Cell Damage-induced Conformational Changes of the Pro-Apoptotic Protein Bak In Vivo Precede the Onset of Apoptosis

Gareth J. Griffiths, Laurence Dubrez, Clive P. Morgan, Neil A. Jones, Jenna Whitehouse, Bernard M. Corfe, Caroline Dive, and John A. Hickman

Cancer Research Campaign Molecular and Cellular Pharmacology Group, School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Abstract. Investigation of events committing cells to death revealed that a concealed NH_2-terminal epitope of the pro-apoptotic protein Bak became exposed in vivo before apoptosis. This occurred after treatment of human Jurkat or CEM-C7A T-lymphoma cells with the mechanistically disparate agents staurosporine, etoposide or dexamethasone. The rapid, up to 10-fold increase in Bak-associated immunofluorescence was measured with epitope-specific monoclonal antibodies using flow cytometry and microscopy. In contrast, using a polyclonal antibody to Bak, immunofluorescence was detected both before and after treatment. There were no differences in Bak protein content nor in subcellular location before or after treatment. Immunofluorescence showed Bcl-x_L and Bak were largely associated with mitochondria and in untreated cells they coimmunoprecipitated in the presence of nonioinic detergent. This association was significantly decreased after cell perturbation suggesting that Bcl-x_L dissociation from Bak occurred on exposure of Bak's NH_2 terminus. Multiple forms of Bak protein were observed by two dimensional electrophoresis but these were unchanged by inducers of apoptosis. This indicated that integration of cellular damage signals did not take place directly on the Bak protein. Release of proteins, including Bcl-x_L, from Bak is suggested to be an important event in commitment to death.

Key words: flow cytometry • etoposide • staurosporine • dexamethasone • protein–protein interactions

One of the central questions regarding the mechanism of engagement of cell death is what biochemical events irreversibly commit the cell to die? These are likely to be changes that precede the execution of the cell. It is also important to understand how signals elicited by diverse toxic stimuli become integrated to initiate commitment to death. Because the suppressers of cell death Bcl-2 and Bcl-x_L inhibit the toxicity of agents with widely different modes of action (reviewed in 29, 42, and 44), it is considered that this family of proteins act at or proximal to some critical point of the convergence of damage signals and close to the point of commitment to death. Recent evidence suggests that a Bcl-2-controlled irreversible commitment to death is an event occurring before the so-called execution phase, characterized by the apoptotic process. This essentially makes the commitment to death an event that is separable from the process of apoptosis itself, an idea that is receiving increasing support (1, 4, 26, 42).

Proteins of the Bcl-2 family have been suggested to interact with each other to set a survival threshold for the cell. These protein–protein interactions are presumed to change as a cell commits to death (42) and enforced interactions between Bax molecules have recently been shown to be lethal (14). It might be predicted that disparate types of cellular perturbation or damage would initiate changes in the topology, thence the associations and thus function of the pro- and anti-apoptotic proteins of the Bcl-2 family and/or of associated proteins. Alterations in Bcl-2 family protein conformation may include those that bring about the proposed changes in the homodimerization and heterodimerization profiles of the promoters and suppressers of apoptosis. It has been suggested that changes in the stoichiometry of partner binding initiates an irreversible commitment to cell death (22). However, from experiments in vivo, which provided appropriate cellular contexts, it has been shown that the pro- and anti-apoptotic Bax and Bcl-2 proteins may act independently of each other (21). In some contexts it is possible that accessory proteins such as Bad, Bid, and Bik, containing only a BH3 domain of the

The first two authors contributed equally to this work.

Address all correspondence to John Hickman, Cancer Research Campaign Molecular and Cellular Pharmacology Group, School of Biological Sciences, G38 Stopford Building, University of Manchester, Manchester M13 9PT, UK. Tel.: 44 161 275 5448. Fax: 44 161 275 5600. E-mail: jhickman@man.ac.uk

conserved Bcl-2 family homology domains, may serve as intermediaries between the major suppressers and promoters of apoptosis (19). Cumulatively, these proteins may be essential components of what has been proposed an "apoptsat," an integrator of pro- and anti-apoptotic signals, where cellular commitment to death or to survival occurs before the activation of caspases (30).

The models whereby different patterns of homo- and heterodimerization of Bcl-2 family members act to control survival and death are based upon observations of protein–protein interactions using either the yeast two-hybrid system or immunoprecipitation of tagged proteins from cellular lysates in the presence of detergents (e.g., 16, 28, 32, 33, 45). Attempts to validate these findings have exploited site directed mutagenesis coupled with studies of function, often after overexpression in a variety of cellular backgrounds. Some of these studies have raised questions regarding the requirements for direct protein–protein interactions between the anti- and pro-apoptotic proteins Bcl-2, Bcl-x_L, Bax, and Bak (7, 35, 45). How Bcl-2 family protein–protein interactions are regulated in response to cellular perturbation and how they then proceed to promote or inhibit cell death is unclear. Recent data suggest that posttranslational modifications of Bcl-2, specifically phosphorylation, may play a role, at least as cells are inhibited in passage through mitosis (13, 15, 18, 24, 25). Additionally, a change in phosphorylation of the BH3 domain protein Bad appears in some cells to be a key component linking survival signalling to the function of Bcl-x_L, so that damage-induced changes in Bad phosphorylation may affect survival (reviewed in 12). Presumably posttranslational modification results in protein conformational changes affecting protein–protein interactions and subsequent activity. It has also been shown that in certain cellular contexts, but not all, cell damage leads to an increase in the amount of proapoptotic family members, such as Bax (27), and that survival signalling can increase the amounts of anti-apoptotic molecules, such as Bcl-2 (31) or Bcl-x_L (6, 37). However, changes in protein synthesis are not universal and appear to be the exception rather than the rule (e.g., 5, 9).

Recently, in vitro experiments using cellular lysates have investigated the interactions between the death suppresser Bcl-x_L and pro-apoptotic Bax protein. It was suggested that the native state of Bax may be monomeric, unbound to either Bcl-x_L or Bcl-2 (17). Using a set of epitope-specific antibodies to different domains of the Bax protein it was concluded that an NH_2-terminal domain of Bax (amino acids 12–24) is concealed unless detergent is added, and that both homodimerization and its heterodimerization with Bcl-x_L only occur after a detergent-induced conformational change. Questions were raised in this study by Hsu and Youle (17) with respect to the validity of previous investigations where fusion proteins had been used to study protein–protein interactions amongst members of the Bcl-2 family as NH_2-terminal fusions may take on the detergent-induced conformation. The suggestion was made that a change in conformation of the native protein may be necessary to expose domains permissive for interaction. In fact, the Bax–Bax interactions first reported by Boise et al. (3) were only obtained under conditions where detergent manipulation was used. The results of Hsu and Youle (17) suggest that Bax, and potentially other members of this family of proteins, is dependent for its interactions and possibly for its activity, on conformational changes involving domains at the NH_2 terminus.

We considered that damage signals, generated after toxin treatment of cells, could initiate conformational changes in Bcl-2 family members to affect their function. We wanted to establish whether any changes in conformation occurred in the native proteins (i.e., not tagged) in intact cells exposed to disparate types of cell damage or perturbation. Consequently, changes in antibody epitope availability, which may reflect conformational change, were monitored in single, drug-treated intact cells by flow cytometry (FCM)[1]. Changes in immunofluorescence measured in this way might represent either changes in epitope availability or in the amount of protein in the intact cell. A direct comparison of Western blotting and flow cytometric analysis of immunofluorescence using monoclonal or polyclonal antibodies to the pro-apoptotic protein Bak (11) was performed. Critically, changes in immunofluorescence were monitored before the appearance of apoptotic cells as we were interested in the events which commit a cell to death rather than its execution. FCM analysis showed that NH_2-terminal epitope-specific changes in Bak immunofluorescence followed early after exposure to very disparate toxic stimuli. This was associated with changes in protein–protein interactions.

Materials and Methods

Cell Culture, Drug Treatment, and Analysis of Apoptosis

The human Jurkat T-leukemic cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (GIBCO BRL). The human CEM-C7A T-leukemic cell line was grown in Optimem 1 medium (GIBCO BRL) supplemented with 5% FBS as were the CEM-C7A cells stably transfected with Bcl-2 (CEM-Bcl-2) or an empty control vector (CEM-Neo; reference 4). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells in a logarithmic phase of growth were resuspended at a density of 3×10^5 cells/ml in fresh medium before drug treatment. Etoposide, staurosporine, and dexamethasone were purchased from Sigma Chemical Co. Stock solutions of drugs in DMSO were stored at −20°C. Control cells received solvent alone. The final concentration of DMSO solvent in the culture medium never exceeded 1% (vol/vol), which was nontoxic to the cells. Mouse IgM anti-CD95 monoclonal antibody (mAb; clone CH-11) was purchased from Coulter Electronics Ltd. An irrelevant mouse IgM antibody (Coulter Electronics Ltd.) was used as negative control. The morphological changes of chromatin condensation, typical of apoptosis, were assessed by fluorescence microscopy after staining of cells with acridine orange (10 μg/ml) and the % apoptosis was scored after scoring at least 200 cells.

