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-CL1 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 nonionic 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 suppressors 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...
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 “apoptsatz,” 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 homodimerization 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-xL, 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-xL, 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-xL (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 suppressor Bcl-xL and pro-apoptotic Bax protein. It was suggested that the native state of Bax may be monomeric, unbound to either Bcl-xL or Bcl-2 (17). Using a set of epitope-specific antibodies to different domains of the Bax protein it was concluded that an NH2-terminal domain of Bax (amino acids 12–24) is concealed unless detergent is added, and that both homodimerization and its heterodimerization with Bcl-xL 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 NH2-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 NH2 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 death 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 NH2-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% CO2. Cells in a logarithmic phase of growth were resuspended at a density of 3 × 105 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-CD 95 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; A mersham Life

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

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

A specific time after drug treatment, cells (10^6) were fixed in paraformaldehyde (PFA; 0.25%) for 5 min at room temperature and washed three times in PBS. Cells were kept at 4°C until analysis, then centrifuged and incubated for 30 min with the anti-human Bak monoclonal antibodies A b-1 or A b-2. The anti-human Bcl-2 mAb (66026E: raised against the peptide sequence amino-acids 1–52 (Dako Ltd.), or A b-2 (A M04; Calbiochem-Novabiochem Ltd.) also made to the same peptide, or murine anti-human Bcl-2 mAb, made to a peptide sequence amino-acids 41–54 (Dako Ltd.). To ensure equal loading and transfer, membranes were also probed with fluorescence using the anti-human mouse monoclonal A C-40 (Sigma Chemical Co.). The immunoreactive proteins were visualized using rabbit horseradish peroxidase-linked goat anti-mouse antibody (Dako Ltd.) and enhanced chemiluminescence (ECL; A mersham Life Sciences).

Subcellular Localization of Bak and Other Proteins

Cells were washed twice in ice-cold PBS and then resuspended at 5 × 10^6/ml in ice-cold lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 15 mM MgCl_2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM MgCl_2, 10 µg/ml aprotinin, and trypsin inhibitor 10 µg/ml). The cell suspension was homogenized and centrifuged at 700 g for 10 min at 4°C. The supernatant was the cytosolic fraction (designated S100). The cytosolic fraction was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting for Bak content. The relative purity of fractions was assayed 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(A-D-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, U niversity of Manchester, U K) to check the purity of the cytosolic fraction.

Immunoprecipitations

Immunglobulins 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 × 10^7) were washed with PBS and then lysed in 2 ml of ice-cold 100 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% NP-40, 40 µg/ml PMSF, 1 µg/ml leupeptin, and apro- tinin. Lysate 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 preclarified 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-xL 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 sedimentsed before use to remove the storage buffer (PBS/thimerosal); Ab-1 (10 µg) and Bcl-xL (20 µg) antibodies were added to 500 µl of lystate. The protein concentration for the Bak polyclonal was unknown and was used at 30 µl per immunoprecipitation.

The immunoprecipitated proteins were recovered from the lystate 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 being subjected to SDS-PAGE analysis, loading 30 µl per lane. The bands 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, preclarified lysates, and immuno-precipitate-depleted lysates were analyzed by SDS-PAGE and Western bloting, 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. Time-points 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 (NH₂-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 NH₂ 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.

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 NH₂ terminus of Bak (A b-1 and A b-2, see Materials and Methods). Although no changes were observed by Western blotting (Fig. 1), it has been suggested elsewhere (2) that

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 (h) | Jurkat | CEM-C7A |
|-----------------------|--------|---------|
| Control               | 0      | 1.2(±0.2) | 0.3(±0.2) |
| Etoposide             | 4      | 7(±1) | 1(±1) |
| Dexamethasone         | 24     | 53(±6) | 81(±13) |
| Staurosporine         | 48     | ND     | 2(±3) |
| Anti-CD95 mAb 1 ng/ml | 24     | 19(±3) | 37(±9) |

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.
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 3 repeat experiments.

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 A b-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-CD 95 antibody (data not shown).

Exposure of the NH₂-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).
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 A b-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.

