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Cytochrome *c* is released in a single step during apoptosis

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Abstract

Release of cytochrome *c* from mitochondria is a central event in apoptotic signaling. In this study, we utilized a cytochrome *c* fusion that binds fluorescent biarsenical ligands (cytochrome *c*-4CYS (cyt. *c*-4CYS)) as well as cytochrome *c*-green fluorescent protein (cyt. *c*-GFP) to measure its release from mitochondria in different cell types during apoptosis. In single cells, the kinetics of cyt. *c*-4CYS release was indistinguishable from that of cyt. *c*-GFP in apoptotic cells expressing both molecules. Lowering the temperature by 7°C did not affect this corelease, but further separated cytochrome *c* release from the subsequent decrease in mitochondrial membrane potential ($\Delta\Psi_m$). Cyt. *c*-GFP rescued respiration in cells lacking endogenous cytochrome *c*, and the duration of cytochrome *c* release was approximately 5 min in a variety of cell types induced to die by various forms of cellular stress. In addition, we could observe no evidence of caspase-dependent amplification of cytochrome *c* release or changes in $\Delta\Psi_m$ preceding the release of cyt. *c*-GFP. We conclude that there is a general mechanism responsible for cytochrome *c* release that proceeds in a single step that is independent of changes in $\Delta\Psi_m$.

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Abbreviations: Act D, actinomycin D; CHX, cycloheximide; cyt. *c*-GFP, cytochrome *c*-GFP; $\Delta\Psi_m$, mitochondrial membrane potential; EDT, 1,2-ethanedithiol; GFP, green fluorescent protein; MOMP, mitochondrial outer membrane permeabilization; STS, staurosporine; TMRE, tetramethylrhodamine ethyl ester; TNF, tumor necrosis factor-α; UV, ultraviolet

Introduction

The permeabilization of the mitochondrial outer membrane to release proteins from the intermembrane space into the cytosol is likely to be the pivotal event in the process of apoptosis.[1] The release of cytochrome *c* triggers the formation of the apoptosome, resulting in caspase activation,[2] and other released proteins, Smac/DIABLO and Omi/HtrA2, enhance the effect by blocking inhibitor of apoptosis proteins. Even if caspase activation is blocked, cell death can follow this release, either through the nuclear effects of apoptosis-inducing factor[3] and endonucleaseG[4] or by the eventual loss of mitochondrial function and ATP production.[5,6]

The process of mitochondrial outer membrane permeabilization (MOMP) is controlled by the Bcl-2 family members, promoted by the multidomain or BH123 proteins Bax and Bak, and antagonized by the antiapoptotic members, for example, Bcl-2 and Bcl-xL.[7,8] While there is wide agreement on this role of the Bcl-2 family and the importance of MOMP in apoptosis, it is here that the consensus ends.

The major controversies revolve around two inter-related issues: (a) the relationship between MOMP and the mitochondrial transmembrane potential ($\Delta\Psi$m), generated by the electron transport chain, and (b) the nature of and requirements for cytochrome *c* release following MOMP. That is, cytochrome *c* may be simply released upon MOMP in a single step, or additional events in the mitochondria may be required for this to occur and apoptosis proceed. These controversies speak directly to the central mechanisms of the apoptotic process and how they are controlled.

Here, we employ single-cell analyses to resolve these issues. Earlier studies in single cells have suggested that the release of cytochrome *c*-green fluorescent protein (cyt. *c*-GFP) is sudden, rapid, and complete, and that it can proceed without changes in $\Delta\Psi_m$,[6,9] and further, that cytochrome *c* and other molecules (Smac/DIABLO) are released simultaneously.[10] These results are at odds with other studies performed using other methods,[11,12] and can be criticized on the grounds that they generally employed only one transformed cell line for each study and rely on the use of chimeric molecules with large fluorescent protein (e.g. GFP). In addressing these criticisms, we have developed alternative approaches to these methods and provide evidence that cytochrome *c* is released during apoptosis in a single, simple step without additional amplification events being required or involved.

Results

In contrast to cytochrome *c*, which is approx. 10.5 kDa, cyt. *c*-GFP is approx. 40 kDa, and therefore its release during apoptosis might not represent the actual release of cytochrome *c*. To address this issue, we employed a recently described technology permitting *in situ* fluorescent labeling of proteins tagged with a short tetracysteine (4CYS)-containing sequence. This tag can be labeled in living cells by exposure to fluorescein- or resorufin-based biarsenical dyes (FlAsH and ReAsH, respectively).[13–15] We therefore generated and expressed cytochrome *c*-4CYS (cyt. *c*-4CYS) (13.3 kDa) in NCI-H1299 and HeLa cells. By comparison with the mitochondrial markers tetramethylrhodamine ethyl ester (TMRE) or Mitotracker Green, we observed that the FlAsH and ReAsH labels efficiently localized to mitochondria in these cells (Figure 1a). Upon induction of apoptosis in response to ultraviolet (UV) radiation, cyt. *c*-4CYS was released in a manner that was sudden, rapid, and complete (Figure 1b and c). In the experiment shown in Figure 1b(i), six NCI-H1299 cells expressing cyt. *c*-4CYS and stained with ReAsH were analyzed and the kinetics aligned with respect to cytochrome *c* release. Simultaneously, $\Delta\Psi_m$ was assessed using TMRE. An example of a trace from an individual NCI-H1299 cell is shown in Figure 1b(ii), in comparison to a similar trace from a HeLa cell expressing cyt. *c*-4CYS (Figure 1b(iii)). Loss of $\Delta\Psi_m$, as well as phosphatidylserine exposure, nuclear condensation, and destruction of membrane integrity all occurred only after cytochrome *c* had been released (Figure 1b and S1). As with cyt. *c*-GFP,[9] the release of cyt.