Analysis of Protein Expression by Western Blotting

After treatment, cells were washed twice in PBS, lysed in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM Na orthovanadate, 0.5% NP-40, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml trypsin inhibitor). Cell lysates (30 μg protein) were separated by SDS-PAGE (12% acrylamide) and transferred to a PVDF membrane (hybond-PVDF; Amersham Life

1. *Abbreviations used in this paper:* Cy3, indocarbocyanine; DMP, dimethylpimelimidate; FCM, flow cytometry; PFA, paraformaldehyde; zVAD-fmk, *N*-benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone.

Sciences). After blocking nonspecific binding sites overnight with 5% nonfat milk in TPBS (PBS, 0.1% Tween-20), the membrane was incubated for 2 h at room temperature with a 1/1,000 dilution of a murine anti–human Bak monoclonal antibody designated as Ab-1 and raised against the peptide sequence amino-acids 1–52 (AM03; Calbiochem-Novabiochem Ltd.), or Ab-2 (AM04; Calbiochem-Novabiochem Ltd.) also made to the same peptide, or murine anti–human Bcl-2 mAb, made to a peptide sequence of amino acids 41–54 (Dako Ltd.). To ensure equal loading and transfer, membranes were also probed for actin using the anti-actin mouse monoclonal AC-40 (Sigma Chemical Co.). The immunoreactive proteins were visualized using rabbit horseradish peroxidase-linked goat anti–mouse antibody (Dako Ltd.) and enhanced chemiluminescence (ECL; Amersham Life Sciences).

Flow Cytometric Analysis of Bak and Bcl-2-associated Immunofluorescence

At specific times after drug-treatment, cells (10^6) were fixed in paraformaldehyde (PFA; 0.25%) for 5 min at room temperature and washed three time in PBS. Cells were kept at 4°C until analysis, then centrifuged and incubated for 30 min with the anti-human Bak monoclonal antibodies Ab-1 or Ab-2, the anti-human Bcl-2 mAb (see above), a rabbit polyclonal anti-human Bak antibody (66026E: raised against truncated protein lacking the COOH terminus; PharMingen), or a mouse IgG2b isotype-specific mouse antibody raised against *Aspergillus niger* glucose oxidase, designated as the irrelevant Ab (Dako Ltd.). All antibodies were diluted 1 in 50 in PBS containing digitonin (500 μg/ml). After three washes in PBS, cells were incubated with FITC-labelled goat anti–mouse or anti–rabbit IgG secondary antibody, diluted 1 in 100 in PBS, for 30 min, washed twice in PBS and resuspended in 1 ml of PBS. Analysis was performed on a FACS® Vantage flow cytometer equipped with an Enterprise laser (Innova Technology, Coherent Inc.) set to excite at 250 mW using the 488-nm laser line. Green fluorescence (FITC, FL-1) was detected at 530 ± 30 nm. Fluorescence was acquired using logarithmic amplifiers. 10,000 cells were analyzed per sample at a flow rate of 300 cells/s.

The effect of coincubation with the broad spectrum caspase inhibitor zVAD-fmk (40 μM) on Bak Ab-1 (NH_2-terminal) immunofluorescence was analyzed by flow cytometric (FCM) in Jurkat cells before and after exposure to etoposide and in CEM cells before and after treatment with dexamethasone, both for 4 h.

CEM-Bcl-2 and CEM-Neo cells (4) were analyzed for immunofluorescence of Bak Ab-1 before and after exposure to etoposide for 4 h.

In order to quantitate the flow cytometric results obtained using Bak Ab-1, the raw data obtained were manipulated in the following way: (a) cells exhibiting a light scatter profile typical of apoptotic cells or cell debris were excluded from the analysis by electronic gating; (b) the median specific Bak-associated fluorescence (*f Bak*) was determined by subtraction of the median fluorescence of the parallel irrelevant antibody control sample (*f IgX*; the fluorescence of samples containing secondary antibody alone was closely similar to, and always less than *f IgX*); (c) *f Bak* was multiplied by the percentage of cells with fluorescence above *f IgX* to generate a figure designated *S*; (d) S_C for control untreated samples at a particular timepoint was subtracted from S_{toxin} for toxin-treated samples to generate a final fluorescence value designated θ. The mean value of θ was determined in two or more separate repeat experiments.

Analysis of Cellular Bak and Bcl-x_L Immunofluorescence by Microscopy

Control and etoposide-treated cells (10 μM) were fixed in PFA 0.25% for 5 min at room temperature and washed three times in PBS. Cells were incubated overnight at 4°C with anti-human Bak mAb (Ab-1, specific to the NH_2 terminus) and/or anti-human Bak polyclonal antibody (pAb-Bak; for details see above) and/or Bcl-x_L using the rabbit anti–human Bcl-x_L polyclonal antibody raised to residues 18–233 (pAb Bcl-x_L; Transduction Laboratories) diluted 1 in 50 in PBS-digitonin (500 mg/ml), washed in PBS and then incubated with either a FITC-conjugated goat anti–mouse IgG antibody diluted 1 in 100 in PBS or a goat anti–rabbit conjugated with indocarbocyanine (Cy3; Jackson Immunochemicals). Nuclei were stained with a solution of Hoechst 33342 (1 μg/ml). Cells were then suspended in Vectashield mounting medium (Vector Laboratories), pipetted dropwise onto a glass slide and observed by fluorescence microscopy using a Zeiss Axioskop microscope equipped with an epiilluminator and appropriate filters.

Subcellular Localization of Bak and Other Proteins

Cells were washed twice in ice-cold PBS and then resuspended at 5×10^7/ml in ice-cold lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 μg/ml aprotinin, and trypsin inhibitor 10 μg/ml). The cell suspension was homogenized in a Dounce homogenizer and centrifuged at 700 *g* for 7 min at 4°C. The pellet containing any remaining intact cells and nuclei (designated as N) was washed once in lysis buffer and the postnuclear supernatant was centrifuged at 10,000 *g* for 15 min. The resulting pellet, designated P10, was washed once in lysis buffer and the supernatant was subjected to ultracentrifugation at 100,000 *g* for 1 h to pellet the remaining membrane, termed P100. The remaining supernatant was the cytosolic fraction (designated S100). The fractions N, P10, and P100 were resuspended in 1 vol of lysis buffer. Each fraction was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting for Bak content. The relative purity of fractions was ascertained by Western blotting using the mouse anti-cytochrome oxidase IV mAb (Molecular Probes) as a marker of the mitochondria, the rabbit anti-calnexin pAb and a rat anti-KDEL peptide (anti-grp78) pAb (both from StressGen) as markers for endoplasmic reticulum, the mouse anti–human poly(ADP-ribose)polymerase mAb (Serotec) as a nuclear marker and the anti-procaspase-3 mAb (Transduction Laboratories) and a rabbit anti-aldolase (a kind gift of Dr. C. Schnarrenberger, University of Manchester, UK) to check the purity of the cytosolic fraction.

Immunoprecipitations

Immunoglobulins were bound to protein A–Sepharose for 1 h at room temperature in the Hepes buffer described below. Beads were sedimented and washed twice with 10 vol of 0.2 M sodium borate, pH 9, then cross-linked with 20 mM DMP for 30 min. Unreacted cross-linker was removed by washing beads with 0.2 M ethanolamine, pH 8. They were resuspended in PBS with 0.01% thimerosal (Sigma).

Jurkat cells were grown to a density of 10^6 cells/ml and treated with etoposide (10 μM) for 20 h, to ensure maximum exposure of the NH_2-terminal epitope of Bak. Control cells received solvent alone. Cells (1.2×10^8) were washed with PBS and then lysed in 2 ml of 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% NP-40, 40 μg/ml PMSF, 1 μg/ml leupeptin, and aprotinin. Lysis was carried out on ice for 30 min, with regular vortexing. A post-nuclear supernatant was prepared by centrifugation at 10,000 *g* for 10 min at 4°C. The resulting lysate was precleared with 200 μl of neat pig-serum, DMP (dimethylpimelimidate) cross-linked to protein A–Sepharose (Pharmacia), rotated at 4°C for 1 h to remove nonspecific IgG binding proteins, and the protein A beads recovered by centrifugation at 10,000 *g* for 5 min at 4°C. The supernatant was further cleared by the addition of 200 μl of protein A–Sepharose slurry (prepared by equilibrating in Hepes buffer according to the manufacturers protocol, and resuspended in two bed volumes), rotated at 4°C for 30 min, and removed by centrifugation at 10,000 *g* for 5 min at 4°C. The resulting cleared lysate was then used for immunoprecipitation of Bak and Bcl-x_L proteins.

