Cardiolipin Is Not Required for Bax-mediated Cytochrome c Release from Yeast Mitochondria*

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Cardiolipin (CL) is an inner mitochondrial membrane phospholipid that contributes to optimal mitochondrial function and is gaining widespread attention in studies of mitochondria-mediated apoptosis. Divergent hypotheses describing the role of CL in cytochrome c release and apoptosis have evolved. We addressed this controversy directly by comparing the spontaneous- and Bax-mediated cytochrome c release from mitochondria isolated from two strains of Saccharomyces cerevisiae: one lacking CL-synthase and therefore CL (ΔCRD1) and the other, its corresponding wild type (WT). We demonstrated that the mitochondrial association of Bax and the resulting cytochrome c release is not dependent on the CL content of the yeast mitochondrial membranes. Bax inserted equally into both WT and ΔCRD1 mitochondrial membranes under conditions that lead to the release of cytochrome c from both strains of yeast mitochondria. Furthermore, using models of synthetic liposomes and isolated yeast mitochondria, we found that cytochrome c was bound more “loosely” to the CL-deficient systems compared with when CL is present. These data challenge recent studies implicating that CL is required for Bax-mediated pore formation leading to the release of proteins from the mitochondrial intermembrane space. In contrast, they support our recently proposed two-step mechanism of cytochrome c release, which suggests that CL is required for binding cytochrome c to the inner mitochondrial membrane.

Cardiolipin (CL, see Fig. 1A) is an unsaturated, anionic phospholipid found exclusively in the inner mitochondrial

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* The abbreviations used are: CL, cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine; ΔCRD1, cardiolipin synthase-deficient S. cerevisiae; WT, wild type S. cerevisiae; LC-MS, liquid chromatography-mass spectrometry; TIC, total ion current; TFP, tetraphenylphosphonium; CCCP, carbonyl cyanide m-chlorophenylhydrazone; ΔΨm, mitochondrial membrane potential; LUV, large unilamellar vesicle; Bax, recombinant his-Bax reconstituted in 1% octylglucoside; LIS, low ionic strength buffer; HIS, high ionic strength buffer; Tp, retention time; m/z, mass to charge ratio.

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In the present study, we addressed this controversy directly by comparing the spontaneous- and Bax-mediated cytochrome c release from mitochondria isolated from two strains of Saccharomyces cerevisiae: one lacking CL-synthase and therefore CL (Δcrd1) and the other, its corresponding wild type (WT). We found essentially that cytochrome c was bound more "loosely" to the Δcrd1 inner mitochondrial membrane compared to the WT control. Furthermore, we showed that Bax-mediated cytochrome c release is not dependent on the CL content of the yeast mitochondrial membranes in that Bax mediated the release of cytochrome c both from WT and Δcrd1 mitochondria.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth**—Standard methods were used for the culture of the homoyogous diploid deletion strain Δcrd1 and the isogenic wild type strain BY4743 (22). Yeast were grown aerobically at 28 °C to mid-logarithmic phase in medium containing a non-fermentable carbon source (2% lactate and 3% glucose). Yeast cells were harvested for mitochondrial isolation when A₅₄₀ was between 1 and 2 absorbance units at full scale.

**Isolation of Yeast Mitochondria**—Yeast spheroplasts were generated from zymolyase-treated cells as described previously (23) using 4 mg of zymolyase/g tissue. After washing, the yeast spheroplasts were resuspended in cold isolation buffer (0.6 M sorbitol, 20 mM Hepes, 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Cells were homogenized with a Potter homogenizer (motor-driven), and intact cells were sedimented at 500 × g for 5 min. The supernatant was transferred to a Sorvall tube (40 ml) while the pellet underwent a second homogenization. Supernatants from both centrifugations were combined and subjected to 10,000 × g for 10 min. The supernatant was discarded while the pellet was resuspended in cold sucrose buffer (20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4). Aggregates were sedimented by a 500 × g spin, and the resulting supernatant was further subjected to 10,000 × g for 10 min. The final mitochondrial pellet was suspended in cold sucrose buffer at a final concentration of ~10 mg of protein/ml. Protein quantification was carried out using the BCA protein kit according to the manufacturer's instructions (Pierce, Rockford, IL).

**Isolation of Yeast Mitochondrial Phospholipids**—Phospholipids were isolated as described above, except that the supernatant was further subjected to 10,000 × g for 10 min. The final mitochondrial pellet was suspended in cold sucrose buffer at a final concentration of ~10 mg of protein/ml. Protein quantification was carried out using the BCA protein kit according to the manufacturer's instructions (Pierce, Rockford, IL).