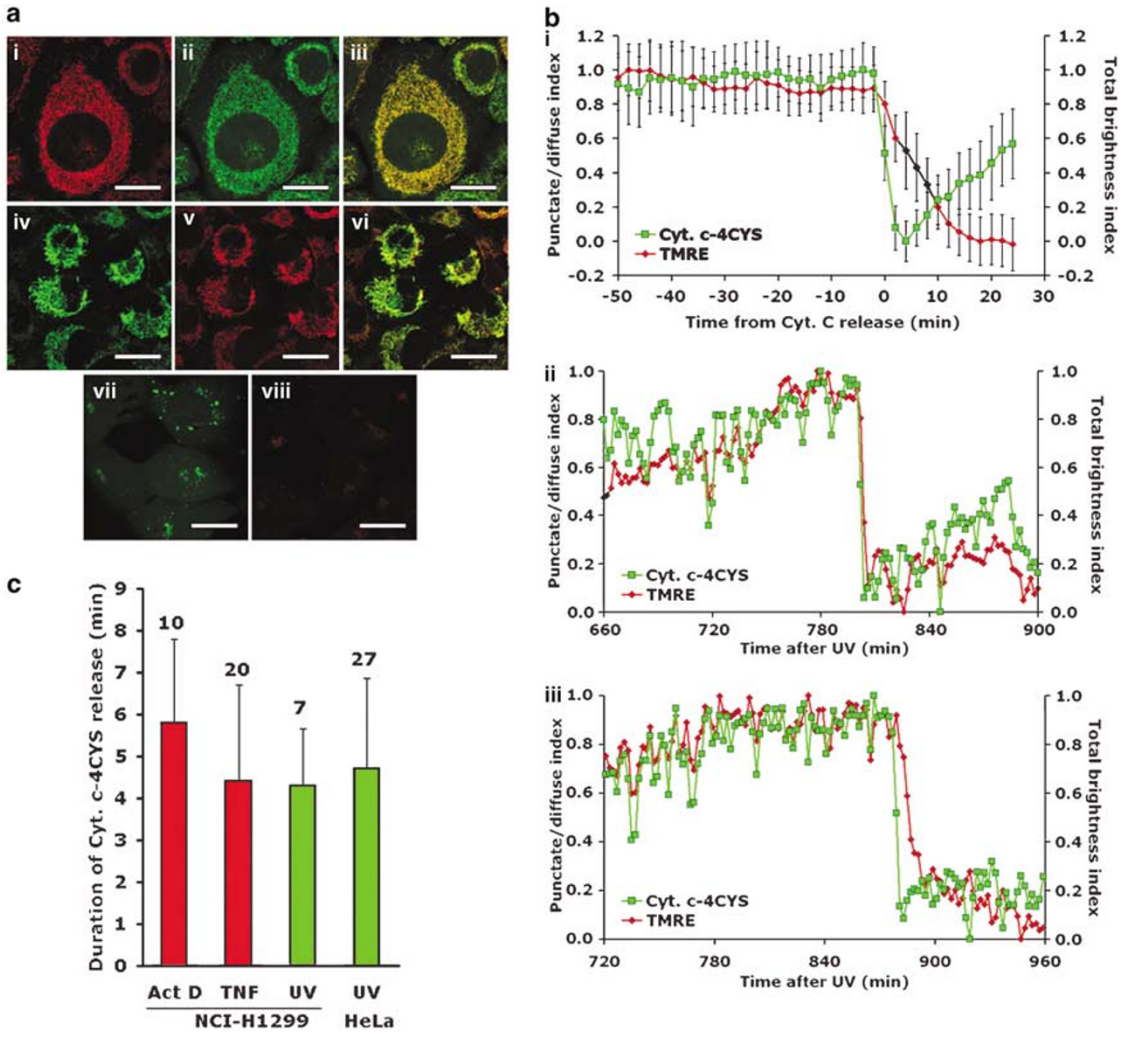

Figure 1 Cytochrome *c*-4CYS is completely released from mitochondria in a single step. (**a**) Cyt. *c*-4CYS colocalizes with mitochondria with little background staining. Cyt. *c*-4CYS expressing NCI-H1299 cells were stained with TMRE (i) and FlAsH (ii), merge (iii) or Mitotracker Green (iv), and ReAsH (v), merge (vi). Control NCI-H1299 cells that did not express cyt. *c*-4CYS were stained with FlAsH (vii) or ReAsH (vii). Images were taken with a $\times$ 40 objective and scale bars represent 50 μm. (**b**) Cyt. *c*-4CYS translocates to the cytoplasm before or coincidentally with mitochondrial depolarization. NCI-H1299 and HeLa cells were costained with FlAsH (cyt. *c*-4CYS) and TMRE ($\Delta\Psi_m$ measure) and exposed to UV light. TMRE intensity and cyt. *c*-4CYS localization are shown on the *Y*-axis labeled total brightness index and punctate/diffuse index (respectively). (i) The traces of six NCI-H1299 cells were aligned by time of cyt. *c*-4CYS release and scaled. Error bars are scaled S.E.M. The single traces of an NCI-H1299 (ii) and a HeLa (iii) cell treated as above. (**c**) The duration of cyt. *c*-4CYS release is about 5 min. The duration from initial to complete release of cyt. *c*-4CYS ReAsH (red bars, left) and FlAsH (green bars, right) was measured in NCI-H1299 cells treated with Act D, TNF and CHX, or UV, and HeLa cells exposed to UV light. Error bars represent S.D. and the number of cells analyzed appears above each column

c-4CYS was complete within an average of approx. 5 min of its initiation (Figure 1c). The release of cyt. *c*-4CYS is shown in the context of other apoptotic events in the Supplemental movie.

As cyt. *c*-4CYS and cyt. *c*-GFP are of significantly different sizes (13.3 and 39.2 kDa, respectively), and because our studies with cyt. *c*-GFP failed to show two-step kinetics suggested by other types of studies,[16,17] we examined the release of these two forms of cytochrome *c* coexpressed in the same cell. As shown in Figure 2a, cyt. *c*-4CYS and cyt. *c*-GFP colocalized in perinuclear compartments in cells that expressed both molecules. When NCI-H1299 cells expressing both forms of cytochrome *c* were induced to undergo apoptosis by exposure to tumor necrosis factor-α (TNF) plus cycloheximide (CHX), redistribution of both labels occurred with identical kinetics (Figure 2b(i)). We then repeated this analysis at a lower temperature (30°C *versus* 37°C). We reasoned as follows: if active changes in mitochondrial function or morphology are necessary for the complete release of cyt. *c*-4CYS, then a decrease in temperature

