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Temporal dissection of Bax-induced events leading to fission of the single mitochondrion in *Trypanosoma brucei*

Anne Crausaz Esseiva[1], Anne-Laure Chanez[1*], Natacha Bochud-Allemann[1], Jean-Claude Martinou[2], Andrew Hemphill[3] & André Schneider[1+]*

[1]Department of Biology/Zoology, University of Fribourg, Fribourg, Switzerland, [2]Departement de Biologie Cellulaire, Sciences III, Genève, Switzerland, and [3]Institute of Parasitology, University of Bern, Bern, Switzerland

The protozoan *Trypanosoma brucei* has a single mitochondrion and lacks an apoptotic machinery. Here we show that expression of the proapoptotic protein Bax in *T. brucei* causes the release of cytochrome *c*, the depolarization of the mitochondrial membrane potential and mitochondrial fission. However, in contrast to mammalian cells, the three events are temporally well separated. The release of cytochrome *c* from the intermembrane space precedes mitochondrial fission, showing that it does not depend on mitochondrial fragmentation. Furthermore, halting Bax expression allows some cells to recover even after mitochondrial fission, the last recorded event, went to completion, indicating that all three Bax-induced events are, in principle, reversible.

Keywords: apoptosis; cytochrome *c*; membrane potential

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INTRODUCTION

The main components of the apopotic death machinery include the pro- and anti-apoptotic members of the Bcl-2 protein family as well as caspases. Mitochondria are central players in apoptosis, as activation of the caspase cascade is often initiated by the release of cytochrome *c* and other intermembrane space components (Desagher & Martinou, 2000; Ferri & Kroemer, 2001; Newmeyer & Ferguson-Miller, 2003). In addition to permeabilization of the outer membrane, a loss of the membrane potential and, in some cases, changes in mitochondrial morphology such as fission are observed (Desagher & Martinou, 2000; Frank *et al*, 2001; Karbowski *et al*, 2002). All effects are initiated by pro-apoptotic proteins such as Bax, which directly interact with mitochondria. However, by which molecular mechanisms they exert their effects and in which order is still unclear.

Some forms of programmed cell death have been described in the parasitic protozoan *Trypanosoma brucei*; however, they are clearly different from the classical apoptosis observed in mammalian cells (Welburn & Murphy, 1998; Ameisen, 2002; Debrabant *et al*, 2003). In agreement with this, a survey of the available *T. brucei* genome did not reveal any homologues for caspases or for members of the Bcl-2 protein family. Genes encoding metacaspases, on the other hand, which show only a limited similarity to bona fide caspases, were found. However, it is unclear at present whether trypanosomal metacaspases have protease activity and whether they have a role in programmed cell death (Szallies *et al*, 2002). Interestingly, *T. brucei*, unlike any other eukaryote, has a single continuous mitochondrion throughout its life and cell cycle (Simpson & Kretzer, 1997; Tyler *et al*, 2001). Its genome is exclusively localized at a precise position in the posterior region of the organelle. Thus, *T. brucei* provides an excellent system to study the effects of pro-apoptotic proteins on the single mitochondrion level in the absence of apoptotic death effectors.

RESULTS AND DISCUSSION

Bax-induced effects on mitochondrial energy metabolism

To take advantage of its unique mitochondrial biology, we have established a transgenic *T. brucei* cell line allowing tetracycline-inducible expression of human Bax. Similar to yeast (Greenhalf *et al*, 1996; Harris *et al*, 2000), expression of Bax inhibits growth and will eventually kill the cells. Is the observed death of physiological significance in that it mirrors the events in apoptotic mammalian cells? To address this question, we have established a trypanosomal cell line that, upon addition of tetracycline, expresses not only Bax but also the Bax antagonist Bcl-x_L. Fig 1 shows that, similar to mammalian cells, Bcl-x_L expression inactivates Bax. The growth rate of the Bax/Bcl-x_L-expressing cell

