

# Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis

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**Mitochondria trigger apoptosis by releasing caspase activators, including cytochrome c (cytC). Here we show, using a pH-sensitive green fluorescent protein (GFP), that mitochondria-dependent apoptotic stimuli (such as Bax, staurosporine and ultraviolet irradiation) induce rapid, Bcl-2-inhibitable mitochondrial alkalinization and cytosol acidification, followed by cytC release, caspase activation and mitochondrial swelling and depolarization. These events are not induced by mitochondria-independent apoptotic stimuli, such as Fas. Activation of cytosolic caspases by cytC *in vitro* is minimal at neutral pH, but maximal at acidic pH, indicating that mitochondria-induced acidification of the cytosol may be important for caspase activation; this finding is supported by results obtained from cells using protonophores. Cytosol acidification and cytC release are suppressed by oligomycin, a  $F_0F_1$ -ATPase/ $H^+$ -pump inhibitor, but not by caspase inhibitors. Ectopic expression of Bax in wild-type, but not  $F_0F_1/H^+$ -pump-deficient, yeast cells similarly results in mitochondrial matrix alkalinization, cytosol acidification and cell death. These findings indicate that mitochondria-mediated alteration of intracellular pH may be an early event that regulates caspase activation in the mitochondrial pathway for apoptosis.**

**T**hough normally stored between the inner and outer membranes of mitochondria, cytC is commonly released into the cytosol following exposure of cells to apoptotic stimuli. Once it is in the cytosol, cytC binds to the caspase-activating protein Apaf-1, inducing formation of an oligomeric complex that recruits and proteolytically activates procaspase-9. Activated caspase-9 then cleaves and activates further downstream caspases, ultimately inducing apoptosis<sup>1</sup>.

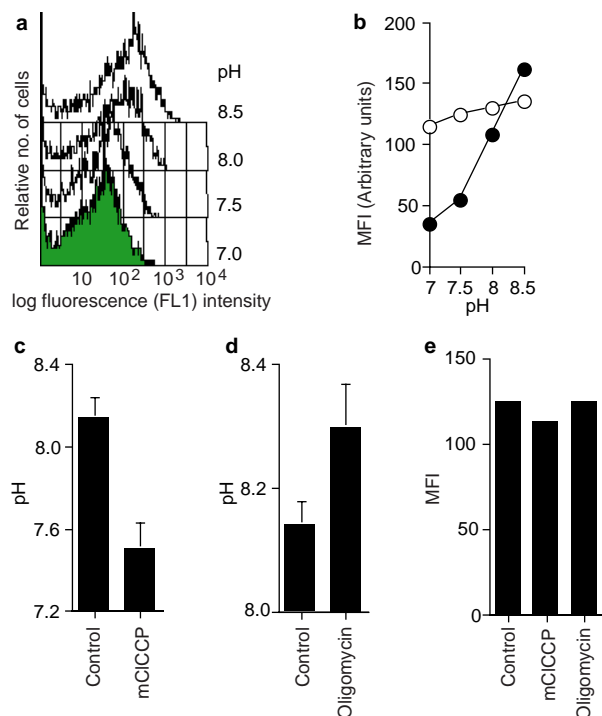
The mechanism by which cytC is released from mitochondria during apoptosis remains unknown. Two competing models have been proposed—first, a volume-dependent mechanism, involving mitochondrial swelling as a result of increased mitochondrial-matrix volume, which causes the subsequent rupture of the outer membrane and release of cytC and other proteins normally sequestered in the mitochondrial intermembrane space (IMS); second, a volume-independent mechanism, in which the permeability of the outer membrane is selectively altered, allowing proteins to leak out (reviewed in refs 2, 3). Two alternative versions of the volume-dependent mechanism for cytC release have been suggested. One model involves ‘mitochondrial permeability transition’ (MPT)<sup>4</sup>, a non-selective increase in the permeability of the inner membrane that is thought to involve a multi-protein complex known as the PT pore. PT-pore opening commonly occurs during apoptosis, and results in depolarization of mitochondria, loss of the  $H^+$  gradient normally present across the inner membrane, and non-selective entry of ions and water into the solute-rich matrix, leading to an increase in volume. Although  $Ca^{2+}$ , oxidants and some other agents that directly affect the PT pore clearly operate through this mechanism<sup>5</sup>, some data indicate that the PT-pore opening induced by many apoptotic stimuli (such as growth-factor deprivation, Bax and DNA-damaging agents) may be a relatively late event that occurs after cytC release and caspase activation (reviewed in ref. 2). In the other proposed model for volume-dependent cytC release, which involves hyperpolarization of mitochondria, inner-membrane permeability remains intact, but unidentified ion channels involved in regulation of matrix volume induce mitochondrial

swelling, without affecting respiration or triggering mitochondrial depolarization<sup>6</sup>.

The pH in localized environments within intact cells can be monitored using engineered mutants of the *Aequorea victoria* GFP that exhibit pH-dependent absorbance and fluorescence-emission properties<sup>7</sup>. Many previous studies of mitochondria and apoptosis have attempted to infer indirectly the status of the  $H^+$  gradient across the inner membrane, using cationic dyes such as rhodamine 123, 3,3'-dihexyloxycarbocyanine iodide (DiOC6<sub>(3)</sub>) and tetramethylrhodamine ester (TMRM). However, these dyes, which partition across membranes in accordance with the Nernst equation, measure voltage potential ( $V_m$ ) rather than pH, and often produce artifacts<sup>8,9</sup>. Here, using a pH-sensitive GFP targeted to mitochondria, we provide evidence that alteration of pH regulation represents an early event in apoptosis, preceding cytC release and PT-pore opening, and facilitating cytC-mediated activation of caspases.

## Results

**Monitoring of mitochondrial-matrix pH using a pH-sensitive GFP mutant.** We used a pH-sensitive GFP mutant, YFP(H148G)<sup>10</sup> (hereafter referred to as pH-GFP) that increases its fluorescence emissions in an almost linear fashion over the pH range 7.0–8.5. This GFP mutant is optimal for measuring mitochondrial-matrix pH because it has a pK<sub>a</sub> of 8, which is close to the matrix pH (ref. 7). To faithfully target the protein to the mitochondrial matrix, we engineered a complementary DNA encoding pH-GFP to contain a mitochondrial-import amino-terminal leader peptide<sup>7</sup>. Transfectants expressing mitochondria-targeted pH-GFP (pH-GFP<sub>mito</sub>) were generated using the Jurkat T-cell leukaemia and HEK293T epithelial-carcinoma cell lines. FACS analysis of cells expressing pH-GFP<sub>mito</sub> that were incubated with ionophores and then equilibrated in buffers of varying pH showed an almost linear increase in fluorescence over the pH range 7.0–8.5 (Fig. 1a, b). Resting matrix pH was estimated at 7.7–8.2 in both 293T and Jurkat cells, which is consistent with previous reports of a pH gradient between mitochondria



