Biphasic translocation of Bax to mitochondria

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Using green fluorescent protein-tagged Bax, we demonstrate that Bax is sequestered from the cytosol of cardiomyocytes in two distinct phases following the induction of apoptosis with staurosporine. In the first phase, lasting several hours, Bax removal from the cytosol was relatively small. In the second phase, Bax was very largely removed from the cytosol and sequestered into large aggregates associated with the mitochondria. To test which of the phases involved cytochrome *c* release, cells were transfected with a red fluorescent protein–cytochrome *c* fusion. The cytochrome *c* fusion protein was accumulated by mitochondria of healthy cells and was released by staurosporine in phase 1. When green fluorescent protein–Bax

was immunoprecipitated from extracts of cells in phase 1 and phase 2, the voltage-dependent anion channel (mitochondrial outer membrane) and the adenine nucleotide translocase (mitochondrial inner membrane) were also precipitated. These data support a two-phase model of Bax translocation in which Bax targets the mitochondrial intermembrane contact sites and releases cytochrome *c* in the first phase, and is then packaged into large aggregates on mitochondria in the second.

Key words: adenine nucleotide translocase, apoptosis, cytochrome *c*, voltage-dependent anion channel.

INTRODUCTION

Mitochondria play an essential part in the apoptotic signalling pathway by releasing proapoptotic proteins to the cytosol. These proteins include cytochrome *c*, Smac/Diablo, apoptosis-inducing factor (AIF), endonuclease G and some procaspases [1–5]. In healthy cells these proteins are retained in the mitochondrial intermembrane space, but during apoptosis the outer membrane becomes ‘permeabilized’ and the proteins are released. The loss of these proteins to the cytosol is controlled by members of the Bcl-2 family. Some members inhibit outer-membrane permeabilization and promote cell survival (e.g. Bcl-2, Bcl-X_L); others induce permeabilization and bring about apoptosis (e.g. Bax, Bak, Bid) [6,7].

Bax has a major role in outer-membrane permeabilization. The mere addition of Bax to isolated mitochondria can release cytochrome *c* [8–10]. Conversely Bax (and Bak)-deficient cells are resistant to cytochrome *c* release [11]. In many cells Bax is located predominantly in the cytosol, but translocates to mitochondria at an early stage in apoptosis [12,13]. Bax binds to mitochondria both as a peripheral and an integral membrane protein. Insertion into the membrane utilizes the hydrophobic C-terminal domain and is accompanied by conformational changes that expose the N-terminal region and the BH3 domain [12,14–16]. These changes are believed to lead to dimerization, possibly mediated by the exposed BH3 domain [17–19]. Higher Bax oligomers have also been described. In particular, Bax complexes (4-mers to 12-mers) have been isolated from apoptotic cells [19,20]. Recently, very much larger Bax aggregates, containing thousands of Bax molecules, have been detected in close proximity to mitochondria during apoptosis [21]. The mechanism of Bax action is unresolved. The voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane may participate in Bax-induced cytochrome *c* release [22,23], although others

have found no evidence for VDAC involvement [19,24], and Bax-alone mechanisms have also been proposed [25,26].

Here we have used green fluorescent protein (GFP)-tagged Bax to monitor Bax movements in single cardiomyocytes exposed to staurosporine. The study reveals the novel finding that Bax translocates to mitochondria in two distinct stages. We present evidence that in the first phase of very limited translocation Bax targets intermembrane complexes of VDAC and the adenine nucleotide translocase (ANT). Cytochrome *c* is released concomitantly. The assembly of large Bax aggregates on mitochondria occurs downstream of these events in the second phase, when Bax sequestration from the cytosol is complete. A two-phase model of Bax sequestration to mitochondria is proposed.

MATERIALS AND METHODS

Expression constructs

Bax and cytochrome *c* were expressed as fusions to the C-terminus of either GFP (GFP–Bax) or the red fluorescent protein (RFP) DsRed2 (RFP–cytochrome *c*). For the Bax fusion, the *Hind*III and *Eco*RI restriction sites were added to full-length human Bax cDNA by PCR and the product was cloned between the same sites of the mammalian expression vector pEGFP-C3 (Clontech). The following primers were used: forward, 5'-CCAGTGAAGCTTATGGACGGGTCCGGGGAGC-3'; reverse, 5'-GCGTCAGAATTCTCAGCCCATCTTCTTCCAG-ATG-3'. The PCR reactions were carried out by 30 cycles of 94 °C (1 min), 60 °C (2 min) and 72 °C (2 min) using *Pfu* DNA polymerase (Stratagene). Cytochrome *c* cDNA was amplified from a human cDNA library by PCR using the forward primer 5'-TAATTGAATTCAATGGGTGATGTTGAGAAA-3' and the reverse primer 5'-CTTCAGGATCCTTACTCATTAGTAG-CTTT-3'. Reactions comprised 30 cycles of 94 °C (1 min), 45 °C (1.5 min) and 72 °C (2 min). The PCR product was cloned

Abbreviations used: ANT, adenine nucleotide translocase; GFP, green fluorescent protein; RFP, red fluorescent protein; t-Bid, truncated Bid; VDAC, voltage-dependent anion channel.

