JCB Article

Spatial and temporal changes in Bax subcellular localization during anoikis

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Bax, a member of the Bcl-2 family, translocates to mitochondria during apoptosis, where it forms oligomers which are thought to release apoptogenic factors such as cytochrome *c*. Using anoikis as a model system, we have examined spatial and temporal changes in Bax distribution. Bax translocates to mitochondria within 15 min of detaching cells from extracellular matrix, but mitochondrial permeabilization does not occur for a number of hours. The formation of Bax oligomers and perimitochondrial clusters occurs concomitant with caspase activation and loss of mitochondrial membrane potential, before nuclear condensation. Cells can be rescued from apoptosis if they are replated onto extracellular matrix within an hour, whereas cells detached for longer could not. The loss of ability to rescue cells from anoikis occurs after Bax translocation, but before the formation of clusters and cytochrome *c* release. Our data suggest that Bax regulation occurs at several levels, with formation of clusters a late event, and with critical changes determining cell fate occurring earlier.

Introduction

Apoptosis is a mechanism for removing unwanted or damaged cells. Its endpoint is the activation of a family of proteases (the caspases) which are responsible for the morphological hallmarks of cell death (Green, 2000). However, the initial decision to embark on the apoptotic pathway occurs considerably earlier, and before a cell can be distinguished from a neighbor that will not die. Diverse insults can set a cell on this pathway. Cytotoxic drugs, UV irradiation, and exposure to ligands for transmembrane death receptors (such as FAS or TNF-R) are essentially positive apoptotic signals from which cells cannot be rescued. Conversely, cells also require the presence of survival signals to suppress apoptosis, such as growth factors or adhesion to ECM (Gilmore et al., 2000). Removal of survival factors leads to apoptosis, but unlike cytotoxic signals, cells are not irreversibly committed, and can be rescued if signaling is restored in time. This provides an opportunity to examine the molecular events associated with commitment.

Proapoptotic Bax and Bak are essential regulators of the mitochondrial pathway of apoptosis (Lindsten et al., 2000; Ranger et al., 2001; Wei et al., 2001). Bak resides permanently on the outer mitochondrial membrane (OMM), whereas Bax is normally found in the cytosol of healthy cells and translocates to the OMM during apoptosis (Hsu et al., 1997; Wolter et al., 1997; Goping et al., 1998; Gross et al., 1998). Both undergo a conformational change, which may be an obligatory step in their proapoptotic function (Desagher et al., 1999; Griffiths et al., 1999; Nechushtan et al., 1999). In the case of Bax, transition to this alternative conformation correlates with translocation to mitochondria.

Bax and/or Bak are required for mitochondrial membrane permeabilization (MMP; Wei et al., 2001). How MMP occurs is controversial, and a number of possible models have been proposed (Martinou and Green, 2001; Zamzami and Kroemer, 2001). During apoptosis, mitochondrial Bax forms large complexes, whereas cytosolic Bax is a monomeric 21-kD protein (Antonsson et al., 2000, 2001; Eskes et al., 2000; Nechushtan et al., 2001). The role of these complexes is unclear, but current models suggest that they regulate mitochondrial morphology and permeability via existing OMM proteins, or by forming de novo channels (Jurgensmeier et al., 1998; Narita et al., 1998; Martinou and Green, 2001; Zamzami and Kroemer, 2001). Ultimately, they cause mitochondria to release a host of factors from the intermembrane space, including cytochrome *c*, SMAC/Diablo, and apoptosis-inducing factor, leading to

The online version of this article includes supplemental material.

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Key words: anoikis; apoptosis; Bax; mitochondria; caspases

Abbreviations used in this paper: MMP, mitochondrial membrane permeabilization; OMM, outer mitochondrial membrane.

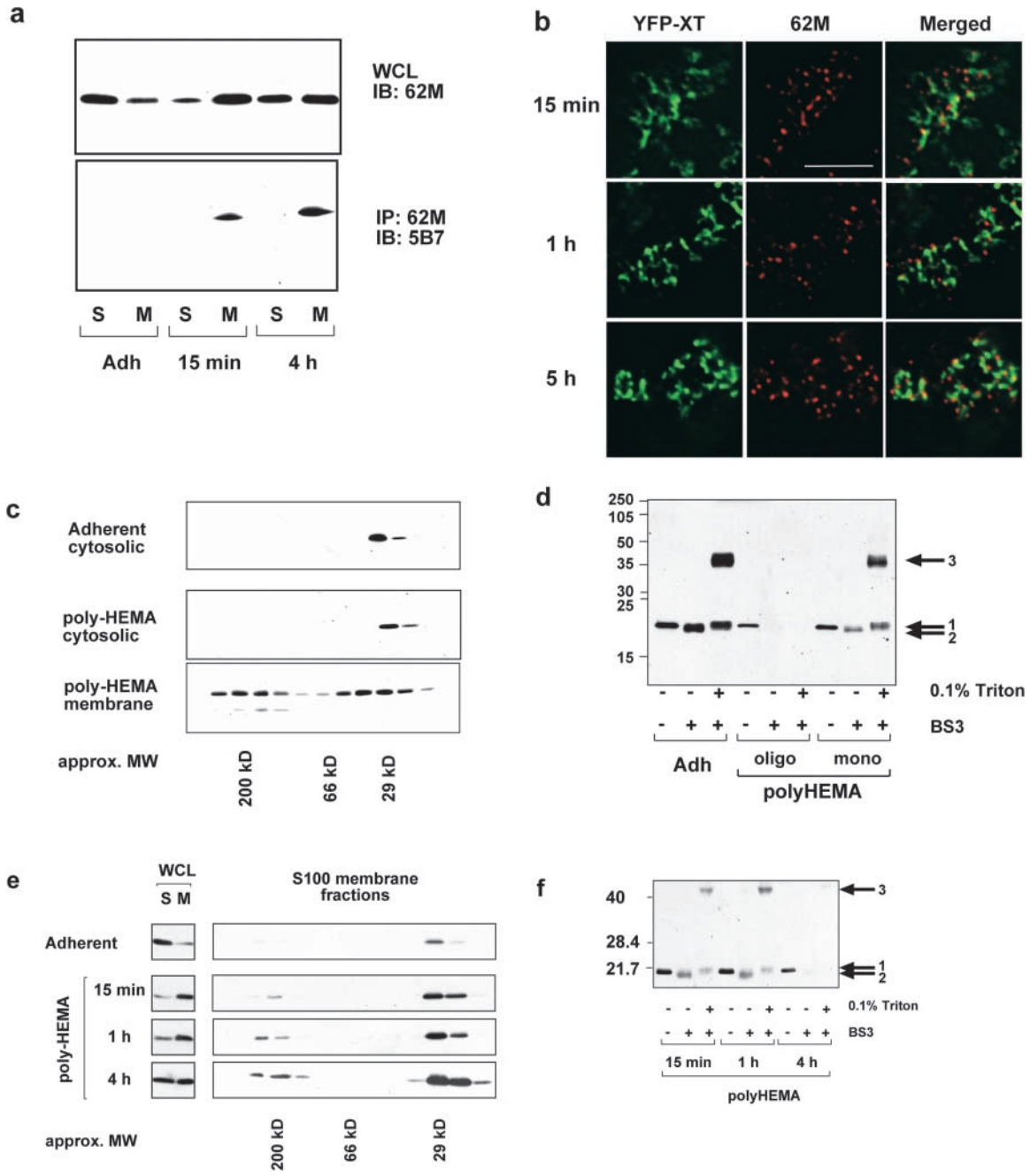


Figure 1. **Bax translocates to mitochondria and forms high mol wt oligomers during anoikis.** (a) FSK-7 cells, adherent or detached (maintained on poly-HEMA for 15 min or 4 h), were separated into soluble (S) and membrane (M) fractions before SDS-PAGE and immunoblotting with polyclonal anti-Bax (top). 4 mM CHAPS was added, and Bax was immunoprecipitated with anti-Bax 62M. Immunoprecipitates were separated by SDS-PAGE and were immunoblotted with anti-Bax 5B7 (bottom). (b) Cells expressing the OMM marker YFP-XT were detached for various times before cytospinning, and were immunostained with the activation-dependent antibody 62M. 62M reactivity is seen at discrete regions on mitochondria at all time points. Bar, 5 μm. (c) Soluble and membrane fractions from adherent cells or cells maintained on poly-HEMA for 4 h were isolated as above. CHAPS was added to a final concentration of 4 mM, and the fractions were concentrated. 5 mg protein from each fraction was loaded onto a Sephacryl S100-HR column (see Materials and methods). Separated fractions were analyzed by SDS-PAGE and immunoblotting for Bax. (d) Membrane fractions from FSK-7 cells detached for 4 h were separated by size-exclusion chromatography as above. Fractions containing high (oligo) and low (mono) mol wt Bax complexes were collected. Fractions were concentrated and separated into three equal parts. These were treated with BS3 (with or without addition of 0.1% Triton X-100). Samples were separated by SDS-PAGE and were immunoblotted for Bax. Cytosolic Bax from adherent cells (Adh) was run for comparison after identical treatment. (e) Membrane fractions isolated from adherent cells or cells detached for various times and separated by size-exclusion chromatography were analyzed by SDS-PAGE and immunoblotting for Bax. Total soluble (S) and membrane (M) fractions show Bax translocation at each time point. (f) Membrane fractions prepared from adherent cells or cells detached for 15 min, 1 h, or 4 h were spilt into three and treated with 5 mM BS3 in the presence or absence of 0.1% Triton-X100. Samples were analyzed by SDS-PAGE and were immunoblotted for Bax.

cell death (Degterev et al., 2001; Waterhouse et al., 2002). Mitochondrial fission also occurs at this time, in which Bax has also been implicated (Frank et al., 2001; Karbowski et al., 2002).