**Isolation of Individual Phospholipids by TLC**—Isolated total phospholipids were first converted to 20 μl of chloroform and loaded onto silica gel TLC plates (10 × 10 cm). The plates were developed in 53:25:6:3 (by vol.) of chloroform/methanol/water/glacial acetic acid in the first dimension followed by 50:10:5:10:20 (by vol.) of chloroform/methanol/water/acetic acid/cone in the second dimension. The TLC plates were dried and exposed to iodine vapor to visualize the double-bond-containing phospholipids, which were then labeled with a pencil. Individual spots were scraped from the glass, and the silica was transferred to a polypropylene microcentrifuge tube (15 ml). The phospholipids were eluted with 2 × 250 μl of the first dimension mobile phase and separated from the silica after each wash by a 5-min, 10,000 × g spin. The supernatant was combined and dried under a stream of N₂(g).

**LC-MS Identification and Characterization of Yeast Phospholipids**—Electrospray MS was performed in negative ion mode with a Micromass Quattro micro™ mass spectrometer (Micromass, Manchester, U.K.). The source temperature was set to 120 °C with a nebulizing gas flow of 160 liters/h and a cone voltage of 35 V. Data were collected between 600 and 850 (to detect PLs and double negative charged CL species) and/or 1380–1480 m/z (to detect single negative charged CL species) with a sweep time of 2 s. Collisional-induced fragmentation of yeast phospholipid species and standards (LC-MS-MS) was effected using argon gas at 4.0 × 10⁻⁵ mbar. Total ion current (TIC) and extracted mass chromatograms showing individual molecular species were generated using MassLynx software and were mean-smoothed.

**Preparation of Large Unilamellar Vesicles**—Lyophilized diacylglycerol phosphatidylcholine (PC, Avanti Polar Lipids, Alabaster, AL) and bovine heart cardiolipin (CL) (Sigma) were dissolved in chloroform to achieve final concentrations of 50, 10, and 10 mg/ml, respectively. Sucrose-loaded vesicles composed of either CL and PC (15%) by weight CL), PL and PC (15% by weight PC), or PC alone were prepared using published protocols (28). Vesicles of uniform size (LUVs) were ensured using a lipid extruder equipped with a 0.1-μm pore-sized polycarbonate filter (Avanti Polar Lipids) and were stored for no longer than 1 week at 4 °C.

**Cytochrome c Binding to Sucrose-loaded LUVs**—Cytochrome c (1 μg/ml final concentration) was incubated with LUVs (500 μm, final lipid concentration) at room temperature for 1 h in either 25 or 150 mM KCl buffer, each with 5 mM Tris, pH 7.4, buffers. These sucrose concentrations were used because they are isosmotic with 25 and 150 mM KCl, respectively, whereas the remaining effluent was diverted to waste. Two batches of LUVs were prepared in either 48 or 256 mM sucrose with 5 mM Tris, pH 7.4, buffers. These sucrose concentrations were used because they are isosmotic with 25 and 150 mM KCl, respectively, whereas the remaining effluent was diverted to waste.

**Verification of the Functional Activity of Isolated Mitochondria**—Mitochondria (1 mg of protein/ml) were incubated in the sorbitol-based mitochondrial isolation buffer described above. Incubations were done at room temperature with continuous stirring to avoid anoxia. Estimation of ΔΨₜₚ was performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP⁺). Energized mitochondria were resuspended in chloroform/methanol (25:75) using a spectrophotometric standard to give a detector flow rate of 20 μl/min, whereas the remaining effluent was diverted to waste.

**Expression, Purification, and Oligomerization of Bax**—The expression and purification of full-length Bax protein were performed as described previously (27) and a detailed description without modifications can be found in our previous report (16). Briefly, the full-length protein of tetramyristoyl (14:0) 4 cardiolipin (Avanti Polar Lipids) and were stored for no longer than 1 week at 4 °C. Cardiolipin Is Not Required for Cytochrome c Release

1101
human Bax cDNA sequence was amplified by standard PCR techniques. The PCR DNA fragment was isolated by the QIAquick kit (Qiagen, Chatsworth, CA) and subcloned into the NcoI and HindIII sites of the plasmid pBAD. The plasmid was transformed into Escherichia coli and transformants were isolated by selection for ampicillin resistance. Cultures of the resistant colony were grown to an \(A_{650} \) of 0.7. After induction, the culture was further incubated for several hours, and cells were harvested by centrifugation. The cells were resuspended in lysis buffer (100 mM Hepes-NaOH, pH 8.0, 100 mM NaCl, 1 mM MgCl\(_2\), 0.1% 2-mercaptoethanol, 1% Triton X-100, a mixture of protease inhibitors, 30 \(\mu\)g/ml DNase I and 50 \(\mu\)g/ml lysozyme) and broken by sonication. After centrifugation, Bax was recovered in the supernatant. The protein was purified by affinity chromatography on nickel-nitrilo-triacetic acid-agarose (Qiagen) followed by ion-exchange chromatography on Q-Sepharose (Amersham Biosciences) performed according to the manufacturer’s instructions. The protein was at least 95% pure as determined by SDS-PAGE, and it was concentrated to 0.4 mg/ml in the presence of 1% octyl glucoside, and aliquots were stored at \(-80^\circ\)C. SDS-PAGE analysis of the thawed aliquot used for this study revealed that \(~50\%\) of the protein was full-length Bax, whereas the remainder was oligomeric. The used concentrations of octyl glucoside did not interfere with mitochondrial functional parameters.