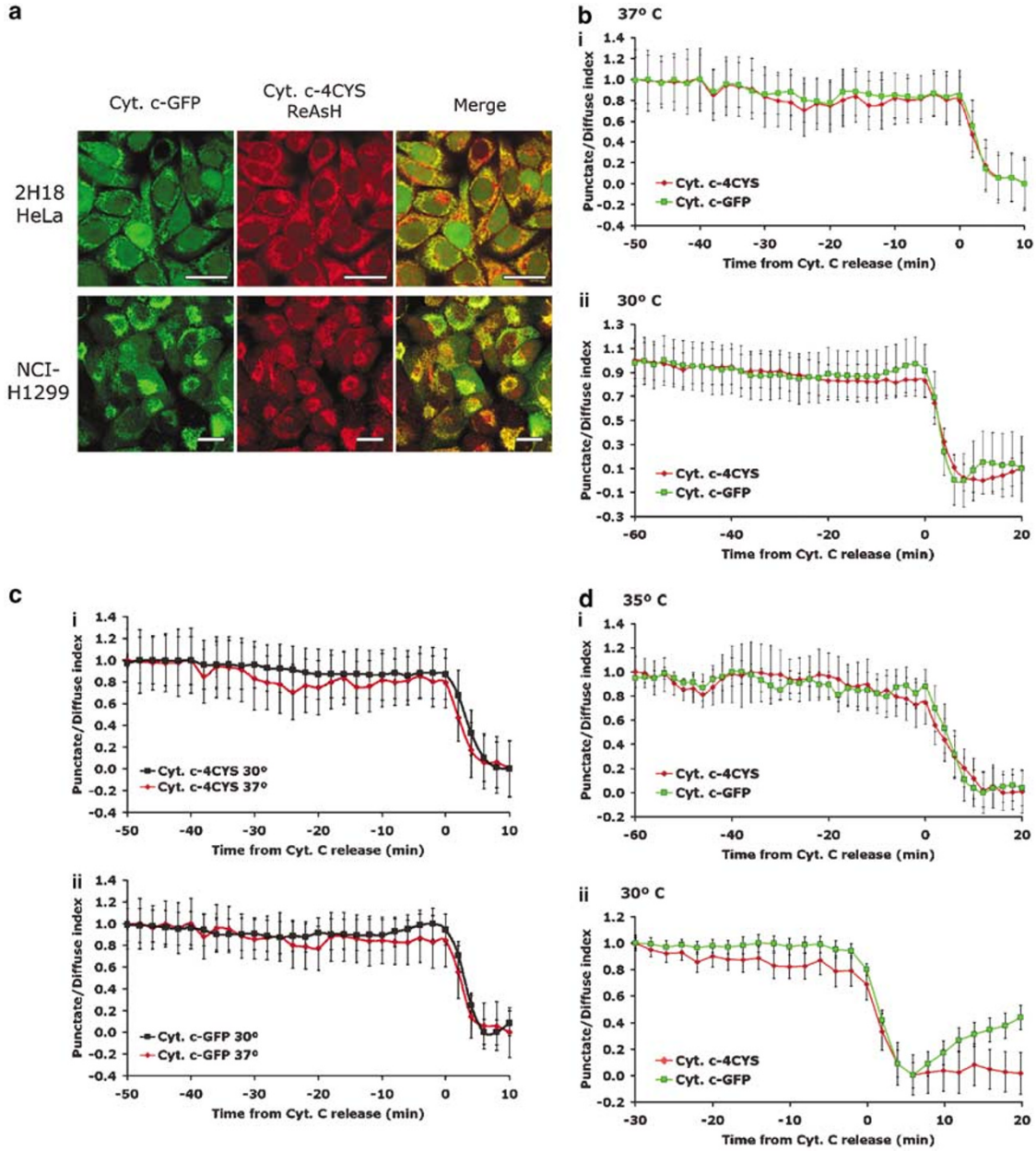

Figure 2 Simultaneous release of GFP- and tetracysteine-tagged cytochrome c. (**a**) HeLa and NCI-H1299 clones expressing both green cyt. *c*-GFP and ReAsH-stained cyt. *c*-4CYS. Images were taken with a $\times$ 40 objective and scale bars represent 50 μm. (**b**) NCI-H1299 cells expressing cyt. *c*-4CYS and cyt. *c*-GFP were induced to undergo apoptosis by TNF and CHX at 37°C (i) or 30°C (ii). Cyt. *c*-4CYS was stained with ReAsH. Data from 14 (i) or 12 (ii) cells were aligned by cyt. *c*-4CYS release and then averaged and scaled. Error bars are S.E.M. multiplied by the scaling factor. (**c**) The same experiments as in (b), comparing the release kinetics of cyt. c-4CYS (i) or cyt. c-GFP (ii) at 30°C and 37°C. (**d**) HeLa cells expressing both cyt. c-4CYS and cyt. c-GFP were treated with Act D and zVAD-fmk at 35°C (i) or 30°C (ii). Data processing were performed as above with six cells (i) and 30 cells (ii) analyzed

should alter the kinetics of the process following its initiation. A difference in this requirement for either form of cytochrome *c* (GFP or 4CYS), for example, due to differences in intramitochondrial location or differential interaction with proteins or lipids in the intermembrane space, would result in a separation in the kinetics of the release of the two labels.

Therefore, NCI-H1299 cells expressing both forms of cytochrome *c* were treated with TNF plus CHX and release was examined at 30°C. As shown in Figure 2b(ii), cyt. *c*-4CYS and cyt. *c*-GFP were released simultaneously even at this lower temperature.

The lack of a temperature effect in the rate of cytochrome *c* release with either method of tracking the protein can be clearly seen when the results are directly compared for the two temperatures. As shown in Figure 2c, cyt. *c*-GFP and cyt. *c*-4CYS are released at nearly identical rates at the two temperatures.

This simultaneous release of the two-labeled proteins was similarly observed in a different cell line, HeLa, treated with actinomycin D (Act D) to induce apoptosis. Again, cyt. *c*-GFP and cyt. *c*-4CYS were released simultaneously at 35 and 30°C (Figure 2d(i and ii)). Therefore, temperature-sensitive events during apoptosis do not differentially affect the mitochondrial release of cyt. *c*-4CYS and cyt. *c*-GFP.

