Bcl-2 Inhibits a Fas-induced Conformational Change in the Bax N Terminus and Bax Mitochondrial Translocation*

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Members of the Bcl-2 family of proteins control the cellular commitment to apoptosis, although their role in Fas-induced apoptosis is ill-defined. In this report we demonstrate that activation of the Fas receptor present on a human breast epithelial cell line resulted in a conformational change in the N terminus of the pro-apoptotic protein Bax. This conformational change appeared to occur in the cytosol and precede Bax translocation to the mitochondria. Overexpression of the anti-apoptotic protein Bcl-2 inhibited both the conformational change of Bax as well as its relocalization to the mitochondria. Bcl-2 overexpression did not, however, inhibit Fas-induced cleavage of both procaspase-8 and the pro-apoptotic protein Bid, indicating that Bcl-2 functions downstream of these events. These results suggest that the mechanism by which Bcl-2 inhibits Bax mitochondrial translocation and subsequent amplification of the apoptotic cascade is not by providing a physical barrier to Bax, but rather by inhibiting an upstream event necessary for Bax conformational change.

Cross-linking of the Fas (CD95) receptor by its ligand or agonistic antibodies results in the formation of a death-inducing signaling complex (DISC), which includes FADD/MORT1 and procaspase-8 (1, 2). Processing and activation of procaspase-8 at the DISC activates a proteolytic cascade with ensuing cell death (3, 4). During this process mitochondrial dysfunction occurs, evidenced by dissipation of the electrochemical gradient, uncoupling of the respiratory chain, and production of reactive oxygen species (5–7). However, recent evidence suggests that the Fas-induced cell death pathway may bifurcate with predominantly mitochondrial-dependent or -independent mechanisms (8, 9).

Members of the Bcl-2 family of proteins interact to set a threshold for cell death, although they may also act independently (10, 11). Recent data indicate that the pro-apoptotic molecule Bax is a monomeric and predominantly cytosolic protein in unstressed cells despite the presence of a C-terminal hydrophobic domain that is required for membrane insertion and Bax-induced cell death (12, 13). During apoptosis Bax translocates to the mitochondrial membrane (12, 14). Insertion of Bax into the mitochondrial membrane appears to be sufficient to induce cytochrome c release, an event that may act to amplify the apoptotic signal (8, 15, 16). Mutations in the C terminus can also result in both constitutively mitochondrial or cytoplasmic Bax protein (17). Deletion of the N terminus, or enforced dimerization of Bax, results in direct targeting of Bax to the mitochondria and enhanced cytotoxicity (18, 19).

Bid is a pro-apoptotic molecule that belongs to a class of the Bcl-2 family that has little homology to other members, containing only a single BH domain (BH3) (20). Bid is unique among the BH3-only class of proteins in that it has been reported to be complex with either pro- or anti-apoptotic members of the Bcl-2 family, potentially enhancing or antagonizing their function (21). Bid, which lacks a C-terminal transmembrane domain, is predominantly cytosolic in living cells. It is cleaved and activated by caspase-8, and the truncated protein translocates to the mitochondria apparently triggering cytochrome c release (22–24). A recent model of Fas-induced apoptosis suggests that Bid causes a conformational change in the N terminus of Bax, resulting in mitochondrial cytochrome c release and amplification of the apoptotic cascade (25). However, it remains unclear whether the conformational change in Bax occurs prior or subsequent to mitochondrial membrane insertion. Hsu and Youle (13, 26) have reported a detergent-induced conformational change in the N terminus of Bax (amines 12–24), which appears to be required for both homodimerization with Bcl-xL. However, the physiological significance and relevance to its role in apoptosis is unknown.