Inhibition of adhesion in epithelial cells leads to apoptosis (anoikis; Frisch and Ruoslahti, 1997; Gilmore et al., 2000). Bax translocates to mitochondria within 15 min of removal of cells from ECM, but they do not die immediately, and it is a number of hours before MMP occurs. Furthermore, if cells are replated, Bax exits the mitochondria and cells survive, suggesting that Bax translocation does not, per se, commit cells to apoptosis (Gilmore et al., 2000). Here, we examine spatial and temporal changes in Bax localization, conformation, and oligomerization during anoikis. Bax initially translocates to mitochondria as an inactive monomer. A fraction of this Bax undergoes a conformational change after translocation, but this precedes MMP and apoptosis by several hours. The formation of prominent Bax clusters is a late event coinciding with activation of all mitochondrial Bax, MMP, and apoptosis. We have also investigated the time window during which epithelial cells can be rescued from anoikis. We find that the point beyond which cells are committed is before Bax forms large perimitochondrial clusters and cytochrome *c* is released. These results suggest that critical events committing a cell to die occur before the detectable changes in mitochondrial permeability.

Results

Bax translocates to mitochondria as an inactive monomer

Adhesion-dependent mammary epithelial cells undergo anoikis after detachment from ECM (Gilmore et al., 2000). Bax translocates to mitochondria within 15 min of detachment, but apoptosis is a slower stochastic event, occurring after several hours. We wanted to determine if this delay between apoptotic signal and death was related to changes in the conformation and organization of Bax.

First, we asked if the conformational change occurs before or subsequent to Bax translocation. FSK-7 mammary epithelial cells were maintained as an adherent monolayer or detached and cultured on a nonadhesive substrate (poly-HEMA) for 15 min or 4 h. Cytosol and membrane fractions were prepared and assayed for the presence of Bax (Fig. 1 a, top). In adherent cells, Bax is predominantly cytosolic. Detaching cells for 15 min results in a significant amount of Bax translocating to the membrane fraction, which persists after longer times in suspension. To determine the conformational state of Bax, we immunoprecipitated from these fractions (in the presence of 0.25% CHAPS) using anti-Bax pAb 62M, which recognizes a BH3 domain–proximal epitope. This antibody only recognizes the proapoptotic conformation of Bax (Pullan et al., 1996; Gilmore et al., 2000). Only Bax from the membrane fraction of detached cells was precipitated by 62M (Fig. 1 a, bottom). 62M-reactive Bax was detected in the membrane fraction of cells detached from ECM for 15 min. No Bax was immunoprecipitated from the cytosolic fraction of detached cells, or from the cytosolic and membrane fractions of adherent cells. This change in reactivity with 62M was not due to covalent modification, as addition of Triton X-100 allowed Bax to be immunoprecipitated from all fractions (unpublished data). These data demonstrate that the Bax molecules that have undergone a conformational change can only be found in the membrane fraction of cells undergoing anoikis.

To determine the subcellular location of activated Bax during anoikis, cells maintained in suspension for various times were cytospun and immunostained with anti-Bax 62M (Fig. 1 b). Previously, we have shown that this antibody does not immunostain adherent FSK-7 cells, but does stain detached cells (Gilmore et al., 2000; Wang et al., 2003). Mitochondria were visualized by transfecting cells with a YFP-tagged marker for the OMM (YFP-XT). At all times after detachment, active Bax was observed in punctate clusters associated with mitochondria. These perimitochondrial clusters appeared identical to those described for GFP-Bax after staurosporine treatment (Nechushtan et al., 2001).

Staurosporine treatment of HeLa cells results in mitochondrial Bax forming high mol wt complexes (Antonsson et al., 2001). Perimitochondrial clusters seen with GFP-Bax (Nechushtan et al., 2001) and here with anti-Bax 62M may represent these complexes. As we observed 62M-reactive Bax only in perimitochondrial clusters, we asked if, during anoikis, membrane-associated Bax was exclusively in large complexes, or whether it initially translocated as a monomer. Cytosolic and membrane fractions were prepared in CHAPS and separated by size-exclusion chromatography. In adherent cells, Bax is cytosolic, and elutes as a single peak consistent with it being a monomer (Fig. 1 c). Cytosolic Bax from cells on poly-HEMA for 4 h is also a single species of monomer, suggesting that, as with the conformational change, oligomerization does not precede translocation. Membrane-associated Bax in detached cells elutes as two peaks, both low and high mol wts. The latter elutes ~200 kD in size, consistent with previous data (Antonsson et al., 2001).

Next, these isolated S100 chromatography fractions were cross-linked using the homo-bifunctional reagent BS3. By SDS-PAGE, Bax from adherent cells runs as a single band (Fig. 1 d, band 1). Bax in both the high and low mol wt S100 fractions of detached cells also run as a single bands under denaturing conditions (Fig. 1 d, band 1). On cross-linking, cytosolic Bax is still a discrete band by SDS-PAGE, but migrates slightly faster (Fig. 1 d, band 2), suggesting internal cross-links, consistent with its folded structure (Suzuki et al., 2000). Addition of Triton X-100 before cross-linking produces a band indicative of Bax homodimers (Fig. 1 d, band 3), also consistent with previous data (Hsu and Youle, 1998). The low mol wt fraction from membranes isolated from detached cells behaves identically to cytosolic Bax when cross-linked with BS3. However, the high mol wt fraction does not show any immunoreactive bands after cross-linking, although some immunoreactive material was seen in the stacking gel (unpublished data). Identical results were seen with a number of anti-Bax antibodies (unpublished data). Furthermore, this fraction was insensitive to the addition of Triton X-100, and no dimer-sized band was induced.

These data show that monomeric and oligomeric forms of Bax are both associated with the membrane fraction of detached cells.

We examined the kinetics of complex formation during anoikis. Membrane fractions isolated from adherent cells or cells on poly-HEMA for various times were extracted in CHAPS and separated by S100 chromatography. Very little Bax is seen in the membrane fraction of adherent cells (Fig. 1 e). After detachment for 15 min, the majority of Bax in the membrane fraction is in the low mol wt, monomeric form. With detachment for longer periods, increasing amounts of Bax are found in the high mol wt complex. Membrane fractions isolated from detached cells were also cross-linked with BS3. Consistent with the gel filtration data, significant amounts of Bax on the membrane after detachment for 15 min is a monomer (Fig. 1 f). In the 15-min sample, Bax was cross-linked into a discrete, faster migrating band (Fig. 1 f, band 2). Triton X-100 produced a cross-linked dimer-sized band (Fig. 1 f, band 3), identical to cytosolic Bax from adherent cells (Fig. 1 d). Bax from cells on poly-HEMA for 1 h was also largely monomeric. However, membrane fractions from cells detached for 4 h showed a significant decrease in the amount of monomeric Bax, as judged by absence of the faster migrating cross-linked band (Fig. 1 f, band 2) and the Triton X-100–induced dimer (Fig. 1 f, band 3).

If cross-linking with BS3 resulted in no 62M immunoreactive bands by Western blotting, why were 62M-positive clusters seen associated with mitochondria by immunofluorescence? It was estimated that GFP-Bax clusters contain thousands of molecules, suggesting a mol wt much greater than 200 kD (Nechushtan et al., 2001). Complexes of this size may be largely insoluble with extraction conditions required to isolate native Bax. To examine this, we separated cells into cytosolic and membrane fractions as before. The membrane fraction was extracted with 0.25% CHAPS, and the CHAPS-insoluble material was solubilized in SDS-PAGE sample buffer. We compared all three fractions by immunoblotting for Bax (Fig. 2 a). In adherent cells, the majority of Bax was found in the cytosolic fraction. All detached cell samples had a proportion of Bax present in the CHAPS-insoluble membrane fraction. However, in cells detached for 4 h, the majority of Bax was associated with the CHAPS-insoluble fraction. The amount of Bax extracted was not increased with higher concentrations of CHAPS (unpublished data). Next, we asked if perimitochondrial Bax clusters represented this CHAPS-insoluble material. Cells expressing YFP-Bax were maintained on poly-HEMA for 5 h, and then either fixed in 4% formaldehyde or extracted with 0.25% CHAPS before fixation. They were immunostained for cytochrome *c* and the mitochondrial membrane protein TOM-20 (Fig. 2 b). TOM-20 was not extracted by CHAPS, whereas cytochrome *c* staining was diminished. However, cells expressing YFP-Bax showed identical perimitochondrial clusters with or without CHAPS extraction. Adherent cells extracted with CHAPS did not have any detectable cytosolic YFP-Bax (unpublished data).