Immunoprecipitations were performed at 4°C for 1–3 h. There was no significant difference in the results if these were left overnight. Each of the antibodies used for immunoprecipitation were cross-linked with DMP (as described above) onto the protein A–Sepharose media. Antibodies were as described above. Both the monoclonal and the polyclonal antibodies were used. In addition, mouse irrelevant (Dako X0931), and rabbit irrelevant (Dako X0903) antibodies were used as controls. The antibody/protein A–Sepharose matrix was sedimented before use to remove the storage buffer (PBS/thimerosal); Ab-1 (10 μg) and Bcl-x_L (20 μg) antibodies were added to 500 μl of lysate. The protein concentration for the Bak polyclonal was unknown and was used at 30 μl per immunoprecipitation.

The immunoprecipitated proteins were recovered from the lysate by centrifugation at 10,000 *g* for 5 min at 4°C. The pelleted Sepharose was washed 3 times with 1-ml vol of the Hepes buffer described above. The immunoprecipitated proteins were eluted from the antibody–bead complex by the addition of 100 μl of 3× SDS-PAGE sample buffer. The beads were vortexed thoroughly, sedimented at 15,000 *g*, and the supernatant boiled before SDS-PAGE analysis, loading 30 μl per lane. The beads could be boiled directly and no difference was seen in protein recovery. However, in some instances immunoglobulin heavy chain was also eluted from the beads. Lysates, precleared lysates, and immuno-precipitate-depleted lysates were analyzed by SDS-PAGE and Western blotting, as described above. The signals from the bands were quantitated using a Bio-Rad GS-700 image densitometer with manufacturer's software.

Two-Dimensional Gel Electrophoresis

Isoelectric focusing tube gels were prepared essentially as in Knowles (20). Tube gels were prepared by water displacement and allowed to polymerize. These were prefocused for 1 h at 200 V. Treated or untreated cells were lysed in urea sample buffer of 9 M urea, 2% ampholine 3-10, 100 mM DTT, 4% NP-40, and 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate). 500 μg of protein was routinely loaded per tube gel. Samples were then electrophoresed at 700 V for 16 h, then fine focused for 1 h at 1,000 V. Gels were incubated in equilibration buffer (0.5 M Tris-HCl, pH 6.8, 1 M glycerol, 1% SDS, with bromophenol blue to color) for 10–15 min before overlaying onto a 15% polyacrylamide slab gel. Two-dimensional electrophoresis was carried out followed by Western blotting, as detailed above.

Results

Kinetics of Apoptosis

The kinetics of apoptosis of Jurkat and CEM-C7A lymphoma cells were characterized after exposure to staurosporine, etoposide, and an agonistic antibody to CD95. Dexamethasone-induced apoptosis in CEM-C7A cells was also measured; the Jurkat cell line used did not respond to dexamethasone. Table I summarizes the apoptotic response of the two cell types to these agents acting at different cellular loci. These were protein kinase inhibition by staurosporine, protein-associated DNA single and double strand breaks induced by etoposide (by inhibition of topoisomerase II), activation of a known death inducing receptor CD95, and activation of glucocorticoid receptors and transcriptional changes by dexamethasone. Timepoints were then chosen for analysis of Bak content and immunofluorescence before and after the appearance of significant numbers of apoptotic cells.

Measurement of Bak Protein Content

Measurements of the cellular content of Bak were made by Western blotting at various times in Jurkat and CEM-C7A cells before and after the death-inducing stimuli. Bak was constitutively expressed in both lines, as determined by the use of the anti-Bak antibody Ab-1 (NH_2-terminal). The cellular content of Bak was unaffected by any of the toxic stimuli over the entire time course (Figs. 1 and 2, top panels). Two other antibodies to the Bak protein (another monoclonal antibody to the NH_2 terminus and the polyclonal, see Materials and Methods) similarly showed no change in the amount of Bak protein by Western blotting (data not shown). There was also no evidence for cleavage of Bak in any experiments. There were also no changes in Bcl-2 protein content in either cell line after toxin treatment (Fig. 1). Actin was used as a loading control.

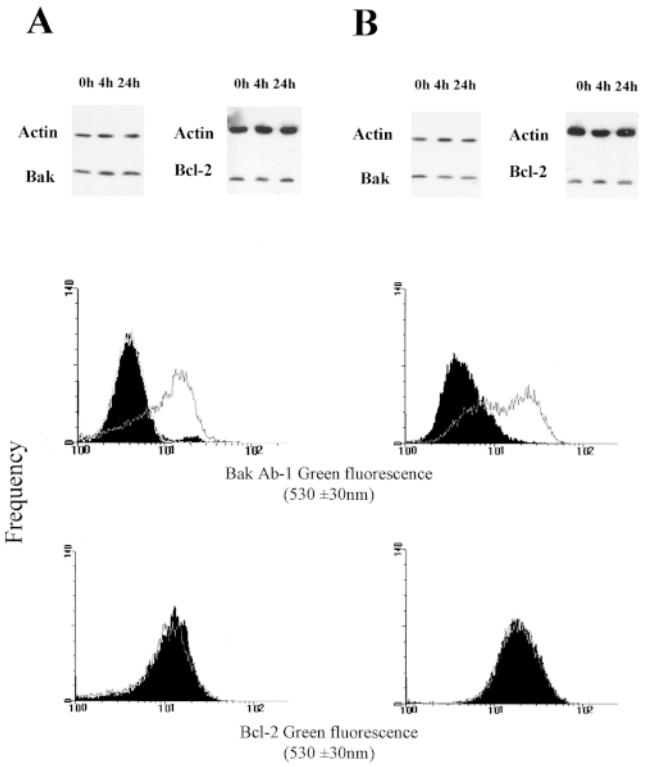

Figure 1. Protein levels of Bak measured by Western blotting using the monoclonal antibody Ab-1 to Bak in CEM-C7A (A, top) or Jurkat cells (B, top) after treatment with STS (250 nM) for the times indicated. Blots for Bcl-2 and actin are also shown. The results are representative of three independent experiments. The middle panels show FCM-generated frequency histograms of Bak Ab-1-associated fluorescence in control, untreated cells (filled histogram) and after STS treatment for 4 h (open histogram). The histogram for the irrelevant antibody control (gray line) is directly superimposable upon the control, untreated histogram. The bottom panels show equivalent data for Bcl-2-associated fluorescence; the histogram for the irrelevant antibody is in the first decade (not shown). Results shown are representative of at least three repeat experiments.

Table I. Kinetics of Apoptosis Induced by Etoposide, Dexamethasone, Staurosporine, or Anti-CD95 Monoclonal Antibody (CH-11) in Jurkat and CEM-C7A T-leukemic Cell Lines

	Time of treatment	Jurkat	CEM C7A
		AO apoptosis	
	h	*%*	
Control	0	1.2(±0.2)	0.3(±0.2)
Etoposide	4	7(±1)	1(±1)
	24	53(±6)	81(±13)
Dexamethasone	24	ND	2(±3)
	48	ND	50(±10)
Staurosporine	4	13(±3)	37(±9)
	24	48(±9)	79(±12)
Anti-CD95 mAb 1 ng/ml	24	55(±3)	22(±6)

Percentage of apoptosis was assessed by fluorescence microscopy after acridine orange (AO) staining of cells. Results are expressed as mean(±SD) of three independent experiments.