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between the *EcoRI* and *BamHI* sites of the mammalian expression vector pDsRed2-C1 (Clontech). Both plasmids were propagated in *Escherichia coli* strain DH5 α (Stratagene).

Cell culture and transfections

Primary cultures of cardiomyocytes were prepared from 14 day-old Sprague–Dawley rats and seeded on to laminin-coated glass coverslips at a density of about 10^5 cells/coverslip [27]. Cells were transfected after culture for 2 days. Optimum transfection was obtained with 1 μ g of plasmid DNA plus 3 μ l of Lipofectamine/coverslip according to the manufacturer's protocol (Lipofectamine 2000 reagent; Life Technologies, Paisley, Scotland, U.K.). The medium was replaced with fresh culture medium after 5 h. Cells were used 1–2 days after transfection.

Immunoprecipitation

GFP–Bax was immunoprecipitated from cell extracts using an anti-GFP monoclonal antibody (clone 1E4; MBL Laboratories, Nagoya, Japan) coupled to Sepharose beads. The coupling reaction was carried out by incubating 30 μ g of antibody with 30 mg of CNBr-activated Sepharose 4B in 0.5 M NaH₂PO₄ (pH 7.5) for 2 h. The beads were then washed in 0.5 M NaH₂PO₄ and 1 M NaCl, and left overnight in 100 mM ethanolamine (pH 7.5) at 4 °C. Cell extracts for immunoprecipitation were prepared by scraping the cells from the coverslips into a lysis medium containing 100 mM NaCl, 10 mM Hepes (pH 7.4), 1 mM EGTA, 1 mM MgCl₂, 1 mM KH₂PO₄, 6% CHAPS, 1 mM PMSF and 2 μ g/ml each of the protease inhibitors aprotinin, pepstatin, leupeptin and antipain. Cells from 10 coverslips were extracted in 0.4 ml of lysis medium. Extracts were centrifuged (10000 g, 5 min) and each supernatant was then gently shaken with 50 μ l of anti-GFP-antibody-coupled Sepharose for 2 h at 4 °C. The supernatant from each incubation was retained (actin and VDAC determinations) and the beads were washed six times with lysis buffer containing 0.6% CHAPS. The beads were then extracted with SDS sample loading buffer at 90 °C for 3 min (100 mM Tris/HCl, pH 6.8, 4% SDS, 10% glycerol and 250 mM mercaptoethanol).

Western blotting

Immunoprecipitated proteins and cell extracts were analysed by standard SDS/PAGE and by Western blotting on to nitrocellulose sheets (Protran B85; Schleicher and Schuell, Dassel, Germany) in buffer containing 48 mM Tris, 39 mM glycine, 20% methanol and 0.1% SDS. The following primary antibodies were used. For Bax, monoclonal antibody clone B-9 (Santa Cruz Biotechnology); for cytochrome *c*, monoclonal antibody clone 7H8.2C12 (PharMingen International); for VDAC, monoclonal antibody clone 31HL (Calbiochem); for ANT, a polyclonal antibody raised against the peptide YDEIKKYV corresponding to the C-terminus of rat ANT-1 (this laboratory); for cyclophilin-D, a polyclonal antibody (this laboratory) raised against the peptide CSDGGARGANSSSQNP corresponding to amino acids 1–16 after cleavage of the mitochondrial targeting sequence. As secondary antibodies we used peroxidase-conjugated goat anti-mouse IgG (Calbiochem) and goat anti-rabbit IgG (Bio-Rad). Bands were developed with the enhanced chemiluminescence reagent from Amersham Biosciences.

Immunofluorescent staining

For imaging the distribution of endogenous Bax, cells were fixed in 4% paraformaldehyde and permeabilized with 0.4% saponin. The cells were reacted with anti Bax B-9 antibody (as above) and

then with fluorescein-conjugated goat anti-mouse IgG (Calbiochem). Cells were visualized by fluorescence microscopy.

