, but not GST alone, efficiently protected HeLa cells from death induced by Fas ligation in the presence of cycloheximide (Fig. 1). Co-injection of a 15-amino acid Bak BH3 peptide (BH3-A78), in which an alanine has been substituted for the leucine at position 78 (numbered according to full-length Bak), did not block Bcl-xL-mediated protection from Fas-induced death. Thus, the ability of these peptides to inhibit the function of Bcl-xL, correlated with their ability to bind to Bcl-xL. Under these conditions, neither peptide had an effect on cell viability in the absence of α-Fas treatment (data not shown). Similar results were obtained with MRC5 human diploid fibroblasts where protection from Fas-induced death by microinjected Bcl-xL was inhibited by co-injection of the wild-type, but not mutant, Bak BH3 peptide (data not shown). These results suggest that Bak BH3 peptides bind to Bcl-xL and interfere with its anti-apoptotic function.

Delivery of BH3 Peptides by Fusion with the Antennapedia Internalization Sequence—To further investigate the activity of this domain in cells, BH3 peptides were synthesized as fusions with the internalization sequence from the Antennapedia (Ant)
protein (Table I). Similar Ant fusion peptides have been shown to be internalized into cells in culture and elicit peptide-specific biological responses (15–21). To determine whether the Ant-BH3 peptide could be internalized, HeLa cells were treated with biotin-tagged versions of the peptides, and peptide uptake was monitored by staining with streptavidin-FITC. While untreated cells or cells treated with non-biotinylated peptides did not stain with streptavidin-FITC (data not shown), cells treated with biotinylated Ant fusion peptides were highly positive within 30 min of addition of peptides to the culture medium (Fig. 2), indicating efficient internalization of the peptides. A biotinylated BH3 peptide lacking the Ant internalization sequence was not internalized.

BH3 Peptides Induce Apoptosis in Intact HeLa Cells—In contrast to the lack of cytotoxicity when BH3 peptides were delivered as a single bolus dose by microinjection, treatment of HeLa cells with the Ant-BH3 peptide, which allows for the delivery of a sustained pool of BH3 peptide, resulted in a dramatic loss of cell viability within 6 h (Fig. 3, C and D). Changes in cell morphology characteristic of apoptosis, including reduction in cell volume, nuclear condensation, and membrane blebbing, were observed as early as 2–3 h following the addition of the fusion peptide. Treatment of cells with peptides comprising just the BH3 domain (Fig. 3D) or the internalization sequence alone (Ant) (Fig. 3D) had no effect on cell morphology or viability, demonstrating that the observed cell killing activity was BH3-dependent and required internalization. Furthermore, the mutant Ant-BH3 peptide (Ant-BH3-A78), which is defective in binding to Bcl-xL (3), did not exhibit significant cell killing activity (Fig. 3, B and D). This underscores the importance of an intact BH3 sequence for the BH3 peptide’s cytotoxic effects and implicates a mechanism involving an interaction with a specific intracellular target, presumably one (or more) of the Bcl-xL-like death suppressors.

Bcl-xL Blocks BH3-induced Apoptosis, and BH3 Treatment Blocks Bcl-xL-mediated Protection from Fas-induced Apoptosis—In consideration of the possible involvement of a Bcl-xL-like death suppressor in BH3-induced apoptosis, we sought to determine the effects of Bcl-xL overexpression on the pro-apoptotic activity of BH3 peptides. HeLa cells were cotransfected with plasmids expressing Bcl-xL and GFP as a marker. Eighteen hours after transfection, cells were treated with the Ant-BH3 peptide for an additional 6 h. GFP-expressing cells were visualized by fluorescence microscopy and scored as either live or dead based on their morphology. Overexpression of Bcl-xL blocked the cell killing activity of the Ant-BH3 peptide in this assay (Fig. 4). Non-transfected cells in the same well (cells not expressing GFP) were also protected from a-Fas-induced death. However, treatment with the wild type Ant-BH3 peptide, but not the loss-of-binding mutant Ant-BH3-A78, resensitized the Bcl-xL-expressing cells to the induction of apoptosis by a-Fas. These findings suggest that interaction with a death suppressor such as Bcl-xL may be an important aspect of the activity of the BH3 domain and its ability to influence a cell’s susceptibility to undergo apoptosis.