Epitope-specific Changes in Immunofluorescence of Bak after Treatment with Toxins Measured by Flow Cytometry

FCM was used to determine whether there were changes in the amount of immunofluorescence associated with Bak using the two epitope-specific antibodies made to the NH_2 terminus of Bak (Ab-1 and Ab-2, see Materials and Methods). Although no changes were observed by Western blotting (Fig. 1), it has been suggested elsewhere (2) that

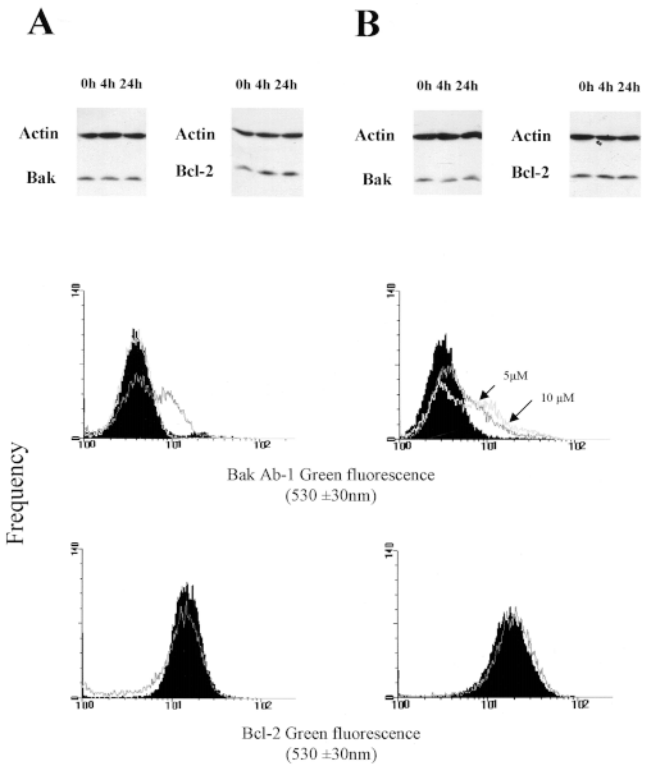

Figure 2. Protein levels of Bak measured by Western blotting using the monoclonal antibody Ab-1 to Bak in CEM-C7A (A, top) or Jurkat cells (B, top) after treatment with etoposide (10 μM) for the times indicated. Blots for Bcl-2 and actin are also shown. The results are representative of three independent experiments. The middle panels show FCM-generated frequency histograms of Bak Ab-1-associated fluorescence in control, untreated cells (filled histogram) and after etoposide treatment for 4 h (open histogram). The histogram for the irrelevant antibody control is directly superimposable upon the control, untreated histogram. The frequency histograms for Jurkat cells treated with two concentrations of etoposide (5 and 10 μM) are shown in the middle right panel. The bottom panels show equivalent data for Bcl-2-associated fluorescence; the fluorescence of the irrelevant antibody is in the first decade (not shown). Results shown are representative of at least three repeat experiments.

this may represent the limitations of a methodology which is population averaged and thus masks cellular heterogeneity. Using FCM there can be two potential explanations for changes in immunofluorescence: firstly they may indeed reflect changes in cell protein content, but secondly they may identify changes in epitope availability in intact cells without a change in protein content. The flow cytometric data shown are displayed as frequency histograms of Bak immunofluorescence, detected using a FITC-conjugated secondary antibody. The data are presented on a logarithmic scale and a shift to the right in the histogram indicates an increase in Bak-associated immunofluorescence. Fluorescence associated with Bak Ab-1 or Ab-2 (NH_2-terminal specific) was undetectable in intact untreated cells by flow cytometry and the histograms are superimposed upon those of an irrelevant antibody control (Fig. 1, middle). Bak Ab-1-associated immunofluorescence became detectable 4 h after treatment of both cell lines with staurosporine (STS; 250 nM; Fig. 1, middle). Similar results were obtained using Bak Ab-2. There was no change in the immunofluorescence associated with Bcl-2 using an antibody to amino acids 41–54 (Fig. 1, bottom).

Similarly, treatment with etoposide (10 μM) that irreversibly commits >90% of CEM-C7A cells to a loss of clonogenic survival after 30 min of exposure (data not shown), resulted in the appearance of Bak Ab-1 NH_2-terminal-associated immunofluorescence by 4 h, without any increase in the amount of protein, measured by Western blotting (Fig. 2 A). This was also seen with Jurkat cells (Fig. 2 B). The increase in NH_2-terminal epitope availability at 4 h was dependent on the concentration of etoposide added to Jurkat cells (Fig. 2 B). Thus increased amounts of etoposide-induced DNA damage, and presumably some DNA damage signal, induced more Bak immunofluorescence associated with an NH_2-terminal epitope of the protein. Again, analysis of Bcl-2-associated immunofluorescence, showed this to be unchanged after etoposide, as it was after STS treatment (compare Figs. 1 and 2). In all FCM experiments, cell debris, the cells that were overtly apoptotic at 4 h and cell aggregates were all electronically excluded (gated out) by virtue of altered light scatter signals, before examination of the Bak immunofluorescence profile. This may not remove all apoptotic cells, particularly those in the early stages. Nevertheless, the changes in Bak-associated immunofluorescence did not appear to be associated with the apoptosis per se, that is, with the execution phase of cell death (see also Table I). Some type of apoptosis-associated proteolytic cleavage of Bak to change epitope availability is also unlikely as we saw no change in the size of Bak protein on the Western blots (Figs. 1 and 2).

Dexamethasone also Changes Bak Ab-1 Immunofluorescence but Ligation of CD95 (Fas, APO-1) Does Not

The immunofluorescence profiles for Bak were examined after the addition of other death stimuli. Fig. 3 A shows that the non-genotoxic drug dexamethasone revealed the Bak Ab-1 epitope in CEM-C7A cells. In contrast, CEM-C7A cells treated with an agonistic antibody to the CD95/Fas/APO-1 receptor did not exhibit Bak immunofluorescence (Fig. 3 B) despite the induction of 22% apoptosis (Table I). Similar results were obtained after treatment of Jurkat cells with the anti-CD95 antibody (data not shown).

Exposure of the NH_2-terminal Epitope of Bak Occurs before Caspase Activation

In experiments using the broad spectrum caspase inhibitor zVAD-fmk, there was no effect on the increase in Bak-associated immunofluorescence (Fig. 3 C). Data were quantitated using an equation to derive the parameter θ (see Materials and Methods and below). The θ values were 583 ± 123 and 578 ± 120 (n = 3) for cells treated with etoposide in the presence or absence of zVAD-fmk, respectively. The caspase inhibitor effectively inhibited apoptosis in etoposide-treated cells to 6 ± 3% (n = 3).

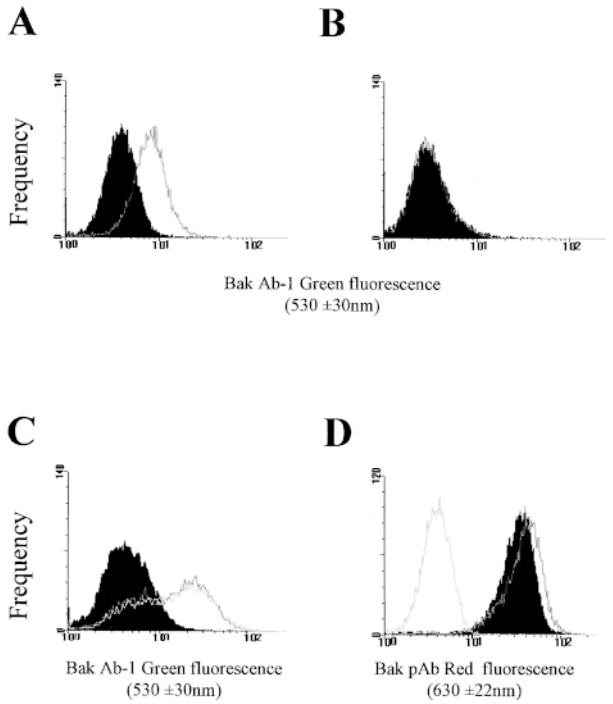

Figure 3. (A) FCM-generated frequency histograms of Bak Ab-1-associated fluorescence in CEM-C7A control, untreated (filled histogram) and after dexamethasone treatment for 24 h (open histogram). (B) FCM-generated frequency histograms of Bak Ab-1 after an anti-CD95 agonistic antibody (open histogram superimposed on control data). (C) FCM-generated frequency histograms showing absence of change in the increase in Bak Ab-1-associated immunofluorescence after staurosporine (STS; 250 nM for 4 h) with or without the caspase inhibitor zVAD-fmk (40 μM; filled histogram, irrelevant antibody; overlapping grey histograms, STS ± zVAD-fmk. (D) FCM-generated frequency histograms of Bak polyclonal antibody (pAb)-associated Cy3 fluorescence for control, untreated (filled histogram) and etoposide-treated cells (10 μM for 4 h; open histogram, in the second decade). The irrelevant antibody control is in the first decade (open histogram). In A–C the histogram for the irrelevant antibody control is directly superimposable upon the histogram for untreated cell. In D the irrelevant antibody is visible in the first decade. Results shown are representative of at least three repeat experiments.

