An intracellular wave of cytochrome c propagates and precedes Bax redistribution during apoptosis

Lydia Lartigue1,*, Chantal Medina1,*, Laura Schembri1, Paul Chabert1, Marion Zanese1,2, Flora Tomasello1, Renée Dalibart1, Didier Thoraval3, Marc Crouzet3, François Ichas1,2,‡ and Francesca De Giorgi1,2

1INSERM U916, Université Bordeaux 2, Institut Bergonié, 229 cours de l’Argonne, 33000 Bordeaux, France
2FLUOFARMA, 2 rue Robert Escarpit, 33600 Pessac, France
3CNRS UMR 5095, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

*These authors contributed equally to this work
‡Author for correspondence (e-mail: francois.ichas@inserm.fr)

Summary

Bax is considered to be pivotal in inducing cytochrome c release (CCR) from mitochondria during apoptosis. Indeed, Bax redistributes to the mitochondrial outer membrane (MOM) upon activation and forms homo-multimers that are capable of permeabilizing the MOM. Our attempts to image this sequence of events in single live cells resulted in unexpected observations. Bax redistribution exhibited two distinct components: an early minor redistribution that was silent in terms of homo-multimerization and a major late redistribution that was synchronous with the formation of Bax multimers, but that proceeded belatedly, i.e. only after caspase 3/7 (C3/7) had already been activated. Intriguingly, neither of these two components of redistribution correlated with CCR, which turned out to be spatially organized, propagating as a traveling wave at constant velocity. Strikingly, propagation of the CCR wave (1) preceded signs of in situ Bax conformational activation; (2) appeared to be independent of autocatalytic loops involving a positive feedback of either C3/7, Ca2+ mobilization or mitochondrial permeability transition; and (3) was triggered by diffuse stimulation with the synthetic Bak activator BH3I-1 but then proceeded independently of Bak activation. Thus, the CCR wave not only questions the exact role of Bax redistribution in cell death, but also indicates the existence of yet unidentified positive-feedback loops that ensure a spatiotemporal control of apoptosis at the subcellular scale.

Introduction

Bax is a cytosolic member of the Bcl2 protein family that remains ‘on hold’ at rest, keeping watch over upstream proapoptotic signals (Oltvai et al., 1993; Wei et al., 2001; Zhang et al., 2000). Once triggered, Bax relocates from the cytosol to the surface of mitochondria (Nechushtan et al., 1999), where it anchors, homo-multimerizes and forms pores going through the mitochondrial outer membrane (MOM) (Antonsson et al., 1997; Dejean et al., 2005; Kuwana et al., 2002). This makes Bax a consensual candidate for causing the terminal release of proapoptotic factors, particularly of cytochrome c (Dejean et al., 2005; Jürgensmeier et al., 1998; Kluck et al., 1997; Kuwana et al., 2002; Yang et al., 1997), from the mitochondrial intermembrane space (Kroemer et al., 2007). However, although the formation of channels by activated Bax is well documented in several systems (Antonsson et al., 1997; Dejean et al., 2005), direct evidences of a causal implication of activated Bax in the normal biology of CCR are elusive. For instance, although it has been demonstrated that Bax activation is generic during apoptosis (Wei et al., 2001; Zhang et al., 2000) and that cytochrome c can diffuse across the pores formed by Bax (Dejean et al., 2005; Jürgensmeier et al., 1998; Kuwana et al., 2002), it is also notorious that CCR and apoptosis can take place all the same in cells devoid of Bax (Wei et al., 2001). Also, at the scale of cell populations, Bax activation and CCR do grossly appear to take place simultaneously, proceeding over several hours (Kashkar et al., 2002). However, the cells within a population are eminently asynchronous, and examining apoptosis at the single-cell scale reveals that CCR is in fact ‘all-or-none’, taking less than 5 minutes from start to completion (Goldstein et al., 2000; Goldstein et al., 2005), whereas the process of Bax activation is slow, developing progressively over several tens of minutes before reaching a plateau (De Giorgi et al., 2002). Another weakness in the involvement of Bax in CCR concerns the structural determinants of its activation: several issues regarding the respective role of its N and C termini, as well as the exact moment of its homo-dimerization during the activation and redistribution sequence, are still actively debated, leading to the co-existence of conflicting models in the literature. For some, Bax dimerization takes place early in the cytosol and is the cause for Bax relocation (Gross et al., 1998), whereas for others, Bax relocates as monomers and multimerizes only secondarily in the MOM (Annis et al., 2005). For some, the C terminus of Bax (α9) and α9 mobility are pivotal for Bax relocation to the MOM (Nechushtan et al., 1999; Schinzel et al., 2004), whereas for others, α9 is simply dispensable in this process (Er et al., 2007).