These experiments demonstrate that Bax is a cytosolic monomer in adherent epithelial cells, and that during anoikis,

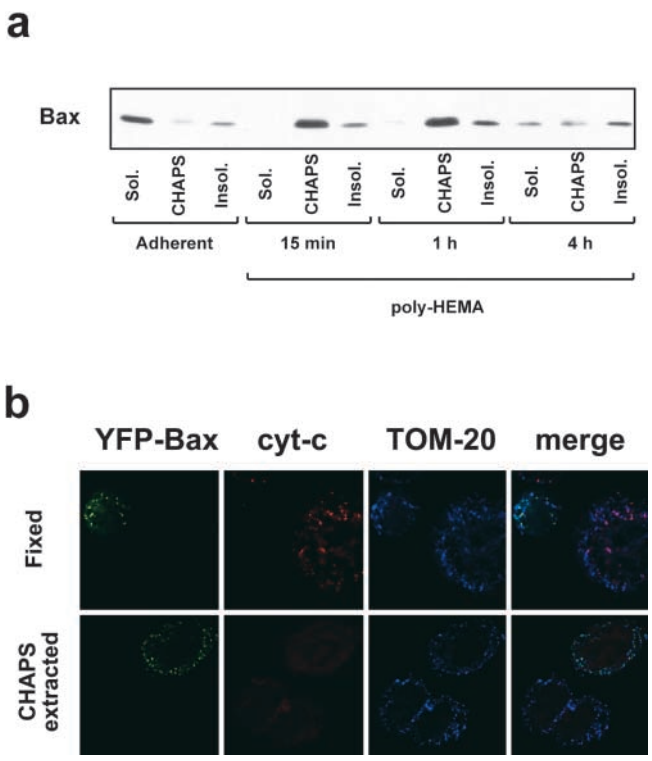


Figure 2. **During anoikis, the majority of Bax becomes resistant to CHAPS extraction.** (a) FSK-7 cells, either adherent or detached for various times, were separated into soluble (Sol) and membrane fractions. The membrane fraction was extracted in 0.25% CHAPS and the insoluble material was removed by centrifugation. The pellet was solubilized in SDS-PAGE sample buffer (Insol). Samples were separated by SDS-PAGE and immunoblotted for Bax. (b) Cells expressing YFP-Bax were detached for 5 h before cytospinning, and either fixed in 4% formaldehyde or extracted at RT in PBS/0.25% CHAPS for 2 min and then fixed. Cells were immunostained for cytochrome *c* and TOM-20. Note that cells with clusters of YFP-Bax do not show mitochondrial cytochrome *c*. The nontransfected cell fixed before permeabilization still has mitochondrial cytochrome *c*.

it redistributes within 15 min to the OMM as a monomer. The conformational change associated with Bax activation is only found with protein associated with the membrane fraction. Subsequent to translocation, Bax forms oligomeric complexes. Over time, Bax progressively associates into larger and more insoluble complexes, ultimately resulting in the large clusters described here and elsewhere (Nechushtan et al., 2001). These complexes are largely insoluble in the conditions required to isolate native Bax, suggesting that the 200-kD complexes isolated in a number of reports (Eskes et al., 2000; Antonsson et al., 2001) represent only a fraction of the oligomeric Bax on apoptotic mitochondria.

Spatial and temporal relationship between Bax activation complex formation and apoptosis

The experiments just mentioned raised a number of questions regarding the spatial and temporal relationship between Bax translocation, the formation of large complexes, and MMP. Furthermore, the requirement of any of these events for anoikis was unclear. To address this, we isolated primary epithelial cells from mid-pregnant Bax-null mice and wild-type

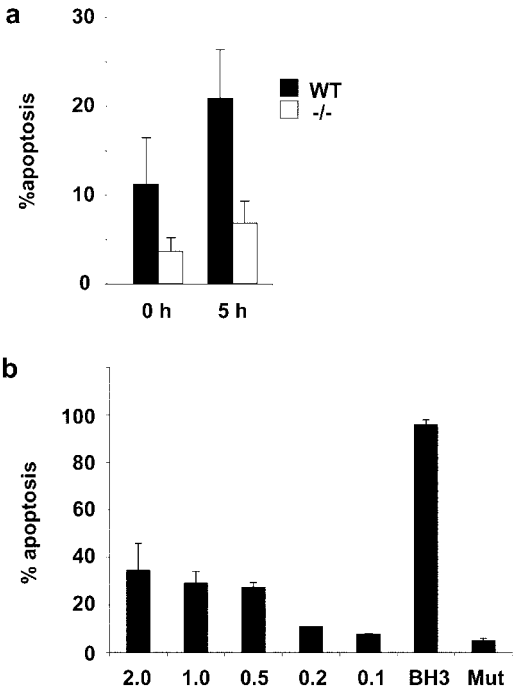


Figure 3. **Bax is required for anoikis, and expressed YFP-Bax does not induce significant apoptosis before detachment.** (a) Primary mammary epithelial cells from wild-type (WT) and Bax-null (−/−) mice were maintained in suspension for 5 h. Apoptosis was assessed by nuclear morphology. (b) Cells transfected with varying amounts of YFP-Bax or 0.5 μg YFP-BH3 (BH3) or YFP-Bax with an inactive BH3 domain (Mut) were stained with Hoescht 18 h after transfection. YFP-positive cells were assayed for apoptosis by nuclear morphology. Error bars are SEM from three experiments.

counterparts. These were detached from ECM for 5 h, and apoptosis was quantified (Fig. 3 a). Bax −/− cells showed a marked delay in the onset of apoptosis, as well as having a lower baseline level of cell death in monolayer culture.

It was unclear if the punctate Bax clusters seen by 62M immunostaining represented all the Bax present on the mitochondrial surface, or whether only a fraction of the protein on the OMM was in the active conformation. One difficulty in addressing this is the scarcity of antibodies that recognize the inactive conformation of Bax. To address this, we used expression of YFP-Bax. This has inherent problems, as overexpression of YFP-Bax alone can induce apoptosis in the absence of an apoptotic stimulus (Wolter et al., 1997). First, we determined what amount of YFP-Bax we could transfect FSK-7 cells with in order to visualize Bax without inducing significant apoptosis and cluster formation in the absence of detachment. FSK-7 cells were transfected with increasing amounts of pEYFP-Bax and grown for 24 h. Both the adherent and floating cells were collected and the number of transfected cells showing apoptotic nuclear morphology were quantified (Fig. 3 b). Cells that were apoptotic invariably showed punctate YFP-Bax clusters. Cells transfected with the Bax BH3 domain alone fused to YFP showed almost 100% apoptosis, whereas <5% of cells expressing YFP-Bax with an inactivating mutation in the BH3 domain were dead. Most cells expressing wild-type YFP-Bax were not apoptotic, though increasing amounts of YFP-Bax increased this proportion. However, the majority of apoptotic cells were found in the supernatant, and were easily removed. Consequently, most transfected cells used in subsequent experiments were not apoptotic and contained cytosolic YFP-Bax before detachment.

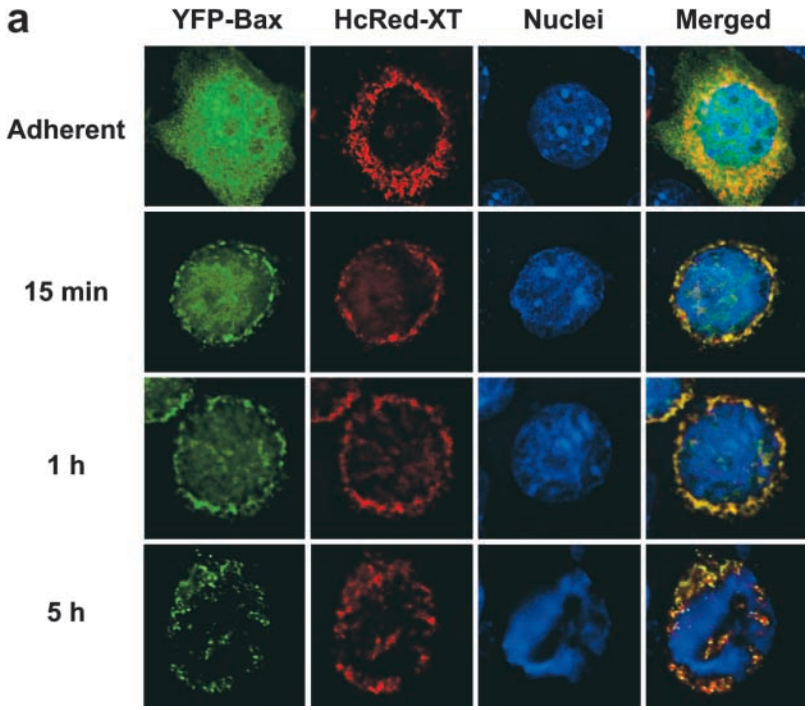


Figure 4. **Spatial and temporal relationship between Bax activation and formation of mitochondrial-associated clusters.** FSK-7 cells transfected with YFP-Bax were fixed while adherent, or were detached for various times before cytospinning. (a) Cell expressing YFP-Bax and HcRed-XT. Bax colocalizes with mitochondria 15 min and 1 h after detachment, but after 5 h, YFP-Bax is restricted to discrete clusters. Bar, 15 μm. (b) YFP-Bax–expressing cells were detached for 15 min, 1 h, and 5 h. After fixation, cells were immunostained with anti-Bax 62M and anti-mtHsp70. At 15 min and 1 h, YFP-Bax colocalizes extensively with mtHsp70, but 62M reactivity is more restricted. At 5 h, YFP-Bax does not colocalize with mtHsp70, but is now completely coincident with 62M staining. Note that the cell at 5 h has a condensed and fragmented nucleus. All cells with this distribution of Bax and 62M reactivity had similar nuclei. Bottom shows boxed areas in greater detail. Bar, 15 μm. (c) Loss of cytochrome *c* occurs along with Bax redistribution into clusters. Cells expressing YFP-Bax were cytospun at various times after detachment and immunostained with monoclonal anti-cytochrome *c* and anti-Bax 62M. Cytochrome *c* is not seen in mitochondria of cells that have complete coincidence of 62M reactivity and YFP-Bax. Cells that have discrete foci of 62M reactivity but extensive YFP-Bax on mitochondria also have mitochondrial cytochrome *c*. No exceptions to this were observed. Bottom shows boxed areas in detail.