**Figure 1 Use of pH-GFP<sub>mito</sub> to estimate mitochondrial-matrix pH.** **a**, Histogram showing FACS data for pH dependence of fluorescence emissions from 293T cells expressing pH-GFP<sub>mito</sub> at the indicated pH values. Emissions were collected at 530 nm (FL1 channel). **b**, Standard curves derived from **a**, showing mean fluorescence intensity (MFI) in the FL1 channel against pH. Closed circles, 293T cells expressing pH-GFP<sub>mito</sub>; open circles, 293T cells expressing a pH-insensitive GFP targeted to mitochondria. **c**, Estimates of matrix pH for 293T cells expressing pH-GFP<sub>mito</sub>, incubated in the absence (control) or presence of mClCCP. Estimates were made using MFI data together with a pH standard curve prepared in parallel. Values are means ± s.e.m. from 10<sup>4</sup> cells. **d**, Estimates of matrix pH for 293T cells expressing pH-GFP<sub>mito</sub> incubated in the absence (control) or presence of oligomycin. Values are means ± s.e.m. from 10<sup>4</sup> cells. **e**, MFI, in the FL1 channel, of 293T cells expressing a mitochondria-targeted pH-insensitive GFP and treated with mClCCP, oligomycin or neither (control).

and the cytosol of ~0.5–1.0 pH units<sup>11</sup>. We treated these cells with protonophore carbonyl cyanide m-chlorophenyl-hydrazone (mClCCP), which dissipates the H<sup>+</sup> gradient across the inner mitochondrial membrane, or with the F<sub>0</sub>F<sub>1</sub>-ATPase/H<sup>+</sup>-pump inhibitor oligomycin, which prevents transport of H<sup>+</sup> back into the matrix from the intramembrane space, and found that the estimated matrix pH decreased or increased, respectively, as expected (Fig. 1c, d). In contrast, levels of fluorescence in cells expressing a pH-insensitive GFP protein (ECFP<sub>mito</sub>)<sup>7</sup> were not significantly altered as a result of treatment with mClCCP or oligomycin (Fig. 1e), a finding that confirms the specificity of this method.

**Mitochondrial-matrix alkalization and cytosol acidification.** We used Jurkat cells expressing pH-GFP<sub>mito</sub> to investigate the regulation of mitochondrial pH during apoptosis. Apoptosis can be induced in Jurkat cells using the kinase inhibitor staurosporine (STS), ultraviolet irradiation, or anti-Fas antibodies<sup>2</sup>. Roughly 50% of these cells underwent apoptosis within 2 h of exposure to 0.5 μM STS, 20 mJ ultraviolet radiation or 0.1 μg ml<sup>-1</sup> CH11 anti-Fas antibody, developing fragmented nuclei with condensed chromatin, as shown by staining with Hoechst 33258 dye (Fig. 2a). STS, ultraviolet radiation and anti-Fas antibody also stimulated detectable increases in caspase activity in these cells within 1 h (Fig. 2b), as measured by cleavage of the fluorogenic substrate benzoyloxycarbonyl-Asp-Glu-Val-Asp-aminofluorocoumarin (Ac-DEVD-AFC)

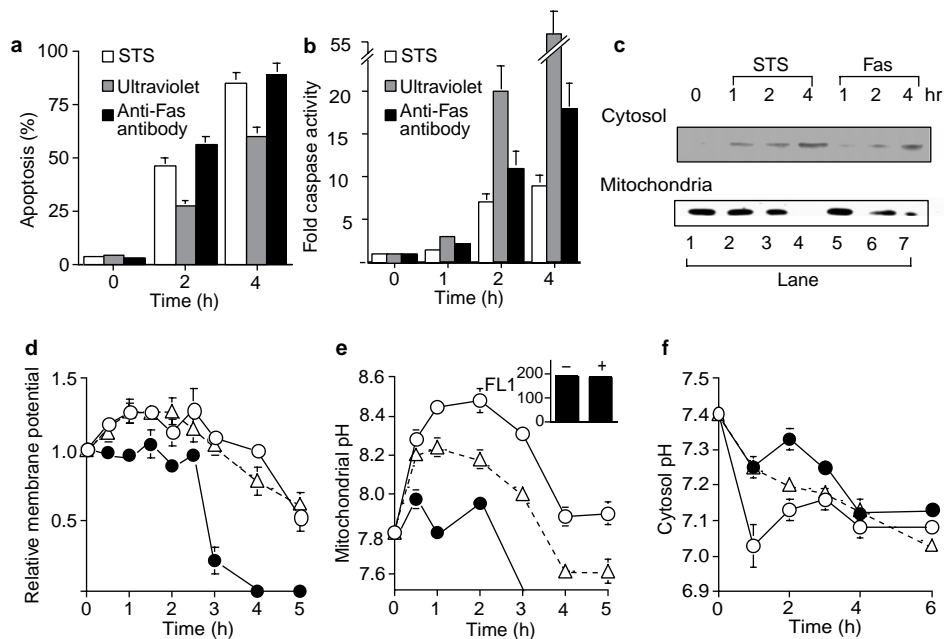
in cell lysates. CytC release from mitochondria was also evident within 1 h or 2 h of treatment with STS or anti-Fas antibody, respectively, as determined by subcellular fractionation. Consistent with previous data<sup>12</sup>, addition of the broad-spectrum caspase inhibitor benzoyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK) to cultures prevented cytC release in cells treated with anti-Fas, but not those treated with STS (data not shown).

We assessed mitochondrial membrane potential (ΔΨ) by FACS analysis using potentiometric dyes, including TMRM and DiOC 6<sub>(3)</sub> (ref. 9). ΔΨ was unchanged in cells treated with anti-Fas antibody for ~2.5 h, then dropped precipitously at ~3 h after treatment (Fig. 2d), indicating that MPT induction may occur as a relatively late event. In Jurkat cells treated with STS, ΔΨ initially increased slightly, then gradually declined from ~3 h after treatment onwards, which is consistent with previous evidence that hyperpolarization can precede induction of MPT in some situations (reviewed in ref. 6). Thus, comparisons of the time courses of apoptosis (Fig. 2a), caspase activation (Fig. 2b), cytC release (Fig. 2c) and ΔΨ change (Fig. 2d) indicate that PT-pore opening in Jurkat cells treated with STS, ultraviolet radiation or Fas is a late event that follows cytC release and caspase activation.

We used pH-GFP<sub>mito</sub> to monitor mitochondrial-matrix pH, and observed striking increases in matrix pH in cells treated with STS and with ultraviolet radiation (Fig. 2e). Matrix alkalization was rapid; marked increases in matrix pH were evident within 30 min and reached maxima at ~2 h after treatment. Matrix pHs then declined (Fig. 2e), consistent with PT-pore opening, as shown by ΔΨ measurements (Fig. 2d). Although STS and ultraviolet radiation induced rapid increases in matrix pH, Fas did not. Rather, in cells treated with anti-Fas antibody, matrix pH remained essentially constant for ~2 h after treatment, then declined to values representative of cytosolic pH. This decline was coincident with induction of PT-pore opening (ΔΨ decrease; Fig. 2d) whereby the inner membranes of mitochondria becomes freely permeable to ions. These data therefore indicate that at least two apoptosis pathways may exist—one represented by STS, in which mitochondrial-matrix alkalization precedes apoptosis, and another typified by Fas, in which matrix alkalization does not occur—despite the fact that caspase activation and generation of reactive oxygen species occur in both paradigms<sup>13,14</sup>.