In conclusion, although it is clear that Bax is experimentally capable of releasing cytochrome c, it is much less clear whether Bax is causal in the normal process of CCR. This prompted us to (1) revisit the process of Bax relocation and multimerization at the single-cell level and (2) try to correlate one or more of these events with CCR that was recorded live.
Results and Discussion

Bax relocalization and cytochrome c release

In a first series of experiments, we imaged Bax activation, relying on its propensity to auto-activate upon simple overexpression (Nechushtan et al., 1999; Oltvai et al., 1993; Xiang et al., 1996). Confocal imaging of HCT116 Bax−/− cells (Zhang et al., 2000) artificially expressing EGFP-Bax revealed different patterns of distribution as expression increased with time since transfection (Fig. 1A). Although the protein initially appeared cytosolic and nuclear (Fig. 1A, basal), after a few hours, EGFP-Bax started relocating to the outer mitochondrial surface in a growing number of cells, reaching 53% of cells at 48 hours (n=50) (Fig. 1A, early). This early relocalization perfectly delineated the mitochondrial surface, with the mitochondrial matrices appearing as voids (Fig. 1A, early). Notably, it only concerned a small fraction of total cellular EGFP-Bax, plateauing at 16.5±6.5% (n=11), with the rest of EGFP-Bax remaining on hold in the cytosolic and nuclear compartments. At distance, a second relocalization phase occurred that concerned all EGFP-Bax this time. It resulted in a total disappearance of the soluble forms of EGFP-Bax – nuclear and cytosolic – which concentrated into clusters (Fig. 1A, late) (93% of cells at 72 hours, n=50). This event was associated with the emergence of an altered mitochondrial morphology and with mitochondrial clumping (De Giorgi et al., 2002; Nechushtan et al., 2001).

Addressed at the single-cell level, these two phases appeared to obey distinct molecular mechanisms (Fig. 1B).

First of all, a study of the mobility of EGFP-Bax by iFRAP (van Drogen and Peter, 2004) revealed that the early relocalization phase corresponded to a docking equilibrium of EGFP-Bax without membrane insertion. Indeed, early relocalized EGFP-Bax was capable of redistributing from mitochondria to the cytosol (Fig. 1B). This contrasted with the case of EGFP fused to a reference MOM tail anchor (EGFP-Cb5TMDRR), which appeared membrane-bound in the iFRAP assay. This also contrasted with the case of late relocalized EGFP-Bax, which, similar to EGFP-Cb5TMDRR, was immobile and incapable of redistributing to the soluble phase, indicating that the process of late relocalization was associated, this time, with membrane insertion.

Interestingly, Bax homo-multimerization that we studied in situ by FRET (Miyawaki et al., 1997) in cells expressing ECFP-Bax and EYFP-Bax (De Giorgi et al., 2002) was not detectable prior to...
3517