Fluorescence imaging

Cells on coverslips were incubated in 100 mM NaCl, 4 mM KCl, 24 mM Hepes (pH 7.4), 1 mM MgSO₄, 1 mM CaCl₂, 1 mM KH₂PO₄ and 11 mM glucose. In some experiments, mitochondria were visualized by staining with either MitoTracker Red CMXRos or MitoTracker Green (Molecular Probes) before induction of apoptosis. For dual imaging, loading with the MitoTracker dyes was kept as low as was practically possible, i.e. 10 ng/ml, 10 min, room temperature. Images were obtained with an Olympus IX-70 fluorescence microscope fitted with a cooled CCD camera (Micromax 140IE; Princeton Instruments), and an excitation filter wheel (Sutter) with band-pass filters for the excitation of GFP and MitoTracker Green (490 nm) and the excitation of RFP and MitoTracker Red (570 nm). Fluorescent light was collected via a polychroic beamsplitter (51006 bs; Chroma) and a two-band-pass emission filter in the filter cube (530 nm, 625 nm; Chroma). The appropriate emission band was then selected by band-pass filters in a simple slider in the emission light path. Data were analysed by Metamorph Software (Universal Imaging) which allowed calculation of mean and S.D. of pixel intensities and the construction of three-dimensional and cross-sectional profiles.

RESULTS

Bax translocation to mitochondria

Figure 1 shows how the distribution of endogenous Bax in cardiomyocytes changes when apoptosis is induced by staurosporine. Whereas endogenous Bax was found throughout the cytoplasm of control cells (Figure 1A), it assumed a largely punctate distribution after the addition of staurosporine (Figures 1B and 1C). This resembles the situation in a number of cell types [12,28,29], but not in others where mitochondrially associated Bax was found in healthy cells [14,19,30]. After 7 h exposure to staurosporine, two kinds of Bax sequestration were discernible, as indicated by the arrows in Figure 1(B). Some cells contained mainly the highly condensed form of sequestration (e.g. Figure 1C).

To elucidate the functional significance of these forms of sequestration we analysed the time course of Bax sequestration in relation to cytochrome *c* release (described below). To do this, we used cardiomyocytes that expressed Bax tagged at its N-terminus with GFP, allowing Bax migration to be followed by fluorescence imaging. Cardiomyocytes transfected with the GFP–Bax fusion construct expressed maximal amounts of GFP–Bax 1–2 days after transfection. Immunoblots of transfected-cell extracts showed a Bax-containing band of the appropriate size for GFP–Bax (50 kDa), which was absent from non-transfected cells (Figure 2A). The same band was also detected with anti-GFP antibodies (not shown). Considerably more GFP–Bax was expressed than endogenous Bax (Figure 2A). Nevertheless, the transfected cells had a negligible tendency to undergo apoptosis in the absence of stimulation by staurosporine. Routinely, < 2% of transfected cells became apoptotic within 60 h, as judged by the absence of pyknotic nuclei and the lack of sequestration of Bax from the cytosol. On addition of staurosporine, however, the majority of transfected cardiomyocytes developed fragmented pyknotic nuclei overnight, characteristic of apoptotic cells (results not shown).

In some experiments, the transfected cells were also treated with MitoTracker Red, which accumulates electrophoretically

Figure 1 Staurosporine-induced translocation of endogenous Bax to mitochondria

Non-transfected cardiomyocytes were fixed at either (A) 0 or (B, C) 7 h after addition of 1 μ M staurosporine, as indicated. Endogenous Bax was imaged after immunofluorescent staining. (B) Shows the two types of Bax sequestration observed, highly condensed (closed arrow) and less condensed (open arrow). In some cells (C), the highly condensed form predominated, whereas in others (B) there was more of the less condensed form. Scale bars, 10 μ m.

Figure 2 Expression of the Bax and cytochrome *c* fusion proteins in cardiomyocytes

Cardiomyocytes were transfected with either the GFP-Bax (A) or the RFP-cytochrome *c* (B) expression constructs. After 2 days, cell extracts were prepared and analysed by Western blotting. The proportions of cells transfected were 26% in (A) and 34% in (B). The numbers refer to M_r markers (kDa). C, control (non-transfected) cells; T, transfected cells.

into mitochondria in response to the high inner-membrane potential. Mitochondria were stained with MitoTracker immediately before induction of apoptosis. Mitochondrial uptake of the dye became impaired at > 3 h after treatment with staurosporine, possibly reflecting the reported decrease in inner-membrane potential at this time [31].

In control non-apoptotic cells, GFP-Bax had a diffuse distribution throughout the cytoplasm and nucleus, with little or no evident association with mitochondria. An example is given in Figures 3(A) and 3(B), showing the distribution of GFP-Bax and MitoTracker, respectively, in the same cell. Control cells generally contained a relatively high concentration of GFP-Bax in the nucleus. Figures 3(C)–3(E) show a typical cell after 3 h of exposure to staurosporine. At this stage, GFP-Bax was partially sequestered into cytoplasmic organelles (Figure 3C). The same organelles were stained by MitoTracker Red (Figure 3D), indicating that the GFP-Bax was associated with mitochondria. Mitochondrial sequestration of GFP-Bax is confirmed in the overlay image (Figure 3E; where co-localization of GFP-Bax and MitoTracker Red appears as yellow). Some regions of GFP-Bax localization did not label clearly with MitoTracker Red, e.g. in the perinuclear region in this particular example.