**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 $\theta$. The absolute increase in Bak A b-1 immunofluorescence, calculating a $\theta$ 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 $\theta$ 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 A b-1 and nuclear morphology by costaining with Hoechst 33342 (Fig. 5 A). Untreated cells did not stain positively for Bak using Bak A b-1 (not shown), confirming the FCM data. Three patterns of
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 A b-1 (b). The remainder displayed Bak A b-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-xL by Fluorescence Microscopy**

To determine whether Bak and Bcl-xL were colocalized in etoposide-treated Jurkat cells, cells were dual stained with Bak A b-1 visualized with a FITC tagged secondary antibody (green) and with Bcl-xL pAb visualized with a Cy3 tagged secondary antibody (red). It was not possible to assess colocalization before etoposide treatment since the Bak A b-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-xL immunofluorescence but no Bak A b-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-xL pAb and Bak A b-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 NH2 terminus. This suggested the possibility that like Bax protein, which moves from cytosol to mitochondrial membranes on exposure of its NH2 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
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-xL by fluorescence microscopy using organelle markers. Fig. 7 B shows that Bcl-xL and Bak predominantly colocalized with the mitochondrial marker cytochrome oxidase IV and that neither Bak nor Bcl-xL 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-xL before and after Toxin Treatment**

We considered that the exposure of the NH2-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-xL 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-xL polyclonal antibody, the polyclonal Bak antibody (which binds Bak in the presence and absence of toxin treatment), and Bak Ab-1 (NH2-terminal epitope cryptic). (B) Treated but pre-apoptotic cell with an intact nucleus showing substantial overlap in location of Bak and Bcl-xL. (C) Etoposide-treated apoptotic cell, with fragmented nucleus showing less overlap of Bak and Bcl-xL signals.
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using a monoclonal or polyclonal antibody to Bak, or a polyclonal antibody to Bcl-xL showed that Bak and Bcl-xL were constitutively associated in control, healthy cells (Fig. 8). Analysis of the immunoprecipitates and superna-

Figure 7. (A) Distribution of Bak in subcellular fractions from Jurkat cells measured by Western blotting. Subcellular fractions were enriched in: N, nuclei; P10, mitochondria; P100, endoplasmic reticulum (ER); and S100, 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-xL (red); j, cytochrome oxidase IV (green); k and l, Bcl-xL (red).

Figure 8. Investigation of the association of Bak with Bcl-xL. 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-xL (upper panel) or to Bak (lower panel).

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).

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

To establish whether the changes in Bak NH2-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 8. Investigation of the association of Bak with Bcl-xL. 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-xL (upper panel) or to Bak (lower panel).
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 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$_2$ terminus of Bak preferentially bound to Bcl-xL (11) and 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 another 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 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$_2$ terminus of Bak preferentially bound to Bcl-xL (11) and in a B cell lymphoma line (our unpublished data). The Journal of Cell Biology, Volume 144, 1999 912
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-xL 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 NH2 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-xL (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-xL, probing the subsequent Western blots showed that Bak-Bcl-xL binding occurred constitutively in healthy cells (Fig. 8). Lysates prepared from these cells treated with etoposide to induce maximal unmasking of the NH2 terminus of Bak, showed a 30–40% decrease in Bak-Bcl-xL 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-xL, which had been reported using murine cell lysates (17). Instead, Bak and Bcl-xL 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 NH2 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-xL 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-xL 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-xL constitutively bound) and where the dissociation of Bak from Bcl-xL correlates with promotion of apoptosis. Using stably transfected Bak mutants it was found that cytotoxic drug-induced apoptosis of FLS.12 cells was accelerated even when the BH3 domain of Bak was mutated, an event that abrogated its binding to Bcl-xL (35). This suggested that Bak works to kill cells independently of its binding to Bcl-xL. In a complementary study, Cheng et al. (7) showed that mutations of Bcl-xL 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 maybe that the Bak responsible for apoptosis is in a cellular pool different from that bound to Bcl-xL and that this is increased after cell perturbation. The questions of Bak population heterogeneity, is all the Bak associated with Bcl-xL and whether the Bak-Bcl-xL 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-xL (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-xL in untreated cells, may be the consequence of detergent use. Significantly, however, they do show that the association of Bak with Bcl-xL 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 NH2 terminus, in complete contrast with the association observed between Bax and Bcl-xL that followed the exposure of the NH2 terminus of Bax (17). The generality of some of the models for changes in heteromerization that preface 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 NH2 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-xL. 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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