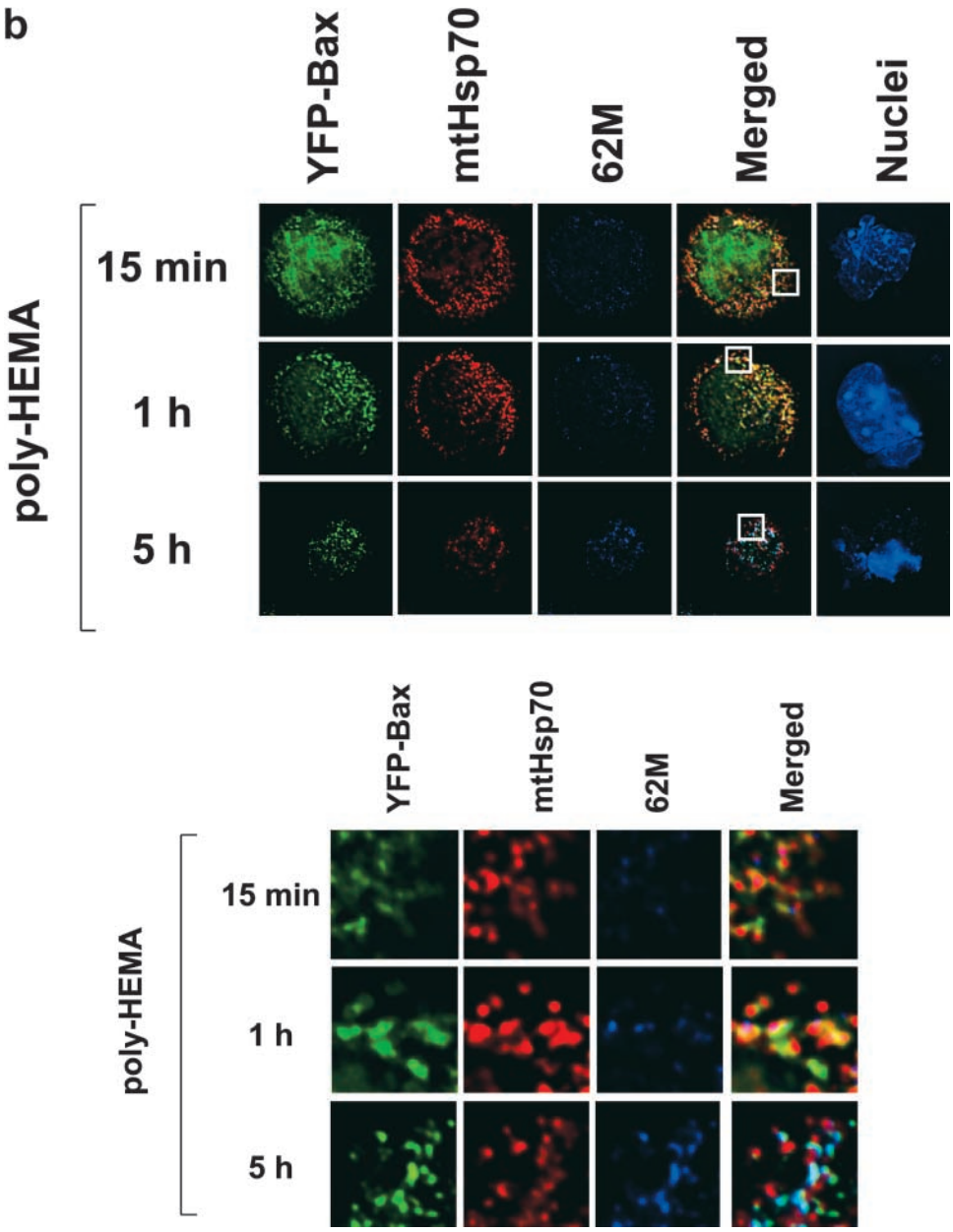


Figure 4 continued.

To assess the spatial and temporal events after Bax translocation, cells were cotransfected with YFP-Bax and a fluorescent marker for the OMM (HcRed-XT). In adherent cells, YFP-Bax showed a diffuse, cytosolic distribution (Fig. 4 a). However, 15 min after detachment, YFP-Bax was localized over the entire surface of the mitochondria, in contrast to the distribution seen with anti-Bax 62M. This distribution changed over time, and after 5 h in suspension, the majority of cells showed YFP-Bax in perimitochondrial clusters. It was striking that all cells that showed this perimitochondrial distribution also had apoptotic nuclei, shown by Hoechst staining.

The Bax that we had previously localized with 62M (Fig. 1 b) was endogenous protein, whereas here, we saw expressed YFP-Bax showing a different localization at early time points after detachment (i.e., uniformly distributed over the mitochondrial surface). To determine if this was an overexpression artifact, cells expressing YFP-Bax were detached for various times and the relative distributions of YFP-Bax and 62M-reactive Bax were examined. Cells were immunostained with anti-Bax 62M and monoclonal anti-mtHsp-70 (Fig. 4 b). Although YFP-Bax was distributed over the entire mitochondrial surface at 15 min and 1 h (with extensive colocalization between YFP-Bax and mtHsp70), 62M reactivity was still only seen at discrete foci (Fig. 4 b, enlarged images). This distribution did not change until cells developed apoptotic nuclei. Morphologically dead cells showed complete colocalization of YFP-Bax and 62M reactivity, and no colocalization with mtHsp70 (Fig. 4 b). This suggests that of the Bax that translocates to the OMM, only a small fraction of it is conformationally altered (i.e., is reactive with 62M). Movement of all the mitochondrial Bax into clusters is associated with it all undergoing this conformational change.

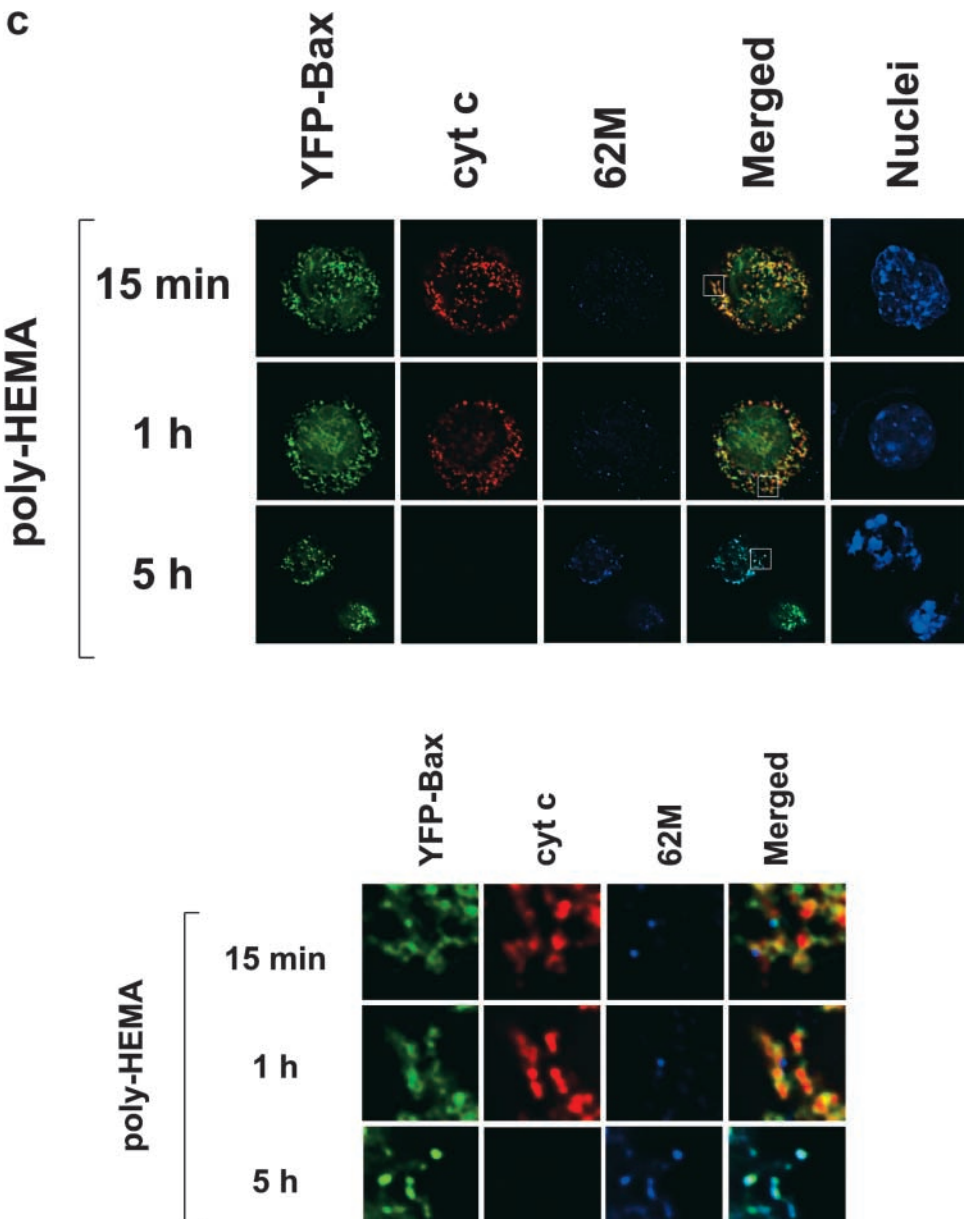


Figure 4 continued.