[1]Department of Biology/Zoology, University of Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland
[2]Departement de Biologie Cellulaire, Sciences III, Quai E. Ansermet 30, CH-1211 Genève, Switzerland
[3]Institute of Parasitology, University of Bern, Laenggass-Strasse 122, 3012 Bern, Switzerland
*These authors contributed equally to this work
+Corresponding author. Tel: +41 26 3008877; Fax: +41 26 3009741; E-mail: andre.schneider@unifr.ch

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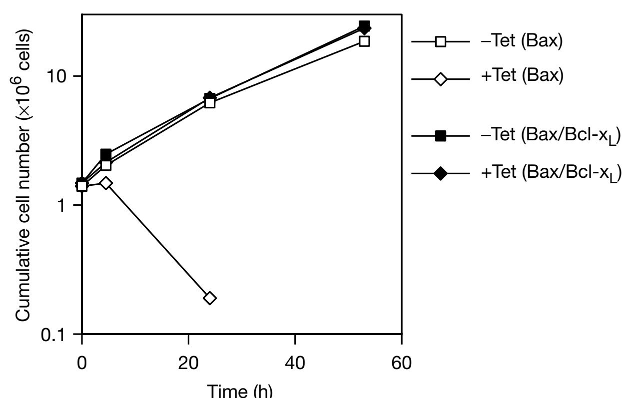

Fig 1 | Effect of Bax expression and Bax/Bcl-x_L coexpression on the growth of *T. brucei*. Growth curves of *T. brucei* cell lines transformed with the human Bax cDNA (Bax) alone or with both the human Bax and Bcl-x_L cDNAs (Bax/Bcl-x_L) together, respectively. Cumulative cell numbers for growth in the absence (−Tet) and presence (+Tet) of tetracycline are shown.

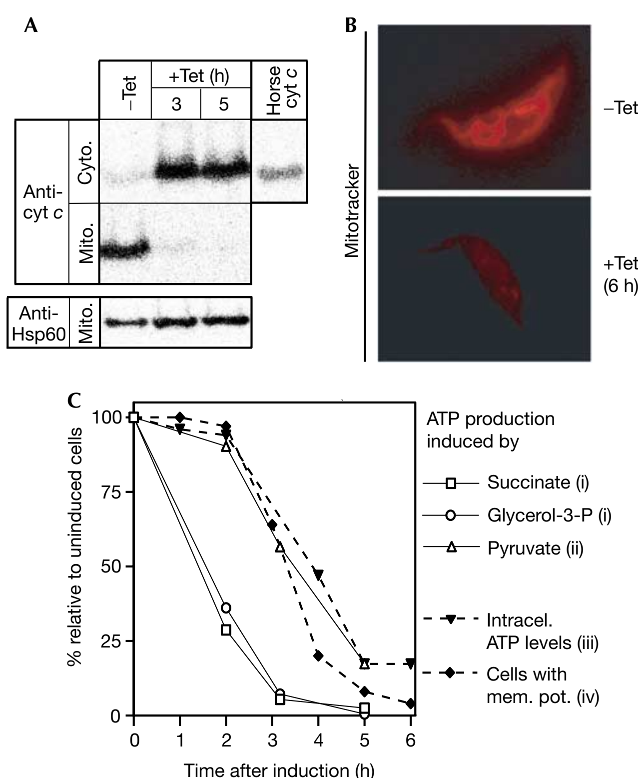

Fig 2 | Bax-induced changes of cytochrome *c* localization and of mitochondrial energy metabolism. (**A**) A transgenic *T. brucei* cell line allowing tetracycline-inducible expression of human Bax was analysed for the release of cytochrome *c*. Using immunoblots, 50 μg each of cytosolic extract (upper panel) and of isotonically isolated gradient-purified mitochondria (middle panel; Hauser *et al*, 1996) from uninduced cells (−Tet) and from cells expressing Bax for 3 or 5 h (+Tet (h) 3 or 5), respectively, were analysed for cytochrome *c* content. All mitochondrial fractions were also analysed for the presence of Hsp60 (lowest panel). (**B**) Mitotracker staining of an uninduced cell with an intact mitochondrial membrane potential (−Tet) and a cell expressing Bax for 6 h that lacks a membrane potential (+Tet, 6 h). (**C**) Time course of Bax-induced changes in mitochondrial energy metabolism. Four parameters were measured at the indicated time points after induction of Bax expression: (i) *in organello* ATP production in response to succinate and glycerol-3-phosphate to monitor oxidative phosphorylation; (ii) *in organello* ATP production in response to pyruvate to measure mitochondrial substrate level phosphorylation (van Hellemond *et al*, 1998; Bochud-Allemann & Schneider, 2002); (iii) intracellular ATP levels (ATP production and ATP content are indicated relative to uninduced cells); and (iv) the presence or absence of a mitochondrial membrane potential in individual cells ($n = 150$–300) as determined by Mitotracker staining (expressed as the percentage of the total population that retains a membrane potential).