Caspases Are Required for BH3-induced Apoptosis—In addi-
Mitochondrial Events Are Not Required for BH3-induced Apoptosis—It has recently become evident that the mitochondria may play an important role in apoptotic signaling (28). Apoptosis induced by a wide variety of stimuli is preceded by a release of cytochrome c from the mitochondrial matrix into the cytosol, where it has been proposed to be involved in the activation of caspasess (29–31). In addition, mitochondrial membrane permeability transition and loss of mitochondrial membrane potential (ΔΨm) are events that are common to many pathways of apoptosis induction. While several members of the Bcl-2 family, including Bax, Bcl-2, and Bcl-xL, are located primarily in the outer mitochondrial membrane, the specific involvement of these proteins in the mitochondrial events associated with apoptosis remains unclear. To analyze mitochondrial events that may be associated with BH3-induced apoptosis, the potential-sensitive fluorescent probe JC-1 (32, 33) was used to detect loss of ΔΨm at 5 h after exposure of cells to various apoptotic stimuli. In untreated cells (Fig. 6A) or cells treated with the inactive Ant-BH3-A78 peptide (Fig. 6E), JC-1 formed the characteristic J-aggregates in the mitochondria which emit photons at 585 nm (orange-red), demonstrating that the mitochondrial membrane potential is intact. Treatment of cells with the proton ionophore carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone, a potent uncoupler of mitochondrial oxidative phosphorylation, causes a rapid loss of membrane potential. Under these conditions, the JC-1 probe failed to aggregate and little or no red fluorescence was observed (Fig. 6B). Etoposide treatment also resulted in a complete loss of ΔΨm at 5 h, indicated by the absence of J-aggregates (Fig. 6D), which preceded caspase activation (see Fig. 5A) and changes in cell morphology. In contrast, no significant loss of ΔΨm was observed during the induction of apoptosis by Ant-BH3 peptide (Fig. 6F) or Fas ligation (Fig. 6C) at 5 h, even after substantial changes in cell morphology (as in Fig. 3) and cleavage of PARP had occurred (Fig. 5A). Fig. 7A shows a quantitative measurement of JC-1 staining obtained on a fluorescence plate reader 3 h after induction of cell death. As expected, both etoposide and staurosporine caused an early (3 h) loss of ΔΨm, which preceded or coincided, respectively, with caspase activation (Fig. 7B), whereas the mitochondrial membrane potential was still unchanged in Ant-BH3-treated cells even after caspases had been activated. The results of these experiments suggest that depolarization of the mitochondrial membrane is likely not required for the induction of apoptosis by the BH3 domain in intact cells. In keeping with these results, treatment of cells with cyclosporine A, an inhibitor of the membrane permeability transition and subsequent loss of ΔΨm (34), did not appear to suppress BH3-induced death.3

The mitochondrial matrix protein cytochrome c is released from the mitochondria to the cytosol during apoptosis either prior to, or coincident with, caspase activation, and is known to be a direct participant in the activation of caspasess in in vitro systems (29–31, 35, 36). To determine whether cytochrome c is released from the mitochondria during BH3-induced apoptosis, the subcellular localization of cytochrome c in treated cells was determined by cell fractionation and Western blot analysis. While staurosporine induced a rapid translocation of cytochrome c (within 3 h) (Fig. 8) that coincided with the cleavage of PARP (Fig. 7B), treatment of cells with the Ant-BH3 peptide did not result in any accumulation of cytochrome c in the cytosol by 3 h (Fig. 8), even though substantial caspase-dependent cleavage of PARP had occurred by this time (Fig. 7B). These data suggest that cytochrome c release is not required for activation of cytosolic caspases by the BH3 domain.

