

Role of cardiolipin on tBid and tBid/Bax synergistic effects on yeast mitochondria

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Abstract

The apoptotic effector Bid regulates cell death at the level of mitochondria. Under its native state, Bid is a soluble cytosolic protein that undergoes proteolysis and yields a 15 kDa-activated form tBid (truncated Bid). tBid translocates to mitochondria and participates in cytochrome *c* efflux by a still unclear mechanism, some of them at least mediated by Bax. Using mitochondria isolated from wild-type and cardiolipin (CL)-synthase-less yeast strains, we observed that tBid perturbs mitochondrial bioenergetics by inhibiting state-3 respiration and ATP synthesis and that this effect was strictly dependent on the presence of CL. In a second set of experiments, heterologous coexpression of tBid and Bax in wild-type and CL-less yeast strains showed that (i) tBid binding and the subsequent alteration of mitochondrial bioenergetics increased Bax-induced cytochrome *c* release and (ii) the absence of CL favors Bax effects independently of the presence of t-Bid. These data support recent views suggesting a dual function of CL in mitochondria-dependent apoptosis.

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Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DiOC6(3), 3,3'-dihexyloxycarbocyanine iodide; $\Delta\Psi_m$, mitochondrial membrane potential; FCCP, fluorocarbonyl cyanide *m*-chlorophenylhydrazine; NAO, nonylacridine orange; TPP⁺, tetraphenylphosphonium

Introduction

Cardiolipin (CL) is a unique phospholipid with four acyl chains. It has been known for long that it is associated with mitochondria and, more specifically, with proteins driving oxidative phosphorylation.^{1,2} CL has been shown to copurify with different mitochondrial proteins like cytochrome *c* oxidase,^{3,4} the adenine nucleotides carrier,^{5,6} the F₀F₁-ATP synthase,^{7,8} the orthophosphate carrier⁹ and the bc₁ complex.⁴ For most of them, CL has also been shown to be required for their optimal function. For example, strong evidences suggest that CL participates in the interaction of cytochrome *c* with the outer face of the inner mitochondrial membrane, thereby ensuring the presence of a pool with limited solubility close to respiratory complexes III and IV, increasing the efficiency of electron transport.¹⁰

During the past decade, it has become unequivocally clear that most proapoptotic stimuli require a mitochondria-dependent step involving the disruption of mitochondrial bioenergetics^{11,12,13,14} and outer membrane permeabilization,^{15,16} leading to the release of apoptogenic factors, including cytochrome *c*.

Bid, a widespread proapoptotic member without a proper mitochondrial targeting sequence, has attracted increasing interest since it was identified as a substrate of caspase-8 in apoptotic pathways triggered by death receptors like Fas.^{17,18} It has also been identified as a target for other regulatory proteases such as granzyme B,¹⁹ cathepsin²⁰ and calpain.²¹ Caspase-8 cleavage of full-length Bid (flBid) generates a 15 kDa fragment corresponding to the C-terminal side of flBid, termed truncated Bid (tBid), which is considered as the active form of the protein, able to interact with mitochondria.^{17,18,22} However, it remains unclear as to how proteolysis activates the protein.²³ The determination of solution structure of Bid showed that it consists of eight α -helices (designated α H1 to α H8), of which α H3 contains the BH3 domain, common to all Bcl-2 family members.^{24–26} α H6 and α H7 form an antiparallel hairpin structure, which is surrounded by the other six α -helices. tBid lacks α H1 and α H2, and the hydrophobic hairpin structure formed by α H6 and α H7 becomes exposed, which may explain their binding to the mitochondrial membranes.²⁷ Compared to flBid, tBid also exhibits a 10-fold higher affinity for Bcl-X_L, and a 100-fold higher capacity to trigger the release of cytochrome *c*.²²

Mutagenesis studies have shown that the translocation of tBid to mitochondrial membranes is independent of its BH3

domain.¹⁷ It has been demonstrated that the successful targeting of tBid depends on the presence of the CL, in what might be a unique structure at the contact sites between the inner and the outer mitochondrial membranes.²⁸ At the contact sites, lipids are likely to form a hexagonal-inverted structure, namely in the presence of calcium ion. It has also been observed that tBid can bind with high affinity to products of CL degradation, namely monolysocardiolipin,²⁹ which are normally present in low amounts in mitochondrial membranes, but increase after the first damage to mitochondria induced during early apoptosis. Uncleaved Bid can also move to mitochondria via a still unclear mechanism, but it remains inactive.³⁰ A new hypothesis³¹ established that tBid interacts with CL at contact sites and that this disturbs the electron transport chain enough to inhibit state-3 respiration in association with a mild uncoupling of the state-4 respiration. These damages may also be linked to superoxide anion production,³² leading to modification of the mitochondrial membrane composition and favor Bax or Bak oligomerization and cytochrome *c* release.³¹

At the mitochondrial surface, tBid participates in the formation and/or activation of channels or pores, in association with other Bcl-2 family proteins such as Bax and Bak: these channels/pores allow the relocalization,²³ from the intermembrane space to the cytosol, of apoptogenic factors, such as cytochrome *c*. The exact nature and mechanism underlying this relocalization is still a matter of debate but might involve, in addition to protein interactions, a reorganization of mitochondrial membranes at the level of contact sites and/or cristae.³³ The role of tBid in this general scheme remains unknown: direct action on Bax/Bak activation and have indirect effects on the organization of mitochondrial membranes has been proposed.

In the present study, yeast was used as a simplified cellular model system to investigate the role of CL in the initial steps of the interaction of tBid with mitochondria and the subsequent bioenergetic changes, by comparing Bid-effects on mitochondria isolated from wild-type and, from CL-less yeast strains. Furthermore, the effect of tBid on Bax-induced release of cytochrome *c* was studied in these two strains, and a model of a self-amplifying loop involving CL was proposed.