Release of cytochrome *c* is a rapid and complete event, occurring over a 5-min period once initiated (Goldstein et al., 2000). These kinetics are similar in response to a range of apoptotic stimuli. To determine the relationship between cytochrome *c* release and redistribution of Bax within mitochondria, YFP-Bax–expressing cells were detached for various times and immunostained with an antibody to cytochrome *c* and anti-Bax 62M. Only cells that had no mitochondrial cytochrome *c* showed complete colocalization of YFP-Bax with 62M immunoreactivity (Fig. 4 c). All cells with released cytochrome *c* also had apoptotic nuclei.

The fact that we did not see any intermediate cells that had Bax present in large clusters but still had mitochondrial cytochrome *c* suggested that the formation of clusters and MMP were rapid and coincident. Given the rapid kinetics of cytochrome *c* release (Goldstein et al., 2000), it was impossible from these experiments to determine if Bax clusters formed before or after MMP, caspase activation, and cell death. To address this, we followed cluster formation in detached cells in the presence or absence of zVAD-fmk. Cells were cytospun and fixed after various times of anoikis, and were immunostained for cytochrome *c* and TOM-20 (Fig. 5 a). As we have previously shown, inhibition of caspases did not prevent Bax association with mitochondria after 15 min (Gilmore et al., 2000). At later times, although nuclear condensation was inhibited by zVAD-fmk, cytochrome *c* release and the formation of YFP-Bax clusters were unaffected, indicating that they were independent of caspase activation.

Next, time-lapse images were taken of FSK-7 cells expressing CFP-Bax, YFP-caspase 3 sensor (which redistributes from the cytosol to the nucleus on cleavage at a DEVD sequence), and HcRed-XT. Activation of caspase 3, loss of mitochondrial membrane potential, and release of cytochrome *c* occur rapidly and simultaneously (Goldstein et al., 2000; Tyas et al., 2000). Furthermore, activation of Bax has been shown to induce loss of mitochondrial membrane potential

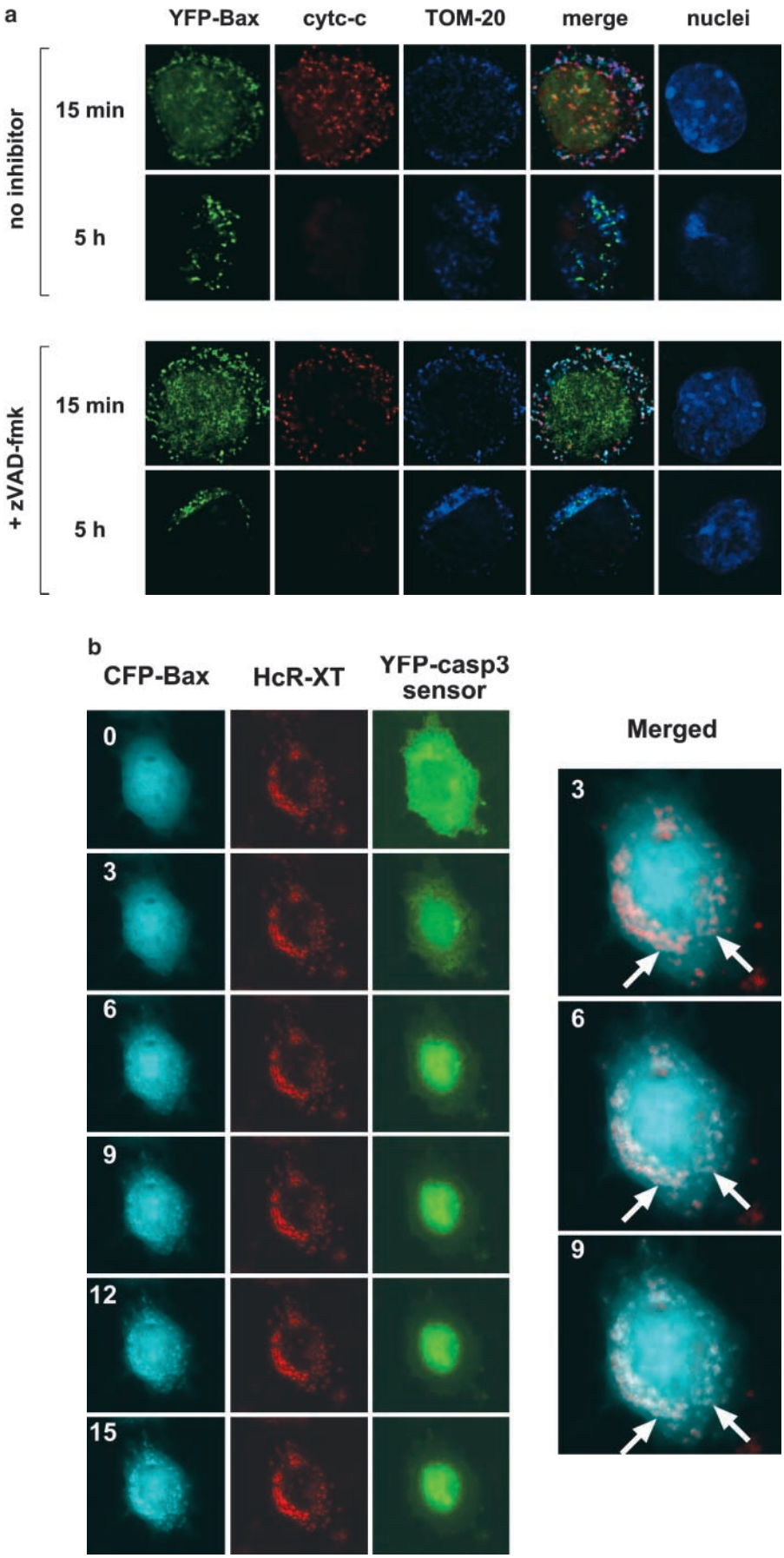
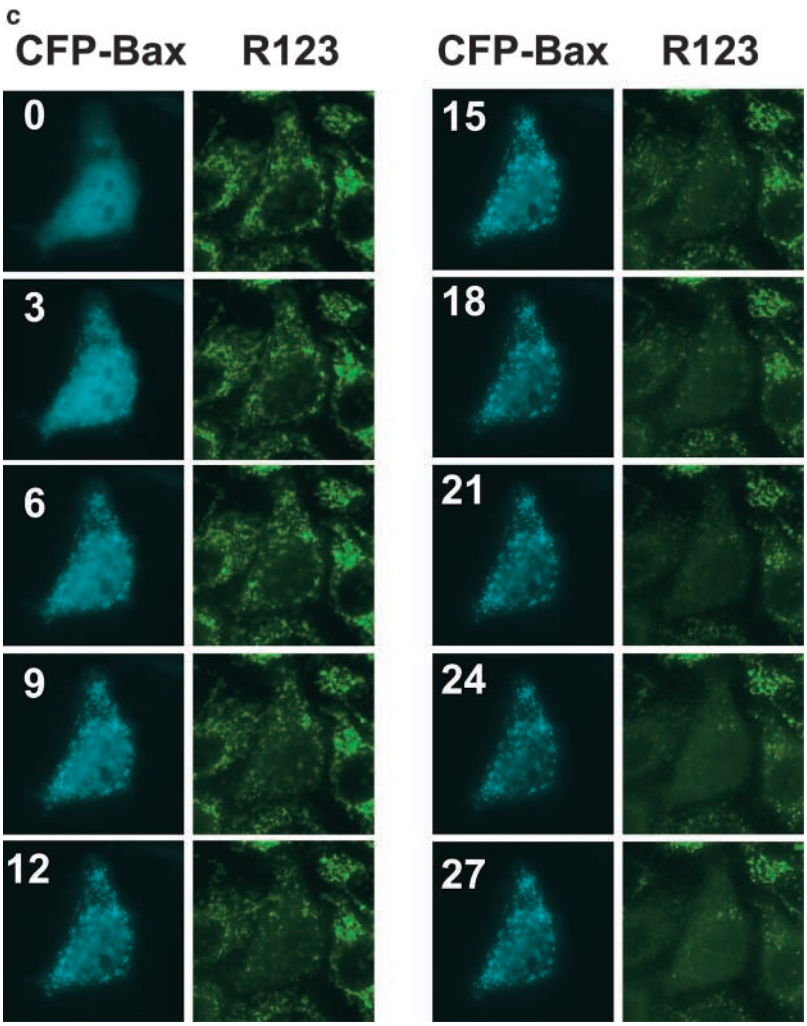


