A Caspase-8-independent Signaling Pathway Activated by Fas Ligation Leads to Exposure of the Bak N Terminus*

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Bak is a pro-apoptotic member of the Bcl-2 family that is activated by apoptotic stimulation: its activation is characterized by conformational changes such as exposure of the N terminus and oligomerization. In death receptor-mediated apoptosis, the activation of Bak depends on activation of caspase-8. However, we found that exposure of the N terminus of Bak (but not oligomerization) can occur in the absence of active caspase-8. Although exposure of the N terminus of Bak without oligomerization is not sufficient to release cytochrome c from the mitochondria and commit cells to apoptosis, this change sensitizes the mitochondria to apoptotic signals (including Bid) and thus sensitizes cells to apoptotic death. Fas-induced, caspase-8-independent exposure of the N terminus of Bak is blocked by staurosporine, a pan protein kinase inhibitor. These results suggest that Fas stimulation not only activates caspase-8, but also a distinct signaling pathway involving protein kinase(s) to induce exposure of the N terminus of Bak.

Mitochondria play a crucial role in many physiological and pathological cell death paradigms in mammals. Most apoptotic stimuli convey death signals to the mitochondria. During apoptosis, the permeability of the outer mitochondrial membrane increases, resulting in the release of proteins into the cytoplasm from the intermembrane space, including several apoptogenic molecules such as cytochrome c, Smac (Diablo), HtrA2 (Omi), and apoptosis-inducing factor. After being released into the cytoplasm, cytochrome c binds to apoptotic protease activating factor 1, which recruits and activates procaspase-9, triggering the activation of the caspase cascade and committing the cell to apoptotic death. Smac (Diablo) and HtrA2 (Omi) facilitate caspase activation by inhibiting inhibitor of apoptosis proteins, which are endogenous caspase inhibitors (1, 2). Mitochondrial membrane permeability is regulated by a group of proteins called the Bcl-2 family. This family of proteins can be divided into anti-apoptotic members (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic members, which consist of multidomain members (Bak and Bax) and BH3-only proteins (Bid, Bim, Bik, Bad, Noxa, and Puma) (3, 4). Multidomain pro-apoptotic Bak and Bax are functionally redundant and play a direct role in increasing mitochondrial membrane permeability, leading to the release of apoptogenic proteins (5, 6). BH3-only proteins are thought to function as death signal sensors. After apoptotic stimulation, these proteins are primarily activated by different mechanism(s) and then activate Bak/Bax by still unknown mechanism(s) or inactivate anti-apoptotic Bcl-2 family members, finally leading to an increase of mitochondrial membrane permeability (3, 4).

Engagement of death receptors such as Fas and tumor necrosis factor α (TNFα)† receptor leads to one of two distinct apoptotic signaling pathways, which mainly differ in the extent of caspase-8 activation. In type I cells, there is extensive caspase-8 activation, which is sufficient to activate downstream execution caspases such as caspase-3 or caspase-7 and commit the cell to apoptotic death. In type II cells, there is less caspase-8 activation, so mitochondrial amplification of the apoptotic signal is required (7). In the latter type of cell, activated caspase-8 cleaves and activates a BH3-only protein, Bid (8, 9), which conveys apoptotic signals to the mitochondria by activating pro-apoptotic Bak or Bax (10–12). Because Bax and Bak are constitutively expressed, and the amount of these proteins usually remains unchanged during apoptosis, it is believed that Bak or Bax activation is regulated at the post-translational level (3, 13, 14). Bak is localized in the cytoplasm of living cells: after apoptotic stimulation, Bak translocates to the outer mitochondrial membrane where it oligomerizes and induces the formation of protein-conducting pores that release apoptogenic proteins (3, 12, 15–17). Bak is present on the outer mitochondrial membrane in a latent form: after accepting an upstream apoptotic signal, it undergoes oligomerization and induces the release of apoptogenic molecules from the mitochondria (11, 18).

Concealed epitopes at the N terminus of Bax or Bak are revealed after apoptotic stimulation, suggesting that a conformational change occurs in Bax and Bak or that a binding protein (which masks the N terminus) undergoes dissociation.