Results

Lipid composition and bioenergetic characterization of $\Delta c1s1$ mitochondria

The investigation of the role of CL on the effects of tBid on mitochondria first required a careful characterization of the properties of $\Delta c1s1$ mitochondria in comparison to wild type. Wild-type strain W303-1B and the $\Delta c1s1$ mutant were grown on a strictly nonfermentable carbon source (glycerol + ethanol) in order to obtain an optimal differentiation status of mitochondria. Mitochondria were isolated and their phospholipid content was analysed (Figure 1a). Under these conditions, CL represented $12 \pm 3\%$ of total phospholipids in wild-type mitochondria, while no CL was detected in $\Delta c1s1$ mitochondria. As previously described, CL was replaced by its precursor monophosphatidylglycerol (PG) since the amount

of (phosphatidylethanolamine(PE) + PG) increased from 31% in wild-type to 49% in $\Delta c1s1$ mitochondria. Interestingly, the proportions of the other phospholipids (phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS)) were not significantly different in wild type and mutant mitochondria, showing that the mutation exclusively modified the repartition between PG and CL. As noted in the introduction, CL is thought to play a crucial role in the existence of contact sites between the inner and outer mitochondrial membranes. In accordance to this hypothesis, the CL proportion in contact sites from wild-type mitochondria was higher than in whole mitochondria ($18 \pm 2\%$ as compared to $12 \pm 3\%$; Figure 1b) and, as expected, the proportion of PG in contact sites of $\Delta c1s1$ strain was also higher than in whole mitochondria (58% PE + PG as compared to 49% ; Figure 1b). Concerning the fatty acid composition of phospholipids, no significant differences on the saturated/unsaturated ratio and the C₁₈/C₁₆ ratio was observed (Figure 1c), showing that differences of the behavior of wild-type and $\Delta c1s1$ mitochondria are exclusively caused by the modification of the polar heads. The absence of CL in $\Delta c1s1$ mitochondria would also be evidenced on whole cells by a cytofluorometric assay on spheroplasts stained with the CL-selective fluorescent probe nonyl acridine orange (NAO; Figure 1d, lower panel). A parallel staining of the cells with the mitochondrial membrane potential ($\Delta\Psi_m$)-sensitive probe DiOC₍₆₎3 showed that the $\Delta\Psi_m$ was reduced, but not completely collapsed, in the $\Delta c1s1$ strain (Figure 1d, upper panel), predicting that the bioenergetic capacity of $\Delta c1s1$ mitochondria is moderately altered.

Bioenergetic characteristics of isolated mitochondria were determined by measuring simultaneously respiration rate, $\Delta\Psi_m$ and ATP synthesis (Figure 2). The basal respiration rate (state 4) of $\Delta c1s1$ mitochondria oxidizing NADH was slightly increased as compared with wild type (150 versus 129 nmol O₂/min/mg protein) in accordance to a slight decrease of $\Delta\Psi_m$ (-171 versus -187 mV). Upon addition of ADP, phosphorylation-coupled respiration (state 3) was significantly lower in $\Delta c1s1$ than in wild type (510 versus 650 nmol O₂/min/mg protein), in accordance with a decreased ATP synthesis (786 compared with 866 nmol H⁺/min/mg protein) and a slight decrease in $\Delta\Psi_m$ (-137 versus -141 mV). The efficiency of coupling between respiration and ATP synthesis was clearly decreased in $\Delta c1s1$ mitochondria, as indicated by the lower respiratory control ratio (3.4 versus 5.0 in wild-type mitochondria) and the lower ADP/O ratio (1.3 versus 1.5 in wild-type mitochondria). These data indicate that $\Delta c1s1$ mitochondria exhibit a slight alteration of their bioenergetic function. It is not caused by the isolation of mitochondria, since the lower $\Delta\Psi_m$ could also be visualized in whole cells (Figure 1d, upper panel). In addition to the effects on mitochondrial transmembrane potential, the absence of CL also induced some alterations in mitochondrial dynamics. Expression of a mitochondria-addressed GFP allows to visualize the mitochondrial network by fluorescence microscopy. In the wild-type strain, the morphology of the network is changing when the cells approach the stationary phase of growth (thread-grain transition). Such a change does not occur in the $\Delta c1s1$ strain (Figure 1e), suggesting that the absence of CL may hamper the reorganization of the inner mitochondrial membrane.