Figure 5. **Formation of Bax clusters occurs concomitant with (but does not require) caspase activation.** (a) Cells expressing YFP-Bax were maintained in suspension for 15 min or 5 h, with or without 100 μM zVAD-fmk. Cells were cytospun and immunostained for cytochrome *c* and TOM-20. Nuclei were stained with Hoechst. Although zVAD-fmk prevented nuclear condensation, it did not inhibit cytochrome *c* release or formation of YFP-Bax clusters. (b) Cells expressing CFP-Bax (Video 1), HcRed-XT (Video 2), and YFP-caspase 3 sensor (Video 3) were treated with 10 μM staurosporine. Images were taken every 3 min. t = 0 represents the first image in the sequence presented, not the point at which staurosporine was added. Appearance of CFP-Bax clusters commences at t = 3, the same point at which YFP is first localized to the nucleus. See Videos 1–3 for complete image sequence. Merged image of CFP-Bax and HcRed-XT indicates Bax is redistributing into perimitochondrial clusters. Examples are indicated by arrows. (c) Cells expressing CFP-Bax (Video 4) were loaded with Rhodamine 123 (R123, Video 5) before adding staurosporine. Images were taken as in b. Loss of R123 localization to mitochondria is first apparent at t = 9, and CFP-Bax clusters form between t = 3 and t = 6. See Videos 4 and 5 for complete image sequence. (d) Cells expressing CFP-Bax were stained with Hoechst, and treated with staurosporine. Images were taken every 5 min. t = 0 represents the first image presented. Bax clusters are apparent at t = 20, and nuclear condensation commences at around t = 35 to t = 40.



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directly (Gross et al., 1998). As we could not follow live imaging of detached cells over the time periods required for apoptosis to occur, we used staurosporine as an apoptotic stimulus. The kinetics of initial Bax translocation after staurosporine treatment appear different to that during anoikis (Capano and Crompton, 2002). Consequently, we concentrated on the appearance of Bax clusters and their relationship to caspase activation, loss of mitochondrial membrane potential, and nuclear condensation. Clusters of CFP-Bax appeared simultaneously with the activation of caspase 3, as judged by YFP redistribution to the nucleus (Fig. 5 b; Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200302154/DC1). Bax redistribution into clusters occurred over 6 min. Colocalization with HcR-XT clearly showed that CFP-Bax was forming perimitochondrial clusters at this time (Fig. 5 b, examples indicated by arrows). The kinetics of cluster formation were unaffected by zVAD-fmk (unpublished data). We examined loss of mitochondrial membrane potential using Rhodamine 123 (Fig. 5 c; Videos 4 and 5). Bax clusters clearly form before loss of membrane integrity. However, a reduction in localized Rh123 began within 3–6 min of CFP-Bax clusters becoming apparent. Complete loss of membrane potential occurred over a longer period than caspase 3 activation. Only partial membrane permeabilization may be sufficient to induce activation of the caspase cascade. Formation of CFP-Bax clusters was also seen consistently before any signs of nuclear condensation (Fig. 5 d).

Together, these results demonstrate that during anoikis, cytosolic Bax translocates to mitochondria within 15 min and remains evenly distributed over the OMM until MMP. Redistribution of Bax into perimitochondrial clusters is followed after several minutes by the formation of apoptotic nuclei. The rapidity of this process agrees with our data obtained with detached, fixed cells, where we did not observe any cells in an intermediate state with Bax clusters, but with cells still showing mitochondrial cytochrome *c*. Thus, the formation of clusters occurs concomitant with, but independent of, caspase activation, suggesting that they form at the same time as MMP.

Epithelial cells undergoing anoikis commit to the apoptotic pathway before formation of large Bax clusters

It is unclear at what point a cell has become irreversibly committed to apoptosis, and whether this relates to the changes in Bax localization and complex formation. Previously, we have shown that if cells are replated after de-

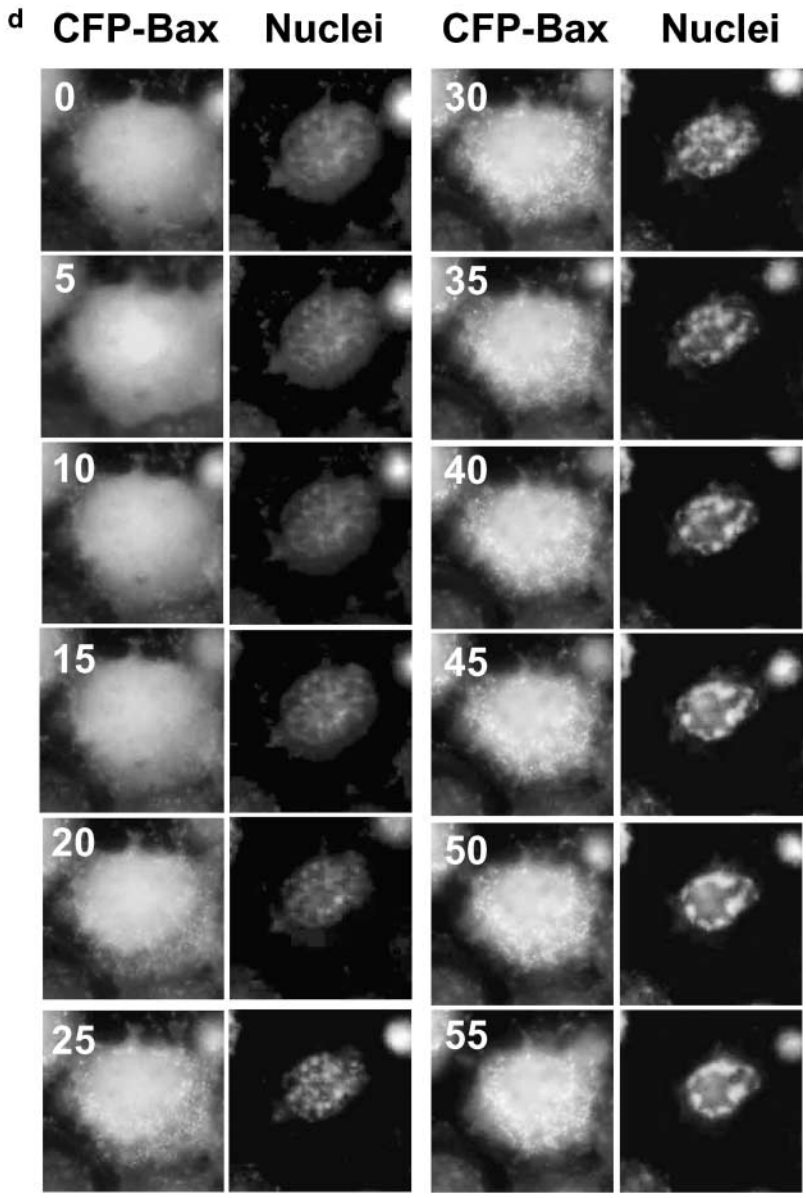

Figure 5 continued.

tachment for <1 h, endogenous Bax is lost from the mitochondria and redistributes to the cytosol (Gilmore et al., 2000). Now, we asked how long a cell needs to be kept in suspension before irreversibly committing to apoptosis, and whether this is related to the formation of Bax complexes. FSK-7 cells expressing YFP-Bax were used to assess complex formation and apoptosis commitment. Cells were detached from ECM for varying times. Cells were then cytospun onto slides, and Bax localization and apoptosis at that time were quantified. Virtually all cells showed mitochondrial YFP-Bax after 15 min detachment from ECM (Fig. 3 a). This did not change over increasing times in suspension. YFP-Bax cells undergo apoptosis only after several hours detachment, even though translocation to mitochondria was rapid. In cells maintained on poly-HEMA, an increase in apoptosis was not apparent until after 3 h in suspension, and occurred in the majority of cells by 5 h (Fig. 6 b).

To determine when cells could no longer be rescued from apoptosis, they were replated onto ECM after being kept in suspension for various times. When cells were maintained in suspension and then allowed to reattach, the majority (>90%) attached within 30 min. If cells were replated after being in suspension for 15 min, the majority survived over the following 18 h (Fig. 6 c). However, if maintained in suspension for 1 or 2 h before replating, >50% of YFP-Bax–expressing cells were apoptotic after 18 h. This occurred despite the level of apoptosis at the time of replating being identical to that at 15 min, when cells could be rescued. These data suggest that the point at which epithelial cells have committed to apoptosis occurs before MMP and release of cytochrome *c*.