Possibly, some mitochondria acquired little MitoTracker; alternatively Bax may associate with another type of organelle. Other workers have also observed sites of Bax accumulation after staurosporine that do not stain for mitochondria [12]. Bax perturbs Ca^{2+} retention by endoplasmic reticulum, indicating that endoplasmic reticulum may also be targeted by Bax [29].

In the longer term, however, Bax was very largely removed from the cytosol and only mitochondria could be detected as organelles containing GFP-Bax. A representative example is given in Figures 3(F)–3(I) (7 h exposure to staurosporine). Here, the cytosolic GFP fluorescence was reduced to near-background levels as essentially all GFP-Bax had been sequestered from the cytosol. All regions of GFP-Bax localization were stained with MitoTracker Red (not shown). Individual, MitoTracker-stained mitochondria were more readily resolved towards the cell periphery, and here the GFP-Bax assumed a bead-like appearance on the mitochondrial filaments. This is shown in the enlarged images of Figure 3(G) (GFP-Bax), Figure 3(H) (MitoTracker Red) and Figure 3(I) (overlay). It is evident that GFP-Bax does not coat the mitochondria uniformly but instead forms aggregates at (or close to) discrete locations on the mitochondrial surface. The proportion of cells that showed Bax aggregates varied between preparations, i.e. between 15% and 45% after 7 h of exposure to staurosporine (30 cardiomyocyte preparations). The aggregates resemble those observed recently by Nechushtan et al. [21] in COS-7 and HeLa cells, and probably correspond to the highly condensed areas of endogenous Bax sequestration (Figures 1B and 1C). The greater number of GFP-Bax aggregates than endogenous Bax aggregates might be expected since the amount of GFP-Bax in transfected cells exceeded the amount of endogenous Bax (Figure 2A). In summary, we can identify two stages in Bax sequestration. In the first stage Bax associates with mitochondria and possibly other organelles. In the second stage, Bax forms large, mitochondrially associated aggregates concomitant with near-complete removal of Bax from the cytosol.

The two phases of Bax sequestration

Figure 4 shows how staurosporine-induced Bax sequestration proceeds via the two stages in a single cardiomyocyte. At the time of staurosporine addition there was little evident association of Bax with intracellular organelles (Figures 4A and 4B). After 4 h of staurosporine treatment, Bax had formed aggregates on the mitochondrial filaments (Figures 4G and 4H) and this state was maintained for at least 1 h. Thereafter (7–9 h), the mito-

Figure 3 Staurosporine-induced GFP-Bax translocation to mitochondria

Cardiomyocytes expressing GFP-Bax were labelled with MitoTracker Red (MTR) and then $1 \mu\text{M}$ staurosporine was added to initiate apoptosis. The images show the fluorescence of GFP-Bax and MTR in cells after different periods of exposure to staurosporine. (A, B) Before addition of staurosporine. (C–E) After 3 h of incubation with staurosporine. (F–I) After 7 h of incubation with staurosporine. Scale bars, $10 \mu\text{m}$ (A, C, F) and $2 \mu\text{m}$ (G).

chondria began to break up and disappear (results not shown). At intermediate stages, 1–3 h after staurosporine addition, the association of GFP-Bax with organelles was clearly discernible (Figures 4C–4F), but no Bax aggregates could be detected at this stage. In this particular cell, then, Bax relocation began within 1 h of staurosporine addition, but a further 3 h elapsed before Bax aggregation became apparent. Once begun, Bax aggregation was complete within 1 h.

Profiles of GFP-Bax distribution within the same cell (as in Figure 4) are given in Figure 5. The three-dimensional plots (Figures 5A–5C; corresponding to Figures 4B, 4F and 4H) and the corresponding cross-sectional profiles of fluorescence intensities (Figures 5D–5F) reveal more clearly the degree of GFP-Bax sequestration at the different stages. After 4 h of exposure to staurosporine the fluorescence emanating from the cytosol differed little from the background fluorescence outside the cell as the GFP-Bax was sequestered almost entirely within the Bax

aggregates (Figures 5C and 5F). At 3 h after staurosporine addition, the degree of GFP-Bax sequestration was very much less (Figures 5B and 5E). Nevertheless, the mitochondrial filaments bearing the Bax aggregates at 4 h after staurosporine (Figure 4H) can also be seen as Bax-coated filaments at 3 h (Figure 4F), although the Bax was not aggregated at this stage. Thus Bax translocates to mitochondria and then forms aggregates some hours later.

In order to collate the time course of Bax sequestration in different cells we looked for a means of grouping the data graphically. In Figure 5(H), we have taken the series of images of Figure 4 and plotted the S.D. of pixel intensities within a selected region of the cell (the region is shown against the 3 h image in Figure 5G). With a near-uniform distribution of Bax (0 h after staurosporine) the S.D. of pixel intensities is small, but the S.D. increases as Bax is sequestered. The increase in S.D. displays two distinct phases, namely at 1–3 h and at 4–5 h after staurosporine.