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* The abbreviations used are: TNF, tumor necrosis factor; DSS, disuccinimidyl suberate; BMH, bismaleimidohexane; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonic acid; siRNA, small interference RNA; GFP, green fluorescent protein; STS, staurosporine; z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone; FACS, fluorescence-activated cell sorting; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; rBid, recombinant human Bid.

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(10, 13, 14, 19). Because it has been reported that exposure of the N terminus of Bax precedes its translocation to the mitochondria and oligomerization (12, 20), it seems that exposure of the N terminus of Bax or Bak represents an intermediate step leading to their activation. Oligomerization of Bax/Bak is thought to reflect its activation, because Bax/Bak oligomerizes after apoptotic stimulation, and this process is correlated precisely with the release of cytochrome c, and oligomerized Bax, but not monomeric Bax, induces cytochrome c-releasing from the isolated mitochondria (11, 12, 15, 21). However, the process of regulation of the activation of Bak during apoptosis, and the relationship between exposure of the N terminus of Bak and oligomerization in the activation process, are still unclear.

In the present study, we found that Fas stimulation elicited a caspase-8-independent signal that induced exposure of the N terminus of Bak. This N-terminal exposure was not sufficient for full activation of Bak, but primed it to become more sensitive to apoptotic signals causing full activation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-Bak (Ab1) monoclonal antibody was purchased from Oncogene Research Products (Boston, MA). Anti-Bax (N20) and anticytochrome c antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D System (Minneapolis, MN), respectively. Anti-cytochrome c (6H2 and 7H8) and anti-mouse Fas (Jo2) monoclonal antibodies were from BD Pharmingen. Anti-human Fas (CH11), anti-caspase-3, and anti-caspase-8 monoclonal antibodies were obtained from MBL (Nagoya, Japan). Anti-glyceraldehyde-3-phosphate dehydrogenase and anti-porin (voltage-dependent anion channel protein) monoclonal antibodies were obtained from Chemicon (Temecula, CA) and Calbiochem (San Diego, CA), respectively. All of the secondary antibodies were purchased from Molecular Probes (Eugene, OR).

The cross-linker disuccinimidyl suberate (DSS) and bismaleimide (BMI) were from Pierce Biotechnology (Rockford, IL). Other chemicals were obtained from Sigma. Recombinant human Bid (rBid) was prepared as described previously (22).

Cell Culture—The human HeLa cell line, human Jurkat T-leukemic cell line and Jurkat caspase-8-deficient subline (J6E) (23) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Mouse embryonic fibroblasts (MEFs) derived from caspase-8-deficient mice were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS.

Immunofluorescence Staining—Cells were fixed in 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After incubation with 2% FBS in phosphate-buffered saline (PBS) for 1 h, the cells were incubated with anti-Bak (Ab1) (1:500) or anti-cytochrome c (1:500) for 1 h. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa 488-conjugated anti-mouse IgG) for 1 h. Then fluorescence was detected under a confocal microscope (Zeiss, LSM510).

Jurkat cells were fixed, permeabilized, and blocked with 20% FBS in PBS and then incubated with anti-Bak (Ab1) antibody followed by a secondary antibody (Alexa 568-conjugated anti-mouse IgG). The cells were subsequently analyzed by a flow cytometer (BD Biosciences, FACS-Calibur) or observed under a confocal fluorescence microscope. In all immunostaining experiments, normal mouse IgG was added instead of the primary antibody (IgG) as a negative control.

In vitro Analysis of Cytochrome c Release—Mitochondria were prepared as described previously (24). Briefly, cells were harvested, washed three times with isotonic buffer, and then incubated with 30 μg/ml digitonin in isotonic buffer for 5 min at 37 °C. After centrifugation at 3000 rpm for 3 min, aliquots of the supernatant (cytosolic fractions) and the pellet (containing the mitochondria) were analyzed by Western blotting with an anti-cytochrome c antibody.