Cytochrome c makes waves in apoptosis

Fig. 2. Differential involvement of α9 reveals that the early and late phases of Bax motion are distinct. (A) Coexpression of EYFP-BaxΔC and ECFP-Bax shows that α9 deletion prevents the early redistribution phase detected at the level of a single mitochondrion. Arrow points to a single mitochondrion. Scale bars: 1 μm. (B) Enforced dimerization of CopGFP-Bax provokes early mitochondrial relocalization without directly causing CCR. Deleting α9 (CopGreen-BaxΔC) prevents this relocalization. Note the appearance of inclusion bodies that are clearly distinguishable from mitochondria. Cc, cytochrome c. Scale bars: 10 μm. (C) α9 constitutively targets EGFP to the MOM in live yeast cells (EGFP-α9). Mitochondria are labeled with DiOC6. Scale bars: 1 μm. (D) Effect of α9 deletion or α9 immobilization by cyclization (cycloBax) (supplementary material Fig. S1) on the proapoptotic potency of Bax. Loss of mitochondrial membrane potential (ΔΨlow) was used as a late apoptosis reporter (Goldstein et al., 2000). **Statistically significant P<0.001 (n=5), t-test. (E) After STS challenge (1 μM, 5 hours), EGFP-BaxΔC bypasses the early relocalization phase and directly undergoes the late one (compare with t-HcRed–Bax in the two upper rows). Direct late relocalization of EGFP-BaxΔC is observed in the absence of full-length Bax (HCT116 Bax−/− cells, lower row); cycloBax (revealed by anti-myc) (supplementary material Fig. S1) behaves similarly to EGFP-BaxΔC. Scale bars: 10 μm. (F) First updated model of Bax relocalization: the early and late relocalization phases are disconnected; CCR is not associated with the early phase.
Fig. 3. See next page for legend.
the late relocalization phase (Fig. 1C), and thus coincided with the terminal anchorage of Bax to the MOM.

Two questions arose from these observations: (1) are the two phases of EGFP-Bax relocalization really distinct or do they constitute a continuum with the late phase corresponding to a progressive recruitment of the fraction of EGFP-Bax already docked to the MOM; and (2) how do these two phases of EGFP-Bax relocalization relate to the apoptotic process, in particular to CCR (Fig. 1D)?

As for the first question, an answer came from studies designed to investigate the impact of α9 on Bax motion. Using the auto-activation model, we observed that, in cells expressing both EGFP-Bax and EYFP-BaxΔC (Bax deprived of α9), the early phase of Bax relocalization was prevented by the deletion of α9 (Fig. 2A). We confirmed this observation in a second model of direct Bax activation relying on its enforced dimerization (Gross et al., 2006). Indeed, HCT116 Baxα9 cells expressing CopGFP-Bax exhibited a constitutive early relocalization of the construct, whereas CopGFP-BaxΔC was unable to reach the MOM and remained cytosolic (Fig. 2B). These results were in good agreement with a ‘mirror image’ experiment: α9 alone, fused to EGFP (EGFP-α9), constitutively associated with the mitochondrial surface in yeast cells that have the distinctive feature to be devoid of Bax and of any other Bcl2 family member (Jin and Reed, 2002) (Fig. 2C).

Thus, in our hands – and at major variance from recent observations (Er et al., 2007) – α9 appeared pivotal, especially because deleting α9 or simply preventing α9 mobility by cyclization (cycloBax) (Williams et al., 2002) (supplementary material Fig. S1) impinged on the proapoptotic function of Bax documented by using mitochondrial depolarization as terminal apoptotic readout (Fig. 2D).

However, when addressing the same issue in an inducer-dependent model of apoptosis, we observed that α9 was dispensable for the late relocalization phase, irrespective of its prominent role during the early one. Indeed, distal to a staurosporine (STS) challenge, cytosolic EGFP-BaxΔC and cycloBax were both eventually recruited in the late mitochondrial clusters without prior docking to the MOM (Fig. 2E).

This shows that the early and late phases of Bax relocalization are not a continuum but are two distinct processes. Notably, α9 is involved in the early docking of Bax, during which neither anchorage nor self-dimerization take place, whereas α9 is dispensable for the direct drain of cytosolic and nuclear Bax that eventually builds up clusters anchored at the mitochondrial surface (Fig. 2F).

Thus, how do the two distinct phases of Bax relocalization relate to apoptosis and CCR? One first indication was already observable in Fig. 2B, in which one could note that the early phase of CopGFP-Bax redistribution was totally silent in terms of CCR, with cytochrome c clearly retained within mitochondria that were covered with CopGFP-Bax. Note, however, that this docking phase of CopGFP-Bax was particularly transient and was rapidly followed by CopGFP-Bax clustering, CCR and apoptosis execution (see Fig. 2D).