Previously, we showed that loss of $\Delta\Psi_m$ occurs following the release of cytochrome *c* in HeLa cells,[6,9,18] but this can subsequently recover if caspase activation is blocked.[6] Unlike the release of cytochrome *c*, we found that this decrease in $\Delta\Psi_m$ immediately after MOMP is influenced by temperature. HeLa cells expressing cyt. *c*-GFP were induced to undergo apoptosis by act D and the loss of $\Delta\Psi_m$ was monitored. We found that as temperature decreased, the lag in drop of $\Delta\Psi_m$ after cytochrome *c* release increased (Figure 3). The same relationship was observed between cyt. *c*-4CYS and TMRE (data not shown). This effect was not due to temperature effects on the loss of staining, as the uncoupler FCCP caused an immediate drop in $\Delta\Psi_m$ as detected by these methods at each temperature (data not shown). In contrast, this reduction in temperature had no effect on the kinetics of cyt. *c*-GFP release once initiated (Figure 3). Similarly, reduction in temperature had no effect on the rate of cyt. *c*-GFP *versus* -4C release in the experiment in Figure 2b(ii) and d(ii). We conclude that the temperature-sensitive drop in $\Delta\Psi_m$ that follows the release event cannot represent a cause of cytochrome *c* release or a factor in its timing.

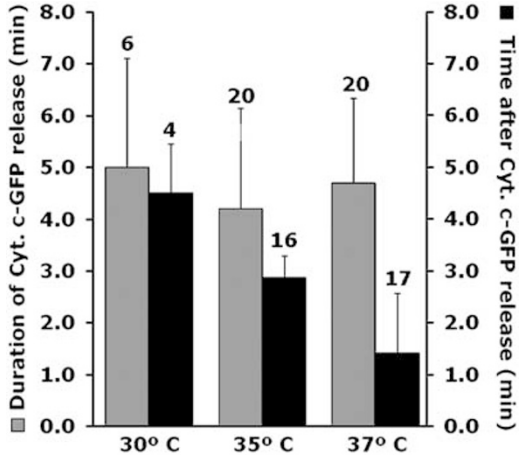

Figure 3 Loss of mitochondrial membrane potential following cyt. *c* release is temperature dependent. HeLa cells expressing cyt. *c*-GFP were costained with TMRE and subjected to Act D and zVAD-fmk. The duration of cyt. *c*-GFP release (gray) and the lag between the start of cyt. *c*-GFP release and onset of depolarization (black) were measured at at 30, 35, or 37°C. Error bars represent S.D. and the number of cells analyzed appears above each column

Of course, it remains possible that tagged cytochrome *c* simply does not localize within the mitochondria in precisely the same way as does native cytochrome *c*. Cytochrome *c* plays an essential role in shuttling electrons from Complex III to Complex IV, and it has been suggested that this function occurs deep in invaginations or crypts formed by the inner mitochondrial membrane.[17,19] We therefore introduced cyt. *c*-GFP into differentiated ES cells from cytochrome *c*-null embryos.[20] Introduction of this chimeric cytochrome *c* effectively restored electron transport (measured as oxygen consumption) to the mitochondria of these cells (Figure 4a). Therefore, cyt. *c*-GFP localizes to those areas of the intermembrane space in which electron transport occurs. Upon induction of apoptosis, cyt. *c*-GFP was released from these mitochondria in the same way we had observed for other cells (Figure 4b), that is, it was sudden, rapid, and complete, and again, $\Delta\Psi_m$ was sustained until following the release of cytochrome *c*. Upon initiation of MOMP, cyt. *c*-GFP was released in approximately 5 min, and this was followed by a drop in $\Delta\Psi_m$ (Figure 4c).

To extend our observations, we introduced cyt. *c*-GFP into several different cell types and induced apoptosis by different means. In each case, we selected cells for proper localization of cyt. *c*-GFP to mitochondria (Figure 5a). Upon induction of apoptosis, cyt. *c*-GFP was released from all mitochondria, and this occurred in approximately 5 minutes upon initiation of MOMP (Figure 5b). The effect was apparently independent of caspase activation, occurring in cells lacking APAF-1 (SAK2) or in the presence of the caspase inhibitor zVAD-fmk, and was similar in adherent and nonadherent cells (Figure 5c). This includes Jurkat cells, where it has been reported that full cytochrome *c* release requires caspase activation.[21,22] Further, cyt. *c*-GFP was released prior to changes in $\Delta\Psi_m$ during apoptosis in both Jurkat and SAK2 cells (Figure 5c).

FL5.12 is a nontransformed IL3-dependent pro-B cell line that has been widely used to study apoptosis induced by growth factor deprivation. Since such factor withdrawal has dramatic effects on cellular metabolism,[23,24] it has been proposed that MOMP occurs under these conditions as a consequence of changes in mitochondrial physiology, manifesting as an increase in $\Delta\Psi_m$.[25] While this increase in $\Delta\Psi_m$ can be readily observed at the population level in a subset of cells,[26] single-cell analysis revealed that no change in $\Delta\Psi_m$ prior to cytochrome *c* release was necessary for MOMP (Figure 5d). In contrast, a rapid increase in $\Delta\Psi_m$ was observed upon addition of nigericin (not shown). Thus, changes in $\Delta\Psi_m$ upon growth factor withdrawal are not an essential component of subsequent MOMP and cytochrome *c* release.

Our analyses do not exclude the possibility that $\Delta\Psi_m$ changes in cells more than several hours prior to MOMP may play a role in the process. However, most models relating $\Delta\Psi_m$ to MOMP predict a rapid cause–effect relationship. For