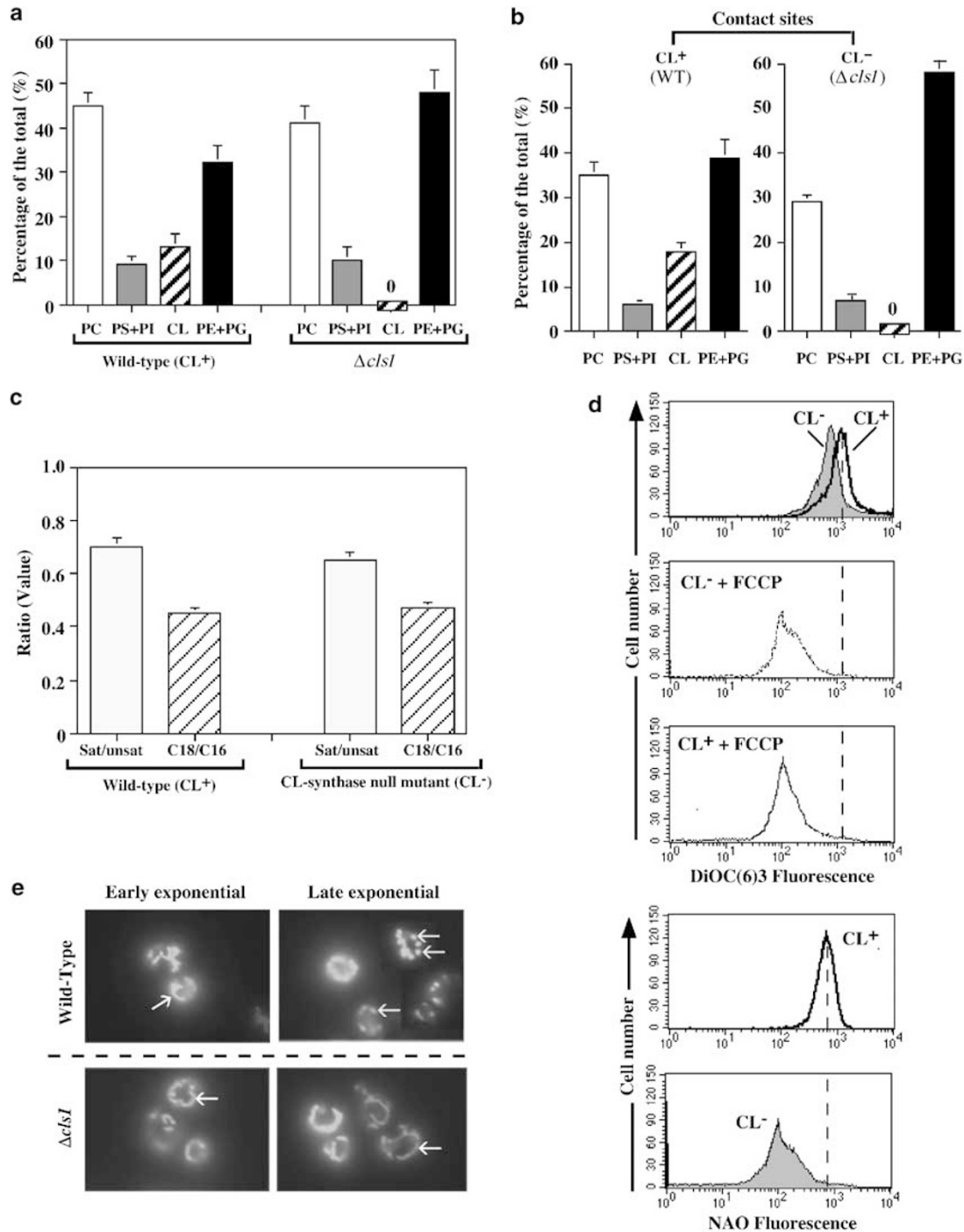


Figure 1 Lipid analyses of wild-type and $\Delta cls1$ mitochondria and their mitochondrial network. **(a)** Proportion of each class of phospholipids in whole mitochondria from wild-type and $\Delta cls1$ strains separated by TLC. Data are means from three independent experiments \pm S.D. **(b)** Proportion of each class of phospholipids in mitochondrial contact sites. Data are means of two independent experiments \pm S.E.M. **(c)** Fatty acid analysis from phospholipids from whole mitochondria. C_{16:0}, C_{16:1}, C_{18:0} and C_{18:1} were separated by gas chromatography-mass spectrometry. Data are means from two independent experiments \pm S.E.M. **(d)** Flow cytometry analysis of spheroplasts for $\Delta\Psi_m$ (DiOC₆(3) (3,3'-dihexyloxacarbocyanine iodide), left panel) and CL content (NAO, right panel). The upper panel describes CL⁺ and CL⁻ mitochondrial membrane potential, whereas the middle panel and the lower panel show the drop in $\Delta\Psi_m$ induced by the uncoupling agent FCCP (at 10 μ M). The upper right panel shows the CL content of the CL⁺ strain via NAO staining, whereas the lower right panel shows the absence of CL in the CL-synthase-less strain (CL⁻). Data are representative from four independent experiments. **(e)** Fluorescence microscopy of the mitochondrial network related to early exponential growing phase or late exponential growing phase. The mitochondrial network appeared as threads during early exponential growth phase, both in wild-type and $\Delta cls1$ strains. When cells reached late exponential growth phase, the network appeared as grains in the wild-type strain but remained as threads in the mutant strain