line is the same as that of uninduced cells, even though a small subpopulation of cells (<1%) still shows a Bax-induced change in mitochondrial morphology (not shown). The Bax protein that is expressed in *T. brucei* is therefore correctly folded and able to interact with its regulators. During the first 12 h after induction of Bax expression, the cell density remains constant and no change in cell morphology is observed. During that period, it is possible to measure the effects of Bax that precede cell death.

The immunoblot in Fig 2A shows that essentially all cytochrome *c* is released within the first 3 h after induction of Bax expression. *In organello* ATP production assays, induced by the respiratory substrates succinate and glycerol-3-phosphate, were used to quantify oxidative phosphorylation (Allemann & Schneider, 2000; Bochud-Allemann & Schneider, 2002). The results in Fig 2C show that, concomitant with the release of cytochrome *c*, a loss of oxidative phosphorylation is observed, suggesting that at early time points Bax permeabilizes the mitochondrial outer membrane and that the release of cytochrome *c* causes the loss of oxidative phosphorylation.

We next investigated the effect of Bax on the mitochondrial membrane potential by using Mitotracker, a membrane-potential-sensitive dye. The Mitotracker staining revealed two types of cells: cells having an intact membrane potential showing the single network-like mitochondrion and cells with a depolarized potential showing a weaker uniform staining (Fig 2B). Fig 2C shows that the loss of oxidative phosphorylation precedes the collapse of the membrane potential by approximately 2 h. These observations are in agreement with reports showing that apoptotic mammalian cells can maintain the mitochondrial membrane potential for a limited time even after cytochrome *c* has been released (Mootha *et al*, 2001; Waterhouse *et al*, 2001). However, unlike as suggested for mammalian systems (Ricci & Gottlieb, 2003), the loss of the membrane potential observed in trypanosomal mitochondria cannot be caused by caspases.

Fig 2C shows furthermore that, most likely as a consequence of the depolarization of the membrane potential, a synchronous loss of the pyruvate-induced mitochondrial substrate level phosphorylation (Bochud-Allemann & Schneider, 2002), a drop of the total cellular ATP content and a decline of flagellar motility occur (not shown).

Bax-induced mitochondrial fission

To monitor directly Bax expression and to investigate its effects on mitochondrial morphology, we performed immunofluorescence

using antibodies directed against Bax and the mitochondrial matrix heat shock protein 60 (Hsp60; Fig 3A). Bax was only detected in induced cells and showed a punctate pattern whose kinetics of appearance coincided with the loss of the membrane potential (Fig 3A,C). The Hsp60 staining shows that, at early time points, cells with detectable Bax exhibit a normal mitochondrial morphology. However, approximately 2.5 h later, the single mitochondrion of uninduced cells becomes fragmented into 7–9 distinct spherical compartments (Fig 3A). The punctate Bax staining colocalizes with the mitochondrial fragments; however, it generally does not completely overlap with mitochondrial vesicles but appears to be concentrated in dots localized at their periphery. Few mitochondrial fragments lacking Bax as well as few Bax-containing structures devoid of Hsp60 are also seen. The highly condensed mitochondrial genome of *T. brucei* is easily detected by DAPI fluorescence and always colocalizes with a mitochondrial fragment.