We observed cytosol acidification in parallel with mitochondrial-matrix alkalization in Jurkat cells treated with STS or ultraviolet radiation, by FACS analysis of cells loaded with the pH-sensitive fluorophore 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), using its acetoxymethyl ester (Fig. 2f). Although decreases in cytosol pH were also evident in Fas-stimulated Jurkat cells<sup>15</sup>, the time course of these decreases was slower than for those in STS-treated cells. Moreover, STS-induced cytosol acidification was caspase-independent, whereas Fas-mediated decreases in cytosolic pH were blocked by z-VAD-FMK (data not shown).

**Bcl-2 inhibits staurosporine-induced mitochondrial-matrix alkalization.** Overexpression of the anti-apoptotic protein Bcl-2 in Jurkat cells expressing pH-GFP<sub>mito</sub> inhibited apoptosis induced by STS but not by Fas (Fig. 3a and data not shown). We therefore compared Neo-transfected (control) and Bcl-2-overexpressing cells, with respect to STS-induced changes in caspase activation, ΔΨ, cytC release, matrix pH and cytosolic pH (Fig. 3). As expected<sup>16,17</sup>, Bcl-2 inhibited STS-induced caspase activation, PT-pore opening (ΔΨ decrease) and cytC release (Fig. 3b–d). Bcl-2 also suppressed the initial mitochondrial hyperpolarization (ΔΨ increase) that precedes PT-pore opening (ΔΨ decrease) in STS-treated Jurkat cells (Fig. 3c). Overexpression of Bcl-2 in Jurkat cells expressing pH-GFP<sub>mito</sub> also suppressed STS-induced matrix alkalization (Fig. 3e) and delayed cytosol acidification (Fig. 3f). In contrast, Fas-induced caspase activation, ΔΨ decrease, cytC release and late cytosol acidification were not inhibited by overexpression of Bcl-2 in these cells (data not shown). Thus, STS-induced changes in regulation of mitochondrial pH are specifically suppressible by Bcl-2.



**Figure 2 Mitochondrial-matrix alkalization is an early event associated with STS-induced, but not Fas-induced, apoptosis.** Jurkat T cells, stably transfected with pH-GFP<sub>mito</sub>, were cultured, treated with staurosporine (STS), ultraviolet radiation or anti-Fas antibody, and analysed at the indicated times after treatment. **a**, Percentage apoptosis, on the basis of staining of nuclei with Hoechst 33258 dye. **b**, Caspase activity, using cell lysates and determined by cleavage of Ac-DEVD-AFC (activity at 0 h designated as 1). **c**, CytC release from mitochondria into cytosol, using cytosolic and mitochondrial cellular fractions (see Methods). **d**, Mitochondrial membrane potential ( $\Delta\Psi$ ), based on FACS (FL1 channel) analysis of untransfected Jurkat cells incubated with 40nM DiOC<sub>6</sub> at 37°C for 20min. Values are relative to untreated cells

(designated as 1.0). Open circles, STS; triangles, ultraviolet; closed circles, Fas. **e**, Mitochondrial-matrix pH, based on FACS (FL1 channel) analysis of cells expressing pH-GFP<sub>mito</sub>, as shown in Fig. 1. Open circles, STS; triangles, ultraviolet; closed circles, Fas. Inset shows data from cells expressing mitochondria-targeted pH-insensitive GFP after culture for 2h in the absence (-) or presence (+) of STS, showing that fluorescence is not affected by STS treatment. **f**, Cytosolic pH, based on FACS (FL1 channel) analysis of untransfected Jurkat cells incubated with 1  $\mu$ M BCECF at 37°C for 30min. pH was estimated from standard curves generated in parallel using cells suspended in buffers of known pH. Open circles, STS; triangles, ultraviolet; closed circles, Fas. Values in **a**, **b**, **d-f** are means  $\pm$  s.d. from at least four separate experiments.

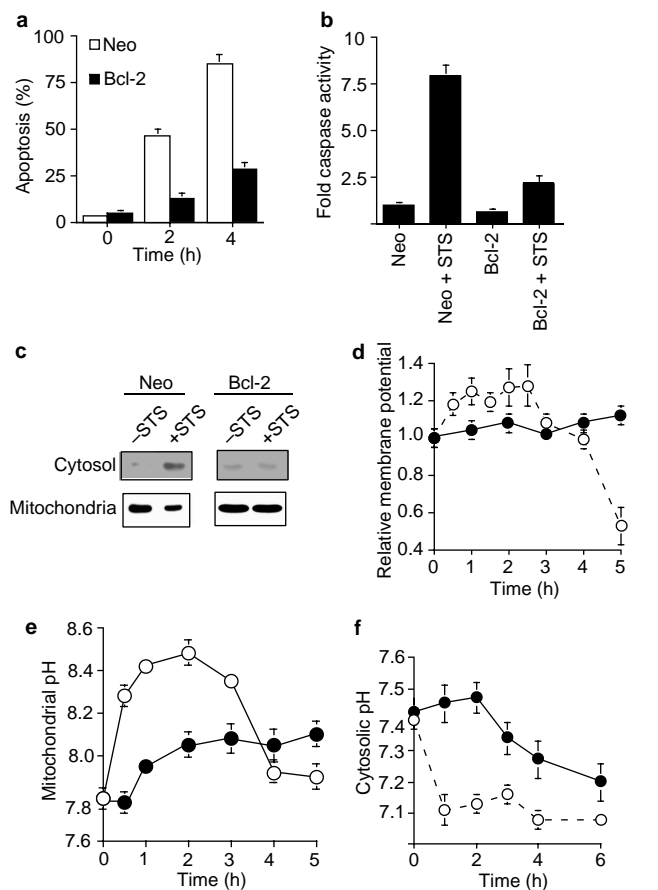
**pH gradients modulate caspase activation induced by STS but not by Fas.** The finding that STS induces rapid mitochondrial-matrix alkalization with concomitant cytosol acidification implies a net efflux of H<sup>+</sup> from mitochondria. Furthermore, the importance of the F<sub>0</sub>F<sub>1</sub>-ATPase/H<sup>+</sup>-pump in apoptosis has previously been demonstrated<sup>18</sup>. Recognizing that this H<sup>+</sup> pump can operate bidirectionally<sup>11,19</sup>, we investigated whether a specific inhibitor of this pump, oligomycin, could alter the changes in mitochondrial pH observed during STS-induced apoptosis. As an alternative approach to exploring the link between pH changes and apoptosis, we also used the protonophore mClCCP, thus equilibrating protons across membranes and preventing net H<sup>+</sup> efflux from mitochondria. Because oligomycin and mClCCP inhibit F<sub>0</sub>F<sub>1</sub>-mediated generation of ATP by mitochondria, we cultured cells in medium containing high concentrations of glucose, to maintain ATP concentrations within 80% of normal levels through anaerobic glycolysis<sup>19,20</sup>. The viability of cells treated with oligomycin or mClCCP remained at >95% for ~6h (data not shown).