Immunofluorescence Using a Polyclonal Antibody to Bak Is Detectable before and after Cell Perturbation

Critically, using the polyclonal antibody to Bak (pAb), raised against all but the transmembrane domain of the protein, immunofluorescence was detectable by FCM in both untreated and etoposide-treated cells (Fig. 3 D). A decade increase in fluorescence above that of the irrelevant antibody control was observed, with a slight increase after etoposide treatment. Further comparison of data obtained with the monoclonal and polyclonal antibodies is described below in Fig. 5. The ability of the polyclonal antibody (pAb) but not Bak Ab-1 antibody to bind Bak in untreated cells supports the idea that a selective change in epitope availability is occurring specifically at the NH_2 terminus of Bak after disparate toxin treatment and that this epitope is concealed in untreated cells. Additionally, the lack of change in immunofluorescence associated with Bcl-2 also supports the idea of a specific damage-induced change at the NH_2 terminus of Bak and not that this was an artefact of FCM.

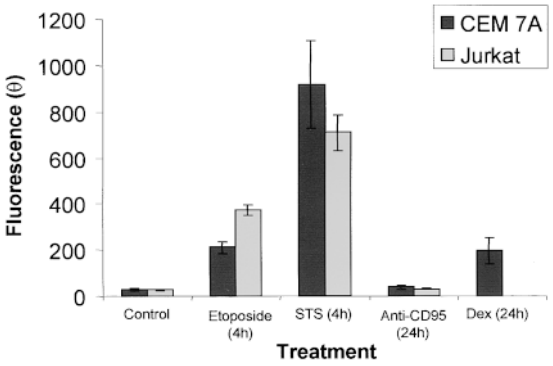

Figure 4. Quantitation of the increases in Bak Ab-1-associated fluorescence after treatment with various toxins as indicated using the equation for θ described in Materials and Methods.

Quantitation of Changes in Bak-associated Immunofluorescence

The changes in immunofluorescence measured by flow cytometry were quantified using an equation (see Materials and Methods) that essentially determines the area under curve (histogram) after subtraction of nonspecific fluorescence signals detected using an irrelevant antibody control to generate a value termed θ. The absolute increase in Bak Ab-1 immunofluorescence, calculating a θ value as described above, was different in magnitude for etoposide, STS and dexamethasone (Fig. 4), possibly reflecting differences in the kinetics of commitment to and onset of apoptosis, as discussed by us previously (40). For example, we have found that while STS induces changes in immunofluorescence throughout the cell cycle, etoposide does this only at specific cell cycle phases, presumably reflecting cell cycle checkpoint-associated signals (manuscript in preparation).

Bak Ab-1-associated immunofluorescence was detected in both CEM-Neo and CEM-Bcl-2 cells after treatment with etoposide. In two preliminary experiments, four hours after drug treatment the θ values for Bak Ab-1-associated immunofluorescence were 154 and 160 for the CEM-Bcl-2 cells compared with 216 and 210 for the CEM-Neo cells.

Microscopy Confirms that the Exposure of the Bak NH_2-terminal Epitope Is a Specific Event Occurring before Apoptosis

In order to test the hypothesis further that the NH_2-terminal epitope of Bak is exposed after perturbation but before the appearance of an apoptotic morphology, we exposed Jurkat cells to toxins and compared by microscopy the immunofluorescence of Bak Ab-1 and nuclear morphology by costaining with Hoechst 33342 (Fig. 5 A). Untreated cells did not stain positively for Bak using Bak Ab-1 (not shown), confirming the FCM data. Three patterns of

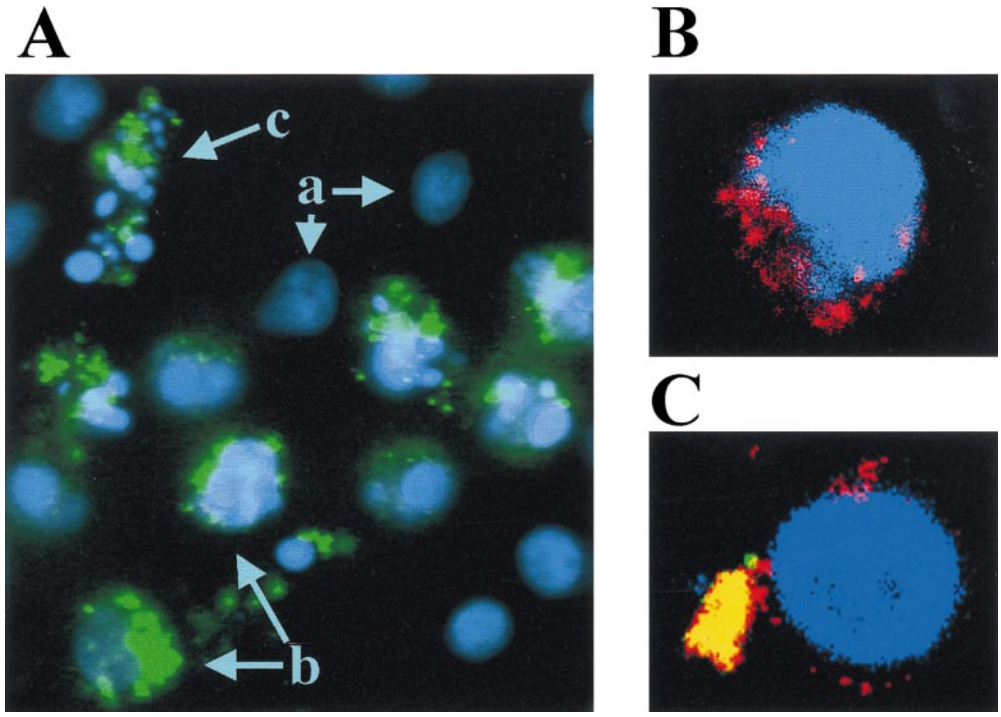

Figure 5. Analysis of Bak-associated immunofluorescence and nuclear morphology using fluorescence microscopy after treatment with etoposide (10 μM) for 4 h. (A) Bak Ab-1-associated fluorescence was visualized using an FITC-conjugated secondary antibody (green) and nuclear morphology was assessed by Hoechst 33342 staining (blue). (a) Cell with an intact nucleus and no Bak Ab-1 staining. (b) Cell with an intact nucleus but which show Bak Ab-1 staining. (c) Cell with a condensed and fragmented nucleus and Bak-associated Ab-1 fluorescence. (B) A control, untreated Jurkat cell dual stained with Hoechst 33342 and Bak polyclonal antibody (red). (C) An etoposide-treated Jurkat cell triple stained with Hoechst 33342, Bak polyclonal antibody (pAb; red) and Bak Ab-1 (green). Coincident staining for Bak Ab-1 and Bak pAb resulted in yellow fluorescence. Results shown are representative of three repeat experiments.

staining were observed 4 h after cells had been treated with etoposide (Fig. 5 A): there were some cells with an intact, non-apoptotic nucleus which remained Bak Ab-1 negative (a); others had an intact nucleus but stained positively for Bak Ab-1 (b). The remainder displayed Bak Ab-1 staining and exhibited fragmented and condensed chromatin (c). These data confirm that Bak Ab-1 fluorescence is induced before morphological indications of apoptosis, characterized by a loss of nuclear integrity. Fig. 5, B and C show Jurkat cells dual stained with both the polyclonal Bak antibody (red) and the Bak Ab-1 (green). B shows an untreated cell: it displays only polyclonal antibody red fluorescence in a punctate distribution. C shows a typical etoposide-treated Jurkat cell with predominantly yellow fluorescence indicating that at 4 h after drug treatment, the majority of Bak protein molecules appear to be dual stained with both antibodies. However, at this time, some Bak molecules remain detectable only by the polyclonal antibody. This may indicate the presence of different pools and/or isoforms of Bak which possess different antibody binding profiles (discussed further below). The staining is no longer punctate, implying the compaction of the mitochondria in a reduced cytoplasm, an established feature of apoptotic cells.

Colocalization of Bak and Bcl-x_L by Fluorescence Microscopy

To determine whether Bak and Bcl-x_L were colocalized in etoposide-treated Jurkat cells, cells were dual stained with Bak Ab-1 visualized with a FITC tagged secondary antibody (green) and with Bcl-x_L pAb visualized with a Cy3 tagged secondary antibody (red). It was not possible assess colocalization before etoposide treatment since the Bak Ab-1 epitope was unavailable (see Fig. 2) nor was it possible to dual stain with polyclonal antibodies for reasons of secondary antibody overlap. Fig. 6 A shows a typical untreated cell exhibiting Bcl-x_L immunofluorescence but no Bak Ab-1 staining. 4 h after etoposide treatment the cell population is heterogeneous, comprised of cells with intact nuclei and cells with fragmented nuclei and condensed chromatin. In Fig. 6 B, in a cell that has not yet undergone apoptosis, Bcl-x_L pAb and Bak Ab-1 are predominantly colocalized (observed as yellow fluorescence). In Fig. 6 C, where the cell is clearly apoptotic, the degree of colocalization appears to be decreased.