DISCUSSION

The BH3 domain plays a critical role in mediating the cell death and protein-binding functions of Bak and related pro-apoptotic proteins. The results of the present study demonstrate that the Bak BH3 domain alone, in the form of synthetic peptides delivered into cells, is biologically active and able to modulate apoptosis. Bak BH3 peptides acted as inhibitors of Bcl-xL in cells, antagonizing its ability to suppress apoptosis induced by Fas ligation. In addition, the Bak BH3 peptide was sufficient to rapidly induce apoptosis when introduced into.

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3 E. P. Holinger, unpublished data.
HeLa cells via fusion to the Antennapedia internalization sequence. Mutant BH3 peptides that fail to bind Bcl-xL were inactive, indicating that the biological activity of BH3 peptides correlates with their capacity to heterodimerize with death suppressors. Taking these findings together, we hypothesize that the cytotoxicity of BH3 peptides is due to their inhibitory interaction with endogenous Bcl-2-related proteins in HeLa cells. Moreover, the ability of Bak BH3 peptides to trigger cell death, even in the absence of other apoptotic stimuli, implies that HeLa cells require the continuous action of one or more Bcl-2 homologs for their survival. In addition to binding Bcl-xL, Bak BH3 peptides interact directly with other Bcl-2-related death suppressors in vitro, including Bcl-2, Mcl-1, A1, and Bcl-w. Thus, the inhibition of other Bcl-2 family members, either alone or in combination, may be responsible for the cytotoxicity of Bak BH3 peptides in HeLa cells. An alternative explanation is that the pro-apoptotic activity of Bak BH3 peptides results from an interaction with a cellular target(s) distinct from Bcl-2-related death suppressors; however, this as yet undefined interaction would have to exhibit the same dependence on specific BH3 residues (e.g. leucine 78).

How might Bak BH3 peptides antagonize the anti-apoptotic function of Bcl-xL? The solution structure of a Bak BH3 peptide/Bcl-xL complex revealed that the BH3 peptide adopts an $\alpha$-helical conformation and binds to a hydrophobic cleft on the surface of Bcl-xL (3). Binding of the BH3 peptide apparently does not induce significant conformational changes in Bcl-xL (3). It is possible that the occupation of this cleft by BH3 competitively displaces a cellular protein whose interaction is critical for the anti-apoptotic function of Bcl-xL. A candidate for such a molecule is Apaf-1, a human Ced-4 homolog implicated as an important target for suppression of apoptosis by Bcl-xL (35, 36). Bcl-xL interacts with Apaf-1 in transfected cells and inhibits the ability of Apaf-1 to promote the activation of caspase-9 (37, 38). Reportedly, Bak and Bik (a “BH3-only” death promoter) interfere with Bcl-xL binding to Apaf-1 (38), consistent with a competition between BH3 and Apaf-1 for the BH3-binding cleft on Bcl-xL. Thus, the BH3 peptide may antagonize Bcl-xL and promote apoptosis by preventing Bcl-xL/Apaf-1 heterodimerization, leaving Apaf-1 free to participate in the activation of caspases. In this respect, the ability of BH3 peptides to act as antagonists of death suppressors may serve as a model for the mode of action of a growing family of death-promoting proteins that share homology only within their BH3 domains. Certain “BH3-only” proteins, such as Bad (9, 13), Bik/Blk (8, 39), Bim (40), and Hrk (41), may promote apoptosis principally through BH3-mediated inhibition of Bcl-2-like death suppressors.