Oligomycin and mClCCP, but not z-VAD-FMK, suppressed STS-induced mitochondrial alkalization and cytosol acidification in Jurkat cells (Fig. 4). Similar results were obtained using an alternative F<sub>0</sub>F<sub>1</sub>-ATPase inhibitor, aurovertin B<sup>21</sup> (data not shown). These results therefore indicate that the F<sub>0</sub>F<sub>1</sub>-ATPase may participate in or modulate changes in H<sup>+</sup> flux during STS-induced apoptosis. Cytosolic pH remained at ~7.4, indicating that cells did not suffer from lactic acidosis over the time course of these experiments, despite their reliance on glycolysis.

We then explored the effect of oligomycin on the transient mitochondrial hyperpolarization that occurs soon (~1 h) after STS treatment, before the induction of PT-pore opening when  $\Delta\Psi$  declines. Culturing Jurkat cells with oligomycin alone induced a slight

increase in  $\Delta\Psi$ , which is consistent with decreased utilization of the H<sup>+</sup> gradient (Fig. 3c). STS, induced a greater hyperpolarization than did oligomycin; however, addition of oligomycin to cultures of STS-stimulated cells prevented a rise in  $\Delta\Psi$  beyond that seen in cells cultured only with oligomycin. Similar results were obtained using aurovertin B (data not shown). Thus, the F<sub>0</sub>F<sub>1</sub>-ATPase may be required for transient hyperpolarization of mitochondria during STS-induced apoptosis. Treatment of cells with mClCCP depolarized mitochondria, preventing STS-induced hyperpolarization. Despite depolarization of their mitochondria, cells remained >95% viable under the culture conditions and showed no evidence of apoptosis in the absence of STS (data not shown). In contrast to oligomycin, aurovertin B and mClCCP, z-VAD-FMK failed to suppress STS-induced hyperpolarization (Fig. 3c).

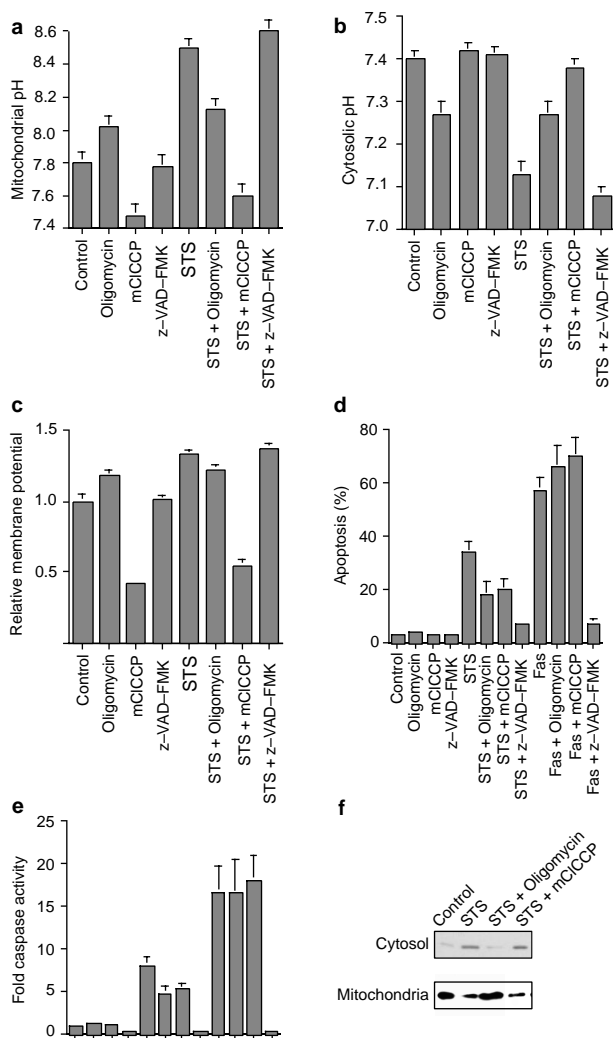
STS-induced apoptosis and caspase activation were partially suppressed by oligomycin (*P*<0.01), aurovertin B (data not shown) and mClCCP (*P*<0.01), whereas Fas-mediated apoptosis was unaffected by these agents (Fig. 4d, e). The fact that Fas-induced apoptosis was unimpaired by oligomycin, aurovertin B and mClCCP provides further evidence that ATP was maintained at adequate levels despite suppression of mitochondrial ATP synthesis, as cytosolic ATP is required for caspase activation induced both by Fas and by STS<sup>20,22</sup>. In contrast to oligomycin and mClCCP, which reduced apoptosis in cells treated with STS but not with anti-Fas antibody, z-VAD-FMK suppressed the nuclear fragmentation and chromatin condensation indicative of apoptosis in both STS- and Fas-stimulated cells (Fig. 4d), which is consistent with evidence that these terminal events of apoptosis are largely caspase-mediated<sup>23</sup>. Together, these data show that STS-induced alteration of intracellular pH correlates with caspase activation. In contrast, although cytosolic pH can decline as a late event in Fas-treated cells (Fig. 2), pH changes



**Figure 3 Bcl-2 inhibits STS-induced matrix alkalization and cytosol acidification.** Jurkat T cells were stably co-transfected with plasmids encoding pH-GFP<sub>mito</sub> and Neo or Bcl-2, cultured in the presence or absence of STS, and analysed at the indicated times. **a**, Percentage apoptosis. **b**, Caspase activity at 4h (ref. 48) relative to activity at 0h. **c**, CytC release into cytosol at 2h. **d**, Mitochondrial-membrane potential ( $\Delta\Psi$ ), using Jurkat-Neo and Jurkat-Bcl-2 cells lacking pH-GFP<sub>mito</sub>. Open circles, Neo; closed circles, Bcl-2. **e**, Mitochondrial-matrix pH. Open circles, Neo; closed circles, Bcl-2. **f**, Cytosolic pH, using Jurkat-Neo and Jurkat-Bcl-2 cells lacking pH-GFP<sub>mito</sub>. Open circles, Neo; closed circles, Bcl-2. Data were obtained as described in Fig. 2; values in **a**, **b**, **d-f** are means  $\pm$  s.d. from at least four separate experiments.

seem to be inconsequential for Fas, as H<sup>+</sup>-pump inhibitors (oligomycin, aurovertin B) and protonophores (mClCCCP) did not interfere with Fas-induced caspase activation and apoptosis. We therefore conclude that STS and Fas trigger activation of effector caspases through pH-dependent and pH-independent mechanisms, respectively.