RESULTS

Caspase-independent Exposure of the N Terminal of Bak Occurs in Fas-stimulated Cells—Using a monoclonal antibody (anti-Bak Ab1) that recognized the N-terminal epitope of human Bak, we examined whether there were any changes to the N terminus of Bak in HeLa cells treated with an agonistic anti-Fas antibody, CH11. As reported previously (13, 19), Bak could not be recognized in living cells by Ab1 antibody, whereas cells exhibited increased immunoreactivity to Ab1 antibody after Fas stimulation (Fig. 1, A and B). Because we could not detect any change of Bak protein content after treatment with CH11 (Fig. 1C), this increased immunoreactivity to Ab1 antibody indicated exposure of the N terminus of Bak. Exposure of the N terminus was detected after 2 h of treatment with CH11, when only a few cells had died (Fig. 1, B and D), consistent with the findings of others that exposure of the N terminus was an early event occurring before morphological changes (13, 19).

In HeLa cells (type II cells), Fas stimulation initiates the activation of caspase-8, which cleaves Bid, a pro-apoptotic member of the Bcl-2 family, and truncated Bid translocates to the mitochondria and activates Bak or Bax by a still unidentified mechanism (9–11). To investigate whether caspase-8 is involved in exposure of the N terminus of Bak, we employed a broad spectrum caspase inhibitor (z-VAD-fmk) to suppress caspase activation. Unexpectedly, we found that z-VAD-fmk (100 μM) did not affect the increased immunoreactivity to Ab1

siRNA Study—All siRNAs were produced by Dharmaco Research, and their sequences were as follows (numbers in parentheses indicate nucleotide positions within the respective open reading frame): mouse Bid (5′-GGCUACGUCCAGGAGCGCACC-3′; mouse Bak (231–252), 5′-AGGAUGAUUGCGCUGGGAC-3′; and GFP (274–294), 5′-GGCUAGCGCAGGAGGGC-3′. Cells were transfected with siRNA by electroporation using an Amaxa System three times at 48-h intervals.

Permeabilized Cells—HeLa cells were cultured in 10-well glass slides. After washing twice with isotonic buffer (25 mM Hepes-KOH (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, 250 mM sucrose), the cells were incubated with isotonic buffer containing 20 μg/ml digitonin for 3 min at room temperature. After washing three times with isotonic buffer, the cells were incubated with cytosol or rBid for 10 min at 37 °C. After washing another three times with isotonic buffer, the cells were subjected to immunostaining.

Preparation of Cytosol—After washing three times with isotonic buffer, cells were suspended in the same volume of isotonic buffer supplemented with protease inhibitors, and then homogenized using a Dounce homogenizer. After centrifugation at 100,000 × g for 1 h, the supernatant was obtained and used as the cytosol.

Subcellular Preparation—Cells were fractionated as described previously (24). Briefly, the cells were harvested, washed three times with isotonic buffer, and then incubated with 30 μg/ml digitonin in isotonic buffer for 5 min at 37 °C. After centrifugation at 3000 rpm for 3 min, aliquots of the supernatant (cytosolic fractions) and the pellet (containing the mitochondria) were analyzed by Western blotting with an anti-cytochrome c antibody.
antibody in CH11-treated cells (Fig. 1, A and B), although the dose of z-VAD-fmk that we used could almost completely suppress the activation of caspases-8, cleavage of Bid, release of cytochrome c from the mitochondria, activation of caspase-3, and cell death (Fig. 1, D–F).

Because Bcl-2/Bcl-xL is known to inhibit the activation of Bax and Bak (26, 27), we also examined whether CH11-induced exposure of the N terminus of Bak was inhibited by Bcl-2. As shown in Fig. 1A, overexpression of Bcl-2 completely inhibited exposure of the N terminus of Bak in CH11-treated cells with or without exposure to z-VAD-fmk.

We next tested whether the same phenomenon also occurred
in other cell lines. As was the case for HeLa cells exposed to CH11, CH11-stimulated Jurkat cells displayed an increase of immunoreactivity to Ab1 antibody, which was not affected by z-VAD-fmk as assessed by FACS and confocal microscopy (Fig. 2, A–C). The dose of z-VAD-fmk used (100 μM) completely inhibited the activation of caspases, cleavage of Bid, and cell death (Fig. 2D and data not shown). Furthermore, we also found that exposure of the N terminus of Bak occurred in Fas-stimulated JB6 cells, a caspase-8-deficient subline of Jurkat cells (Fig. 2E). These results suggest that Fas stimulation could activate a caspase-8-independent signaling pathway and induce exposure of the N terminus of Bak, but this change of
Fas Induces Dual Signals Causing Bak Conformational Change