Subcellular Localization of Bak Protein after STS

The change in Bak immunofluorescence induced by etoposide and other agents represents the exposure of an otherwise cryptic epitope at the NH_2 terminus. This suggested the possibility that like Bax protein, which moves from cytosol to mitochondrial membranes on exposure of its NH_2 terminus (39), Bak may also change its cellular location following a damage signal. Therefore, we attempted to investigate the subcellular location of Bak and whether it was translocated in Jurkat cells after STS using cell fractionation methods similar to those used previously (36, 39). However, we were concerned about the purity of the mitochondrial (P10) and endoplasmic reticulum (P100) enriched fractions using these methods and consequently

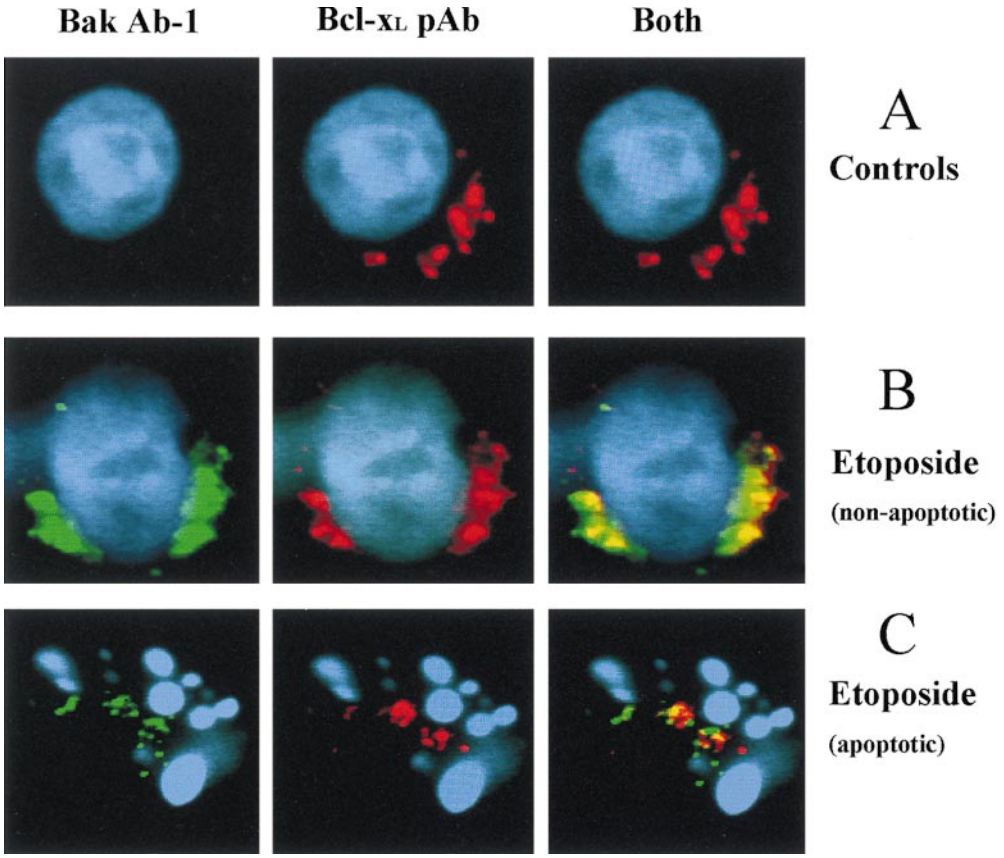

Figure 6. Immunofluorescence of Bak (Ab-1) visualized with a FITC-tagged secondary antibody (green) and Bcl-x_L (pAb) visualized with a Cy3 tagged secondary antibody (red) in Jurkat cells with or without etoposide (10 μM) for 4 h. (A) Untreated cells with an intact nucleus and staining for Bcl-x_L, showing no immunofluorescence associated with Bak (NH_2-terminal epitope cryptic). (B) Treated but pre-apoptotic cell with an intact nucleus showing substantial overlap in location of Bak and Bcl-x_L. (C) Etoposide-treated apoptotic cell, with fragmented nucleus showing less overlap of Bak and Bcl-x_L signals.

examined markers for each fraction. This showed that, using these methods, P10 and P100 both contained calnexin and grp78, both indicative of the presence of endoplasmic reticulum. Additionally, there was also some contamination of the nuclear fraction by the endoplasmic reticulum. However, procaspase 3 and aldolase were only present in the cytoplasmic fraction (S100). It is thus only possible to conclude that Bak protein was absent from the cytosolic fraction, before and after STS treatment, and that it was membrane associated (Fig. 7 A). Importantly, this differs from reports of the location of the congener Bax which is claimed to be cytosolic before signals which initiate apoptosis (17, 39). Due to the lack of purity in cellular subfractions generated by this protocol we also examined the cellular location of Bak and Bcl-x_L by fluorescence microscopy using organelle markers. Fig. 7 B shows that Bcl-x_L and Bak predominantly colocalized with the mitochondrial marker cytochrome oxidase IV and that neither Bak nor Bcl-x_L colocalized with the endoplasmic reticulum markers grp78 or calnexin, supporting the idea of mitochondrial association for both molecules.

Assessment of Changes in Binding of Bak to Bcl-x_L before and after Toxin Treatment

We considered that the exposure of the NH_2-terminal epitope of Bak after cellular perturbation might represent a conformational change and/or the release of a Bak binding partner to change epitope availability. Since Bcl-x_L has been shown to bind to Bak (7, 8, 11, 35) and we have shown that they colocalized in Jurkat cells by immunofluorescence (see above), we examined whether these two proteins were physically bound in untreated cells and whether their association was altered by toxin treatment. Immunoprecipitations were carried out using the Bcl-x_L polyclonal antibody, the polyclonal Bak antibody (which binds Bak in the presence and absence of toxin treatment), and Bak Ab-1 (NH_2 terminus, which only binds to Bak after toxin treatment). In initial experiments we attempted to coimmunoprecipitate Bak with Bcl-x_L in the absence of any detergent, which would mimic the conditions used for FCM where cells are fixed before detergent is added. Hsu and Youle (17) cautioned that the use of detergents in protocols for the immunoprecipitation of Bcl-2 family members might initiate conformational changes promoting altered patterns of protein–protein interactions. However, we were unable to immunoprecipitate either Bak or Bcl-x_L in the absence of detergent, as the proteins were not solubilized from the membrane fraction.

Using a low concentration of detergent (0.1% NP-40) it was possible to immunoprecipitate Bak using the Ab-1 NH_2-terminal antibody in control untreated cells (Fig. 8). Using Bak Ab-1 there was no difference in the ability to immunoprecipitate Bak comparing untreated cells with those treated with etoposide for 20 h to maximize epitope exposure at the NH_2 terminus (Fig. 8). These data are in contrast to the inability to detect Bak using this antibody in untreated cells by FCM and microscopy (Figs. 1, 2, and 4) and most likely reflect the ability of detergent to expose the Bak Ab-1 epitope. Immunoprecipitations performed