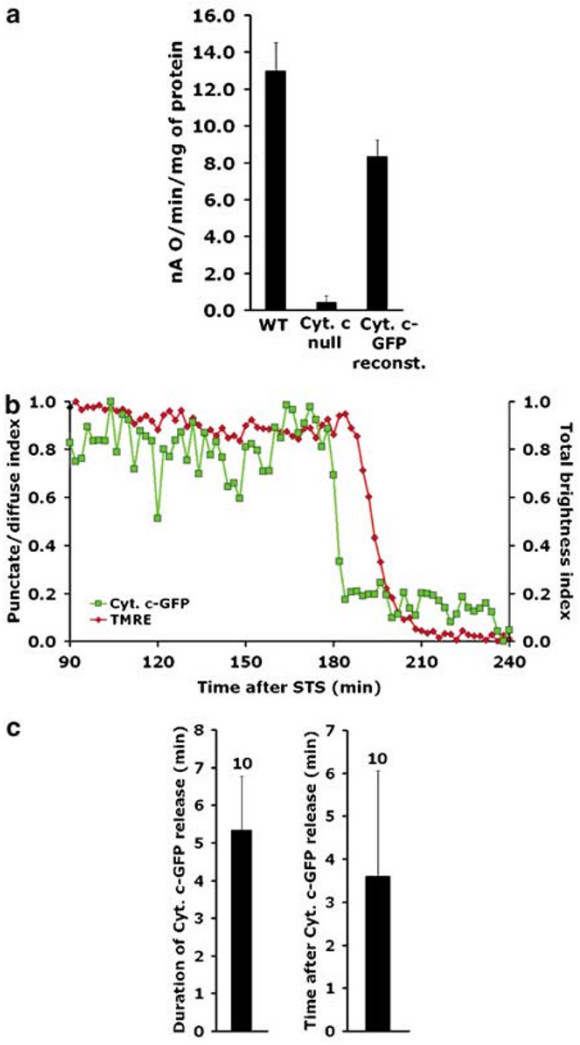

Figure 4 Cyt. c-GFP rescues respiration in cytochrome *c* null cells and is released in an all-or-nothing manner. (**a**) Oxygen consumption of WT MEFs, cyt. *c*-null cells, cyt. c-null cells expressing cyt. c-GFP was measured in the presence of succinate. In (**b**) and (**c**), cyt. c-GFP reconstituted-cytochrome c-null cells were treated with STS in the presence of zVAD-fmk. (**b**) Trace of cytochrome *c* release (punctate/diffuse index) and mitochondrial membrane potential (total brightness index) in a typical cell. (**c**) Duration of cyt. *c*-GFP release (left) and lag between the start of cyt. *c*-GFP release and the beginning of mitochondrial depolarization (right). Error bars represent S.D. and the number of cells appears above each column

example, loss of $\Delta\Psi_m$ associated with a mitochondrial permeability transition in isolated mitochondria can result in cytochrome *c* release within minutes.[27–29] No such relationship was found in any of the cell lines we tested, or in any of the conditions for induction of cytochrome *c* release we examined. We conclude that mitochondrial events associated with changes in $\Delta\Psi_m$ are not required for the complete release of cytochrome *c* upon MOMP.

Discussion

Our results support a model of cytochrome *c* release in which the permeabilization of the mitochondrial outer membrane is sufficient to allow all of the cytochrome *c* to be released from all of the mitochondria in the cell, without requirements for additional, temperature-sensitive events to permit or facilitate this process. In particular, events that involve a change in $\Delta\Psi_m$, as measured by potentiometric dyes such as TMRE, are unlikely to play a role at or around the time of release. This idea, that cytochrome *c* is released upon a single event (such as outer membrane permeabilization), without recourse to amplification or facilitation, is basically a 'single-step' model of release. The simplest form of this model is that upon permeabilization of the mitochondrial membrane, cytochrome *c* diffuses out of the intermembrane space without additional changes being necessary.

This conclusion is not dependent on the cell type employed, nor to the form of cytochrome *c* we use for these observations. Further, we found that cyt. *c*-GFP that is actively involved in mitochondrial respiration (in cells lacking endogenous cytochrome *c*) can be released in the same manner and with the same properties as we observe in other situations. Therefore, the phenomenon of single-step cytochrome *c* release appears to be general.

MOMP occurs as a consequence of the activation of Bax and/or Bak and the formation of openings in the outer membrane.[30] These may occur as a direct effect of the proapoptotic Bcl-2 family proteins alone,[31] interacting with membrane lipids,[32] or through interactions with other proteins such as VDAC.[26,33] Our data in this paper do not distinguish between these possibilities *per se* but do argue that, if additional molecules are involved, their roles are unlikely to extend beyond MOMP to additional temperature-sensitive effects involved in the release of cytochrome *c*. For example, our data appear to be inconsistent with models in which changes in the morphology of the mitochondria following or accompanying MOMP are necessary for complete cytochrome *c* release,[17,21,22] and models in which the first mitochondria to undergo MOMP trigger other mitochondria to do so, by release of calcium.[34]

We and others have shown mitochondrial proteins linked to GFP translocate from mitochondria during apoptosis.[9,10] However, the use of large fusion molecules risks missing phenomena where molecular size is important. For instance, molecular size may determine if proteins are successfully imported into cristae and, therefore, may be subject to different release mechanisms as has been proposed.[17] Using smaller fluorescent tags, we have reduced the size of observable molecules that are released from the mitochondria to about 13 kDa and measured corelease with much larger molecules of size 40 kDa in the same cell. We propose that the same mechanism is responsible for the release of both molecules, validating work that showed molecules of up to 2 MDa can be released when MOMP is induced *in vitro*.[32] This is strong evidence against cytochrome *c* being released through a small pore of approximately 20 Å[35] and, coupled with the inability to visualize a large pore in the absence of mitochondrial swelling,[36,37] supports the model that cytochrome *c* is released through a lipidic pore[38] or similar sort of opening in the outer mitochondrial membrane.

Rigorous studies on cytochrome *c* release in isolated mitochondria have convincingly demonstrated that cytochrome *c* resides in two pools, the major one being