Fig. 3. Oligomerization of Bak in Fas-stimulated Jurkat cells, and its inhibition by z-VAD-fmk. A, Jurkat cells were treated without or with CH11 (0.5 μg/ml) in the presence or absence of z-VAD-fmk (100 μM) for 2 h, harvested, and incubated with a cross-linker, BMH (uncleavable), or Me₂SO (the solvent) for 30 min at room temperature. Because its oligomerization is correlated with the release of cytochrome c from the mitochondria (11), it is thought to represent activation. Oligomerization of Bak can be detected either by using a protein cross-linker or by gel filtration column chromatography (11, 15). With these procedures, we showed that Bak was oligomerized in CH11-treated cells and this change was inhibited by z-VAD-fmk (Fig. 3, A–C), indicating that its oligomerization was dependent on the activation of caspases. These results suggested that caspase-independent exposure of the N terminus of Bak was separable from its oligomerization.

Bak was not sufficient for full activation.

**Fas-induced Caspase-independent Exposure of the N Terminus of Bak Is Separable from Oligomerization**—Previous studies indicated that Bak undergoes homo-oligomerization in apoptotic cells (11, 18, 28). Because its oligomerization is correlated with the release of cytochrome c from the mitochondria (11), it is thought to represent activation. Oligomerization of Bak can be detected either by using a protein cross-linker or by gel filtration column chromatography (11, 15). With these procedures, we showed that Bak was oligomerized in CH11-treated cells and this change was inhibited by z-VAD-fmk (Fig. 3, A–C), indicating that its oligomerization was dependent on the activation of caspases. These results suggested that caspase-independent exposure of the N terminus of Bak was separable from its oligomerization.

We further confirmed caspase-8-independent exposure of the N terminus of Bak by gel filtration. Lysates of Jurkat cells treated with or without CH11 in the presence or absence of z-VAD-fmk were incubated with Ab1 antibody or normal mouse IgG before being subjected to gel filtration column chromatography. If Bak binds to Ab1 antibody, it would be eluted in the fractions that correspond to the larger molecular size proteins. As shown in Fig. 3D, when the sample from untreated cells was incubated with Ab1 antibody and then applied to the column, Bak was detected in the fractions corresponding to low molecular weight proteins, as was the case without incubation with Ab1 antibody (Fig. 3C) or after incubation with control normal mouse IgG (Fig. 3D, upper panel), indicating no exposure of the N terminus of Bak. When the sample from CH11-treated cells was incubated with Ab1 antibody, Bak shifted to the larger molecular weight fractions, which were larger than that when the sample was incubated with normal mouse IgG or without Ab1 antibody (Fig. 3, C and D), suggesting that the N terminus was exposed in apoptotic cell lysates. When lysates from cells treated with CH11 in the presence of z-VAD-fmk were incubated with Ab1 antibody, the Bak peak was found in fractions that corresponded to both high and low molecular weight proteins (Fig. 3D, lower panel), indicating that Bak was recognized by Ab1 antibody in cells treated with CH11 in the presence of z-VAD-fmk. This result is consistent with our immunostaining data. Taken together, these observations suggest that Fas stimulation induces exposure of the N terminus of Bak in a caspase-8-independent manner and that N-terminal exposure is separable from the oligomerization of Bak.