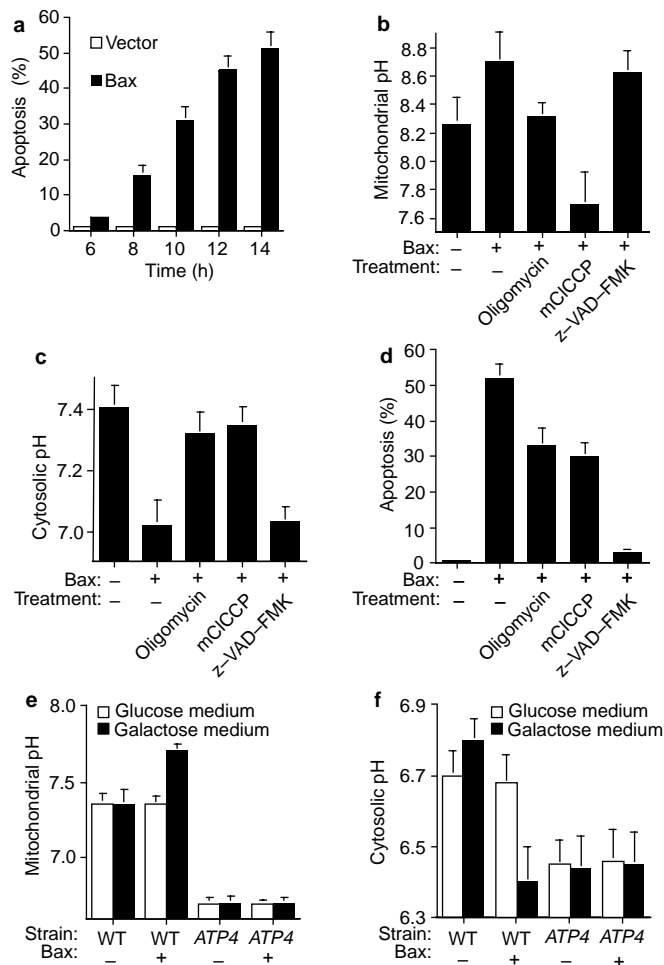
In addition to its effects on caspase activation, oligomycin also suppressed mitochondrial cytC release in STS-treated (Fig. 4f), but not Fas-stimulated (data not shown), cells, indicating that the F<sub>0</sub>F<sub>1</sub>-ATPase may control cytC release. In contrast, mClCCCP inhibited apoptosis and caspase activation (Fig. 4d), but did not suppress STS-induced cytC release (Fig. 4f). Under our culture conditions, mClCCCP was not sufficient to induce cytC release in the absence of STS (data not shown), indicating that mitochondrial depolarization may be insufficient to trigger release of this caspase-activating protein. The caspase inhibitor z-VAD-FMK also failed to suppress STS-induced cytC release from mitochondria<sup>12</sup>, although it did prevent Fas-induced CytC release (data not shown). Together, these results indicate that oligomycin-sensitive components of the F<sub>0</sub>F<sub>1</sub>-ATPase may participate in or modulate STS-induced cytC release,



**Figure 4 Effects of oligomycin, mClCCCP, and z-VAD-FMK on mitochondrial alkalization and cytosol acidification induced by STS and Fas.** Control or pH-GFP<sub>mito</sub>-expressing Jurkat cells were cultured in high-glucose-containing medium<sup>18,44</sup> in the presence or absence of STS or anti-Fas antibody. Where indicated, oligomycin, mClCCCP or z-VAD-FMK was added to cultures 30min before stimulation with STS or anti-Fas antibody. Cells were analysed after 2h incubation. **a**, Mitochondrial-matrix pH. **b**, Cytosolic pH. **c**, Mitochondrial-matrix potential ( $\Delta\Psi$ ). **d**, Percentage apoptosis. **e**, Caspase activity relative to activity at 0h. **f**, CytC release into cytosol. Data were obtained as described in Fig. 2; values in **a-e** are means  $\pm$  s.d. from at least four separate experiments.

but that the release of cytC may not require matrix alkalization or cytosol acidification, as mClCCCP treatment failed to prevent release.

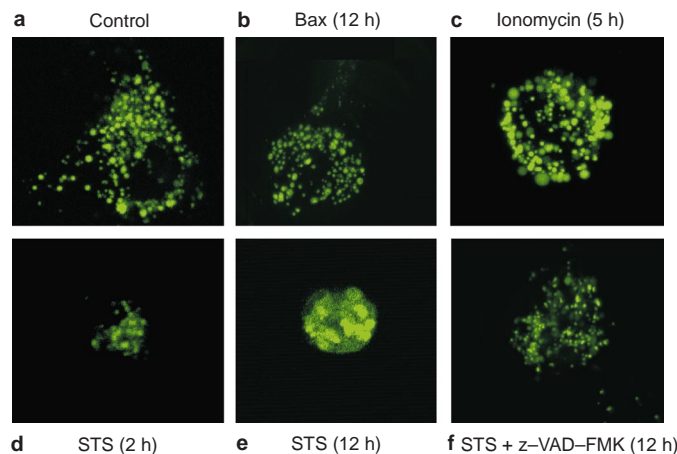
**Bax induces matrix alkalization in mammalian cells and yeast.** Bax is a pro-apoptotic protein from the Bcl-2 family that targets mitochondria (reviewed in ref. 24). Transient overexpression of Bax in 293T cells expressing pH-GFP<sub>mito</sub> caused apoptosis within 8–10h of transfection (Fig. 5a), and resulted in increased matrix pH (Fig. 5c) and decreased cytosolic pH (Fig. 5c). Oligomycin and mClCCCP attenuated Bax-induced mitochondrial alkalization and cytosol acidification, and reduced Bax-induced apoptosis (Fig. 5b–d). In contrast, z-VAD-FMK did not suppress Bax-induced mitochondrial alkalization or cytosol acidification. Although z-VAD-FMK blocked nuclear manifestations of Bax-induced apoptosis (Fig. 5d), it did not prevent subsequent cell death through non-apoptotic mechanisms, as determined by exclusion assays using



**Figure 5 Bax expression induces mitochondrial alkalization and cytosolic acidification in human cells and yeast.** **a–d**, Untransfected or pH-GFP<sub>mito</sub>-expressing 293T cells were transfected with either a plasmid encoding human Bax or an empty vector, and cultured in high-glucose-containing medium. Where indicated, oligomycin, mClCCCP or z-VAD-FMK was added 3h after transfection. Cells were analysed for percentage apoptosis at the indicated times (**a**), mitochondrial-matrix pH at 7h (**b**), cytosolic pH at 7h (**c**) and percentage apoptosis at 12h with the indicated treatments (**d**). Data were obtained as described in Fig. 2. **e**, Cells from the D273A/B (wild type; WT) and PVY10 (*ATP4* mutant; *ATP4*) yeast strains were transformed with pGilda (-) or pGilda-Bax (+) plasmids, in which Bax protein expression is controlled from a galactose-inducible GAL1 promoter<sup>18</sup>, together with a plasmid encoding pH-GFP<sub>mito</sub> under control of the GPD promoter. After 6h of culture in the indicated medium, cells were analysed by FACS; pH was estimated from a standard curve generated using cells suspended in various buffers of known pH containing 10 μM mClCCCP. **f**, Cells from the wild-type and *ATP4*-mutant strains described above, lacking the pH-GFP<sub>mito</sub>-encoding plasmid, were cultured in the indicated medium for 6h, incubated with the pH-sensitive fluorescent dye SNARF<sup>47</sup>, and analysed by FACS for determination of cytosolic pH, which was estimated from a standard curve. Values are means ± s.d. from at least four separate experiments.

trypan-blue dye (data not shown)<sup>25</sup>. Thus, caspases are not required for Bax-induced changes in mitochondrial and cytosolic pH. Moreover, these observations indicate that Bax-induced changes in mitochondrial and cytosolic pH may be correlated with apoptosis, as prevention of these pH alterations, using either oligomycin or mClCCCP, attenuated Bax-induced apoptosis (Fig. 5d; *P* < 0.01) and cell death (data not shown).