We have previously demonstrated that activation of the Fas receptor leads to Bax translocation from the cytosol to the mitochondria that can be inhibited by Bcl-2 overexpression (27). In the present report we demonstrate that activation of the Fas receptor results in a conformational change in the N terminus of Bax, which appears to precede its translocation to the mitochondria. Our findings that overexpression of Bcl-2 does not inhibit Fas-induced cleavage of procaspase-8 or Bid, but does inhibit both the Bax conformational change and mitochondrial translocation, provide further insight into the Fas apoptotic pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Fas Treatment—Mid-passage MCF10A1 cells, a spontaneously immortalized non-tumorigenic human breast epithelial cell line, were obtained from the Barbara Ann Karmanos Cancer Institute. The transfection, selection, and growth conditions used for both control vector transfected (N10) and stable Bcl-2-expressing (B30) clones have been described previously (27). Anti-Fas monoclonal antibody (clone
CH11, Upstate Biotechnology) was stored at −20 °C and diluted directly into tissue culture medium. Cells were exposed to 100 ng/ml anti-Fas antibody plus 1 μg/ml cycloheximide (Calbiochem).

**Sample Preparation and Immunoblotting**—For separation of subcellular cytosolic and nuclear/membrane fractions, 10° cells were washed twice with PBS at 4 °C and resuspended in extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37 °C. After microscopic examination with trypan blue verified that >95% cells were lysed, the crude lysate was centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant, which consisted of the cytosol, was separated from the pellet that contained the cellular membrane and organelles, and the pellet was resuspended in 1 ml of extraction buffer. Immunoblotting for the nuclear protein topoisomerase I (clone C-21, kindly provided by Dr. Y.-C. Cheng, Yale University School of Medicine, New Haven, CT) and the mitochondrial protein COX VIc (clone 3G5-P7-G3, Molecular Probes) revealed no cross-contamination of these organelles in the cytosolic fraction by this method of fractionation. As a cytosolic marker, a lactate dehydrogenase assay kit (Sigma) was used according to the manufacturer’s instructions, and experiments were only deemed valid if >95% of the total dehydrogenase activity was detected in the cytosolic fraction.

Whole cell extracts were prepared by lysing 10° cells in 1 ml of either Nonidet P-40 buffer (ph 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 50 mM NaF, 5 mM EDTA, 0.1 mM orthovanadate, plus protease inhibitor mixture (Sigma)) or CHAPS lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS, plus protease inhibitor mixture) on ice for 30 min. The crude lysate was then centrifuged at 10,000 × g for 10 min at 4 °C and the supernatant stored at −80 °C.

Protein concentration was estimated by the bicinchoninic acid assay method (Pierce) using a BSA standard. Equal amounts of protein, or equal volumes of cytosol and membrane fractions, were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Primary antibodies used were rabbit polyclonal antiserum raised against human Bcl-2, Bax (PharMingen), and actin (Sigma); a goat polyclonal antibody raised against a peptide corresponding to amino acids 176–195 of human Bid (Santa Cruz); and a mouse monoclonal antibody corresponding to mouse monoclonal anti-Bax, which recognizes a detergent-induced conformational change of Bax during this translocation we used the 6A7 monoclonal antibody, which recognizes a detergent-induced conformational change of Bax, but not native Bax (13, 17, 26).

**Immunoprecipitation**—Equal amounts of protein from whole cell lysates (1 mg) or cytosolic fractions (750 μg) were used for immunoprecipitation. The KC1 concentration of the cytosolic lysates was adjusted to 150 mM, and all samples were brought to a final volume of 450 μl with 10 mM Tris, pH 7.4, 150 mM NaCl. Samples were rotated for 2 h at 4 °C with 6 μg of monoclonal anti-Bax 6A7 (Trevisen) or 7 μl of polyclonal anti-Bax (PharMingen). Antigen-antibody complexes were immobilized by rotation for 2 h at 4 °C with protein G-agarose (Pierce) for polyclonal anti-Bax or protein A-Sepharose (Amerham Pharmacia Biotech) for polyclonal anti-Bax. The complexes were centrifuged and the supernatant removed with an aliquot being stored for subsequent analysis. The complexes were then washed five times with the same buffer used for the immunoprecipitation and subjected to SDS-PAGE and immunoblotted as described above.