**Exposure of the N Terminus of Bak Makes Cells More Sensitive to Apoptotic Stimuli**—The results described above showed that Fas-dependent/caspase-independent exposure of the N terminus of Bak did not lead to its full activation. Did such exposure of N terminus have any biological significance? We hypothesized that full activation of Bak requires at least two steps, i.e. exposure of the N terminus, followed by oligomerization, and that Bak with N-terminal exposure might be in a “primed state.” It should be noted that exposure of the N terminus can be mediated by both caspase-8-dependent and independent mechanisms. If this hypothesis is correct, cells in which the N terminus of Bak is exposed may be more sensitive to subsequent death stimuli. To test this possibility, Jurkat cells were pretreated with CH11 in the presence of z-VAD-fmk for 2 h to “prime” Bak, and then were exposed to 10 μM VP-16 (etoposide) in the presence of z-VAD-fmk. z-VAD-fmk did not inhibit transmission of VP-16-induced death signals to the mitochondria or release of cytochrome c from the mitochondria.
FIG. 4. Increased susceptibility to VP-16-induced apoptosis of cells possessing Bak with N-terminal exposure. A, Jurkat cells were treated with or without CH11 (0.5 μg/ml) in the presence of z-VAD-fmk (100 μM) for 2 h, followed by incubation with VP-16 (10 μM) in the presence of z-VAD-fmk for the indicated times. The cells were fractionated into cytosolic (cytosol) and organellar (pellet) fractions, and then analyzed by Western blotting with anti-cytochrome c antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and voltage-dependent anion channel protein (VDAC) were monitored to verify successful fractionation and as loading controls. B, cytosol was prepared from caspase-8−/− MEFs treated without (control) or with Jo2 (0.5 μg/ml) for 16 h and was added to HeLa cells that had been permeabilized by incubation with digitonin (20 μg/ml) for 3 min at room temperature. After incubation for 10 min at 37 °C, immunostaining was done with anti-Bak Ab1 antibody. As a negative control, isotonic buffer was added instead of cytosol. Scale bar = 10 μm. C, caspase-8−/− MEFs were treated with Jo2 (0.5 μg/ml) or TNFα (10 ng/ml) for 16 h, followed by incubation with 10 μM VP-16 for the indicated times. Apoptotic cells were detected by Annexin-V staining. Data are shown as the mean ± S.E. (n = 3). D, caspase-8−/− MEFs were pretreated as described in C, followed by incubation with 10 μM VP-16 for 24 h, and then stained with Hoechst 33342. Scale bar = 10 μm. E, caspase-8−/− MEFs were treated with Jo2 (0.5 μg/ml) or TNFα (10 ng/ml) for 16 h, followed by incubation with 10 μM VP-16 for 18 h. The cells were fractionated into cytosolic (cytosol) and organellar (pellet) fractions, and then extracts were subjected to Western blotting with anti-cytochrome c antibody. F, caspase-8−/− MEFs were treated with Jo2 (0.5 μg/ml) or TNFα (10 ng/ml) for 16 h, followed by incubation with 10 μM VP-16 for 18 h. After lysis, the activation of caspase-3 was assayed by Western blotting.

(data not shown). VP-16 induced the release of only a small amount of cytochrome c from the mitochondria in the cells without CH11 pretreatment (Fig. 4A, lanes 4, 7, and 10). In contrast, a large amount of cytochrome c was released after cells were pretreated with CH11/z-VAD-fmk and then exposed to VP-16 (Fig. 4A, lanes 3, 6, and 9). This result indicates that, although CH11 alone could not induce cytochrome c release in the presence of z-VAD-fmk (Fig. 4A, lanes 2, 5, and 8), it sensitized cells to subsequent treatment with VP-16, suggesting that Bak with N-terminal exposure might represent “primed Bak.”

We also examined whether Fas-induced caspase-independent exposure of the N terminus of Bak could sensitize cells to apoptotic death. Because z-VAD-fmk inhibits apoptosis, we could not use it in experiments that assayed cell death. Therefore, we employed caspase-8-deficient murine embryonic fibroblasts (MEFs), which are completely resistant to death receptor-mediated apoptosis (29). First, we investigated whether stimulation of Fas with agonistic anti-mouse Fas antibody Jo2 induced exposure of the N terminus of Bak in caspase-8-deficient MEFs. Because no antibody was available to recognize a conformational change of mouse Bak, we employed an in vitro assay system using HeLa cells permeabilized by digitonin. Lysates derived from caspase-8−/− MEFs treated with or without Jo2 were added to digitonin-permeabilized HeLa cells, and then immunostaining was performed with Ab1 antibody. As shown in Fig. 4B, lysate from Jo2-treated caspase-8−/− MEFs, but not lysate from untreated cells, caused an increase of Ab1 immunoreactivity. This indicated that, as in Jurkat and HeLa cells, one or more factors that induced exposure of the N terminus of Bak existed in the lysates of Jo2-treated caspase-8−/− MEFs and, although not proved, suggested that Bak entered a primed state in caspase-8−/− MEFs after Jo2 treatment.