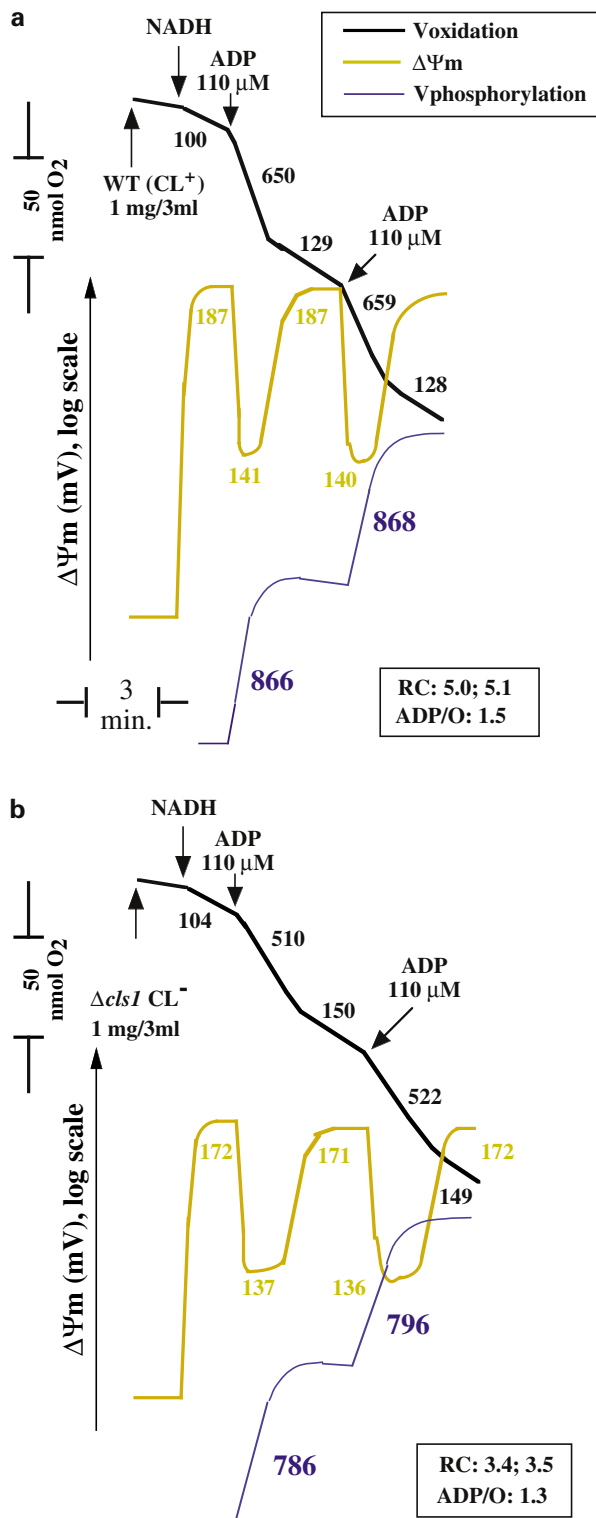


Figure 2 Bioenergetic characteristics of wild-type and $\Delta cls1$ mitochondria. (a, b) On-line recordings of oxygen concentration (black line, respiration values in nmol O₂/min/mg protein), concentration of TPP⁺ (green line, $-\Delta\Psi_m$ values in mV) and proton production linked to ATP synthesis (blue line, values in nmol H⁺/min/mg protein) for wild-type (a) and $\Delta cls1$ (b) mitochondria

CL-dependent effects of tBid on bioenergetic function of isolated mitochondria

The effect of 20 nM tBid on the bioenergetic function of wild-type mitochondria was measured. Both state-3 respiration and ATP synthesis were decreased (from 650–470 nmol O₂/min/mg⁻¹ protein, and from 866 to 715 nmol H⁺/min/mg protein, respectively) (Figure 3a). Interestingly, $\Delta\Psi_m$ values (both under state 4 and state 3) were not affected showing that the effect of tBid is not a simple detergent-like effect on the membranes. This effect of tBid was completely absent when the protein was assayed on $\Delta cls1$ mitochondria (Figure 3b). A titration of tBid has been carried out, which demonstrates that the tBid-binding sites, that is, CLs, were hardly saturable (Figure 4a). Effectively, almost all the tBid added to the mitochondrial suspension (0.5–20 nM with reference to 25 nM tBid alone as a 100% for the Western blot reference) is recovered at the mitochondrial level. Concerning the changes in mitochondrial respiration, the state-3 inhibition by tBid is detected at 5 nM and above, whereas the mild uncoupling leading to state-4 enhancement is only apparent at 10 nM (Figure 4a). flBid at 200 nM is without effect on such parameters.

The rate of ATP synthesis was directly measured with a bioluminometric method (Figure 4b): tBid dramatically inhibited the rate of ATP synthesis in wild-type mitochondria, whereas it had no effect on $\Delta cls1$ mitochondria.

These demonstrate that, in the absence of any other Bcl-2 family protein, tBid alters mitochondrial function in a CL-dependent way.

CL-dependent synergetic action of tBid and Bax on cytochrome c release

The main proapoptotic function of tBid is thought to occur via its stimulating effect on the conformational change of Bax leading to the acquisition of a structure able to trigger the release of cytochrome c.²³ This early step of apoptosis can be reproduced in yeast, following the expression of a Bax variant that has already acquired the active conformation: this was simply obtained by adding a *c-myc* tag at the C-terminal end of the protein.³⁴ This Bax-variant targets yeast mitochondria, is inserted in the outer membrane and is able to form a large cytochrome c-permeable channel, identical to that observed in apoptotic mammalian cells.³⁵

This simplified cellular system allows testing the effect of tBid on the function of Bax, independently of the apoptotic network. Following the expression of the proteins alone or in combination, mitochondria were isolated and the spectrophotometric measurement of the amount of mitochondrial cytochromes allows visualization of the release of cytochrome c, while the amounts of membrane-attached cytochromes *b* and *aa₃* serve as internal controls (Figure 5a). Quantitative results of these series of experiments are given in Figure 5b.

Full-length Bid (flBid) or tBid were expressed under the control of the strong promoter *GAL1/10*: none of them were able to induce any release of cytochrome c. When expressed in yeast, flBid does not spontaneously localize to mitochondria. On the contrary, tBid is localized in mitochondria and this localization is higher when tBid is coexpressed with