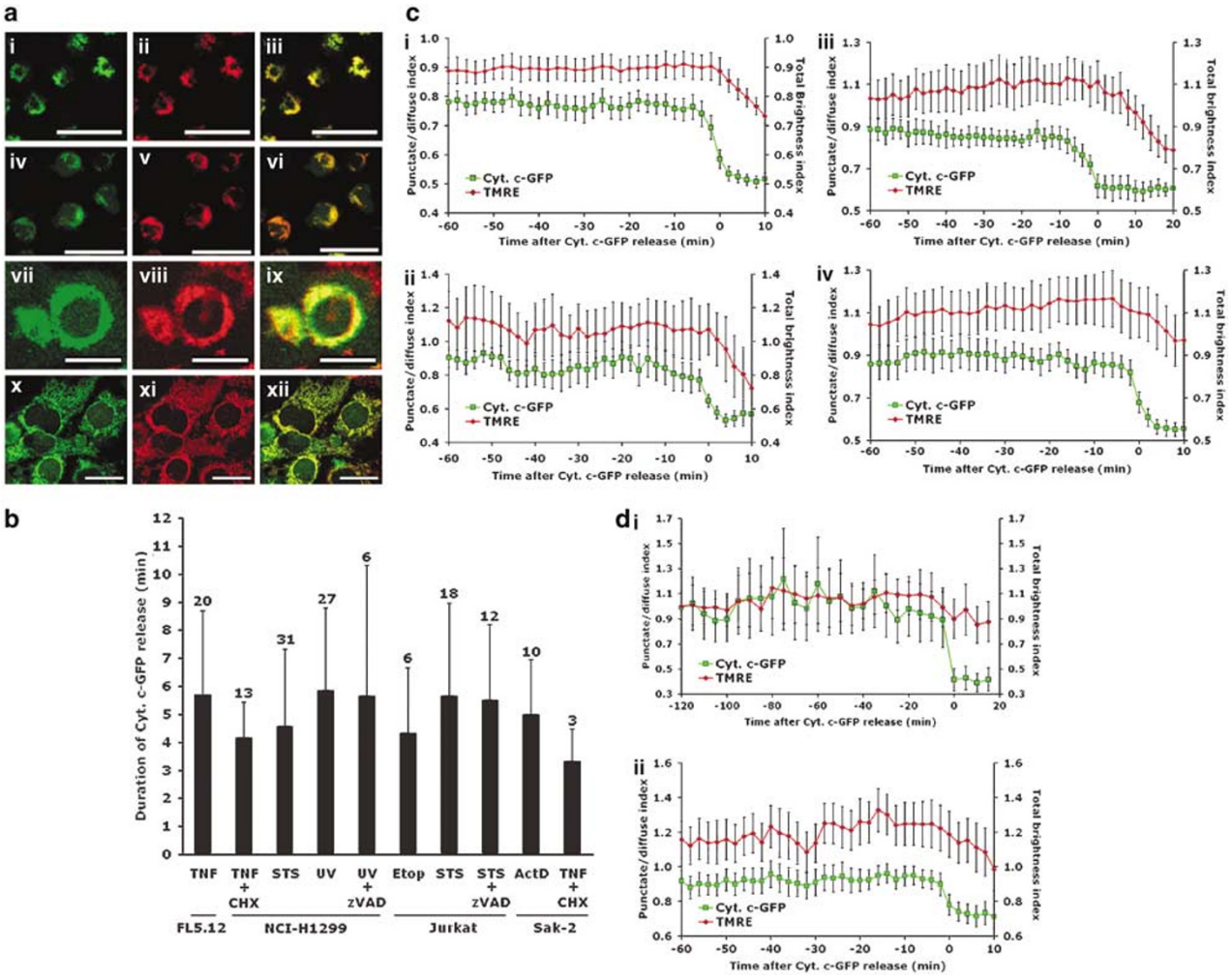

Figure 5 Kinetics of cytochrome *c* release are general and there is little or no change in mitochondrial membrane potential preceding this event. (**a**) cyt. *c*-GFP expressed in FL5.12 cells (i), Jurkat cells (iv), NCI-H1299 cells (vii), and APAF-1$^{-/-}$ SAK2 cells (x); mitochondria were stained with TMRE (ii, v, vii, and xi), merge (iii, vi, ix, and xii). Images were acquired with a $\times$ 40 objective and scale bars represent 50 μm. (**b**) The duration of cyt. *c*-GFP release during apoptosis is about 5 min in diverse cell lines treated with various apoptosis-inducing agents. Error bars represent standard deviation. The number of cells in each condition appears above each column. (**c**) Cyt. c-GFP expressing SAK2 (i) and Jurkat (ii–iv) cells were costained with TMRE and induced to undergo apoptosis by Act D (i), etoposide (ii), STS (iii), or STS and zVAD-fmk (iv). (i) $n = 10$, (ii) $n = 6$, (iii) $n = 11$, or (iv) $n = 9$. Cells were aligned by time of cyt. c-GFP release and averaged. Error bars represent S.E.M. Cyt. c-GFP distribution and TMRE staining were measured by the punctate/diffuse index and total brightness index, respectively. (**d**) FL5.12 cells expressing cyt. c-GFP were stained with TMRE and subjected to IL-3 withdrawal, $n = 7$ (i) or TNF, $n = 13$ (ii). Experiments were processed as above

sequestered so that permeabilization of the outer membrane fails to release the protein unless it is redistributed by a morphological change in the inner membrane.[17] Confounding these studies, however, is the difficulty in interpreting differences in cytochrome *c* release in a population of isolated mitochondria. By imaging mitochondria in a cell, we can differentiate between some of the mitochondria releasing all of their cytochrome *c* and all of the mitochondria releasing some of their cytochrome *c*. Furthermore, our system can accurately measure permeability transition-related events that have been proposed to be the cause of mitochondrial remodeling (e.g. change in $\Delta\Psi_m$). Our data suggest that this remodeling is not required for complete cytochrome *c* release in intact cells. In particular, the induction of a permeability transition and the resulting change in $\Delta\Psi_m$ and mitochondrial morphology is highly temperature sensitive,[39] and the lack of a temperature effect on the dynamics of cytochrome *c* release following MOMP in our studies argues against this being a requisite step involved in remodeling the mitochondria for cytochrome *c* release in cells.

Recently, it was demonstrated that loss of $\Delta\Psi_m$ and changes in mitochondrial morphology can occur as a consequence of caspase activation, and the impact of the active caspases on the permeable mitochondria.[40] A caspase substrate accessible to the intermembrane space was identified (p75/NDUSF1), and the caspase cleavage site was mutated to render the protein noncleavable. Cells expressing this mutant showed normal cyt. *c*-GFP release during apoptosis, but both the drop in $\Delta\Psi_m$ and the ultrastructural changes in the mitochondria were dramatically inhibited. Such changes, at least in some cases, may therefore be a downstream consequence of MOMP and caspase activation rather than a contributor to cytochrome *c* release.

A similar argument may apply to enzymatically triggered mitochondrial fission, which has been suggested to be critical for cytochrome *c* release.[41] Although not examined directly in our studies, it is reasonable to expect that such fission should be temperature sensitive. If this were required as an additional step after MOMP for cytochrome *c* release to occur, we should have expected to have seen a temperature effect in our system.

Previous studies have suggested that in some cell lines, for example, Jurkat-, etoposide-, and staurosporine (STS)-induced cytochrome *c* release is dependent on caspase activity.[21,22] However, we found that under similar conditions, cytochrome *c* was released in a single step that was resistant to the effects of the caspase inhibitor zVAD-fmk, and therefore is likely to be independent of caspase-mediated amplification. A possible resolution to this contradiction could be that whereas others studies report a decrease in the amount of cytochrome *c* released in a population, we observed individual cells that still completely release all of their cytochrome *c*. It is possible that the inhibition of one pathway, in cells induced to die by more than one pathway, results in complete cytochrome *c* release in a subset of the cells via the second pathway. Time-lapse microscopy enables us to measure the events in this subset, which raises the exciting possibility of a method that could be employed to study a death pathway that is not fully engaged in every instance. For example, Jurkat cells express Fas ligand in response to some forms of stress, and the ensuing Fas-mediated cytochrome *c* release and death in a subset of cells[42] would be expected to be caspase dependent.