To examine whether Jo2-treated caspase-8−/− MEFs, in which Bak was considered to exist in a primed state, were more sensitive to death stimuli, we pretreated caspase-8−/− MEFs with Jo2 alone for 16 h, followed by exposure to 10 μM VP-16. Note that cycloheximide was not added here, because cycloheximide inhibited VP-16-induced apoptosis in MEFs (data not shown). As shown in Fig. 4 (C and D), pretreatment with Jo2 enhanced VP-16-induced apoptosis. The same result was also obtained with TNFα instead of Jo2 (Fig. 4, C and D). After 18 h of exposure to VP-16, Jo2- or TNFα-treated cells exhibited release of cytochrome c into the cytosol and activation of caspase-3, whereas untreated cells did not show any release of cytochrome c or caspase-3 activation at this time (Fig. 4, E and F). Consistent with a previous report (29), Jo2 or TNFα alone did not induce cytochrome c release (data not shown) or death of caspase-8−/− MEFs (Fig. 4, C and D). These results indicate...
that Fas stimulation without caspase-8 activation could sensitize cells to apoptotic death.

_Cytosol from Cells with Primed Bak Enhances Bid-induced Release of Cytochrome c from Isolated Mitochondria—_To assess whether Fas stimulation without caspase-8 activation (leading to exposure of the N terminus of Bak) could sensitize the mitochondria to apoptotic signals, we used an _in vitro_ system with mitochondria isolated from rat livers. The mitochondria were incubated for 5 min with cytosol derived from caspase-8−/− MEFs with or without Jo2 treatment, followed by incubation with rBid (30 μg/ml) for an additional 20 min. Release of cytochrome c was detected by Western blotting. As shown in Fig. 5, treatment with rBid induced the release of cytochrome c from isolated mitochondria, and preincubation with cytosol obtained from untreated cells only slightly promoted the release of cytochrome c, whereas preincubation with cytosol from treated cells markedly promoted its release. The incubation of isolated mitochondria with cytosol alone only induced slight release of cytochrome c (Fig. 5). These results indicate that Fas stimulation without caspase-8 activation induces a signal that could sensitize the mitochondria to apoptotic stimuli.

_Bak Has an Essential Role in the Increased Susceptibility to VP-16 of Cells Possessing Bak with Caspase-8-independent N-terminal Exposure—_To investigate whether the increased susceptibility to VP-16 of Jo2- or TNFα-pretreated caspase-8−/− MEFs was due to primed Bak, we used RNA interference to silence the expression of Bak in caspase-8−/− MEFs. Cells were transfected with Bak siRNA, or GFP siRNA (as a negative control), and then the expression of Bak was examined by Western blotting. As shown in Fig. 6A, Bak expression decreased to a nearly undetectable level after treatment with siRNA for Bak, and the silencing effect lasted for more than 72 h, whereas the Bax level remained unchanged. As observed above, Jo2- or TNFα-pretreated caspase-8−/− MEFs displayed an increase of susceptibility to VP-16-induced apoptosis when transfected with control GFP siRNA, whereas Jo2 or TNFα could not sensitize these cells to VP-16 after Bak was eliminated by siRNA treatment (Fig. 6B). It should be noted that silencing of Bak did not affect VP-16-induced apoptosis (Fig. 6B), which was probably mediated by Bax. In contrast, when Bax was eliminated by using Bax siRNA (Fig. 6C), the susceptibility of Jo2- or TNFα-pretreated caspase-8−/− MEFs to VP-16 was unchanged (Fig. 6D). Taken together, these data indicate that Bak plays an important role in the increased susceptibility to VP-16 of Jo2- or TNFα-treated cells.