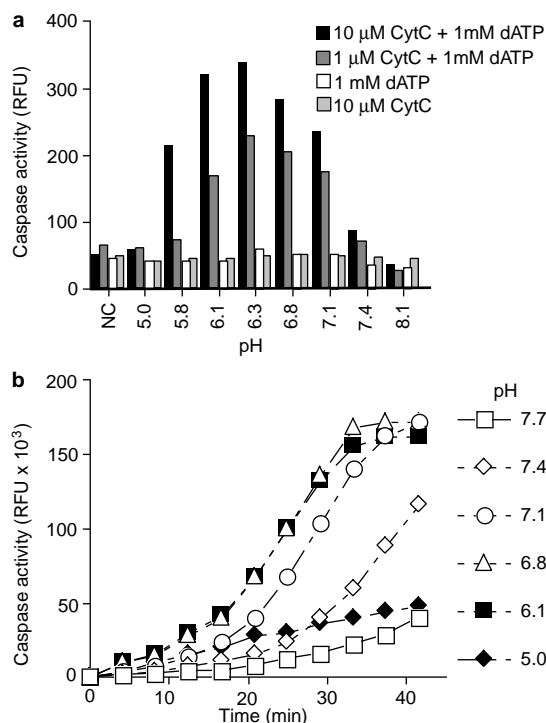
Although oligomycin is a specific inhibitor of the F<sub>0</sub>F<sub>1</sub>-ATPase at



**Figure 6 Analysis of mitochondrial size using mitochondria-targeted GFP.** 293 cells expressing pH-insensitive GFP targeted to mitochondria were cultured with a transfected Bax-encoding plasmid (**b**) or the indicated reagents (**c–f**). Cells were analysed by confocal ultraviolet microscopy at the indicated times after treatment. **a**, Control. **b**, Note the absence of mitochondrial swelling in this shrunken, apoptotic cell. **c**, Note the presence of swollen mitochondria. **d**, Note the absence of mitochondrial swelling in this shrunken, apoptotic cell. **e**, Note the presence of swollen mitochondria. **f**, Mitochondria remain at a normal size as a result of z-VAD-FMK treatment.

the concentrations used here<sup>26</sup>, we sought independent evidence of the function of this H<sup>+</sup> pump in Bax-induced cell death. Ectopic expression of Bax in the yeast *Saccharomyces cerevisiae* induces cytC release, mitochondrial membrane hyperpolarization and depolarization, and cell death<sup>27,28</sup>. Bax-induced cell death in yeast is inhibited by ablation of genes encoding subunits of the F<sub>0</sub>F<sub>1</sub>-ATPase/H<sup>+</sup>-pump, such as *ATP4*<sup>18</sup>. We therefore expressed the pH-GFP<sub>mito</sub> protein in both wild-type and *ATP4*-deficient haploid yeast, and verified its targeting to mitochondria. For these experiments, Bax was expressed from a plasmid, driven by the *GAL1* promoter, that permits induction in galactose-containing media and repression in glucose-containing media<sup>18</sup>. Induction of Bax resulted in matrix alkalization and cytosol acidification in wild-type (*P* < 0.01), but not *ATP4*-deficient yeast (Fig. 5e, f). Although both matrix and cytosolic pH values were initially lower in *ATP4*-deficient yeast than in the wild type, as a result of suppression of respiration and compensatory glycolysis, Bax failed to affect the pH of either the matrix or the cytosol of *ATP4*-deficient cells. As these data are derived from a non-mammalian system, they indicate that changes in mitochondrial pH regulation may be a conserved feature of Bax-induced cell death, and provide further evidence of a function of the F<sub>0</sub>F<sub>1</sub>-ATPase in this process.

**Mitochondrial swelling is not required for pH changes induced by Bax or STS.** Mitochondrial swelling is not required for pH changes induced by Bax or STS. It has been proposed that cytC release and transient mitochondrial hyperpolarization during apoptosis may occur by mechanisms that involve mitochondrial swelling in some instances but not in others<sup>2</sup>. In attempting to address this issue, we observed that mitochondria-targeted GFP provides a convenient method for monitoring the size of mitochondria by confocal microscopy. In cells expressing pH-insensitive GFP targeted to mitochondria, mitochondria of various sizes can be observed by this method (Fig. 6a). Mitochondria in cells induced to undergo apoptosis by overexpression of Bax were of similar size to control cells (Fig. 6b), even 12 h after transfection with Bax, when many cells exhibited typical nuclear manifestations of apoptosis. In contrast, enlarged and swollen mitochondria were clearly evident in cells treated with the Ca<sup>2+</sup> ionophore ionomycin, which is known to cause Ca<sup>2+</sup>-mediated swelling of mitochondria (Fig. 6c). In cells treated with STS,



**Figure 7 pH-dependence of cytC-mediated activation of caspases.** Cytosolic extracts were prepared from Jurkat T cells and adjusted to the indicated pH values. **a**, Ac-DEVD-AFC-cleaving caspase activity in pH-adjusted extracts subjected to the indicated treatments, on the basis of fluorimetric detection of released AFC (relative fluorescence units; RFU) after 10 min incubation at 30 °C with 100 μM substrate. NC, negative control, in which extracts were incubated without CytC and dATP in each of the four groups of experiments, shown here for pH 7.1 although similar results were obtained at all tested pH values (data not shown). **b**, Enzyme progress curves for pH-adjusted extracts treated at 0 min with 10 μM CytC and 1 mM dATP, showing AFC release (RFU) against time.

mitochondria remained unchanged in size for ~2 h (Fig. 6d), but, thereafter, cells containing grossly swollen mitochondria began to accumulate in cultures (Fig. 6e). Addition of z-VAD-FMK to STS-treated cultures prevented this late-stage mitochondrial swelling (Fig. 2f), indicating that mitochondrial swelling may represent a post-caspase-activation event. In contrast, z-VAD-FMK did not prevent ionomycin-induced swelling of mitochondria (data not shown), which is consistent with the ability of Ca<sup>2+</sup> to directly induce PT-pore opening<sup>29</sup>. Thus, overt swelling of mitochondria is not required for matrix alkalization induced by STS or Bax, or for cytosol acidification or cytC release.