Figure 4 Bax is sequestered from the cytosol in two stages

GFP-Bax was imaged in a single cardiomyocyte at different times (0–5 h) after addition of 1 μ M staurosporine, as indicated. The image at each time point (left-hand column; scale bar, 10 μ m) is accompanied by an enlargement of part of the image (right-hand column; scale bar, 2 μ m); the part enlarged is boxed in (A).

These two phases correspond to the pre- and post-Bax aggregation phases (Figure 4) and with the fluorescence intensity profiles showing small and large gradients between sequestered and cytosolic Bax (Figures 5B–5F). From this we conclude that S.D. measurements are a reasonable means of representing the sequestration of GFP-Bax graphically.

Figure 6 compares GFP-Bax sequestration (S.D. of pixel intensities) in different cells. To facilitate comparison between the cells, the S.D. values have been divided by the mean pixel intensity value for each data point. This minimized cell-to-cell differences due to different degrees of GFP-Bax expression from cell to cell. For any particular cell, there was little change in mean

pixel intensity as Bax sequestration progressed (results not shown), i.e. the GFP-Bax was simply shifted from one location to another. The collated data show that the two-phase pattern of Bax segregation was observed generally. However not all cells showing phase 1 progressed to phase 2 (Bax aggregation) within the time scale shown. Occasionally, cells in phase 1 remained in this state for > 6 h, but were found to be in phase 2 the following day. Thus the onset of phase 2 was rather variable. Nevertheless, phase 2 was never observed before 4 h incubation with staurosporine, and no cell examined (from a total of > 100) entered phase 2 without previously showing the partial sequestration characteristics of phase 1.

Other properties of the two phases of Bax sequestration

A fundamental question is whether Bax-induced permeabilization of the mitochondrial outer membrane occurs in phase 1 or in phase 2. This was investigated by imaging the release of cytochrome *c* using cells transfected with a RFP-cytochrome *c* expression construct. Immunoblots of the transfected cell extracts showed a cytochrome *c*-containing band that was the expected size of the RFP-cytochrome *c* fusion protein (40 kDa), which was absent from non-transfected cells (Figure 2B). There was also a minor band of lower M_r (about 25 kDa) in the transfected cells, but not in the control cells, which may be a breakdown product of the fusion protein. The cardiomyocytes expressed relatively little of the fusion protein in comparison with native cytochrome *c* (12 kDa). From the relative densities of the 40 and 12 kDa bands (5.7:1), and the percentage of cells that were visibly transfected (34%; fluorescence microscopy), we estimate that the molar ratio of endogenous cytochrome *c*: fusion protein was about 6:1.

Figures 7(A)–7(C) show the intracellular distribution of the RFP-cytochrome *c*. The RFP-cytochrome *c* had a punctate distribution (Figure 7A), which co-localized with mitochondria labelled with MitoTracker Green (Figure 7B; see the overlay in Figure 7C). Very little RFP fluorescence could be detected outside the mitochondria. It appears, then, that the fusion protein was efficiently imported into the mitochondria.

Figures 7(D)–7(G) show the effect of staurosporine on the distribution of RFP-cytochrome *c*. In order to allow for the possibility that expression of GFP-Bax might influence the kinetics of cytochrome *c* release, the effects of staurosporine were investigated on cells that had been co-transfected with both the RFP-cytochrome *c* and GFP-Bax constructs (see the Materials and methods section). Thus the cell shown in Figures 7(D)–7(G) expressed both RFP-cytochrome *c* and GFP-Bax. The excitation/emission combination used for RFP-cytochrome *c* (570 nm/625 nm; see the Materials and methods section) produced no detectable fluorescence from GFP, so that the actual measurements of RFP-cytochrome *c* distribution were unaffected by the presence of GFP-Bax. (The converse was not true, however; a small fraction of the RFP signal did contaminate GFP-Bax measurements, which are not shown.) As shown in Figures 7(D)–7(G), staurosporine caused the punctate distribution of RFP-cytochrome *c* to become considerably more diffuse, indicating that RFP-cytochrome *c* was released to the cytosol. The release was essentially complete after 2 h of exposure to staurosporine, in that longer incubation gave no further release. These images are representative of 10 such experiments in which the release of RFP-cytochrome *c* from mitochondria was complete within 3 h of addition of staurosporine. It is concluded that Bax-induced cytochrome *c* release from mitochondria occurs in phase 1. This means that the very large Bax

Figure 5 Analyses of GFP-Bax sequestration

(A–F) Analysis of the images of Figures 4(B), 4(F) and 4(H), corresponding to 0, 3 and 4 h after the addition of staurosporine, as indicated. (A–C) Three-dimensional plots of GFP-Bax fluorescence intensities. (D–F) Cross-sectional profiles of GFP-Bax fluorescence intensities, where the cross section was taken at the position indicated by the arrows in (A). bg, background fluorescence. The left and right sides of the Figures correspond to the edges of the cell. (G, H) Images of Figure 4 were used to determine S.D. of pixel intensity values. (G) The standard area analysed at each time point is shown by the boxed region of the 3 h image. (H) The S.D. values at the different times after addition of staurosporine.

aggregates appearing in phase 2 are unlikely to play a role in outer-membrane permeabilization.