To confirm that the observed mitochondrial fragmentation is a specific consequence of Bax expression and not just associated with any kind of cell death, we made use of an RNA interference (RNAi) cell line ablated for succinyl-CoA synthetase, a mitochondrial enzyme essential for survival of insect-stage *T. brucei* (Bochud-Allemann & Schneider, 2002). The right panel of Fig 3A shows that the mitochondrial morphology in these cells remains normal at a time point when the cells are already irreversibly committed to death.

Electron micrographs show that the outer and the inner membranes can clearly be distinguished in the fragmented mitochondria of induced cells, indicating that Bax expression does not cause a global rupture of the mitochondrial outer membrane (Fig 3D). After 8 h of Bax expression, the entire cell

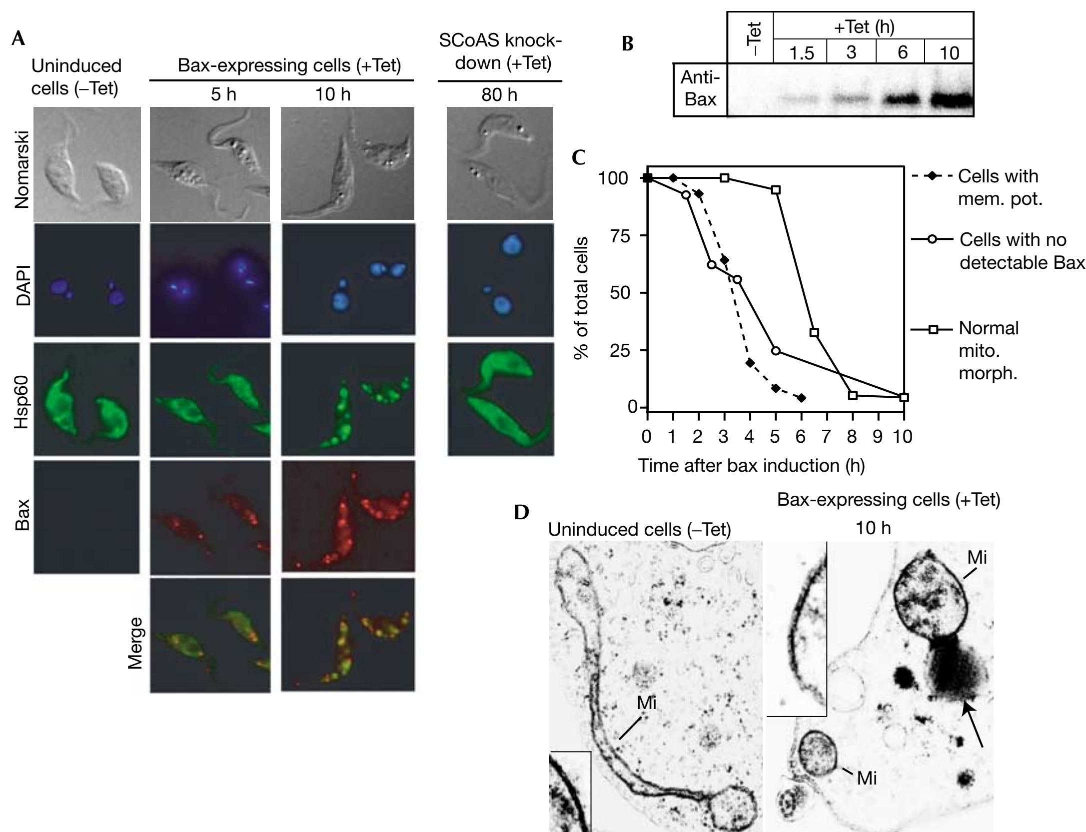

Fig 3 | Bax-induced changes of mitochondrial morphology. (**A**) Left panel: the *T. brucei* cell line transformed with the human Bax cDNA was analysed for Bax expression and mitochondrial morphology using double immunofluorescence with Hsp60 and Bax antisera, respectively. Left column: uninduced cells (−Tet); middle and right columns: Bax-expressing cells induced (+Tet) for 5 and 10 h, respectively. Right panel: Hsp60 staining of an RNA interference (RNAi) cell line downregulated for the essential mitochondrial enzyme succinyl-CoA synthetase (SCoAS) 80 h after induction of RNAi (Bochud-Allemann & Schneider, 2002). At this time, the culture is dying but no mitochondrial fission is observed. The length of a *T. brucei* cell is approximately 20 μm. (**B**) Time course of Bax detection on immunoblots. (**C**) Time course of the appearance of the punctate Bax staining and mitochondrial fission. Double immunofluorescence of cells from a culture induced for Bax expression was used to score the presence of the punctate Bax staining and the occurrence of mitochondrial fission. The relative proportions of each cell culture ($n = 150$–300 cells), which at the indicated times after induction of Bax expression still show the phenotypes of uninduced cells, namely absence of Bax and a single mitochondrion, are indicated on the y-axis. For comparison, the disappearance of the mitochondrial membrane potential (same curve as shown in Fig 2C) is shown as well. (**D**) Electron micrographs of uninduced cells and cells grown in the presence of tetracycline for 10 h. The double mitochondrial membranes are shown in the insets. Electron-dense material attached to mitochondrial vesicles (Mi) seen in induced cells is indicated by the arrow.

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culture essentially only consists of cells that have a fragmented mitochondrion (Fig 3C). However, a low number of cells (approximately 5%) still show normal mitochondrial morphology even though most of them express Bax.

Mitochondrial fission observed during mammalian apoptosis is due to recruitment of dynamin-related protein 1 (Drp1) and Mfn2, two proteins that are involved in fission of the mitochondrial outer membrane in normal cells (Frank *et al*, 2001; Karbowski *et al*, 2002). Furthermore, overexpression of a dominant-negative mutant of Drp1 was shown to inhibit the loss of the membrane potential as well as the release of cytochrome *c*, suggesting that mitochondrial fission is required for both processes (Frank *et al*, 2001). In *T. brucei*, however, the observed