**Cytosolic acidification enhances cytC-induced caspase activation.** What function might mitochondria-induced acidification of the cytosol have in apoptosis? To address this question, we investigated the pH-dependence of arguably the best-understood mechanism for coupling of mitochondrial damage to apoptosis—the Apaf-1-mediated activation of cytosolic caspases induced by cytC<sup>1</sup>. We prepared cytosolic extracts and buffered them to various pHs, then added cytC alone or in combination with the Apaf-1 cofactor dATP. Caspase activation induced by the combination of cytC and dATP exhibited pH-dependence; optimal activation occurred at pH 6.3–6.8. At pH 7.1, which represents the approximate cytosolic pH observed in cells undergoing apoptosis induced by STS or Bax (Figs 2–4), the efficiency of cytC/dATP-stimulated caspase activation was ~3–4-fold higher than at physiological pH 7.4 (Fig. 7a). The pH-dependent differences in cytC/dATP-induced caspase activation were not attributable to faster substrate depletion or to other arte-

facts, as determined by evaluation of enzyme progress curves (Fig. 7b). Moreover, progress curves obtained at pH 7.4, pH 7.1 and pH 6.8 exhibited roughly similar slopes, but with a pH-dependent delay in the time of onset of caspase activity following addition of cytC, indicating that a lower pH may accelerate the rate of formation of an active Apaf-1 complex, as opposed to increasing the  $V_{max}$  of cytC-activated caspases. We therefore conclude that at least one effect of mitochondria-mediated acidification of the cytosol during apoptosis may be to facilitate activation of caspases by cytC.

## Discussion

At least two principal pathways for apoptosis have been described—one requiring the participation of mitochondria, which activate caspases by releasing cytC (the so-called ‘intrinsic pathway’), and another in which mitochondria are bypassed and caspases are activated directly (the ‘extrinsic pathway’, of which TNF-family death receptors represent an example (reviewed in refs 30, 31)). Using pH-sensitive GFP as a probe, we have observed that a very early event in mitochondria-dependent apoptosis involves a change in cellular pH regulation, characterized by mitochondrial alkalization and concomitant cytosol acidification. The mitochondria-dependent (STS, Bax) and independent (Fas) pathways for caspase activation and apoptosis are differentially sensitive to suppression by Bcl-2, the H<sup>+</sup>-pump inhibitors and protonophores. Moreover, whereas Bcl-2, oligomycin and mClCCP suppress mitochondrial-matrix alkalization induced by STS and Bax, the caspase inhibitor z-VAD-FMK does not, indicating that pH alterations may occur independently of these proteases.

pH-dependent GFP technology has a number of advantages over cationic dyes for assessing the integrity of the mitochondrial inner membrane in intact cells, and provides a means to monitor local pH in the mitochondria of cells undergoing apoptosis<sup>7,10</sup>. Previous studies of apoptosis have relied on cationic dyes to determine whether the inner membrane remains intact (polarized). However, the amount of dye accumulation in the matrix is influenced not only by  $\Delta\Psi$ , but also by matrix volume<sup>9,32</sup>, thus complicating the interpretation of results. By an independent method, our data support the idea that the mitochondrial inner membrane remains intact even under circumstances where cytC has been released (reviewed in refs 2, 6). Moreover, by monitoring mitochondria size using GFP, we observed no evidence of gross mitochondrial swelling until late in the apoptotic process, after caspase activation. Although swelling-dependent mechanisms for cytC release clearly function in response to certain apoptotic stimuli (such as Ca<sup>2+</sup> overload), our data provide further evidence for a pathway that permits cytC release independently of swelling. In this regard, recent reports have proposed a reversible mechanism for releasing cytC, which does not necessarily commit cells to death, provided that caspase activation is blocked<sup>33,34</sup>; this implies a pathway for cytC release that is independent of mitochondrial swelling or rupture.

The efficiency of caspase activation by cytC was found to be pH-sensitive, with a pH optimum of 6.3–6.8 *in vitro*. Furthermore, when changes in cytosolic pH induced by STS or Bax were prevented by use of F<sub>0</sub>F<sub>1</sub>-ATPase inhibitors or protonophores, caspase activation was impaired and fewer cells underwent apoptosis. Thus, mitochondria-induced acidification of the cytosol promotes cytC-mediated activation of caspases. Although further analysis is required, assembly of the caspase-activation complex, consisting of oligomers of cytC, Apaf-1, and procaspase-9, may occur faster at acidic pH. Identifying the optimum pH for this cytC-dependent reaction in an acidic range could serve as a safety mechanism for avoiding inappropriate activation of cytosolic caspases, such as that caused by accidental release of small amounts of cytC during normal mitochondrial turnover. Interestingly, several growth and survival factors induce cytosol alkalization<sup>35</sup>. It has been shown that some types of cell, such as sympathetic neurons, require a period of survival-factor (for example NGF) deprivation before becoming

competent to activate caspases in response to cytC<sup>36</sup>. Similarly, reports that Bcl-2 and Bcl-X<sub>L</sub> block caspase activation downstream of cytC<sup>37,38</sup> may also be explained in terms of Bcl-2/Bcl-X<sub>L</sub>-mediated suppression of cytosol acidification, rather than of a direct interaction between Apaf-1 and Bcl-2 or Bcl-X<sub>L</sub>. Although cytosol alkalization was recently reported in association with apoptosis<sup>39</sup>, acidification is more generally observed<sup>15,40–42</sup>.

Given that the matrices of mitochondria in cells treated with STS and with Bax undergoes alkalization in parallel with cytosol acidification, and that these events can be suppressed by inhibition of the F<sub>0</sub>F<sub>1</sub>-ATPase, we speculate that reverse operation of this H<sup>+</sup>-pump is triggered in this process. What, then, induces this change in the direction of the H<sup>+</sup> pump? The F<sub>0</sub>F<sub>1</sub> complex can pump protons in reverse from the matrix, across the inner membrane, into the IMS (which is essentially continuous with the cytosol where ions are concerned) under circumstances where the matrix ratio of ATP concentration to that of ADP and phosphate (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) becomes high, and ADP or phosphate is therefore in limited supply<sup>11,19</sup>. Under these conditions, the F<sub>0</sub>F<sub>1</sub> complex consumes ATP, resulting in extrusion of protons from mitochondria. Exchange of ATP for ADP between the mitochondrial matrix and cytosol may be impaired early in apoptosis (reviewed in ref. 6). This exchange is mediated largely by the adenine nucleotide translocator (ANT) in the inner membrane, in collaboration with the voltage-dependent anion channel (VDAC) in the outer membrane<sup>5</sup>. Physical and functional interactions have been reported between anti- and pro-apoptotic Bcl-2-family proteins and the ANT, and genetically modified yeast strains that lack the ANT are resistant to Bax-induced cell death<sup>43</sup>. Thus, genetic evidence indicates that these ADP/ATP-exchanging proteins may be required for the cytotoxic mechanisms by which Bax kills yeast. If exchange of ATP for ADP by the ANT is shut off, one would expect matrix ratios of ATP to ADP to rise, favouring reverse operation of the F<sub>0</sub>F<sub>1</sub>/ATPase. Moreover, ATP hydrolysis, generating ADP and phosphate, would be expected to further contribute to matrix alkalization by driving the mitochondrial hydroxide/phosphate antiporter, which exports phosphate in exchange for hydroxide ions<sup>19</sup>.