_A Staurosporine-sensitive Signal Elicited by Fas Ligation Is Responsible for Caspase-independent Exposure of the N Terminal of Bak—_In addition to the well characterized caspase-8-de-
dependent apoptotic pathway, Fas stimulation has also been shown to activate other signaling pathways that lead to the activation of extracellular signal-regulated kinase (ERK) (30–32), c-Jun N-terminal kinase (JNK) (33), and tyrosine kinases (34). To obtain some insights into the signaling pathways involved in exposure of the N terminus of Bak in a caspase-8-independent fashion after Fas stimulation, we employed several kinase inhibitors to examine whether N-terminal exposure induced by CH11 in the presence of z-VAD-fmk could be blocked in HeLa cells. Among the inhibitors tested, PD98059 (ERK), SP600125 (JNK), and genistein (tyrosine kinases) did not affect Fas-induced caspase-8-independent exposure of the N terminus of Bak (data not shown). However, the pan kinase inhibitor staurosporine (STS) (10 nM) impaired the increase of anti-Bak Ab1 immunoreactivity induced by CH11 in the presence of z-VAD-fmk (Fig. 7A), although STS did not inhibit the increase of anti-Bak Ab1 immunoreactivity induced by CH11 alone (Fig. 7A). This indicates that at least two independent pathways are involved in exposure of the N terminus of Bak after Fas stimulation, one that is caspase-8/Bid-independent and involves an STS-sensitive kinase and another that is the well-characterized caspase-8/Bid pathway. The inhibitory effect of STS was also confirmed by our in vitro permeabilized cell system. As shown in Fig. 7B, consistent with the in vitro results, cytosol derived from cells treated with CH11 in the presence of z-VAD-fmk together with STS could not induce exposure of the N terminus of Bak, whereas cytosol from cells treated with CH11 in the presence of z-VAD-fmk and rBid induced exposure of the N terminus. Furthermore, cytosol from cells treated with CH11/z-VAD-fmk/STS also failed to enhance rBid-induced cytochrome c release from isolated mitochondria (Fig. 7C). These results indicate that an STS-sensitive kinase plays a role in caspase-independent exposure of the N terminus of Bak after Fas stimulation.

DISCUSSION

In response to various apoptotic stimuli, Bax and Bak undergo conformational changes such as exposure of their N terminus and homo-oligomerization: the homo-oligomers are considered to be active forms that induce permeabilization of the outer mitochondrial membrane, a process that is central to apoptotic death (3, 4). During death receptor-mediated apoptosis (type II cells), formation of the active forms of Bax and Bak depends on activation of caspase-8 and subsequent cleavage of Bid, because both caspase-8 and Bid are essential for these modes of apoptosis (11, 29, 35). In the present study, however, we showed that, in the absence of caspase-8 activa-

![Figure 5](image1.png)

**Fig. 5.** Enhancement of Bid-induced release of cytochrome c from isolated mitochondria by cytosol from cells with prior exposure of the N terminus of Bak. Cytosol derived from untreated caspase-8−/− MEFs (lane 2) and caspase-8−/− MEFs treated with 0.5 μg/ml Jo2 for 16 h (lane 3) were incubated with mitochondria isolated from rat livers for 5 min, followed by incubation with (lower panel) or without (upper panel) rBid (30 μg) for a further 20 min. As a negative control, isotonic buffer was added instead of cytosol (lane 1). Release of cytochrome c was determined by Western blotting.

![Figure 6](image2.png)