**Immunofluorescence and Confocal Microscopy**—Cells were grown on glass coverslips under the same conditions described above. Prior to Fas activation, cells were labeled with Mitotracker Red CM-H2XRos (Molecular Probes). Preliminary experiments had determined the minimum concentration and time required for optimal Mitotracker Red staining of MCF10A1 cells to be 2 μM for 45 min at 37 °C. Treatments with Fas-activating antibody were carried out as described above. At the indicated time points, cells were washed twice in PBS, fixed for 30 min in 3% formaldehyde, and permeabilized for 2 min with 0.2% CHAPS/PBS. The samples were then incubated with mouse monoclonal antibody (Trevisen) diluted 1:300 in 3% BSA/PBS for 1 h at 37 °C in a humidified chamber. Excess antibody was removed by washing the coverslips six times with PBS. Cells were then incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Zymed Laboratories Inc.) diluted 1:20 in 3% BSA/PBS, for 1 h protected from light. After washing six times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). Control slides were stained with secondary antibody alone. Immunofluorescence of total Bax was performed using a rabbit anti-Bax polyclonal antibody, as we have described previously (27). Cells were viewed under a Zeiss Axioskop 20 fluorescence microscope and imaged using the Bio-Rad MRC 1024 Laser Confocal Imaging System. For each time point, at least 300 cells were counted and the numbers reported represent the average and S.E. of two experiments.

**RESULTS AND DISCUSSION**

We have shown previously that treatment of MCF10A1 N10 cells with anti-Fas antibody results in a redistribution of Bax from the cytosol to the mitochondria, which is inhibited by overexpression of Bcl-2 (B30 cells) (27). To study the conformation of Bax during this translocation we used the 6A7 monoclonal antibody, which recognizes a detergent-induced conformational change of Bax, but not native Bax (13, 17, 26). Fig. 1 shows immunofluorescence with anti-Bax 6A7 (green) either alone (A–C, G, and H) or merged with mitochondrial staining (red) (D–F, I, and J). Untreated N10 and B30 cells were negative for staining with 6A7 (A, D, G, I, and J), consistent with previous results using COS-7 cells (17).

Treatment of N10 cells with anti-Fas induced a conformational change in Bax, resulting in a punctate staining pattern (B, C, E, F). At early time points (2 h, B and E) a proportion of conformationally changed Bax appeared not to co-localize to the mitochondria. In some cells there was extremely little colocalization as indicated by the green color observed in Fig. 1E, while in other cells there was a mixture of non-co-localized and co-localized Bax indicated by green and yellow, respectively (Fig. 1E). At a later treatment time point (4 h) a significant proportion of 6A7 staining was yellow (Fig. 1F), indicating its co-localization to the mitochondria, while apoptotic morphology was also observed in some cells. The remainder of the intracellular green staining observed in Fig. 1F is consistent with our previous repopulation experiments (35–40% of total Bax had translocated to mitochondria by 4 h following Fas activation (27)).

Overall, these data raise the possibility that the conformational change of Bax precedes translocation to the mitochondria. Notably, the Bax conformational change was observed before morphological evidence of apoptosis, and once associated with the mitochondria, Bax maintained this altered conformation.
Bax Conformation Change and Mitochondrial Translocation

**Fig. 2.** Bax conformational change and membrane translocation. N10 and B30 cells were treated with anti-Fas and separated into cytosolic and membrane fractions. The integer above each lane denotes the treatment time. A, immunoprecipitations of cytosolic fractions with monoclonal anti-Bax 6A7, immunoblotted with polyclonal anti-Bax. IP, immunoprecipitate; Super, supernatant. Nonidet P-40 (NP-40) and CHAPS are untreated N10 whole cell lysates prepared with buffer containing 0.2% Nonidet P-40 or 1% CHAPS, respectively. The immunoprecipitate was exposed to radiographic film three times longer than the Super. B, immunoblot of cytosolic (cyto) and membrane (mem) fractions of N10 and B30 cells, probed for Bcl-2 (p26) and Bax (p21). COX-V1 (p11) demonstrates the presence of mitochondria in the membrane fractions only.

As the cells executed apoptosis. Strikingly, overexpression of Bcl-2 inhibited the conformational change of Bax (Fig. 1, H and J). Fig. 1K shows graphically the time-dependent increase in immunoreactivity with anti-Bax 6A7 in N10 cells, while the conformational change is inhibited in B30 cells.

The proportion of N10 and B30 cells exhibiting a punctate Bax staining pattern, indicative of mitochondrial translocation, was assessed as we have described (27) using a polyclonal antibody that recognizes both native and conformationally active Bax (total Bax, Fig. 1K). The increase in the proportion of cells with punctate total Bax staining showed a similar time course, albeit slightly delayed, as that for conformationally changed Bax (6A7).