The very limited Bax translocation in phase 1 raises the question of whether Bax targets a mitochondrial receptor during this phase. This question was approached by examining whether GFP-Bax interacts with mitochondrial proteins in either of the two phases (Figure 8). Cells transfected with GFP-Bax were extracted at 0, 2 and 6 h after addition of staurosporine, corresponding to zero time, phase 1 and phase 2 (for 32 % of the cells; Figure 8B). GFP-Bax was precipitated from the extracts with anti-GFP antibodies bound to Sepharose beads (see the Materials and methods section). The immunoprecipitates were then examined for the presence of co-immunoprecipitated proteins.

In silver-stained gels (Figure 8A), the GFP-Bax fusion protein (50 kDa) co-immunoprecipitated a band at about 30 kDa in the 2 and 6 h (after staurosporine) extracts, but not in zero-time controls. The identity of the co-immunoprecipitated protein(s) was probed in the immunoblots of Figure 8(B). Near-equal amounts of transfected cells were extracted at the different times as judged by the amount of immunoprecipitated GFP-Bax and non-immunoprecipitated actin. VDAC (32 kDa) was detected using a commercial monoclonal antibody showing a high specificity for VDAC (Figure 8C). Whereas barely detectable amounts

of VDAC co-immunoprecipitated from extracts of control (zero time) cells, VDAC clearly co-immunoprecipitated at 2 h (phase 1) and 6 h (phase 2) after staurosporine. Conversely, there was less VDAC remaining in the supernatant (i.e. non-immunoprecipitated) at 2 and 6 h (after staurosporine) with respect to the control (zero time). In extracts of cells transfected with GFP alone, there was no immunoprecipitation of VDAC. These data indicate that GFP-Bax interacts with VDAC in phase 1. GFP-Bax may also bind to VDAC in phase 2, but this cannot be ascertained since not all cells in phase 1 had progressed to phase 2 at 6 h after staurosporine.

Outer-membrane VDAC can bind to the ANT in the inner membrane, thereby establishing contact sites between the inner and outer membranes (reviewed in [32]). Accordingly, we also examined whether ANT immunoprecipitated with the GFP-Bax and VDAC. We used a polyclonal antibody, raised against the C-terminus of rat ANT (see the Materials and methods section), which reacted reasonably selectively with a band corresponding to the size of ANT (32 kDa) in blots of mitochondrial proteins (Figure 8C). As shown in Figure 8(B), ANT was also co-immunoprecipitated from 2 and 6 h (after staurosporine) cell extracts, but not from control extracts. These data indicate that Bax interacts not only with VDAC, but also with VDAC-ANT complexes in phase 1. VDAC-ANT complexes can also

Figure 6 Comparison of GFP-Bax sequestration in different cells

The sequestration of GFP-Bax was induced by 1 μ M staurosporine and measured as in Figure 5(H). The S.D. values were divided by the mean pixel intensity values to allow for the different degrees of GFP-Bax expression between cells. Eight cells were used to follow the first phase of Bax sequestration and a second group of eight cells the second phase. The boundary values (dotted lines) for unstimulated, phase 1 and phase 2 cells are the limits observed in this series of experiments. Thus the S.D./mean values for the second group of cells before addition of staurosporine were all within the range (unstimulated) shown in the Figure for the first group of cells. The appearance of large Bax aggregates on mitochondria is indicated by $\circ$.

recruit cyclophilin D from the mitochondrial matrix to produce a complex that forms the permeability transition pore under pathological conditions [33]. Therefore, we also tested for the presence of cyclophilin D in the immunoprecipitates, but none was detected. In summary, our data suggest that Bax targets intermembrane contact sites established by VDAC-ANT complexes at an early stage in apoptosis, concomitant with cytochrome *c* release, but preceding the formation of large Bax aggregates.