release of cytochrome *c* and the depolarization of the membrane potential are not caused by mitochondrial fission, as they precede mitochondrial fragmentation by 4.5 and 2.5 h, respectively (Figs 2C and 3B). At present, we cannot explain this discrepancy. However, it is interesting to note that the *T. brucei* mitochondrion has 'discoidal cristae' (flat with pinched bases) whereas mammalian mitochondria contain 'flattened cristae'. Discoidal cristae are found in few protozoa and are thought to represent the most ancestral cristae type (Taylor, 1999). The only physiological fission event predicted for the *T. brucei* mitochondrion is expected to occur during cell division and must therefore be tightly regulated temporally and spatially. It is possible that expression of Bax activates this putative mitochondrial fission machinery at the wrong sites and at the wrong time.

Bax-induced effects are reversible

Removing tetracycline from the cell culture stops Bax expression. Interestingly, all three described Bax-induced transitions are, in principle, reversible, as a *T. brucei* population that was expressing Bax for 10 h and therefore consisted of 95% of cells having fragmented mitochondria was able to resume normal growth after a short lag phase (Fig 4A). The lag phase is most likely caused by the fact that outgrowth of the cells requires degradation of the remaining Bax protein. Alternatively, the lag phase might be due to selection of cells that are unable to express Bax. However, we believe this is unlikely as the outgrowing population, on addition of tetracycline, will re-express Bax and respond to it in the same way as the original population (Fig 4A, broken lines). Nevertheless, it is difficult to exclude that the growth of the population is due to selection of the low number of Bax-expressing cells whose mitochondrion never became fragmented. However, Mitotracker staining of the recovering cell culture reveals two populations of mitochondrial membrane-potential-positive cells, one showing the expected single mitochondrion and the other showing unfused mitochondrial vesicles exhibiting a membrane potential (Fig 4B). As the latter are never seen during induction of Bax expression (Fig 2B), these results indicate that at least some cells that have a fragmented mitochondrion are able to re-establish a membrane potential. The detection of these putative intermediates indicates that there is a population of cells in which, after halting of Bax expression, the fragmented mitochondrial vesicles fuse to rebuild the single mitochondrion. The *T. brucei* mitochondrion must therefore have a functional mitochondrial fusion machinery even though fusion is never observed under physiological conditions. Fig 4C shows that cells recovering from 10 h of Bax expression simultaneously restore the membrane potential and the network-like mitochondrial morphology. These results suggest that restoration of the membrane potential precedes mitochondrial fusion, and are in agreement with results from mammalian cells showing that mitochondrial fusion requires an intact membrane potential (Legros *et al*, 2002; Mattenberger *et al*, 2003). Thus, within the first 10 h, all the observed Bax-induced effects are, in principle, reversible, suggesting that neither of the three described events irreversibly damages the mitochondrion. In agreement with this, it was shown that mitochondria of sympathetic neurons after