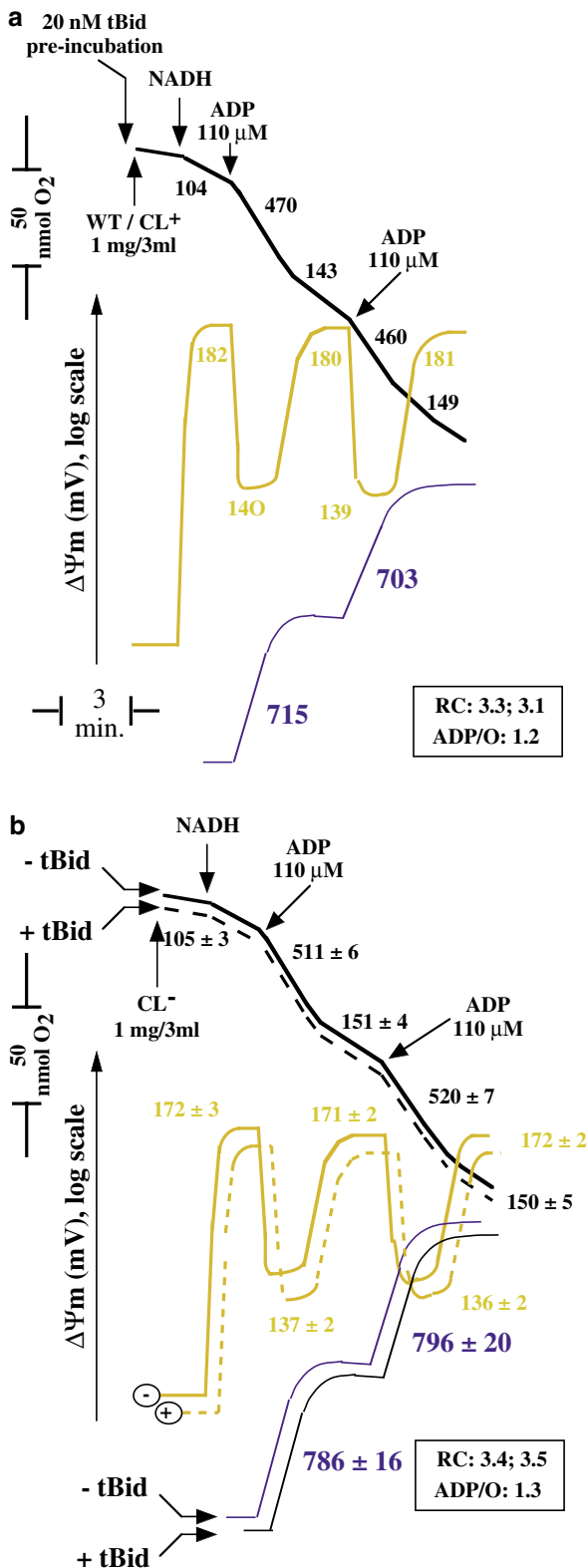


Figure 3 Effect of tBid addition on bioenergetic characteristics of isolated mitochondria. Wild-type mitochondria (a) and $\Delta cls1$ (b) mitochondria were preincubated for 20 min in the presence of 20 nM purified recombinant tBid and 200 μM ATP prior to bioenergetics measurements

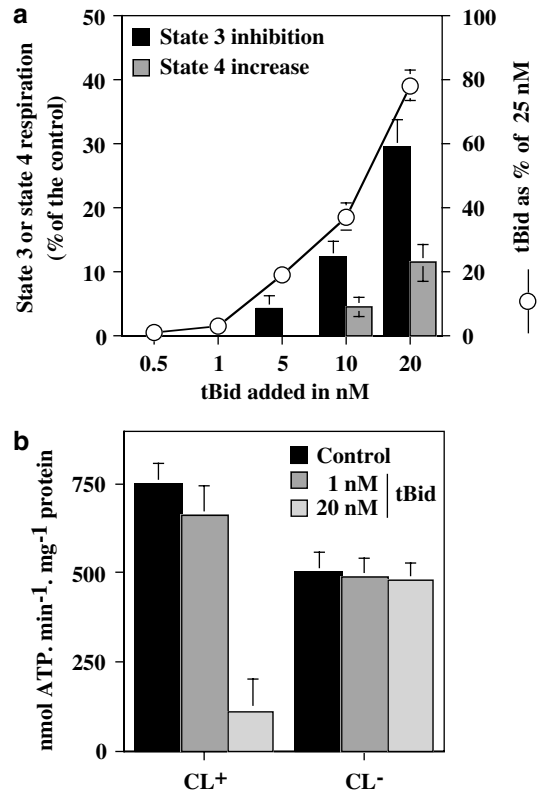


Figure 4 Effect of tBid addition on the mitochondrial respiration and ATP synthesis. (a) Wild-type mitochondria were preincubated for 20 min with different concentration of tBid (0.5–20 nM). The Western blots with a 25 nM tBid as a reference were scanned and the amount of tBid bound to mitochondria was estimated by comparison to the reference. The plots presented are the mean ± S.D. of five blots. The data concerning the state-3 inhibition and the state-4 increase induced by tBid were selected from seven different experiments conducted with the Clark electrode (cf. Materials and methods). (b) For the ATP synthesis, the experimental conditions were identical to those in Figures 2 and 3 except that ADP concentration was 1 mM. Aliquots were taken out at 0, 15, 30 and 45 s after ADP addition for the measurement of ATP by bioluminescence

Bax-c-myc,³⁴ suggesting that both proteins are able to interact in the mitochondrial membrane. Bax was first expressed in the wild-type strain under the control of the low-strength promoter *tet-off*, under conditions where it does not induce a significant release of cytochrome *c*. The combined expression of fBid with weakly expressed Bax did not induce any release either. On the contrary, the combined expression of tBid with weakly expressed Bax induced the release of cytochrome *c* (which is also visualized in Figure 5a). This result is a clear support to the hypothesis that the presence of tBid actually helps in the acquisition of the ultimate active conformation of Bax.

The same series of experiments was carried out in the $\Delta cls1$ strain (Figure 5b, gray bars). Like in wild-type strain, neither weakly expressed Bax nor strongly expressed Bid or tBid induced any release of cytochrome *c*. The combination of weakly expressed Bax and strongly expressed tBid induced a release of cytochrome *c*, with a higher extent than in wild-type mitochondria. Accordingly, the coexpression of weakly expressed Bax and strongly expressed Bid induced a slight but significant release of cytochrome *c* in $\Delta cls1$ strain, while this combination was inefficient in wild type.