To complete this observation, we simultaneously imaged the single-cell dynamics of EGFP-Bax motion and of caspase 3/7 (C3/7) activation after STS challenge using a new positional C3/7 recombinant biosensor (Schembri et al., 2007) (Fig. 3A). Kinetic analysis clearly indicated that (1) the early phase of Bax activation preceded C3/7 activation, and that (2) the late relocalization phase proceeded long after C3/7 had already been fully activated (Fig. 3A).

We also noted a strong imbalance regarding the internal kinetics of the two processes: C3/7 activation was fast, reaching completion in less than 10 minutes, whereas the late relocalization of EGFP-Bax was particularly slow, proceeding for ~40 minutes in this cell (Fig. 3A).

This experiment made it clear that the late relocalization of Bax, a process associated with the dimerization of the protein and with its anchorage to the MOM, could simply not be causal in the activation of C3/7 because it occurred later. It even raised the theoretical possibility that the late relocalization of Bax could be dependent on activated C3/7. We tested this possibility and observed, in agreement with the literature (Xiang et al., 1996), that full caspase inhibition with zVAD-fmk did not prevent the late relocalization of Bax (supplementary material Fig. S2). Thus, although most Bax relocalization is late and occurs second to C3/7 activation, there is no positive feedback of C3/7 on Bax.

These observations indicated that what is generally meant as the hallmark of Bax activation, i.e. anchorage to the MOM and multimerization, occurs second to C3/7 activation (Fig. 3B), which itself follows CCR (supplementary material Fig. S3). To confirm this sequence, we simultaneously imaged the kinetics of CCR and Bax relocalization after STS challenge in single cells expressing cytochrome-c–EGFP (Goldstein et al., 2000) and t-HeRed–Bax (Fig. 3C,E). As was expected based on the previous experiments, CCR initiated and completed prior to the late mitochondrial recruitment of Bax. Again, an imbalance between the internal kinetics of the two processes was noted: in agreement with earlier reports (Goldstein et al., 2000; Goldstein et al., 2005), CCR was ‘all-or-none’ and completed in less than 5 minutes, whereas t-HeRed–Bax relocalization appeared as a slow, progressive process, plateauing only after several tens of minutes (Fig. 3C).

These results, indicating that CCR preceded evident signs of Bax motion, were puzzling, and we thus reasoned that some ‘undetectable’ relocalization of Bax could perhaps be responsible for CCR. However, we observed that the early relocalization of Bax that was artificially elicited by enforced dimerization (Fig. 2B), i.e. easily detectable in this case, did not cause CCR. We interpreted these data as a likely indication that, if Bax played any role in CCR, it rather corresponded to a pure in situ microactivation concerning the small fraction of Bax already loosely docked to the MOM than a process requiring the massive and de novo relocalization that is generally described.

Fig. 3. CCR precedes Bax redistribution and propagates within cells. (A) Simultaneous single-cell monitoring of EGFP-Bax and C3/7 activity with the mom-C3/7 probe (Schembri et al., 2007). Frames are numbered in minutes. Ellipse: nuclear region used to detect late EGFP-Bax relocalization (drop in green fluorescence) and t-HeRed released by C3/7 from mitochondria (rise in red fluorescence) as shown in the graph. Late EGFP-Bax relocalization slowly develops after C3/7 activity has already plateaued. (B) Second updated model of Bax relocalization: the late relocalization phase occurs secondary to C3/7 activation, which itself precedes CCR (supplementary material Fig. S3). (C) Simultaneous single-cell monitoring of t-HeRed–Bax motion and of cytochrome-c–EGFP release. Frames are numbered in minutes. Enclosed region shows the group of mitochondria that was used to quantify early and late t-HeRed–Bax recruitment (red-fluorescence increase) and cytochrome-c–EGFP release (green-fluorescence decrease) as shown in the graphs. (D) Simultaneous single-cell monitoring of t-HeRed–Bax motion and of cytochrome-c–EGFP release shows a polarized and propagating CCR. The experiment and conclusions are identical to those in C, with CCR and Bax motion quantified this time using a punctuate/diffuse index (Goldstein et al., 2000). In the first five frames, polarized CCR is observable (from left to right). (E, left) Subcellular quantification in mitochondrial regions 1 to 8 (R1-R8) shows a propagating wavefront of CCR. Left panel: in a HeLa cell, R1-R8 indicate a wave propagating perpendicularly to the cell main axis. (Center) Duration of elemental CCR in R1-R8 (blue bars) is fairly constant. (Right) The CCR wave elicited by STS in a HCT116 Baxα9 cell.
Fig. 4. See next page for legend.
Spatiotemporal organization of CCR