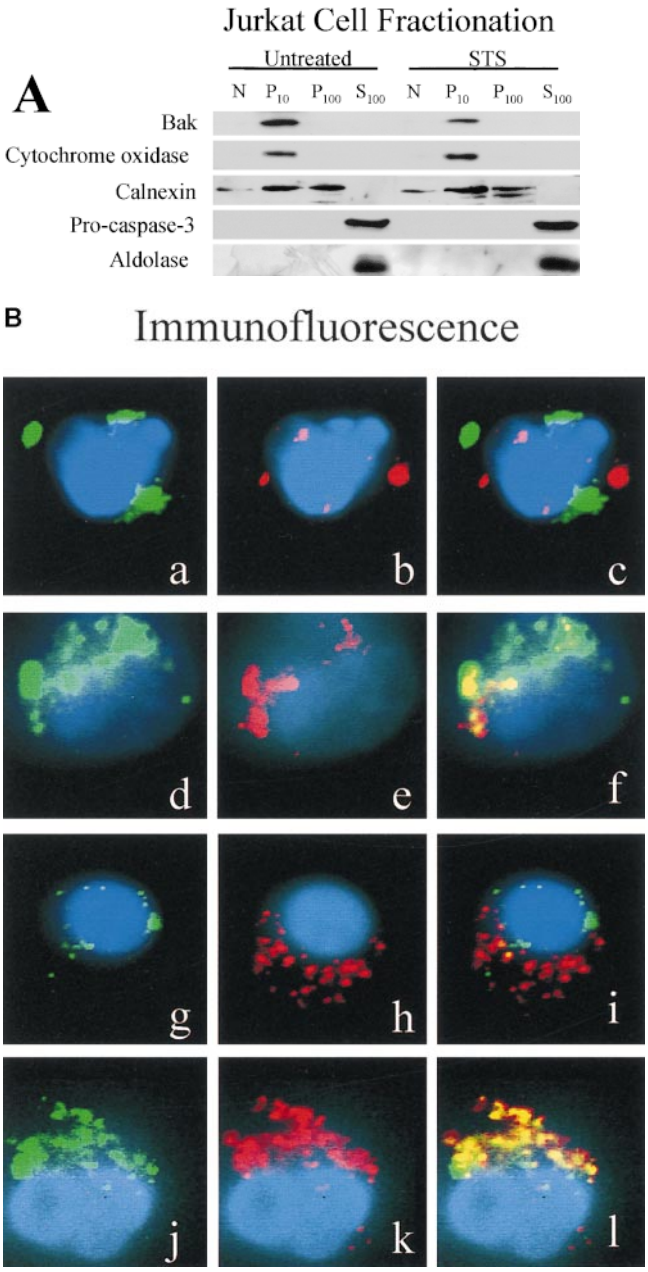

Figure 7. (A) Distribution of Bak in subcellular fractions from Jurkat cells measured by Western blotting. Subcellular fractions were enriched in: N, nuclei; P_{10}, mitochondria; P_{100}, endoplasmic reticulum (ER); and S_{100}, cytosol. Purity of the fractions was examined using markers of these organelles (cytochrome oxidase for mitochondria, calnexin, and grp78 for ER, and procaspase 3 and aldolase for cytosol). Results shown are representative of three repeat experiments. (B) Images of the immunofluorescence of Bak and from marker molecules characteristic for various subcellular compartments. a, grp78 (green); b and c, Bak pAb (red); d, cytochrome oxidase IV (green); e and f, Bak pAb (red); g, grp78 (green); h and i, Bcl-x_L, (red); j, cytochrome oxidase IV (green); k and l, Bcl-x_L, (red).

using a monoclonal or polyclonal antibody to Bak, or a polyclonal antibody to Bcl-x_L showed that Bak and Bcl-x_L were constitutively associated in control, healthy cells (Fig. 8). Analysis of the immunoprecipitates and supernatant lysates showed that using the Bak polyclonal antibody 100% of Bak was bound to Bcl-x_L. In contrast, analysis of the supernatants after immunoprecipitation with the Bak pAb showed that ~40% of Bcl-x_L remained unbound to Bak and was therefore in excess. When these immunoprecipitations were performed using etoposide-treated cells, the association between Bak and Bcl-x_L was clearly reduced (Fig. 8). In two independent experiments where we obtained very clearly defined bands after Western blotting, densitometry showed that a 30% and a 41% reduction of binding had occurred. The irrelevant antibody controls indicate that immunoprecipitations were epitope specific and not due to the type of immunoglobulin used (data not shown).

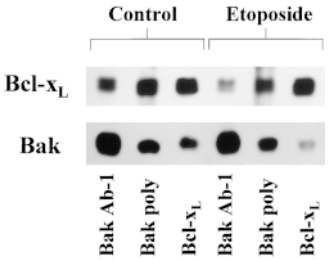

Figure 8. Investigation of the association of Bak with Bcl-x_L. Immunoprecipitations were performed using 0.1% non-ionic detergent (NP-40) before or after treatment of Jurkat cells with etoposide. The antibodies used are shown and are as described in Materials and Methods. The subsequent Western blots of the immunoprecipitated proteins were probed with either an antibody to Bcl-x_L (upper panel) or to Bak (lower panel).

Bak Exists in Multiple Isoforms Identified by Two-Dimensional Gel Electrophoresis and Is Unchanged after STS Treatment

To establish whether the changes in Bak NH_2-terminal-associated immunofluorescence were associated with a post-translational modification of Bak, we performed two-dimensional electrophoresis and Western blotting for Bak after treatment of Jurkat cells with STS, which produced the most substantial change in epitope availability. Fig. 9 shows that Bak exists in multiple forms based on charge differences in untreated cells, without major changes in mass. There were no discernible changes in protein mobil-

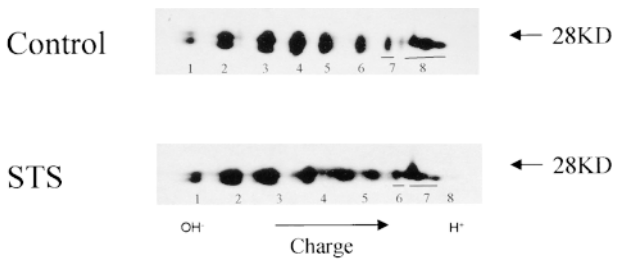

Figure 9. Representative Western blots from two-dimensional gel electrophoresis of cell lysates from control, untreated Jurkat cells, and those treated with STS (250 nM for 4 h), probed with anti-Bak mAb-1. The numbers show what were considered to be either discrete protein spots (1 to 7) or a group of unresolved proteins (8).

ity observed after STS treatment. Taken together, protein spots before or after treatment at positions 1 to 6 were very reproducible. However, the more acidic proteins at positions 7 and above were more variable from sample to sample, even in untreated cells, and we cannot exclude the possibility that subtle changes may be occurring which we are unable to resolve sufficiently. This is the subject of further experimentation. Similar data were obtained when cells were treated with etoposide.

Discussion

We have found that very different types of cellular perturbation, all of which induced apoptosis (Table I), brought about exposure of a specific epitope at the NH_2 terminus of the Bak protein. The epitope was completely concealed from antibodies raised to the NH_2 terminus in untreated cells (Figs. 1 and 2). Importantly, the damage-induced exposure of the epitope occurred before detection of both morphological and biochemical changes typical of apoptosis. This is particularly exemplified by the 24 h data for dexamethasone (Fig. 3 A) where we showed previously that caspase activity was undetectable until after 30 h of exposure (4). These alterations in Bak-associated immunofluorescence induced by dexamethasone also preceded the changes in mitochondrial membrane potential, occurring before caspase activation (4). Thus, the change in the availability of the NH_2 terminus of Bak is not a component of the execution phase per se, but presages it and is an early indicator that the cells had been perturbed or damaged. The specificity of this change in epitope availability was indicated by the findings that while the two different antibodies to the NH_2 terminus showed significant changes in immunofluorescence (Fig. 5 and Results), this was not observed when a polyclonal antibody was used (Fig. 3 D). The observation that totally disparate types of cellular perturbation initiated this change also suggests that either Bak itself or an associated molecule was acting as an integrator of damage signals arising from different types of disturbance in the cell, generated at different locations. The change in epitope availability observed here in lymphoid cells has been reproduced for Bak in both neuronal and epithelial cell lines and for an NH_2-terminal epitope of Bax in a B cell lymphoma line (our unpublished data).

In experiments where cells were treated with etoposide, which rapidly commits them to apoptosis, simultaneous probing with both the polyclonal and NH_2 terminus–targeted monoclonal antibodies to Bak showed that the immunofluorescence mostly overlapped (Fig. 6). There was a small proportion of Bak, recognized by the polyclonal antibody, that did not show toxin-induced exposure of the Bak NH_2 terminus. Whether this was a temporal phenomenon, arising from the cell cycle specificity of the damage signals generated by etoposide, or whether this represents some subpopulation of the Bak protein remains to be determined. Immunoprecipitation showed that, in the presence of some detergent, all of the Bak was bound to Bcl-x_L but this does not preclude heterogeneity. As discussed below, Bak protein is present in multiple, posttranslationally modified forms and it is possible that only some of these represent the active species, with others forming latent or inactive subpopulations, perhaps in the complex with Bcl-x_L.

Interestingly, the ligation of CD95, an effective initiator of apoptosis (Table I) did not bring about a change in the availability of the epitope at the NH_2 terminus of Bak (Fig. 3 B). This suggests that ligation of CD95 (APO-1/Fas) must act in some other way from the other toxins used to stimulate cell death. Whereas CEM-C7A cells transfected to overexpress *bcl*-2 were afforded significant delay in the onset of apoptosis induced by etoposide, and complete protection of the loss of clonogenicity induced by dexamethasone (4), over expression of Bcl-2 protein completely failed to inhibit apoptosis induced by the ligation of CD95 (our unpublished data). It has been shown recently that apoptosis induced by CD95 ligation may proceed either via activation of CD95-associated FADD and FLICE (caspase 8), with subsequent caspase activation, a process which is not suppressed by Bcl-2, or via Daxx and the activation of the JNK pathway. The Daxx pathway to apoptosis is Bcl-2 inhibited in certain cells (43). We are unable to state how CD95 ligation activated apoptosis occurs in these CEM-C7A cells. Whatever the precise pathway used by CD95, unlike the damage signals arising from treatment with etoposide, dexamethasone and staurosporine (Figs. 1–4), it did not bring about a change in the immunofluorescence of Bak. This supports the idea that the signalling cascade for the initiation of apoptosis by CD95 differs from that imposed by the toxins.