Apoptosis is a dynamic process in which cells commit to die, release cytochrome *c* and other proteins from mitochondria, and activate caspases in an asynchronous manner.[43] The precise order and timing of the key events can only be rigorously examined *in vivo* at the single-cell level. Our studies have shown that these events are remarkably similar in diverse cell types induced to undergo apoptosis by different stimuli, and point to a single step in cytochrome *c* release upon MOMP. The regulation of apoptosis, therefore, is most likely to occur upstream or downstream of this event, but not during it. One important aspect of this idea is that the release of other intermembrane space proteins will coincide with cytochrome *c* release or follow it if the second protein is somehow held in the mitochondria. While some studies challenge this idea,[11] investigations at the single-cell level support it.[10] We can find no evidence that cytochrome *c* release can depend on amplification steps downstream of MOMP (e.g. dependent on caspases or effects of other released proteins). Admittedly, one limitation of our analysis is that we restrict our view to events that are sensitive to changes in temperature or depend on changes in $\Delta\Psi_m$. However, since essentially all biologically relevant catalytic events or structural changes are likely to be temperature sensitive, our conclusion is likely to be correct.

Materials and Methods

Culture conditions and the induction of apoptosis

Adherent cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 200 mg/ml penicillin, and 100 mg/ml streptomycin sulfate in a humidified atmosphere of 5% CO_2/95% air. Cytochrome *c*-null cells were also supplemented with 55 μM 2-mercaptoethanol. Adherent cells were subcultured 1 or 2 : 10 by incubating them in 0.25% trypsin (Gibco) when they were 70% confluent and resuspending cells in growth medium.

Jurkat cells were grown in RPMI (Gibco) 10% FCS, 2 mM L-glutamine, 200 mg/ml penicillin, and 100 mg/ml streptomycin sulfate. FL5.12 murine pro-B lymphoma cells were grown as described earlier[44] in RPMI supplemented with 10% conditioned media from WEHI 3B cells plus 10% FCS, 20 mM HEPES (pH 7.2), 2 mM L-glutamine, 200 mg/ml penicillin, and 100 mg/ml streptomycin sulfate.

Apoptosis was induced with 1 μM STS, 1 μM act D, 40 μM etoposide, and 10 ng/ml human TNF-α, together with 10 μg/ml CHX (FL5.12 cells did not require CHX). UV light (UVC) doses were 90 mJ/cm^2(HeLa) and 180 mJ/cm^2(NCI-H1299 cells). IL-3 withdrawal-induced apoptosis was induced by washing the cells 2 $\times$ in PBS and resuspending the cells in FL5.12 media lacking WEHI 3B conditioned media. After a 1-hour incubation, the cells were washed an additional two times and again resuspended in the IL-3-deficient media. zVAD-fmk (100 μM) was added 30 min before the apoptotic stimulus.

Derivation of cell lines expressing cytochrome *c* fusions

Cytochrome *c*-4CYS

NCI-H1299 and HeLa cells were infected with three (HeLa) or five (NCI-H1299) rounds of pBABE mouse cyt. *c*-4CYS virus.

Cytochrome *c*-GFP

HeLa, SAK2 MEFs, Jurkat, and NCI-H1299 cells were infected with three rounds of pBABE mouse cyt. *c*-GFP (LTR promoter). Jurkats were centrifuged for 30 min at 200 $\times$ *g* with the virus each round. A total of 20–80% of the cells, depending on the cell type, had cyt. *c*-GFP localized in the mitochondria. Cells were sorted for GFP expression and cloned by limiting dilution. Bright clones with mitochondrial localization were selected. FL5.12 murine pro-B lymphoma cells expressing cyt. *c*-GFP were obtained by electroporating 1 $\times$ 10^7 FL5.12 at 960 μF and 250 mV with (rat) cyt. *c*-GFP plasmid DNA[45] followed by selection in medium containing 1 mg/ml G418. Single-cell clones were obtained from transfectant pools by limiting dilution cloning in 96-well microtiter plates. Cytochrome *c*-null ES cells (ATCC) were infected with three round of pBABE mouse cyt. *c*-GFP (SV40 promotor) virus, and a GFP-expressing population was selected by sorting.

Cytochrome *c*-4CYS and cytochrome *c*-GFP

2H18 HeLa cells that express GFP-cyt. *c*[9] and cyt. *c*-GFP expressing NCI-H1299 were infected with three rounds of pBABE mouse cyt. *c*-4CYS virus. In bulk populations, many cells favored mitochondrial cyt. *c*-4CYS localization at the expense of a diffuse localization of cyt. *c*-GFP. The cells were cloned by limiting dilution and clones that had the brightest expression of both molecules in the mitochondria were chosen.

Virus production

Viruses were made by a standard calcium phosphate DNA coprecipitation transfection method using the retroviral vector, Gag-pol, and the VSVG envelope protein into the Phoenix packaging line (a gift from G Nolan). At 12 h after transfection, cells were incubated in 10 mM *n*-butyric acid (Sigma-Aldrich) for 10 h. Supernatants were collected at 12, 24, and 48 h

following the butyrate shock. Polybrene (5 μg/ml) (Sigma-Aldrich) was added to the viral supernatants after collection.