DISCUSSION

Bax performs an essential role in apoptosis by eliciting the release of cytochrome *c* and other proapoptotic proteins from the mitochondrial intermembrane space. In accomplishing this, Bax translocates from the cytosol to mitochondria, undergoes conformational change, and inserts into the mitochondrial outer membrane [12,14,15,19,20,28,34]. The mechanisms that control the retention of Bax in the cytosol of healthy cells and that direct its translocation to mitochondria during apoptosis are unknown. Here we report the novel finding that Bax translocation is a two-stage process. In the first phase, lasting > 3 h, GFP-Bax removal from the cytosol is far from complete. This is best seen in Figure 5(B), showing little variation in fluorescence intensity between different parts of the cell, and in the corresponding cross-sectional profile of Figure 5(E), where the fluorescence inside the cell was always considerably higher than the level of background fluorescence. By contrast, in the second phase of GFP-Bax redistribution, shown in Figures 5(C) and 5(F), the fluorescence values between the 'peaks' of mitochondrial fluorescence were close to background as nearly all the GFP-Bax was sequestered from the cytosol. From visual (Figure 4) and S.D. analyses (Figures 5 and 6), the first phase of GFP-Bax sequestration attained a near plateau that was maintained for several hours.

Thus GFP-Bax was accumulated discontinuously by mitochondria with an abrupt transition between the two phases. The characteristics of the two phases provide a basis for analysing Bax action as follows.

Cytochrome *c* was released from mitochondria in phase 1 when only a small part of the GFP-Bax had been recruited by mitochondria. The long plateau in GFP-Bax sequestration in phase 1 shows clearly that only a fraction of the Bax was utilized for outer-membrane permeabilization. In part, this may be a result of the markedly increased content of Bax (as GFP-Bax) in transfected cells, so that the fraction of total Bax actually required for cytochrome *c* release may be considerably lower after transfection. Nevertheless, the fact remains that there was a limited translocation of Bax to mitochondria during cytochrome *c* release. Evidently, there are mechanisms operative in phase 1 which limit the amount of Bax sequestered by mitochondria. Either only a small part of the cytosolic Bax is made available for transfer to mitochondria at this time or mitochondria themselves limit the amount of Bax bound, conceivably by restricting the binding to receptor proteins on the mitochondrial surface. Here we present evidence that the VDAC-ANT complex may act as receptor.

Tsujimoto and co-workers [22,23] have proposed that VDAC provides the outer-membrane structure of the cytochrome *c*-permeable pore, which is then 'opened' (to cytochrome *c*) by Bax and 'closed' by anti-apoptotic Bcl-X_L. However, the actual mechanism of cytochrome *c* release is unresolved and 'Bax-only' mechanisms have also been put forward. These include the assembly of Bax oligomers in the mitochondrial outer membrane to form cytochrome *c*-permeable pores [19,24,25,35] and Bax-induced destabilization of the phospholipid bilayer, leading to transient breaks in the outer membrane [26]. Here we show that ANT from the mitochondrial inner membrane was also precipitated with Bax and VDAC. We cannot tell whether the co-immunoprecipitation of ANT was adventitious, merely reflecting the ability of VDAC to bind ANT [32], or whether it points to the formation of a stable Bax-VDAC-ANT complex with a discrete role in Bax action. But the latter is favoured by other lines of enquiry that also implicate ANT in apoptosis. Thus, overexpression of the ANT-1 isoform promoted apoptosis in a number of cell types [36]. This was unconnected with the basic function of ANT in adenine nucleotide transport, since overexpression of mutated, transport-deficient ANT was also effective. Also, certain virally encoded proteins have been identified that influence apoptosis and that bind to ANT, i.e. vMIA (cytomegalovirus [37]) and Protein R (HIV [38]). When taken together, these and the present findings suggest that Bax targets VDAC-ANT complexes (rather than VDAC alone) at intermembrane contact sites. The complexes may act as mitochondrial 'receptors' for Bax, directing the translocation of Bax to these organelles and limiting, initially, the amount of Bax bound. The amount of mitochondrially bound Bax may be limited further by cytosolic proteins that can bind to VDAC. Among these, hexokinase (isoenzymes I and II) binds to VDAC in a manner modified by VDAC association with ANT [39]; this generates a microdomain that facilitates the use of mitochondrially generated ATP for glucose phosphorylation. Hexokinase binds to VDAC in competition with Bax [40,41]. The competition is believed to be a factor suppressing apoptosis of transformed cells with greatly increased expression of hexokinase II [40]. The competition may also be a feature of growth-factor-mediated cell survival since protein kinase B, an important link in this pathway, promotes the association of hexokinase with mitochondria [41].

Intriguingly, there is evidence that the contact sites are also targeted by Bid, a proapoptotic Bcl-2 family protein that is

Figure 7 RFP–cytochrome *c* localizes to mitochondria and is released by staurosporine

(A–C) A cardiomyocyte transfected with RFP–cytochrome *c* was labelled with MitoTracker Green (MTG). The fluorescence images show the location of RFP–cytochrome *c* (A) and MTG (B). Co-localization is shown by yellow in the overlay (C). (D–G) Fluorescence images of RFP–cytochrome *c* in a cardiomyocyte at different times (0–3 h) after addition of 1 μ M staurosporine. This cardiomyocyte expressed both RFP–cytochrome *c* and GFP–Bax (see text). Scale bars, 10 μ m.