Going further and looking more carefully at the intracellular kinetics of CCR, we observed that what we described so far as a coordinated and ‘all-or-none’ phenomenon seemed in fact to be polarized, and to spread or propagate within cells (Fig. 3D,E). For instance, in the cell of Fig. 3D, CCR clearly initiated in the mitochondria populating the left cell extremity, and progressively propagated up to the mitochondria located in the extreme right zone of the cytoplasm. CCR recorded at the level of small individual groups of mitochondria positioned along the cell axis exhibited constant internal kinetics, but was triggered gradually, suggestive of a traveling wave of CCR (Fig. 3E). This phenomenon preceded Bax motion – evidenced either by computing the standard deviation of pixel intensity (Goldstein et al., 2000) (Fig. 3D, right plot) or by direct intensity measurement within mitochondrial regions of interest (not shown) – was not distinctive of CCR induced by STS and was also observed after FasL challenge (see supplementary material Movie 1).

In order to better characterize this unexpected feature of CCR, we had to overcome a technical issue: following an apoptotic challenge, the exact moment of the onset of CCR in a given cell is unforeseeable and, thus, increasing the frequency of image acquisition to a value capable to catch the fine kinetics of CCR most of the time resulted in probe bleaching or in cellular photodamage before any CCR had taken place. We thus reasoned that a technical alternative could consist of keeping image sampling unforeseeable and, thus, increasing the frequency of image acquisition to a value capable of catching the fine kinetics of CCR.

We thus created giant syncytia made of cytochrome-c-EGFP-expressing cells (Goldstein et al., 2000) by inducing plasma-membrane fusion in confluent cultures (Fig. 4A). CCR could be elicited in such syncytia by FasL, indicating that the physiological apoptosis signaling machinery was in working order even after fusion of several tens of cells. Here, again, CCR was polarized, exhibited a wavefront and propagated from one mitochondrion to another until all cytochrome c was released in the syncytium. Supplementary material Movie 2 shows the syncytium shown in Fig. 4A,B live. Supplementary material Movie 3 shows a CCR wave in a smaller syncytium as well as within an adjacent single cell. The propagation speed was constant around a mean value of 17.7±3.3 μm.minute⁻¹ (37°C, n=5) and was not significantly affected by lowering the temperature to 27°C (15.2±4.9 μm.minute⁻¹, n=4). The constancy of the speed indicated that the process was not simply diffusive, but rather corresponded to a sustained autocatalytic propagation. The temperature insensitivity suggested that the autocatalytic spread involved the diffusion of a triggering agent and not an enzymatic process (Goldstein et al., 2005), and seemed to indicate that temperature-sensitive MOMP promoters such as the BH3-only proteins Bad, Bid, Bim and Puma (Madesh et al., 2002; Uren et al., 2007), or even the mitochondrial permeability transition pore (PTP) (García et al., 2005), were involved in the activation process. The temperature insensitivity thus creates the conditions for a second wave process.

Concerning Bax, using syncytia we intriguingly observed that the CCR wave preceded measurable signs of endogenous Bax activation. Thanks to the extremely large size of the syncytia, we were indeed able to recover from fixed populations syncytia with a CCR wave that was ‘frozen’ by the fixative while it was in the course of propagation (Fig. 4C). In those syncytia, detection of endogenous activated Bax by immunofluorescence suggested that Bax conformational activation took place behind the CCR wavefront. Although we cannot completely exclude an artifact resulting from the relative sensitivity of the two antibodies, still, this observation casts doubts on our previous conclusion that an in situ microactivation of Bax could be the cause of CCR. One could note that Fig. 3E (right panel) already challenged the idea of a participation of Bax in the CCR wave because the latter could still be observed in the few HCT116 Bax⁻/⁻ cells that responded to an apoptotic challenge by releasing cytochrome c. Interestingly enough, the syncytia experiments also showed that Bax conformational activation was not stochastic, delineating what seemed to be a second wavefront (Fig. 4C). This suggested that the CCR wave could either spatially prime Bax or, alternatively, progressively unmasked affinity sites at the mitochondrial surface capable to attract Bax (Kagan et al., 2005), thus creating the conditions for a second wave process.