Bak BH3 peptides triggered the rapid activation of caspases, and pharmacological inhibition of caspases prevented BH3-induced apoptosis. These observations are consistent with a mechanism whereby BH3 liberates caspase-activating factors, such as Apaf-1, from an inhibitory interaction with Bcl-2-like proteins (as proposed above). BH3 peptide-induced apoptosis, however, occurred in the absence of a detectable decrease in mitochondrial $\Delta \Psi_m$ or release of cytochrome $c$, the latter of...
which is a factor required for the caspase-activating function of Apaf-1 at least in \textit{in vitro} systems (29, 42). If the proposed mechanism of BH3 peptide-induced apoptosis is correct, sufficient activation of Apaf-1 must occur either in the absence of cytochrome c release, or in the presence of cytochrome c at levels below the limits of detection in our assays, to initiate apoptosis \textit{in vivo}. Caspase activation in the absence of cytochrome c release has also been observed during Fas-induced death (43, 44).

The biological effects of BH3 peptides observed here appear to account for only a part (caspase activation) of the pro-apoptotic activities that have been previously described for the BH3-containing Bcl-2 homologs Bak or Bax. The induction of cell death by Bax has been shown to require, at least in some cases, the loss of $\Delta \Psi_m$, and the release of cytochrome c from the mitochondrial matrix (45–47). Cyclosporine A, a known inhibitor of the membrane permeability transition (34), prevents both the loss of $\Delta \Psi_m$ and cytochrome c release, as well as Bax-induced death (45). Indeed, Bax is capable of promoting the release of cytochrome c from isolated mitochondria, though the ability of BH3 peptides to substitute for the full-length protein in these assays was inconsistent (48, 49). BH3-mediated cytochrome c release may depend on the presence of cytosolic factors as demonstrated using extracts from \textit{Xenopus laevis} oocytes (10). The BH3 peptide in our assays with intact cells was not sufficient (although it may be necessary) to trigger the mitochondrial events described for Bax, suggesting that Bax delivers pro-apoptotic signals apart from BH3-mediated effects. Additional Bax function may be related to its membrane pore-forming activity observed \textit{in vitro} (50–53).

The importance of caspase activation in the induction of cell death by the death-promoting members of the Bcl-2 family remains controversial. While caspase activation is a consistent hallmark of Bax overexpression, the ability to block Bax-induced death by caspase inhibitors is not (45–47, 54, 55). Xiang \textit{et al.} (47) have suggested that Bax may activate two pathways, a caspase-dependent and a caspase-independent pathway. The latter pathway may involve crucial alterations in mitochondrial function, such as loss of $\Delta \Psi_m$, and increased production of reactive oxygen species. Based on our results using the Anti-BH3 peptide, BH3-mediated cell death may utilize a caspase-dependent pathway. The relative contribution of each pathway to the induction of cell death may be dependent on cell type or context. Therefore, the requirement of caspase activation for Bax-induced death would be dependent on the specific cellular conditions.

This dual pathway hypothesis may also help explain contradictory evidence regarding the importance of the BH3 domain in the induction of cell death by Bax and Bak (6, 56, 57). Depending on the cellular context, death induction by Bax or Bak may require the activation of caspases and be BH3-dependent, or proceed through caspase-independent alterations in mitochondrial function leading to cell death that may not require BH3-mediated processes. The relative ability of the death promoters of the Bcl-2 family, such as Bax and Bak, to activate either pathway under specific conditions may determine the apparent dependence on BH3 for cell death.

The apoptotic activity exhibited by a small peptide taken from the BH3 domain of the Bak protein suggests a novel pharmacological approach for manipulating a cell’s susceptibility to apoptosis. Small non-peptidyl compounds designed to mimic the activity of the BH3 peptide may impact the fate of the cell via mechanisms similar to those employed by the BH3 peptide. As it has been proposed that up-regulation of anti-apoptotic proteins such as Bcl-2 or Bcl-x, contributes to the tumorigenesis and resistance to drug treatment in certain types of cancers (58, 59), these results suggest that antagonizing these death suppressors with BH3 mimics may prove to be effective in promoting apoptosis in tumor cells that depend on such anti-apoptotic proteins for survival.

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