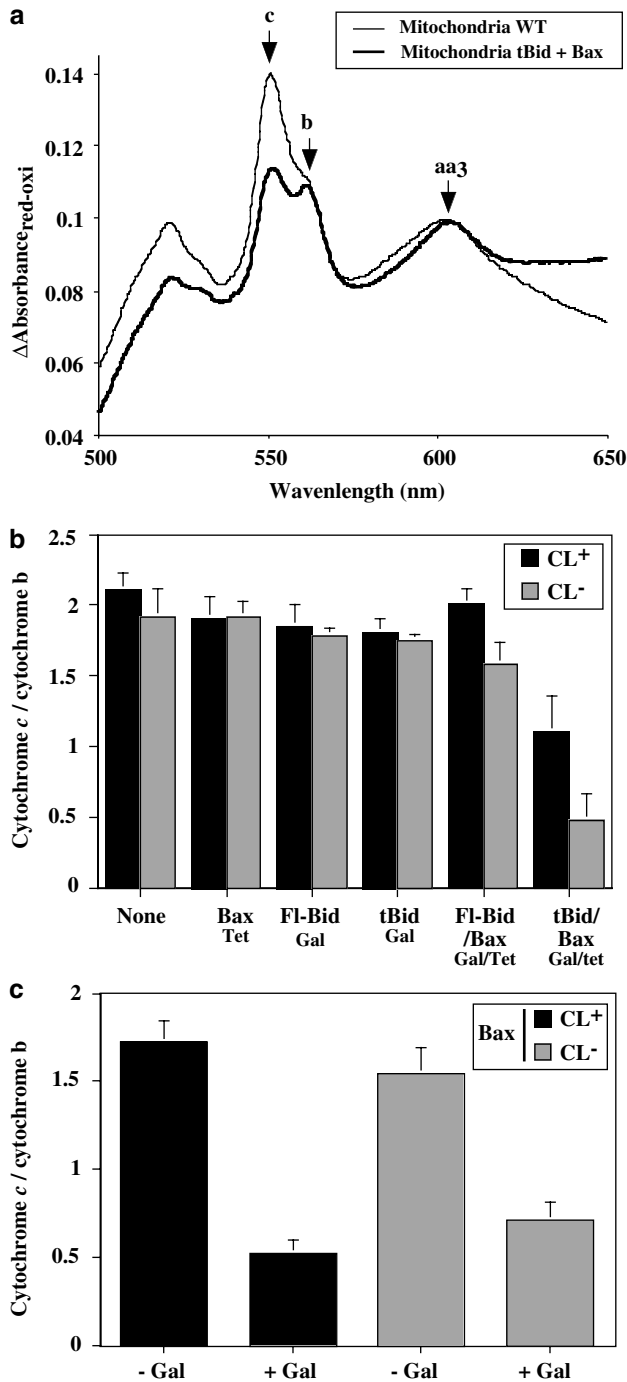


Figure 5 Effect of tBid on Bax-induced cytochrome *c* release. **(a)** Redox spectrum of mitochondria from the wild-type strain (thin trace) or mitochondria from the wild strain coexpressing tBid and Bax (bold trace). The peaks of absorbance difference corresponding to cytochromes *c*, *b* and *aa₃* are indicated. **(b)** Quantification of cytochrome *c* in mitochondria isolated from wild-type or $\Delta cls1$ strains expressing Bax, Bid and tBid. Data are expressed as the ratio cytochrome *c*/cytochrome *b*, which is an unreleased membrane protein and is thus taken as an internal standard. Data are means from three to seven independent experiments \pm S.D. Bid and tBid were expressed under the control of the strong promoter *GAL1/10*, while Bax was expressed under the control of the weak promoter *tet-off*.^{39,40} **(c)** Quantification of cytochrome *c* in mitochondria isolated from wild-type or $\Delta cls1$ strains expressing Bax under the control of the strong promoter *GAL1/10*. Data are expressed as in **(b)** and are means of three independent experiments \pm S.D.

A recent paper described that the killing effect of Bax expression in yeast does not require CL.³⁶ To support this observation, we expressed Bax under the control of the high-strength promoter *GAL1/10* in wild-type and $\Delta cls1$ strains (Figure 5c): under these conditions, Bax alone was able to induce the release of cytochrome *c*, independently of the presence of CL.

Discussion

Data reported in this paper are a strong support to the hypothesis that CL plays a crucial role in the effects of tBid on mitochondria. Yeast provides a simplified cellular system that allows testing of the interaction of tBid and Bax with mitochondria and a possible synergy between them, independently from the apoptotic network, and evaluates the role of CL in this interaction.

In vitro experiments demonstrated that, in the absence of any other Bcl-2 family members, tBid is able to target the mitochondrial membrane and to induce moderate but significant alterations of oxidative phosphorylation. An important observation is the lack of effect on $\Delta\Psi_m$, which shows that tBid does not act via a simple detergent-like effect. The second important characteristic of this *in vitro* effect of tBid is the requirement for CL.

The extensive bioenergetic characterization of CL-less mitochondria showed that, although slightly altered, these mitochondria are still able to support vigorous oxidative phosphorylation. This is an important point since it demonstrates that the lack of effect of tBid is not caused by a dramatic collapse of the bioenergetic function. This was particularly apparent from direct measurements of ATP synthesis (Figure 4): tBid induced a dramatic collapse of ATP synthesis in wild-type mitochondria and was almost inefficient on $\Delta cls1$ mitochondria.