However, if this hypothesis is correct, then other mechanisms must also be involved, as reverse operation of the F<sub>0</sub>F<sub>1</sub>/H<sup>+</sup>-pump would eventually cause a steady-state condition to be reached, in which H<sup>+</sup> influx and efflux are equalized. Thus, the changes in pH described here are probably not entirely due to reverse operation of the F<sub>0</sub>F<sub>1</sub>/H<sup>+</sup> pump, and may derive from other parallel events, such as trapping of organic bases in the mitochondrial matrix, counterion movements, increased exchange of hydroxide for phosphate resulting from ATP hydrolysis, or other processes<sup>6</sup>. Regardless of the specific mechanisms, the findings reported here provide evidence that an imbalance in intracellular pH regulation represents an evolutionarily conserved mechanism that is closely linked to the site of action of Bcl-2 proteins. Moreover, these pH alterations precede PT-pore opening, represent the earliest measurable event in the mitochondrial pathway for induction of apoptosis, and facilitate activation of cytosolic caspases following cytC release. □

## Methods

### Plasmids.

pH-sensitive enhanced yellowish GFP containing an H148G mutation (pH-GFP) was created by a two-step polymerase-chain-reaction (PCR) method<sup>19</sup>. pH-GFP and ECFP cDNAs were fused with the N-terminal 12 amino acids of the presequence of subunit IV of cytC oxidase signal sequence, to form pH-GFP<sub>min</sub> and ECFP<sub>min</sub>, respectively. pH-GFP<sub>min</sub> and ECFP<sub>min</sub> (pH-insensitive GFP<sub>min</sub>) were subcloned into the *Hind*III and *Xho*I sites of the following plasmids: pcDNA3-neo, pcDNA3-hygro, p426-GPD1 and p426-GPD1-zeocin.

### Cell cultures and transfections.

Jurkat cells were transfected with the following plasmids using DMRIE-C reagent (Life technologies, Gaithersburg, Maryland): pcDNA3-neo-pH-GFP<sub>min</sub>, pcDNA-hygro-pH-GFP<sub>min</sub>, pcDNA3-neo-ECFP<sub>min</sub>, pRC/CMV or pRC/CMV-Bcl-2. Stable transfectants were selected by culturing in medium containing 0.5 mg ml<sup>-1</sup> G418 and/or 0.5 mg ml<sup>-1</sup> hygromycin. 293 and 293T cells (2 × 10<sup>6</sup>) were transfected with 5 μg pcDNA3-pH-GFP<sub>min</sub> or pcDNA3-ECFP<sub>min</sub>, as described<sup>18,44</sup>. Other transfections and treatments with apoptotic agents were carried out as described<sup>18,44,45</sup>.

### Yeast procedures.

D273-10B/A (wild-type) and PVY10 (*ATP4*-mutant)<sup>46</sup> strains were transformed by the lithium acetate method, using 1 μg of plasmid DNA (pGilda, pGilda-Bax, p426-GPD-zeocin-pH-GFP<sub>min</sub>), and then selected in histidine-deficient medium containing 5 μg ml<sup>-1</sup> zeocin. Induction of Bax protein was accomplished by switching cells from glucose-based to galactose-based medium<sup>18</sup>.

### Mitochondrial-matrix pH.

Mitochondrial-matrix pH was estimated using pH-GFP<sub>min</sub><sup>7</sup>. Correct targeting of the pH-GFP protein to mitochondria was confirmed by two-colour immunofluorescence confocal microscopy, using specific antibodies against Hsp60 (human) or Bax (yeast), and by subcellular fractionation methods<sup>45</sup>. Fluorescence from cells expressing pH-GFP<sub>min</sub> or ECFP<sub>min</sub> was measured using flow cytometry (excitation 480 nm, emission 530 nm), and the mean fluorescence intensity (MFI) of 10,000 cells (mammalian) or 50,000 cells (yeast) was determined. Pilot experiments confirmed that the MFI of cells expressing ECFP<sub>min</sub> did not significantly change as a function of pH or as a consequence of stimulation with STS, Fas or Bax over the time course of experiments (<12 h). Titrations of pH were carried out by placing cells in buffers of various pH, ranging from 7.0–8.5 (293T cells) or 7.5–8.9 (Jurkat cells), containing 10 μM nigericin and 10 μM monensin, as described<sup>7</sup>. FACS-based measurements of MFI at these pHs were used to generate a standard curve for each experiment and to estimate matrix pH for each cell sample. For experiments with yeast, cells transformed with p426-GPD-zeocin-pH-GFP<sub>min</sub> were similarly analysed by FACS, without gating, and using 10 μM mClCCP instead of nigericin/monensin for pH standard curves. Treatment of cells with Ca<sup>2+</sup> ionophore (5 μM ionomycin for 20 min) did not significantly change the MFI of cells equilibrated in buffers with fixed pH, indicating that fluorescence of the mitochondria-targeted pH-GFP protein does not respond to changes in Ca<sup>2+</sup>. Examination of cells expressing pH-GFP<sub>min</sub> by fluorescence confocal microscopy showed mostly homogeneous fluorescence of mitochondria at various pHs, excluding the possibility of significant contributions to total fluorescence by a small fraction of the total mitochondrial population.

### Cytosolic pH measurements.

Cytosolic pH of mammalian cells was estimated by FACS analysis using BCECF<sup>40</sup>. Standard curves for converting MFI data into pH estimates were generated as described above, using buffers containing 10 μM nigericin and 10 μM monensin. All pH measurements were carried out within 10 s of removing cells from incubation with 5% CO<sub>2</sub> at 37°C. For experiments with yeast, cytosolic pH was estimated by FACS analysis using 1-carboxy-seminaphthorhodafouor-1 (acetoxymethyl ester) (SNARF)<sup>47</sup>. Titration of cytosolic pH of yeast cells was carried out by incubating cells at 30°C for 8 min in glucose-based or galactose-based culture media of defined pH, containing 10 μM mClCCP and 30 mM HEPES.

### Cell extracts.

Cytosolic extracts<sup>48</sup> were mixed in a ratio of 10:1 with various concentrations of NaOH or HCl solutions. CytC (1 or 10 μM) and/or 1 mM dATP (final concentrations) were then added on ice and pHs were measured using aliquots. To initiate protease activation, 10-μl aliquots of the extracts were incubated at 30°C for 15 min, and then placed on ice to stop reactions. For caspase-activity measurements, 4 μl of the mixture (30 μg protein) was brought to 100 μl in caspase buffer (final concentrations: 50 mM Tris-HCl, 100 mM KCl, 10% Sucrose, 0.1% CHAPS, 5 mM dithiothreitol pH 7.5) together with 100 μM Ac-DEVD-AFC. AFC release was measured by fluorometry in 96-well plates at 37°C. To monitor the kinetics of cytC activation of caspases, 100 μl of cell extract was used; AFC release was monitored continuously for 45 min at 37°C. CytC release was measured by subcellular fractionation and immunoblot analysis of fractions; enhanced chemiluminescence was used for detection and results were normalized for cell equivalents<sup>49</sup>.

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