believed to interact functionally with Bax. Bid is retained in the cytosol of healthy cells, but early in apoptosis it becomes cleaved by caspase-8 and the truncated Bid (t-Bid) migrates to mitochondria [42]. Immunogold labelling shows that t-Bid is located mainly at mitochondrial intermembrane contact sites in apoptotic cells [43], where it is believed to bind to cardiolipin [44]. Cardiolipin is found in the mitochondrial inner membrane, but the close apposition of the inner and outer membranes at the contact sites may facilitate interaction between the t-Bid and cardiolipin. t-Bid needs Bax (or Bak) to exert its pro-apoptotic role [45]. Gathering data suggest that Bid facilitates the changes in Bax conformation required for its insertion in the outer mitochondrial membrane [16,46,47]. Thus Bid–Bax interactions probably precede Bax integration into the outer membrane to form cytochrome *c*-permeable pores. Whether or not these interactions and the associated conformational changes in Bax occur at the intermembrane contact sites remains to be seen, but our finding that Bax interacts with VDAC–ANT in phase 1 is clearly consistent with this. Moreover, the fact that cytochrome *c* is released during the period when the amount of mitochon-

drially bound Bax remains limited (i.e. in the plateau of phase 1) suggests that Bax permeabilizes the outer membrane while still associated with VDAC–ANT, and that the intermembrane contact sites are also the sites of cytochrome *c* release.

Phase 2 was characterized by the near-complete sequestration of Bax from the cytosol into large aggregates associated with the mitochondria. Smaller Bax aggregates, undetectable by fluorescence microscopy, may occur at an earlier stage. Antonsson et al. [19] have described the isolation from staurosporine-treated cells of Bax oligomers of 96 and 260 kDa (monomeric Bax is 23 kDa), and the existence of intermediates between these and the large Bax aggregates cannot be ruled out. But from the present study it seems clear that the formation of the large Bax aggregates visible under the fluorescence microscope occurs downstream of cytochrome *c* release. Accordingly, the large Bax aggregates cannot be responsible for the release of cytochrome *c*. This raises the obvious question of why mitochondria assemble these aggregates when the job of cytochrome *c* release has already been accomplished. Either the aggregates carry out a further, as yet unrecognized, role of Bax, or the aggregates

Figure 8 GFP-Bax co-immunoprecipitates VDAC and ANT from extracts of apoptotic cells

(A) GFP-Bax-transfected cells were stimulated with $1 \mu\text{M}$ staurosporine and extracted 0, 2 and 6 h later as indicated (0, 2, 6). GFP-Bax was immunoprecipitated and the immunoprecipitates were analysed by SDS/PAGE (silver stained). (B) GFP-Bax-transfected cells were extracted 0, 2 and 6 h after staurosporine as indicated. GFP-transfected cells were extracted 6 h after staurosporine. GFP-Bax and GFP were immunoprecipitated and the immunoprecipitates (IP) analysed for GFP-Bax (50 kDa), VDAC (32 kDa) and ANT (32 kDa). The non-immunoprecipitated VDAC and actin in the supernatant (SN) were also measured. The 2 and 6 h extractions correspond to phase 1 and phase 2, respectively, as confirmed by the proportion of cells showing GFP-Bax aggregates (fluorescence microscopy). (C) Shows the selectivity of the anti-ANT polyclonal and anti-VDAC monoclonal antibodies used on mitochondrial extracts (Coomassie-stained track). The positions of M_r markers (21, 35 and 51 kDa) are indicated.

provide a step in the processing of Bax subsequent to its role in permeabilizing the mitochondrial outer membrane. In any event, aggregate formation was characterized by a sudden onset after several h of phase 1. It appears, then, that on completion of phase 1 the cell receives a signal to remove residual Bax from the cytosol and to package the Bax into large aggregates. Conceivably, the signal is the release of intermembrane space proteins or a related contemporaneous event. Nechushtan et al. [21] found that the formation of large Bax aggregates was inhibited by overexpression of anti-apoptotic Bcl- X_L , suggesting a correlation between aggregate formation and cytotoxicity. However, these observations can also be accounted for by the present model which ascribes no cytotoxic role to the large Bax aggregates. Since Bcl- X_L inhibits Bax-induced cytochrome *c* release [48] it presumably acts in phase 1 and would also be expected to suppress the signal (see above) for progression to phase 2, when aggregate assembly takes place.

In conclusion, our data support a two-phase model of Bax translocation to mitochondria in which mitochondrial Bax sequestration is initially limited while cytochrome *c* release takes place. This limitation may be imposed by binding to junctional VDAC-ANT complexes. Only when the outer membrane is permeabilized to cytochrome *c* is the second phase of Bax translocation initiated in which Bax is quantitatively channelled into the large mitochondrially associated aggregates. The model should facilitate experimentation into the mechanisms that control Bax translocation in the two phases.

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