Since the proapoptotic action of tBid is thought to involve the activation of Bax towards a conformation changeable to trigger the release of cytochrome *c*,³⁰ the role of CL on this activation was investigated. It clearly appeared that tBid was actually able to activate the cytochrome *c*-release activity of Bax, and that the absence of CL further amplified this stimulating effect of tBid. This last point is confirmed by the fact that the less-efficient flBid, which had no stimulating effect in wild type, gained a weak but significant stimulating effect in $\Delta cls1$ mutant. This observation is in apparent contradiction with the effect of tBid *in vitro* but is consistent with the hypothesis that a first damage to mitochondria (visualized by the effect on oxidative phosphorylation) further helps the action of Bax. It has been shown that tBid exhibits a strong affinity for CL²⁸ and even more for by-products of CL degradation, namely monolysocardiolipin.²⁹ It has also been shown that Bax expression, even at low-expression levels, induced the oxidation of mitochondrial lipid, including CL, an effect which is prevented by hydrophobic antioxidants, such as α -tocopherol and resveratrol.³⁷ It is important to notice that yeast expression of tBid alone has no effects on the whole yeast cell respiration (data not shown) nor on cell growth.³⁴ However, in the presence of Bax-*c-myc*, the expression of tBid increased the kinetics of Bax-*c-myc*-induced cell death,³⁴

showing that tBid is able to amplify the killing function of an active form of Bax.

Considering these observations and data reported herein, the following self-amplification loop model can be proposed.

- (1) tBid binds to mitochondria in a CL-dependent fashion. This initial injury to mitochondria has been suggested to induce the oxidation of lipid,²⁹ leading to the formation of lysophospholipids, including monolysocardiolipin. This might help the further binding of tBid.²⁹
- (2) The presence of tBid has destabilizing effect on mitochondrial membranes, as evidenced by the effects on oxidative phosphorylation reported in the present paper. The nature of this effect remains unclear. It is not an acute detergent-like effect, but might be a more subtle reorganization of the membranes induced by CL reorganization (like change in membrane curvature³⁸) and subsequent destabilization of the CL/enzymatic complexes in the electron transport chain, such as has been observed in apoptotic cells.³¹
- (3) The destabilizing effect of tBid on mitochondrial membranes leads to the activation of Bax towards its final active conformation, resulting in its oligomerization, which is able to trigger the release of cytochrome *c*. It should be noted that the binding of tBid to yeast mitochondria did not increase the binding of Bax (while the binding of Bax did increase that of tBid).³⁴ This further supports the view that tBid acts by favoring the activating conformational change of Bax, which allows the acquisition of the capacity to release cytochrome *c*, but that it does not act by increasing the amount of Bax bound to mitochondria.

It should be noted that, both *in vitro* and in yeast, tBid is not absolutely required for Bax function since Bax alone is able to target mitochondria, form a channel in the outer mitochondrial membrane and trigger the release of cytochrome *c*, but this does not preclude a crucial role *in vivo*, in a genuine apoptotic network. Data presented in this paper are now a basis for further investigation into the molecular mechanisms involved in the interaction between tBid, Bax and CL, since yeast represents a simplified living model for evaluating the action of Bid and Bax mutants in the absence or in the presence of CL.

Materials and Methods

Strains and plasmids

The strain WKEN011B (*matx, ade2, his3, leu2, trp1, ura3, cls1::kanMX4*, termed $\Delta cls1$ in the following) was derived from the wild-type strain W303-1B, and was obtained from Euroscarf (Frankfurt, Germany). The wild-type strain HT444 (*mat a, his3, leu2, lys2, ura3*) expressing Bax-*c-myc* under the control of *GAL1/10* promoter was described previously.³⁹ The strain SM400 was derived from HT444 by introducing the *cls1::kanMX4* construction, amplified by PCR from the WKEN011B genome, at the original *CLS1* locus. The construction of the plasmid pCM189/bax, expressing Bax-*c-myc* under the control of the *tet*-off promoter was described in Priault *et al.*⁴⁰ The construction of the plasmids pYES3/Bid and pYES3/tBid, expressing Bid and tBid under the control of the *GAL1/10* promoter, was described in Priault *et al.*³⁴ PCR, cloning, and transformations were performed by standard procedures.

Mitochondria isolation and bioenergetic measurements

Cells were grown aerobically in a complete medium YPGE (1% yeast extract, 1% Bacto-peptone, 0.1% potassium phosphate, 0.12% ammonium sulfate, 3% ethanol, 3% glycerol, pH 5.0) until mid-exponential growth phase. The use of glycerol/ethanol as a carbon source ensured a full differentiation of mitochondria. Mitochondria were isolated from zymolase-treated cells, as described in Law *et al.*⁴¹

For bioenergetic measurements, mitochondria (0.33 mg/ml) were suspended in respiration buffer (0.6 M mannitol, 5 mM tris/maleate, 2 mM EGTA, 3 mM potassium phosphate, 5 μ M tetraphenylphosphonium bromide, pH 6.7) in a thermostatically controlled chamber (28°C) connected to three electrodes, allowing the simultaneous measurement of oxygen concentration, tetraphenylphosphonium (TPP⁺) concentration⁴² and pH, respectively.⁴³ Respiration noncoupled to ADP-phosphorylation (state 4) was initiated by the addition of 1 mM NADH (yeast mitochondria are able to oxidize directly cytosolic NADH via two intermembrane space-facing NADH:ubiquinone oxidoreductases). Respiration coupled to ADP phosphorylation (state 3) was initiated by the addition of 110 μ M ADP. Respiration rates were calculated from the slopes of oxygen concentration *versus* time curves. $\Delta\Psi_m$ was calculated from TPP⁺ concentration according to the Nernst equation corrected for TPP⁺ binding.⁴⁴ The rate of ATP synthesis was extrapolated from the rate of acidification caused by the extrusion of the scalar proton in the reaction of ADP phosphorylation.⁴³ Alternatively, ATP synthesis was measured directly by a bioluminometric method. Aliquots (100 μ l) were sampled at different times after ADP addition and precipitated in 100 μ l of 50 mM EDTA in 7% perchloric acid. After immediate centrifugation (3 min, 12 000 g), the pH of the supernatant was adjusted at 7.0 with KOMO (2 M MOPS, 0.2 N potassium hydroxide). ATP was monitored with a luciferin/luciferase kit (BioOrbit) in an LKB bioluminometer against ATP standard solutions.