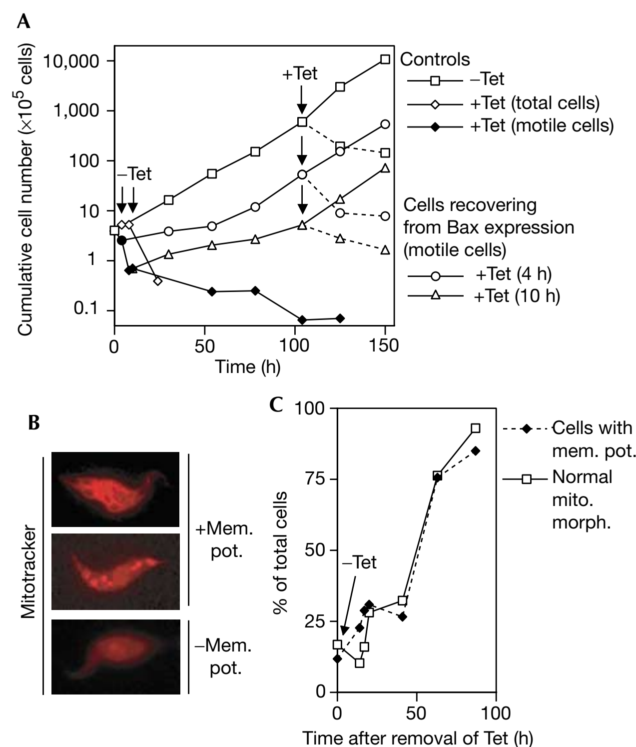

Fig 4 | Recovery from Bax-induced changes. (**A**) Controls: −Tet, growth curves of the *T. brucei* cell line transformed with the human Bax cDNA grown in the absence of tetracycline, all cells are motile; +Tet (total cells) and +Tet (motile cells), same cell line grown in the presence of tetracycline (1 μg/ml) to induce Bax expression, total cell number and motile cells only are indicated, 24 h after induction all cells were immotile. Cell cultures recovering from Bax expression: +Tet (4 h) and +Tet (10 h), growth curves of cell cultures induced for Bax expression for 4 or 10 h, after which expression was stopped by the removal of tetracycline (arrows, −Tet); motile cells only were scored. Broken lines: growth curves of recovering cell cultures in which tetracycline (1 μg/ml) was readded (arrows, +Tet) 95 h after the stopping of Bax expression; motile cells only were scored. (**B**) Three types of Mitotracker staining observed in cultures recovering from Bax expression: top panel, single mitochondrion staining of a completely recovered cell; middle panel, membrane-potential-positive mitochondrial fragments, a staining exclusively observed in recovering populations; lowest panel, membrane-potential-negative cell. (**C**) Time course of the restoration of the membrane potential scored by Mitotracker staining and the normal mitochondrial morphology measured by immunofluorescence using Hsp60 antiserum. The cell culture expressed Bax for 10 h before removal of the tetracycline.

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complete release of cytochrome *c* were able to recover fully in the presence of caspase inhibitors (Martinou *et al*, 1999). Prolonged expression of Bax (more than 24 h; Figs 1 and 4A) will kill trypanosomes even in the absence of death effectors. However, the observed Bax-induced killing of *T. brucei* is most likely not of apoptotic nature, but can be explained by the lack of oxidative phosphorylation, a process essential for the survival of procyclic *T. brucei*.