This intriguing result seemed to indicate that Bax was not a major player in the functional loop causing the spatial spread of the CCR wave. We thus immediately considered the Bax homolog Bak. Bak permanently resides in the MOM, is capable of causing CCR similarly to Bax and appears to constitute a redundant alternative to Bax (Wei et al., 2001). Studying Bak function at the single-cell level is limited by the absence of protein movements characterizing Bak operation. Moreover, the poor signal-to-noise ratios exhibited by conformation-dependent antibody that detects the activated form of Bak prevented us establishing a correlation between the signs of Bak activation and the CCR wavefront in giant syncytia (not shown). To substantiate this observation without changing cellular model, we considered two possible approaches: one based on Bak silencing with siRNAs, the other based on Bak activation with the small-molecule BH3 mimic, BH3I1 (Degterev et al., 2001). The siRNA approach appeared inconclusive because of partial silencing and the impossibility to control the extent of the latter at the level of individual live cells (not shown). By contrast, Bak activation with BH3I1 was more informative. This molecule is a cell-permeant mimic of Bid. Because Bid activation during apoptosis is diffuse and exhibits no
particular subcellular spatial patterning (Ward et al., 2006), we reasoned that challenging cells with BH3I should mimic the direct operation of Bak triggered by Bid. Strikingly enough, and in spite of the diffuse nature of the stimulus, BH3I caused a rapid CCR under the form of a CCR wave (Fig. 4D). This result supports the idea that Bak activation is a prerequisite to and primes the CCR wave. To test the participation of Bak in the subsequent CCR-wave-propagation process, we created chimeric synctia made of a mixture of human cytochrome-c–EGFP-expressing cells and of mouse Bax/Bak double-knockout embryonic fibroblasts (Wei et al., 2001). To our surprise, clusters of Bax<sup>−/−</sup>/Bak<sup>−/−</sup> cells appeared to have no impact on the process of CCR-wave propagation because the wave crossed the clusters within the synctia without loss of velocity (Fig. 4E).

However, to account for the autocatalytic propagation of a CCR wave, one has to identify at least one complete functional loop that is capable of reconnecting CCR back to itself, i.e. a self-amplification loop. Thus, we challenged the different candidate self-amplification loops capable of functionally connecting cytochrome c back to its own release.

As to the possibility that cytochrome c could trigger its own release (Kagan et al., 2005), it can be noticed that, as propagation proceeded, the basal concentration of cytochrome c ([cytochrome c]), progressively rose throughout the giant cell, making it clear that there was no direct connection between the value of [cytochrome c], and the triggering of CCR (Fig. 4B). This indicated an absence of threshold for CCR in terms of [cytochrome c], i.e. the cytochrome c released from one mitochondrion was not directly causing CCR in the neighboring mitochondria. This is in agreement with earlier observations showing that a local release of cytochrome c was able to elicit further CCR in the neighboring mitochondria (Khodjakov et al., 2004).

Only two other families of self-amplification loops have been identified so far, and are initiated either by C3 and/or by Ca<sup>2+</sup>. Indeed, once released, cytochrome c is capable of (1) causing and/or promoting Ca<sup>2+</sup> release from the endoplasmic reticulum (ER) by direct interaction with the inositol (1,4,5)-trisphosphate receptor (Boehning et al., 2003) and (2) causing the activation of C3/7 through caspase 9 (Kluck et al., 1997; Yang et al., 1997). C3/7 and Ca<sup>2+</sup> can then retroact more or less directly with mitochondria, providing candidate amplification mechanisms of CCR (Boehning et al., 2003; Kroemer et al., 2007; Pacher and Hajnóczky, 2001). Briefly, both C3/7 and Ca<sup>2+</sup> could exert positive feedback on the PTP (Boehning et al., 2003; Pacher and Hajnóczky, 2001), whereas C3/7 could alternatively be able to promote the retro-activation of BH3-only pro-apoptotic proteins such as Bid or Bim by elevating caspase 8 or other upstream substrates (Kroemer et al., 2007).