**Fig. 6.** Requirement of Bak for the Jo2-induced increased susceptibility to VP-16 of caspase-8−/− MEFs. A, caspase-8−/− MEFs were transfected with Bak siRNA (10 μg) or GFP siRNA (10 μg). Expression of Bak, Bax, and glyceraldehyde-3-phosphate dehydrogenase was detected at the indicated times after the last transfection of siRNA by Western blotting. B, siRNA-transfected caspase-8−/− MEFs were treated with Jo2 (0.5 μg/ml) or TNFα (10 ng/ml) for 16 h, followed by incubation with 10 μM VP-16 for the indicated times. Apoptotic cells were detected by Annexin-V staining. Data are shown as the mean ± S.E. (n = 3). C, caspase-8−/− MEFs were transfected with Bax siRNA (10 μg) or GFP siRNA (10 μg). Expression of Bax and glyceraldehyde-3-phosphate dehydrogenase was detected 24 h after the last transfection by Western blotting. D, siRNA-transfected caspase-8−/− MEFs were treated with Jo2 (0.5 μg/ml) or TNFα (10 ng/ml) for 16 h, followed by incubation with 10 μM VP-16 for 24 h. Apoptotic cells were detected by Annexin-V staining. Data are shown as the mean ± S.E. (n = 3).
tion, stimulation of Fas and TNF receptor still induced exposure of the N terminus of Bak, suggesting that N-terminal exposure and homo-oligomerization were separable, which is consistent in principle with the previous report that activation of Bak proceeds in multiple steps (19). Similar observations have also been reported for Bax (36, 37). Importantly, we also provided evidence that Bak with N-terminal exposure is in a primed state, because cells possessing Bak with N-terminal exposure were more sensitive to subsequent apoptotic stimulation. This may imply that exposure of the N terminus of Bax/Bak is a prerequisite for homo-oligomerization. Because it is known that exposure of the N terminus of Bax/Bak and oligomerization can be induced by tBid (11, 12), which is generated from Bid by active caspase-8 during death receptor-mediated apoptosis, it needs to be determined whether caspase-dependent exposure of the N terminus of Bak (induced via tBid) and caspase-independent exposure during death receptor-induced apoptosis are identical or not. It is also to be determined how significantly caspase-8-independent exposure of the N terminus of Bak contributes to death receptor-mediated apoptosis. This would be answered once a signaling molecule involved in this process has been identified.

In our present study, we found that Fas stimulation induced exposure of the N terminus of Bak not only in HeLa cells, but also in Jurkat cells. This does not seem consistent with the previous observation of Griffiths et al. (13). These authors showed that CH11 (1 ng/ml) could not cause exposure of the N terminus of Bak in Jurkat cells. This discrepancy may be due to a difference in CH11 concentration used. In fact, we found that exposure of the N terminus of Bak did not occur when CH11 concentration was lower than 10 ng/ml (data not shown), suggesting that exposure of the N terminus of Bak requires strong signals from death receptors.

What signals are elicited by stimulation of Fas and TNF
receptor, leading to caspase-8-independent exposure of the N terminus of Bak? Recently, caspase-10 has been identified as an alternative mechanism of death receptor-induced cell death (38). However, it is not likely that caspase-10 induced exposure of the N terminus of Bak, because the caspase inhibitor z-VAD-fmk could not suppress Fas-induced exposure of the N terminus in HeLa and Jurkat cells. Furthermore, the caspase-10 gene has not yet been identified in mice. It has been reported that engagement of Fas activates different signaling pathways, leading to activation of ERK (30–32), JNK (33), tyrosine kinases (34), or phosphatidylinositol-specific phospholipase C (39). It is possible that any of these caspase-independent signaling pathways could transmit a signal to mitochondrial Bak. The present study showed that a nonspecific protein kinase inhibitor, STS, actually inhibited Fas-induced exposure of the N terminus of Bak in the presence of z-VAD-fmk, but not inhibitors of ERK, JNK, or tyrosine kinases. Although further studies are necessary for identification of the kinase(s) responsible for Fas-induced caspase-8-independent exposure of the N terminus of Bak, our results indicate that stimulation of death receptors can elicit two independent signals targeting the mitochondria that regulate Bak activation, one via caspase-8/ Bid and the other via a caspase-8-independent pathway that possibly involves one or more kinases.

In death receptor-induced apoptosis, activation of Bak is mainly mediated by the caspase-8/Bid pathway. Because we showed that Fas-induced caspase-8-independent exposure of the N terminus of Bak occurred in MEFs in the absence of cycloheximide, which was suggested to overcome the inhibitory effect of cFLIP or vFLIP on death-inducing signaling complex-dependent activation of caspase-8 (40), the caspase-independent signaling pathway that primes Bak might contribute to some death paradigms such as elimination of cells infected with vFLIP-producing virus by cooperation with other intrinsic apoptotic signals targeting the mitochondria.

In conclusion, a death receptor-activated caspase-independent signal induces exposure of the N terminus of Bak. Although this change is not sufficient for activation, it primes Bak for full activation. Thus, our results have revealed a novel signaling pathway involved in death receptor-mediated apoptosis.

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