Discussion

Translocation of Bax is associated with both a conformational change and oligomerization into high mol wt com-

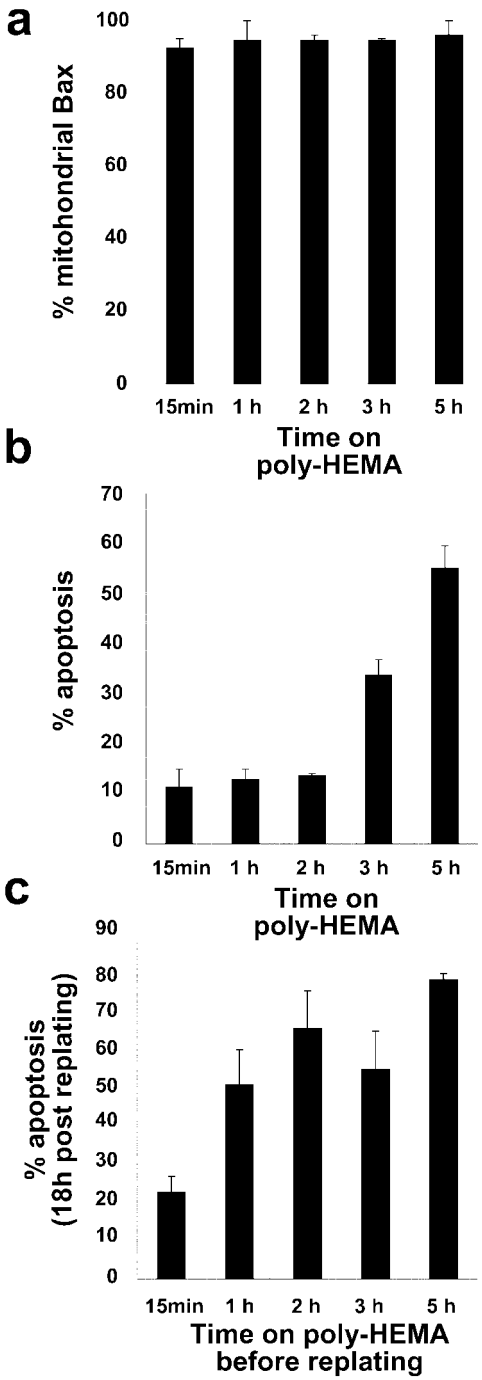

Figure 6. **Cell commitment to apoptosis follows Bax translocation, but is before formation of perimitochondrial clusters.** (a) Cells transiently expressing YFP-Bax were detached for various times before cytospinning onto slides. Cells were immunostained for TOM-20, and the percentage of transfected cells showing mitochondrial YFP-Bax was determined. Error bars are SEM from three experiments. (b) Cells were stained with Hoechst (as in a). Apoptosis in YFP-Bax–positive cells was quantified. Error bars are SEM from three experiments. (c) YFP-Bax–expressing cells were replated onto ECM at the same time points as cells were cytospun in b. Cells were then cultured for a total of 18 h from the start of the experiment. All cells were collected, and were stained with Hoechst. Apoptosis was quantified in YFP-positive cells. Error bars are SEM from three experiments.

plexes (Goping et al., 1998; Desagher et al., 1999; Gilmore et al., 2000; Antonsson et al., 2001). These events have been linked with its activation from a latent, cytosolic form, to a proapoptotic one on the OMM (Gross et al., 1998; Antonsson et al., 2000; Korsmeyer et al., 2000; Kuwana et al., 2002). However, the relationship between Bax translocation, its conformational change, the formation of large mitochondrial clusters, and their requirement for downstream apoptotic events is not clear.

In adhesion-dependent epithelial cells, Bax translocation occurs rapidly after detachment from ECM (Gilmore et al., 2000; Wang et al., 2003). Despite this, the release of cytochrome *c* and morphological apoptosis occur in a stochastic manner over several hours. This delay between apoptotic signal and death prompted us to investigate the kinetics of Bax translocation and oligomer assembly, and how these relate to mitochondrial permeabilization. We have shown that Bax translocates to mitochondria as an inactive monomer. Oligomerization occurs after association with the OMM. Although activated Bax is detectable on mitochondria immediately after detachment, this is a fraction of the total protein and is restricted to small foci. Only when mitochondria permeabilize is the majority of Bax found in perimitochondrial clusters. Time-lapse experiments show that formation of these clusters is concomitant with caspase activation, and before nuclear condensation. Both MMP and cluster formation occur in the presence of caspase inhibitors.

Translocation of Bax to mitochondria precedes both its conformational change and formation of large complexes

Our data clearly indicate that Bax translocation and conformational change into its "active" form are distinct molecular events, although they are often described as being one and the same. We only detected active Bax on the membrane fraction of detached cells, and the small proportion of Bax present in the membrane fraction of adherent cells was not immunoprecipitated with conformation-specific antibodies. Furthermore, cytosolic Bax could not be immunoprecipitated from detached cells, at either early (before MMP) or late stages of anoikis. These data indicate that after a physiological death stimulus, translocation must occur before, and not as a consequence of, the conformational change. This is supported by data from HeLa cells, where Bax can be constitutively associated with mitochondria, but not in the active conformation (Desagher et al., 1999). The COOH-terminal tail appears to drive mitochondrial targeting, and mutations within it can uncouple translocation from apoptosis (Wolter et al., 1997; Nechushtan et al., 1999). The three-dimensional structure of Bax shows remarkable similarity to anti-apoptotic Bcl-X_L (Muchmore et al., 1996; Suzuki et al., 2000). Unlike Bcl-X_L, the structure of Bax was solved with the COOH-terminal mitochondrial-targeting sequence (helix 9) present, lying within a hydrophobic groove along the surface of the molecule. The similarity between the structures of Bax and Bcl-X_L suggests that occupation of this groove is not required for helices 1–8 to fold correctly. Therefore, one can envision that altering the orientation of helix 9 may regulate translocation without affecting the con-

formation of the NH_2 terminus (Suzuki et al., 2000). Our data, showing that only a fraction of the YFP-Bax on mitochondria of detached cells colocalizes with 62M reactivity, supports this hypothesis.

Large oligomers of Bax are clearly associated with apoptosis, though their function and composition is far from clear (Antonsson et al., 2001). Conversely, cytosolic Bax is a monomer. Oligomerization and dimerization have been suggested as mechanisms that regulate Bax association with and insertion into the OMM (Antonsson et al., 2000; Korsmeyer et al., 2000; Suzuki et al., 2000; Kuwana et al., 2002). Forcing cytosolic Bax to dimerize induced translocation in FL5.12 cells (Gross et al., 1998). However, these enforced dimers did not result in release of cytochrome *c*, suggesting that they were not functioning in the same way as endogenous Bax. The BH3-only protein BID can induce the formation of Bax dimers and trimers, leading to insertion into isolated mitochondria (Eskes et al., 2000). We clearly saw that in the initial stages of anoikis, Bax had translocated primarily as a monomer. Our cross-linking data also indicated that dimers were not required for translocation, though we could see dimers induced by addition of Triton X-100. Interestingly, dimers, trimers, and tetramers were not seen in our cross-linking experiments with detached cells at any time point, suggesting that if they do occur and play a role in MMP, then they must exist very transiently in only those cells in which it is occurring. Larger complexes appear over time in suspension, being more apparent at 1 h and particularly at 4 h. However, the analysis of Bax complexes by gel filtration does come with caveats, as they represent an average of a cell population in which cell death occurs in a stochastic manner. This is seen with other death-inducing stimuli, where MMP appears to occur randomly within a population of cells that will all ultimately undergo apoptosis (Goldstein et al., 2000). Therefore, from these experiments, it is impossible to determine whether complexes are forming in all cells over time, or if they occur only in those cells that have undergone MMP and have become apoptotic. Our single-cell analysis of cells expressing fluorescently tagged Bax suggests that large clusters only form in cells that are undergoing MMP, and that their formation is rapidly followed by downstream apoptotic events.

Do large perimitochondrial clusters represent the functional Bax complex?

Bax has been shown to form a variety of different-sized complexes, and this oligomerization has been shown to be required for Bax to permeabilize membranes in cell-free systems (Antonsson et al., 2000). Formation of dimers and tetramers occur in a concentration-dependent manner in vitro, the latter of which can form cytochrome *c*–conducting channels in isolated mitochondria and lipid vesicles (Saito et al., 2000; Pavlov et al., 2001). Yet larger Bax complexes (>200 kD) are seen by gel filtration in CHAPS-extracted mitochondria here and elsewhere (Antonsson et al., 2001). Analysis of YFP-Bax clusters has suggested much larger complexes, containing thousands of Bax molecules (Nechushtan et al., 2001). However, recent data suggests that very large Bax complexes do not have permeabilizing activity, whereas smaller complexes (~100 kD) can release proteins up to megadalton sizes (Kuwana et al., 2002). We found that much of the Bax present in cells after several hours of anoikis was resistant to CHAPS extraction, as were the perimitochondrial clusters of YFP-Bax in cells with apoptotic nuclear morphology. Therefore, very large Bax clusters must be different from the 200-kD complexes seen by gel filtration of CHAPS-extracted mitochondria, and may represent the endpoint of mitochondrial permeabilization. Therefore, the active complex that releases intermembrane proteins may be extremely transient, and may result in large, insoluble aggregates of Bax after permeabilization. Time-lapse imaging of single cells undergoing apoptosis in response to staurosporine indicates that cluster formation is rapid, occurring over a few minutes exactly when caspase activity can be first detected at a single-cell level. MMP is also a sudden, complete, and dramatic event, releasing cytochrome *c* within 5 min of commencement (Goldstein et al., 2000). This is consistent with our inability to identify intermediate complexes by cross-linking. Together with our data, it appears that formation of Bax clusters occurs rapidly at the same time as MMP.