To examine the possible role of the family of loops triggered by C3/7, we imaged simultaneously C3/7 activity and CCR in cells expressing both a recombinant C3/7 probe (mom-C3/7) (Schembri et al., 2007) and cytochrome-c–EGFP. We found that, at the single-cell level, CCR initiated and completed several minutes before C3/7 activity became detectable, thus casting serious doubts on a possible involvement of any C3/7-dependent loop in CCR-wave propagation (supplementary material Fig. S3). This was confirmed by the observation that the pan-caspase inhibitor, zVAD-fmk (60 μM), did not prevent the appearance or spread of the CCR wave triggered by STS (not shown). The latter result ruled out the contribution of other caspases as well, such as, for instance, caspases 9 and 8, and their possible downstream-effector substrates belonging to the Bcl2 family. It also excluded that caspase modulators such as SMAC (also known as DIABLO) that are released from mitochondria together with cytochrome c could influence the spread of the CCR wave by virtue of their ability to unlock C3/7 (Kroemer et al., 2007).

As for the Ca<sup>2+</sup>-dependent loops, we were first of all unable to confirm the previous observations regarding its active participation in CCR induced by STS (Boehning et al., 2003) (Fig. 4F, left panel). Second, and at variance from previous hypotheses (Boehning et al., 2003; Pacher and Hajnóczky, 2001), we observed that Ca<sup>2+</sup>-mobilization from the ER or even Ca<sup>2+</sup> entry from the extracellular medium had no role to play in the propagation of the CCR wave. Indeed, the CCR wave could be normally observed in cells in which the intracellular Ca<sup>2+</sup> stores had been emptied, the cytosolic [Ca<sup>2+</sup>] buffered, and the plasma-membrane Ca<sup>2+</sup>-entry channels blocked (Fig. 4F, center and right panels). The propagation speed of the CCR wave in these cells was identical to the one observed in control conditions [17.6±2.7 μm.min<sup>−1</sup> ([n]=3) vs 17.7±3.3 μm.min<sup>−1</sup> ([n]=5), respectively]. Strikingly, however, we noted that, under such conditions, released cytochrome c exhibited a clear tendency to eventually relocate back to mitochondria (Fig. 4F, center and right panels).

We finally could exclude any mechanism relying on the PTP (De Giorgi et al., 2002; Kroemer et al., 2007), because the CCR wave proceeded in the absence of any change of the inner-mitochondrial-membrane electrical potential detected with the potentiometric probe TMRM (20 nM) in dual-channel live-cell-imaging experiments (supplementary material Fig. S4).

In conclusion, our observations unveil the existence of an underlying autocalytic mechanism, independent of known positive-feedback loops, propagating gradually from one mitochondrion to another and supporting a traveling wave of CCR during apoptosis. Discovering the meaning and mechanisms of this CCR wave constitutes a new challenge.

Materials and Methods

EGFP-Bax and variants
EGFP-Bax, EYFP-Bax and ECFP-Bax were obtained or constructed as previously described (De Giorgi et al., 2002; Nechushtan et al., 1999). EGFP-BaxAC (EGFP-Bax–171) was synthesized by PCR using the reverse primer (forward) 5′-CCCAAAGCTTTCACTGGCAAGTCGCTCC-3′, and was cloned into pEGFPc3 and pYFPVC3 vectors. EGFP-ε9 was constructed as previously described (Nechushtan et al., 1999). To express EGFP-ε9 in yeast, the cDNA fragments coding for the last 21 aa of Bax were excised by HindIII-SalI restriction digestion and inserted in fusion with the EGFP sequence in the yeast low-copy plasmid pUG36 under the control of a regulated MET25 promoter. Fusions of Bax or of BaxAC with mHcRed or mGFp were obtained by cloning them into the MCS of mCopGFP and pt-HcRed vectors (Evrogen).

cycleBax and cycloEGFP-Bax
Split-inverted intein of Synecochocystis sp. PCC6803 was amplified from pNW1118 (Williams et al., 2002), and cloned in pCMSEGFP (Clontech) and pCDNA3.1. cycloEGFP-Bax and cycloECFP-Bax were constructed by cloning the PCR products of Bax and EGFP-Bax in the EcoRI-MluI sites of the split-inverted intein cloned, respectively, in pCMSEGFP (Clontech) and pCDNA3.1.