Constructs

Mouse cyt. *c*-GFP

Mouse cytochrome *c* was amplified with the sense primer 5′-ACGTGTCGACCTAATATGGGTGATGTTGAAAAAGG and antisense primer 5′-ACAGATCTTTCTCATTAGTAGCCTTTTTAAG. The fragment was ligated into pIB/V5-His Topo vector (Invitrogen, Carlsbad, CA, USA) and then cut with *Sal*I and *Bgl*II. This fragment was ligated into pEGFP-N1 (Clontech, PaloAlto, CA, USA) at *Xho*I and *Bam*HI sites.

pBABE mouse cyt. *c*-GFP (LTR promotor)

Cyt. *c*-GFP (above) was amplified with the sense primer 5′-GAGCTGGTTTAGTGAAC and antisense primer 5′-AGTAGAATTCCTA CAAATGTGGTATG and ligated into pYes2.1 V5-His Topo (Invitrogen, Carlsbad, CA, USA). This vector was verified by sequencing and cutting with *Bgl*II and *Eco*RI. The resulting insert was cloned into pBABE-puro at *Bam*HI and *Eco*RI sites in the multiple cloning region.

pBABE mouse cyt. *c*-GFP (SV40 promotor)

Cyt. *c*-GFP (in pEGFP-N1 vector, above) was amplified with the sense primer 5′-ACGTAAGCTTGCCACCATGGGTGATGTTGAAAAAGGCAAG and the antisense primer 5′-TATCGAATCTACTCGGTCTTCACGCAAC and ligated into pcDNA3.1/V5-His Topo vector (Invitrogen, Carlsbad, CA, USA). Cyt. *c*-GFP was cut out with *Cla*I and *Hind*III and ligated into pBABE in place of the puro resistance cassette, which had been removed by a *Cla*I and *Hind*III codigestion.

pBABE mouse cyt. *c*-4c

Mouse cytochrome *c* was amplified with the sense primer 5′-ACGTGGATCCGCCACCATGGGTGATGTTGAAAAAGG and the antisense primer 5′-ATGAATTCTACTCGGTCTTCACGCAACAGCCGGGG CAACAGGCCTCTCTAGCGGCCTCATTAGTAGCCTTTTTAAGATAAGC TATTAGG. The antisense primer added the peptide sequence AAREA CCPGCCVKTE to the C-terminus of translated cytochrome *c*. The placement of Pro-Gly between the Cys pairs increases the affinity of biarsenical binding.[13] The PCR fragment was ligated into pYes2.1 V5-His Topo and verified by sequencing. Cyt. *c*-4CYS was excised by *Bam*HI and *Eco*RI digestion and ligated into pBABE-puro at identical restriction sites.

Confocal microscopy

For time-lapse analysis, adherent cells were grown in 35 mm glass-bottomed microwell dishes (MatTek), and treated in 3 ml of phenol-red free DMEM (Gibco) supplemented with 10% FCS, 20 mM HEPES (pH 7.3), 2 mM L-glutamine, 200 μg/ml penicillin, 100 μg/ml streptomycin sulfate, and 55 μM 2-mercaptoethanol. For experiments with suspension cells, 2–5 $\times$ 10^6 cells were spun down at 200 $\times$ *g* for 5 min and then resuspended in 0.2 ml of phenol-red free RPMI supplemented as described above. This was added to 0.5 ml of 2 $\times$ RPMI, and 0.5 ml of 2% low melting point agar kept liquid by submersion in a 37°C water bath. The mixture was mixed well and then centrifuged in 35 mm glass-bottomed microwell dishes at 200 $\times$ *g* for 10 min, until the low melting point agar set. Following centrifugation, 2 additional ml of microscope media were added to the top. This treatment kept the cells from moving out of the field of view during prolonged imaging, but did not prevent them from rotating freely.

After treatment with the apoptotic stimuli, cells were returned to an incubator for 3–10 h. TMRE and Mitotracker Green (Molecular Probes) were added at a concentration of 40 and 50 nM, respectively. Annexin V–Alexa 647 (Molecular Probes) was added at a concentration of 0.75/100 (v/v) in the presence of 2.5 mM $CaCl_2$. The media were overlaid with 2 ml mineral oil (Sav-On, Buena Park CA, USA) and the dish was then placed in a Leiden Micro-Incubator coupled to a Bipolar Temperature Controller (Harvard Apparatus). Images were taken every 2 min.

Images were acquired using a Nikon Eclipse TE 300 microscope and a Biorad MRC 1024 confocal laser unit. Cyt. *c*-GFP and TMRE were excited simultaneously using a 488-nm line from an Ar/Kr laser attenuated at 92–88%, and detected through a 522DF35 bandpass filter (cyt. *c*-GFP) or a 585EFLP filter (TMRE). Annexin V–Alexa Fluor 647 was excited using a 647 laser line attenuated at 94–96%, and detected through a 680DF32 filter. Images were taken with a $\times$ 40 oil objective. Data from three Kalman-averaged scans were obtained using Lasersharp 2000 Software (Biorad). Images were analyzed with Metamorph 4.0 (Universal Imaging, West Chester, PA, USA) by drawing regions around individual cells and then computing standard deviation (punctate/diffuse) and integrated brightness (total brightness). The punctate/diffuse index and the total brightness index were calculated by dividing each value by the average of the first five values. When noted, data were scaled by the following formula: scaled point = 1−((Max−x)/Maxdifference) where Max equals the maximum value in the series, *x* equals the point of interest, and MaxDifference equals the Maximum minus the Minimum value in series. Error bars were scaled by multiplying each error bar by 1/MaxDifference.

Cytochrome *c* labeling with biarsenical ligands

The staining media were prepared by the addition of a premixed DMSO stock solution to give a final concentration of 2.5 μM of the green dye FlAsH–1,2-ethanedithiol $(EDT)_2$ or the red dye ReAsH–EDT_2[13] and 10 μM EDT (Fluka) in DMEM. Cells were incubated at 37°C in 5% CO_2/95% air for 2 h. Cells were then rinsed in glucose-containing Hanks' buffered saline solution (HBSS, Gibco) and incubated for 10 min at room temperature in HBSS containing EDT 62.5 μM. Cells were washed three times with HBSS and returned to the incubator for at least 30 min before treatments.

Oxygen electrode measurement

Two independent Clark oxygen electrodes (Instech Laboratories, Plymouth Meeting, PA, USA) with two independent thermo-jacketed chambers were used. This dual system allowed us to analyze two samples in parallel. Cells were permeabilized using 30 μg/ml of digitonine until $>$95% of cells were trypan blue positives. After washing, cells were resuspended in the respiration buffer (RB: 250 mM sucrose, 2 mM EDTA, 30 mM KH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris (pH 7.4) and 2 mM ADP). The volume corresponding to 400 μg of protein was injected into the chambers containing 600 μl of air-saturated RB prewarmed at 37°C. Respiration was induced by the addition of 5 mM succinate and was recorded for 4 min. Then 1 μM antimycin A was added and respiration was recorded for 2 min. Oxygen concentration was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer.[46] Rates of oxygen consumption are expressed as ng-atoms of oxygen/min/mg of proteins.

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