When *bcl*-2 overexpressing CEM-C7A cells (4) were challenged with etoposide there was only a 30% reduction in the exposure of the NH_2-terminal epitope of Bak, measured by a fall in the θ value (see Results). Thus overexpression of *bcl*-2 somehow either attenuates the damage signal that reveals the Bak NH_2-terminal epitope or it changes the protein–protein interactions between Bak and its partners. Bak preferentially binds to Bcl-x_L (11) and in immunoprecipitations of Bak we found no association with Bcl-2 (Savory, P., unpublished results). However, Bcl-2 overexpression may change the stoichiometry of the protein complex containing Bak, but until the components of that complex have been identified, no mechanistic explanation can be offered to explain the reduction of the exposure of the Bak NH_2 terminus by Bcl-2.

How does the NH_2-terminal epitope of Bak, recognized by Ab-1, become exposed after damage? This could be the result of direct changes in the conformation of Bak and/or due to the release of a protein(s) that conceals the NH_2-terminal epitope. Is Bak itself the recipient of damage signals, perhaps becoming posttranslationally modified, so as to change conformation? Or, are damage signals received by accessory proteins, which then bring about the dissociation of protein(s) from the NH_2-terminal domain of Bak, exposing this epitope? And how do these changes contribute to the irreversible commitment of the cell to subsequent apoptosis? Hsu and Youle (17) reported changes in epitope availability of murine Bax, again specifically at the NH_2 terminus, after addition of nonionic detergent to cell lysates. They proposed a model whereby the detergent-induced changes in Bax epitope availability, observed in lysates, equated to a change in conformation. This brought about different patterns of protein–protein interactions. An open NH_2 terminus of Bax promoted Bax homodimerization and heterodimerization with Bcl-x_L (17). Additionally, it was suggested that the detergent-induced confor-

mational change in Bax mimics the conformation that Bax adopts when it becomes located in the mitochondrial membrane after translocation from the its monomeric form in cytosol to a membrane-bound, dimeric form. When associated with the mitochondrial membrane, Bax-Bcl-x_L heterodimerization may then occur (17). Elegant studies of murine Bax by Gross et al. (14) confirm that aspects of this model for Bax translocation from cytosol to mitochondria occur in whole cells. Using protein cross-linking agents they showed that monomeric Bax translocated to mitochondria after an apoptotic stimulus (IL-3 withdrawal) where it then homodimerized. Manipulation of Bax to enforce homodimerization resulted in translocation to mitochondrial membranes and in apoptosis (14).

Our results, which examine endogenous human Bak in intact cells, show that there are distinct differences between the phenomena observed with Bak compared with those reported for the murine Bax protein either in lysates or murine cells. As discussed above, in cells that were fixed before the use of any detergent, the NH_2 terminus of Bak became available to the antibody Ab-1 after different types of cell damage. We considered that this might promote a change in its subcellular location in a manner similar to that suggested for Bax using cell lysates and, subsequently, a change in its association with Bcl-x_L (17). The subcellular fractionation experiments and immunofluorescence showed that Bak was not cytosolic before or after toxin treatment (Fig. 7 A), rather both were more closely associated with mitochondria, before and after perturbation (Fig. 7 B). Taken together our data show that, unlike the scenario for murine Bax, an apoptotic stimulus does not induce a change in subcellular location of Bak. Signals derived from cellular perturbation by toxins are thus presumably integrated at intracellular membrane locations.

When we performed immunoprecipitations, with a low concentration of nonionic detergent present, using either Ab-1 or the polyclonal antibody to Bak or the polyclonal antibody to Bcl-x_L, probing the subsequent Western blots showed that Bak-Bcl-x_L binding occurred constitutively in healthy cells (Fig. 8). Lysates prepared from these cells treated with etoposide to induce maximal unmasking of the NH_2 terminus of Bak, showed a 30–40% decrease in Bak-Bcl-x_L binding (Fig. 8). Increasing the detergent concentration from 0.1 to 1% did not alter this result (data not shown). Therefore, toxin treatment did not recapitulate for Bak the detergent-mediated promotion of Bax association with Bcl-x_L, which had been reported using murine cell lysates (17). Instead, Bak and Bcl-x_L appear to be constitutively bound in healthy cells and a proportion of Bak became dissociated as the cells committed to apoptosis, although it remained membrane bound. The damage induced conformational change in Bak, either at the NH_2 terminus itself, or as other domains then undergo subsequent topological changes, therefore modulates the stoichiometry of binding of these particular pro- and anti-apoptotic molecules.

What changes are taking place in this Bak-Bcl-x_L complex as the cells commit to apoptosis? And how are these involved in the commitment of cells to death? Using transient transfections, a mutational analysis of Bak had suggested that its BH3 domain was required both for binding to Bcl-x_L and for its pro-apoptotic activity, implying that heterodimerization may be necessary for the induction of apoptosis (8). Our data are compatible with a model in which death may be suppressed by heterodimerization (Bak and Bcl-x_L constitutively bound) and where the dissociation of Bak from Bcl-x_L correlates with promotion of apoptosis. Using stably transfected Bak mutants it was found that cytotoxic drug-induced apoptosis of Fl5.12 cells was accelerated even when the BH3 domain of Bak was mutated, an event that abrogated its binding to Bcl-x_L (35). This suggested that Bak works to kill cells independently of its binding to Bcl-x_L. In a complementary study, Cheng et al. (7) showed that mutations of Bcl-x_L could be made which retained most of their anti-apoptotic effect yet did not bind Bax or Bak as effectively in immunoprecipitations. These latter studies support growing evidence that heterodimerization of some of the pro- and anti-apoptotic members is not required for function (10, 21, 38). Thus, although the heteromeric binding of these two molecules has been elegantly modeled in vitro (34) it may be that the Bak responsible for apoptosis is in a cellular pool different from that bound to Bcl-x_L and that this is increased after cell perturbation. The questions of Bak population heterogeneity, is all the Bak associated with Bcl-x_L and whether the Bak-Bcl-x_L interaction actually does occur in vivo, are difficult to resolve. The essential use of detergent to immunoprecipitate these membrane proteins has been shown to induce conformational changes that promote heteromeric associations, at least in lysates containing Bax and Bcl-x_L (17). It might be argued that the results of the immunoprecipitations shown in Fig. 8, and our finding that all the Bak was constitutively associated with Bcl-x_L in untreated cells, may be the consequence of detergent use. Significantly, however, they do show that the association of Bak with Bcl-x_L is changed after toxin treatment. And, critically, we observed a dissociation of pro- and anti-apoptotic molecules following damage and the exposure of Bak's NH_2 terminus, in complete contrast with the association observed between Bax and Bcl-x_L that followed the exposure of the NH_2 terminus of Bax (17). The generality of some of the models for changes in heteromerization that presage apoptosis are therefore questionable.

Two-dimensional electrophoretic analysis of Bak, which surprisingly showed considerable posttranslational modification of Bak, allows speculation that different isoforms may contribute to a heterogeneity of associations in membranes (Fig. 9). This remains to be determined. We had originally considered that toxin-induced exposure of an epitope at the NH_2 terminus of Bak may induce or alter some of the posttranslational modifications of the Bak protein. This would represent an event whereby there was an integration of signals arising from different types of cellular damage directly at the Bak molecule itself which would then contribute to changes in conformation and/or protein–protein interactions. However, we could detect no obvious change in the pattern of isoforms after toxin treatment, for example with STS (Fig. 9) or etoposide (data not shown). This suggests that damage signals are not received directly by Bak but at some associated protein(s) which may promote a change in Bak conformation and/or interactions of Bak with other proteins, including Bcl-x_L. The association of accessory protein(s) in a complex with Bak

is presumably changed once the NH_2 terminus has been exposed.

In summary, FCM has permitted, for the first time, an analysis of changes in protein conformation and/or of protein–protein binding in intact cells expressing endogenous levels of native Bak protein. Events at the NH_2 terminus of Bak involving changes in protein–protein interactions are suggested to be important for the integration of damage signals and to the subsequent commitment of these human lymphoid cells to apoptotic death. Precise characterization of the protein(s) bound to the NH_2 terminus may clarify both how damage signals become integrated at the locus of Bak and how this subsequently commits a cell to die.

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