mom-C3/7 probes

Interfacial C3/7 probes were constructed and expressed as previously described (Schembri et al., 2007). Briefly, the cDNA encoding the mom-C3/7 probe was generated by insertion of a DEVD coding sequence by site-directed mutagenesis (QuikChange, Stratagene) of the EGFP–mom-TMD cDNA (Schembri et al., 2007). Briefly, the cDNA encoding the mom-C3/7 probe was generated by insertion of a DEVD coding sequence by site-directed mutagenesis (QuikChange, Stratagene) of the EGFP–mom-TMD cDNA (Schembri et al., 2007). To obtain spectral variants, the DEVDe–mom–TMD sequence was cloned into mCopGFP or pt-HcRed-tandem (Evrogen).

Cell biology

HCT116 Bax<sup>−/−</sup> and 2H18 HeLa cells were cultivated and transfected as previously described (Goldstein et al., 2000; Zhang et al., 2000). For western blotting, membranes were revealed using an anti-Bax antibody (Bax N-20; Santa Cruz Biotechnology) at a 1:200 dilution. For immunofluorescence (IF), cells were fixed in 3.7% formaldehyde for 10 minutes at room temperature before permeabilization in 0.5% Triton. Aspecific binding was blocked by incubation in 0.2% gelatin/PBS, and staining was performed...
using specific primary antibodies against Bax (Bax N-20; Santa Cruz Biotechnology) and and an Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and imaged with an LSM 510 META confocal microscope (Carl Zeiss). Giant synctia were obtained by treating confluent monolayers of HeLa cells or mixtures (2/3 to 1/3, respectively) of HeLa and Bax−/Bax+ mouse embryonic fibroblasts for 40 seconds with PEG 8000 50% (w/v) plus DMSO 10% (v/v) in PBS. The monolayers were then immediately washed three times with DMSO 10% in PBS, and finally incubated for 4 hours in DMEM supplemented with 4.5 g/l glucose, and 10 mM pyruvate at 37°C, 5% CO₂ before imaging. For addressing the contribution of the monolayers were then immediately washed three times with DMSO 10% in PBS, or mixtures (2/3 to 1/3, respectively) of HeLa and and anti-cytochrome-c antibody. Anti-cytochrome-c was prepared by immunizing female New Golden, Chin, K., Parvin, E. I., and Mazur, L. J. (2001). Bax/Bak channel-forming activity by bcl-2 and anti-bak channel-forming activity by bcl-2.

Imaging

Acquisition and processing of images were performed using an LSM image-analysis software piloting an LSM 510 META confocal microscope (Carl Zeiss). Additional image analysis was performed using MetaMorph 7 (Molecular Devices). For quantification of the fraction of relocalized ECFP-Bax, volume stacks were generated and the fluorescence associated with mitochondria was integrated and divided by total fluorescence. IFRAP experiments were performed by bleaching ~80% of the cell section with a 488-nm Argon laser and by monitoring the resulting fluorescence decay in the unbleached mitochondrial region (van Drogen and Peter, 2004). For FRET measurements, cells were co-transfected with the ECFP and/or EYFP fusions of Bax, incubated, and eventually fixed with 4% paraformaldehyde. FRET was evaluated by the acceptor photobleaching method and quantified by the dedicated FRET module of the LSM image-analysis software (Carl Zeiss).

Yeast experiments

Saccharomyces cerevisiae strain BY4742 (MATa his3; leu2; lys2; ura3) was from the Euroscarf collection (Frankfurt, Germany). After transformation with the plasmids p363EGFP and p363EGFP-eyt, yeast transformants were grown overnight in synthetic dextrose inducible medium (low methionine). Cells were harvested for analysis during the exponential phase of growth. Vital labeling of yeast mitochondria was performed with DiOOC6(3), EGF-positive cells stained with DiOC6 were then spectrally imaged with the LSM 510 META, and EGF and DiOOC fluorescence signals were deconvoluted by elementary spectra unmixing using the META module of the LSM image-analysis software (Carl Zeiss).

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References