Flow cytometry measurements and fluorescence microscopy

All measurements were carried out with a FACScalibur (Becton-Dickinson) equipped with a 488 nm laser. For $\Delta\Psi_m$ measurements, spheroplasts obtained from zymolase treatment of the cells were incubated for 30 min in the presence of 5 nM DiOC₍₆₎3 in the absence or in the presence of 10 μ M fluorocarbonyl cyanide *m*-chlorophenylhydrazine (FCCP), and fluorescence was detected at 530 \pm 30 nm in the FL1 channel. For CL-content measurements, spheroplasts were incubated for 30 min in the presence of 1 μ M NAO and fluorescence was detected at 585 \pm 40 nm in the FL2 channel.

For fluorescence microscopy experiments, cells were transformed with plasmid pGAL-CLbGFP, which allows the galactose-driven expression of GFP fused downstream the mitochondria-addressing sequence of mitochondrial citrate synthase.⁴⁴ Cells were grown aerobically on a medium supplemented with 2% galactose and observed during the early (O.D.550 nm = 1) or late (O.D.550 nm = 10) exponential phases of growth, with a Leica fluorescence microscope connected to an SIS camera.

Heterologous expression of proapoptotic proteins and measurement of cytochrome *c* release

The expression of *c-myc*-tagged Bax was achieved with two systems giving different levels of expression.^{34,35} High-level expression was carried

out with the GAL1/10 promoter as follows: HT444/Bax and SM400/Bax cells were grown aerobically in a complete medium YPL (1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, 2% lactate, pH 5.0) until the early-exponential growth phase. Galactose (1%) was added to induce Bax expression, cells were harvested after 90 min and mitochondria were isolated for cytochrome *c* measurement. Low-level expression of Bax, in the presence of Bid or tBid, was carried out as follows: W303-1B and WKEN011B cells, carrying or not pCM189/Bax, pYES3/Bid and pYES3/tBid plasmids, were grown aerobically on a minimal medium YNBGal (0.17% yeast nitrogen base, 0.1% potassium phosphate, 0.5% ammonium sulfate, 0.2% Drop-Mix, 0.01% auxotrophic requirements, 2% galactose, pH 5.0) with 10 μ g/ml doxycyclin: these conditions allowed the expression of flBid or tBid and the repression of Bax. When cultures reached mid-exponential growth phase, cells were harvested, washed three times and resuspended in the same medium with 0.1 μ g/ml doxycyclin to induce a low level of Bax expression.³⁴ Cells were harvested after 12 h and mitochondria were isolated for cytochrome *c* measurement.⁴⁵

Cytochrome *c* content of mitochondria was measured spectrophotometrically. Mitochondria suspension (5 mg/ml) was poured into the two cuvettes of a double-beam spectrophotometer (Aminco DW2000). The reference cuvette was oxidized with potassium ferricyanide and the sample cuvette was reduced with sodium dithionite. Difference spectra were acquired between 500 and 650 nm. The amount of each cytochrome was measured from the difference in the difference spectra between maxima and isobestic points: 550–540 nm for cytochrome *c* ($\Delta\epsilon = 18\,000/\text{M}$), 561–575 nm for cytochrome *b* ($\Delta\epsilon = 18\,000/\text{M}$) and 603–630 nm for cytochromes *a* + *a*₃ ($\Delta\epsilon = 24\,000/\text{M}$). Previous reports have shown that the amount of cytochrome *c* which disappeared from mitochondria following Bax expression was found in the postmitochondrial supernatant.³⁹

Contact sites, outer and inner mitochondrial membranes were isolated according to Simbeni *et al*⁴⁶ and Pon *et al*⁴⁷, respectively, from isolated mitochondria.

Synthesis of tBid

Full-length His-tagged flBid and tBid were purified as described by Desagher *et al*.³⁰ The protein was stored in 25 mM tris-HCl, 100 mM NaCl, 1% octyl D-glucopyranoside, 0.2 mM dithiothreitol, 30% glycerol, pH 7.5, at -80°C . Stock solution of tBid (1.9 mg/ml) was diluted in the nM range in the respiration buffer. An identical dilution of the storage buffer has no effect on mitochondrial functions.

Lipid extraction and fatty acid analysis

Mitochondria were suspended in methanol (10 mg/ml) and lipids were extracted twice by a chloroform/methanol/water (2/1/1) (v/v/v) mixture. The organic phases were pooled, evaporated to dryness and resolubilized in 50 μ l chloroform/methanol (2/1) (v/v). Polar lipids were resolved by one-dimensional thin layer chromatography on 10 \times 10 cm silica gel plates (Merck) using the solvent system as described.⁴⁸ Lipids were located by spraying the plates with a solution of 0.001% primuline in 80% acetone, visualized under UV light, scrapped and quantified by UV absorbance versus known standards.

For fatty acid analysis, aliquots were mixed with 900 μ l methanol containing 2.5% H₂SO₄ and 10 μ g C_{17:0} as an external standard. Transesterification was carried out at 80°C for 1 h and, after cooling on ice, methyl ester fatty acids were extracted with 1 ml hexane. Separation of methyl ester fatty acids was carried out by gas chromatography (Hewlett-

Packard 5890 series II) on a 15 m \times 0.53 mm Carbowax column (Alltech). The oven temperature is programmed for 1 min at 160°C, followed by a 20°C/min ramp up to 190°C, a 5°C/min ramp up to 210°C and a final 5 min at 210°C. Methyl ester fatty acids were identified and quantified by comparison of their retention time with those of standards.

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