Do these large clusters have a role in apoptosis? It has been hypothesized that Bax may act as a protein translocase, rather than by forming a permanent membrane channel (Lazebnik, 2001). Some evidence for this exists. The transport of megadalton dextrans across the OMM has been shown to occur without the formation of permanent pores or membrane disruption (Kuwana et al., 2002). If this requires Bax to unfold and form transient pores, then it may subsequently form large aggregates adjacent to mitochondria. Indeed, unfolding purified Bax with detergents induces it to form large aggregates (Suzuki et al., 2000). Mitochondria can regulate protein unfolding, and this may have a role in apoptosis regulation (Matouschek et al., 2000; Thress et al., 2001). Therefore, large perimitochondrial clusters might represent a by-product of a process involving Bax unfolding during MMP. Clusters may have other roles, and several proteins have also been identified in them, including Drp-1 and Mfn-1 (Karbowski et al., 2002). These regulate mitochondrial fission during apoptosis, although inhibition of Drp-1 did not prevent Bax itself from forming large complexes (Frank et al., 2001). Interactions with these or other mitochondrial proteins may be required for mitochondrial fragmentation, but it is unclear if they are required for Bax apoptotic activity (Roucou et al., 2002).

Epithelial cells commit to apoptosis before the appearance of Bax oligomers

It has been presumed that the point at which a cell has irreversibly committed to apoptosis equates with release of cytochrome *c*, SMAC/Diablo, and other proapoptotic factors (Zamzami and Kroemer, 2003). Unlike many model systems used to study the kinetics of apoptosis, detached cells can be rescued if they are allowed to reattach to ECM (Gilmore et al., 2000). We have used this system to determine the temporal relationship between the molecular events associated with Bax activation and commitment to apoptosis. Our data suggest that, although the formation of large Bax clusters is associated with release of cytochrome *c*

and apoptosis, cells actually pass the point of commitment before these events. The majority of epithelial cells kept in suspension for >1 h could not be rescued by replating them onto ECM, eventually showing apoptotic morphology. However, cells replated after only 15 min of anoikis were rescued. At the time of replating, YFP-Bax was present on the mitochondria of all cells, but had not yet formed large clusters or resulted in cytochrome *c* release. The conclusion must be that neither Bax translocation nor complex formation mark the point of commitment. This raises interesting questions regarding events occurring on the mitochondrial surface after an apoptotic stimulus but before MMP. The answers to these questions may explain the stochastic nature of this process in response to diverse cellular insults.

Materials and methods

Antibodies and reagents

The polyclonal anti-Bax antibody 62M has been described previously (Pullan et al., 1996; Gilmore et al., 2000). Polyclonal anti-Bax recognizing the whole molecule was purchased from Santa Cruz Biotechnology, Inc. Monoclonal anti-Bax 5B7 was purchased from Sigma-Aldrich. Anti-mitochondrial Hsp-70 (JG1), was purchased from Affinity BioReagents, Inc. The TOM-20 antibody was a gift from David Breckenridge and Gordon Shore (McGill University, Montreal, Quebec, Canada). Monoclonal anti-cytochrome *c* was purchased from BD Biosciences. Triton X-100 and CHAPS were purchased from Sigma-Aldrich. BS3 was purchased from Pierce. Rhodamine 123 was obtained from Sigma-Aldrich.

Expression constructs

The Bax cDNA was amplified from mouse embryo cDNA with Pfu polymerase (Promega) using oligonucleotide primers to the 5′ and 3′ ends of the coding sequence, cloned into pCRScript (Stratagene), and confirmed by sequencing. To generate YFP-Bax, PCR primers were used that incorporated restriction endonuclease sites and the Bax start and stop codons. The product was ligated into either pEYFP-C1 or pECFP-C1 (CLONTECH Laboratories, Inc.). The BH3 domain construct was generated using PCR primers based on the three-dimensional structure of Bax, and restriction sites were incorporated into each primer. This was cloned into pEYFP-C1. The inactive BH3 mutant was constructed using PCR primers to incorporate a double point mutation (L64 to E and G68 to E). pHcRed1-XT and pEYFP-XT were made by PCR from a Bcl-X_L cDNA (a gift from Richard Youle, National Institutes of Health, Bethesda, MD), using primers that encompassed the coding sequence for the last 25 amino acids including the stop codon. pEYFP-caspase 3 sensor was purchased from CLONTECH Laboratories, Inc. All constructs were confirmed by DNA sequencing and immunoblotting.

Cell culture and transfection

FSK-7 cells (Kittrell et al., 1992) were grown in DME/Ham's F12 (2% FCS, 5 ng/ml EGF, and 880 nM insulin). Transfection of FSK-7 cells with LipofectAMINE™ plus (Life Technologies) has been described previously (Gilmore et al., 2000). In brief, cells were plated at 10^5 cells/cm^2 on glass coverslips 18 h before transfecting with 0.5 μg DNA in 12-well culture plates. For detachment assays, cells were trypsinized and replated on 35-mm dishes coated with poly-HEMA (Sigma-Aldrich). Cells on poly-HEMA were collected by centrifugation (5,000 *g*, 30 s). For immunostaining, cells were resuspended in culture medium and centrifuged onto polylysine slides (Merck) using a centrifuge (Cytospin; Shandon).

Cell fractionation, chromatography, cross-linking, and immunoprecipitation

Manipulations were performed on ice unless otherwise stated. Cells were resuspended in hypotonic buffer (10 mM Hepes, pH 7.6, 10 mM NaCl, 1.5 mM/$MgCl_2$, 4 mM NaF, 100 μM sodium orthovanadate, and protease inhibitors) and were lysed with 20 strokes in a Dounce homogenizer (Wheaton), followed by addition of NaCl to 150 mM. Cytosol and membrane fractions were separated by centrifugation (100,000 *g* for 30 min). For size-exclusion chromatography, CHAPS was added to the cytosol fraction to 4 mM. To extract the membrane fraction, the 100,000-*g* pellet was resuspended in 10 mM Hepes/Cl, pH 7.6, 150 mM NaCl, 4 mM CHAPS, 4 mM NaF, 100 μM sodium orthovanadate, and protease inhibitors, and was sonicated. The solubilized membrane fraction was centrifuged at 100,000 *g*. The insoluble material left at this stage was solubilized by boiling in SDS-PAGE sample buffer. Lysates were concentrated in Centricon 10 concentrators (Amicon), and 5 mg protein in 0.5 ml was loaded onto a Sephacryl S100-HR column (1.5 × 25 cm, Amersham Biosciences; equilibrated in 10 mM Hepes/Cl, pH 7.6, 150 mM NaCl, and 4 mM CHAPS). The column was resolved at 4°C, flow rate 0.1 ml/minute. Calibration was with mol wt standards from Sigma-Aldrich (12.4 kD, cytochrome *c*; 29 kD, carbonic anhydrase; 66 kD, BSA; 200 kD, β-amylase; void volume, blue dextran). Column fractions were concentrated with TCA, separated by reducing SDS-PAGE, transferred to nitrocellulose (Bio-Rad Laboratories), and immunoblotted. For cross-linking experiments, lysates were incubated with 5 mM BS3 for 30 min at RT. Where indicated, Triton X-100 or CHAPS was added 30 min before BS3. Reactions were stopped with SDS-PAGE sample buffer. Reactions were separated by SDS-PAGE. For immunoprecipitation of active Bax, soluble and membrane fractions isolated in 4 mM CHAPS buffer were clarified by centrifugation. 1 mg protein was precipitated with 1 μg/ml anti-Bax antibody 62M on protein A–Sepharose (Roche) at 4°C for 2 h, and was washed three times in CHAPS buffer. Proteins were recovered by boiling the beads in SDS-PAGE sample buffer before immunoblotting with anti-Bax 5B7. Immunoblots were visualized with SuperSignal® chemiluminescence (Pierce Chemical Co.).

Immunofluorescence microscopy and live-cell imaging

Cells fixed in 3.7% PFA/PBS were permeabilized in PBS/0.5% Triton X-100. Primary antibodies were diluted in PBS/0.1% Triton X-100/0.1% horse serum (37°C for 1 h). After washing in PBS, secondary goat anti–mouse or goat anti–rabbit Cy5 or Cy3 conjugates were incubated in above buffer (37°C for 30 min). Cells were counterstained with 1 μg/ml Hoescht 33258. Coverslips were mounted in ProLong® (Molecular Probes, Inc.). Images were collected on an inverted microscope (model IX70; Olympus) using a 100× Plan-Apo 1.4 NA objective, equipped with a DeltaVision imaging system. Images were processed by constrained iterative deconvolution on softWoRx™ v3.0 software (Applied Precision). For live imaging, cells expressing fluorescently tagged proteins were incubated with 1 μg/ml Hoescht 33258 and/or 5 μM Rhodamine 123 for 30 min before washing in growth medium, and 10 μM staurosporine was added. Images were captured using a Leica AS-MDW workstation (37°C, 5% CO_2) with an HCX Plan-Apo 63×/1.3 NA glycerol objective.

Online supplemental material

Videos 1–3 show FSK-7 cells treated with staurosporine (images taken every 3 min), and are the complete sequence from which Fig. 5 b was compiled. Videos 4 and 5 show FSK-7 cells loaded with Rhodamine 123 and treated with staurosporine, and are the complete sequence from which Fig. 5 c was compiled. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200302154/DC1.

The authors are grateful to Alex Verkhratsky for advice on mitochondrial membrane potential, and Dr. Peter March with live-cell imaging. We would like to thank Dr. Keith Brennan and Dr. Vasken Ohanian for critical reading of the manuscript.

This work was supported by the Wellcome Trust. A.P. Gilmore is a Wellcome Trust Research Career Development Fellow.

Submitted: 25 February 2003
Accepted: 8 July 2003

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