In summary, our work shows that expression of Bax in *T. brucei* causes (i) the release of cytochrome *c* and the loss of oxidative phosphorylation, (ii) the depolarization of the membrane potential and a decline of the intracellular ATP concentration, and (iii) mitochondrial fission. All three events are induced by Bax alone and do not require other apoptotic factors. Furthermore, in contrast to mammalian systems in which the three effects essentially occur simultaneously (Frank *et al*, 2001), they can be temporally separated in *T. brucei* and are, in principle, reversible.

METHODS

Bax-expressing and Bax/Bcl-x_L-coexpressing cell lines. Bax-expressing *T. brucei* cells were prepared as follows: a DNA fragment derived from the cDNA of the wild-type human Bax mRNA was inserted into the trypanosomal expression plasmid pLew100, which carries a tetracycline-inducible procycline promoter (Wirtz & Clayton, 1995; Wirtz *et al*, 1999). The construct was transfected into the insect-stage *T. brucei* strain 29-13, which expresses the tetracycline repressor. Transfection, selection with phleomycine and cloning were performed as described (Beverley & Clayton, 1993). Bax expression was induced by the addition of tetracycline to 1 μg/ml. The Bax/Bcl-x_L-coexpressing cells are based on the Bax-expressing cell line, which was transfected with the Bcl-x_L-expressing plasmid. This plasmid is identical to the Bax-expressing plasmid, except that it contains the human Bcl-x_L cDNA as an insert and a puromycine resistance gene for selection. To remove tetracycline, the cells were reisolated and washed once in media without tetracycline and then resuspended in the same volume of media.

Release of cytochrome *c*. Purification of mitochondria having an intact outer membrane was carried out as described (Hauser *et al*, 1996). Mitochondrial and cytosolic protein extracts from uninduced cells and cells induced for Bax expression for 3 and 5 h, respectively, were resolved on 16% SDS–PAGE, blotted to nitrocellulose and probed with polyclonal rabbit antisera directed against trypanosomal cytochrome *c*. The rabbit antiserum was produced by Eurogentec using the peptides PPKERAALPPGDAVR and QERADL IAYLETLKD as antigens.

ATP production assays. ATP production assays were carried out as described (Allemann & Schneider, 2000; Bochud-Allemann & Schneider, 2002), and a detailed analysis of the different ATP production pathways in *T. brucei* and how they can be distinguished has been published (Bochud-Allemann & Schneider, 2002). Oxidative phosphorylation was measured using either succinate or glycerol-3-phosphate as substrate. Trypanosome-specific mitochondrial substrate level phosphorylation was measured in the presence of antimycine using a combination of pyruvate and succinate as substrates. For all substrates, the reactions were also performed in the presence of atractyloside, a specific inhibitor of the ADP/ATP translocator. Atractyloside blocks the import of ADP and is used to prove that the detected ATP productions are mitochondrial.

To measure the total cellular ATP content, aliquots taken at the indicated times and containing equal cell numbers were treated with 0.01 U/μl of apyrase at 4°C for 5 min and processed in the same way as described above.

Immunofluorescence. Immunfluorescence was performed as described (Sherwin *et al*, 1987). Fixation was carried out using 4% (w/v) of paraformaldehyde in 1 × phosphate-buffered saline (PBS) for 10 min, and cells were permeabilized for 2 min using PBS containing 2% (w/v) of Triton X-100. Expression and localization of Bax were determined using 2 μg/ml of the monoclonal anti-human Bax antibody 2D2 (NeoMarkers, Fremont, CA). To visualize the mitochondrion, a 1:100 dilution of a polyclonal anti-Hsp60 antiserum raised against the isolated yeast protein was used.

Immunoblots. To detect Bax expression on immunoblots, we used a 1:1,000 dilution of the polyclonal rabbit anti-human Bax antiserum (BD Pharmingen) and the SuperSignal West Femto Max Sensitivity Substrate from Pierce for detection.

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