In an attempt to confirm the immunofluorescence results, immunoprecipitation with 6A7 was performed on the cytosolic fractions of N10 and B30 cells that had been lysed in the absence of detergents. As controls, immunoprecipitations were performed on cells lysed with 0.2% Nonidet P-40 or 1% CHAPS. Consistent with the results of Hsu and Youle (13), lysis with Nonidet P-40 induced a conformational change in Bax, allowing its immunoprecipitation with 6A7 (Fig. 2A, lane a). In contrast, CHAPS lysis did not induce a Bax conformational change, prohibiting immunoprecipitation with 6A7 (lane b). In the cytosolic fraction of untreated N10 or B30 cells lysed in the absence of detergents, Bax was in its native conformation and therefore was not immunoprecipitated by anti-Bax 6A7 (Fig. 2A, lanes c and f), in agreement with a previous study using mouse L929 cells (17). After 3- and 6-h treatment with anti-Fas, the conformationally changed Bax was detected in the immunoprecipitate of the cytosolic fractions of N10 cells (Fig. 2A, lanes d and e). Fig. 2B shows Bax relocalization from the cytosol (lanes a–c) to the membrane compartment (lanes d–f), and subsequent proteolysis (lane f) during Fas activation, demonstrating that the decreased amount of Bax protein in the 6-h supernatant from the immunoprecipitation (Fig. 2A, lane c) reflects its relocalization to the membrane compartment.

Thus, we have demonstrated by two different methods that the conformational change in Bax can be detected in the cytosol of anti-Fas-treated N10 cells. These results differ from those of Desagher et al. (25) who described a conformational change in Bax but concluded that Bax is constitutively associated with the mitochondria and undergoes a conformational change within that location. The apparent differences in Bax localiza-

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interacting with Bax or Bcl-2 have used recombinant proteins in vitro or cells expressing ectopic Bid that were lysed with Nonidet P-40 (20, 25). Our data suggest that endogenous cellular Bid and Bax do not dimerize to any significant degree.

In conclusion, we have demonstrated that activation of the Fas receptor results in a conformational change in the N terminus of native Bax protein (amino acids 12–24). The conformational change appeared to precede its translocation to the mitochondria. This conformational change, which can be induced by detergents, may be necessary for Bax to interact with other members of the Bcl-2 family (13, 26).

The structure of Bax is currently unknown; however, x-ray crystallography of the related family member Bel-2 suggests that the N terminus and BH3 domain are adjacent to the C-terminal amino acid 197 (31). The Bax C terminus is known to be required for redistribution during apoptosis (12), while the N terminus negatively regulates Bax membrane insertion (18). Based on these and other studies it has been hypothesized that in its native conformation the N terminus of Bax masks domains at the C terminus that are required for mitochondrial translocation (17). Bax molecules containing C-terminal deletions or substitutions that cause its constitutive localization to mitochondria do not undergo N-terminal conformational change until the application of an apoptotic stress (17). In the same study, the temporal relationship between N-terminal conformational change and mitochondrial translocation of wild-type Bax was not delineated (17). Our data support a model in which, following application of an apoptotic stress such as Fas activation, Bax undergoes an N-terminal conformational change in the cytosol, thereby unmasking the C-terminal hydrophobic domain allowing insertion into the mitochondrial membrane. In support of this model, high pH was recently shown to induce an N-terminal conformational change of cytosolic Bax in lymphoid cells (32).

It is unlikely that Bcl-2 can inhibit the Bax conformational change by direct interaction with Bax, since the two proteins appear to be in different subcellular locations when the conformational change occurs. Furthermore, Bcl-2/Bax interactions are not observed in immunoprecipitation experiments in the absence of nonionic detergents (13). Our data demonstrate that overexpression of Bcl-2 does not sequester Bid to the membrane compartment, nor does it prevent Bid cleavage during Fas-induced apoptosis. Thus, Bcl-2 appears to function downstream of caspase-8 and Bid activation. It is possible, however, that Bcl-2 may inhibit activation of other caspases that are responsible for the Bax conformational changes. These mechanisms are currently under investigation.

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