Fig. 1. Isolation and characterization of CL from S. cerevisiae. Both standard and WT yeast mitochondrial phospholipids were separated by TLC. The spots corresponding to standard CL and WT CL were scraped off from the TLC plates, eluted into an appropriate solvent, and analyzed by electrospray LC-MS and LC-MS-MS methods. The source temperature was set to 120 °C with a nebulizing gas flow of 180 liter/h and a cone voltage of 35 V. Data shown were collected between 1380 and 1480 m/z with a sweep time of 2 s. Collisional-induced fragmentation of yeast phospholipid species and standards (LC-MS-MS) was effected using argon gas at 4.0 \(\times\)10\(^{-3}\) mbar. A, structures of CL derived from bovine heart and Saccharomyces cerevisiae and their major fragments. B, TIC (left-hand side, mass spectrum inset) and LC-MS-MS spectrum (right-hand side) of the WT CL isolated by TLC. C, TIC (left-hand side, mass spectrum inset) and LC-MS-MS spectrum (right-hand side) of the bovine heart CL isolated from other phospholipid standards by TLC.
Bax Association and Cytochrome c Release

Isolated yeast mitochondria (1 mg/ml) were resuspended in either LIS or HIS buffers described above in the presence or absence of 800 ng of Bax in a final incubation volume of 100 μl. When testing for Bax insertion, the incubations were centrifuged at 10,000×g for 5 min. The supernatants were removed, and the pellets were resuspended in 0.2 ml of 0.1 M sodium carbonate, pH 11.5. After a 20-min incubation at 4°C, the samples were centrifuged for 10 min at 100,000×g, and the pellets were analyzed by Western blotting using the anti-Bax polyclonal antibody. When testing for the release of cytochrome c, mitochondria were centrifuged at 10,000×g for 5 min. Supernatants were centrifuged once more to avoid mitochondrial contamination. The resulting supernatant and pellet were assessed for the presence of cytochrome c using Western blotting.

Western Blot Analysis—Supernatants or pellets obtained from 20 μg of mitochondrial protein were boiled for 5 min in Laemmli loading buffer. Proteins were separated by 15% SDS-PAGE at 120 V followed by electroblotting to nitrocellulose membrane at 100 V for 2 h. Membranes were blocked with 5% nonfat milk in phosphate-buffered saline and probed with either a monoclonal anti-cytochrome c antibody (BD Pharmingen, 1:2500) or polyclonal anti-Bax polyclonal antibody (Santa Cruz Biotechnology, 1:1,000). After rinsing the membranes, bound antibodies were detected using enhanced chemiluminescence (Amersham Biosciences, UK) according to the manufacturer’s instructions. The cytochrome c band densities were quantitated using Quantity One software (version 4.2.3, Bio-Rad Laboratories). A two-tailed Student’s t test was used to compare control from Bax-treated cytochrome c release.

RESULTS

Identification and Characterization of Yeast CL—We initially verified the absence of CL in the ΔCRD1 mitochondrial CL by two-dimensional TLC, as previously described (28). Silica plates were loaded with either standard, WT, or ΔCRD1 phospholipids. The WT phospholipids developed a spot with the same RF as the standard CL (derived from bovine heart), whereas this spot was absent from the developed ΔCRD1 phospholipids. We also employed a sensitive and specific LC-MS method to detect mitochondrial phospholipids. Phospholipids isolated from the WT yeast mitochondria were analyzed for the presence of CL. There were peaks with roughly the same RT as the mammalian standard, however, they did not have the same mass. In fact, extracting m/z 1447 (the negative ion mass of bovine heart CL, M−H) from the total ion current data (TIC) of the WT phospholipid analysis revealed no peaks (data not shown). Thus it was clear that, despite their similar RF values, the CL species from our strain of yeast was not the same as the mammalian standard, and so we set out to identify the fatty acid composition of the CL from WT mitochondria.

Both standard and WT phospholipids were separated by TLC. The spots corresponding to standard CL and WT CL were scraped off from the TLC plates, eluted into an appropriate solvent, and analyzed by LC-MS and LC-MS-MS methods (Fig.
Cardiolipin Is Not Required for Cytochrome c Release

1, B and C). As expected, the standard and WT CL have similar chromatographic properties in that they both have $T_R$ of $\sim$17 min. However, in contrast to the $m/z$ of the standard CL (1447.4, M – 1), the $m/z$ of the major peak from the WT sample was 1399.4. Collision-induced fragmentation of the standard CL revealed four prominent peaks, each of which could easily be assigned due to loss of the 18:2 acyl-linked fatty acids ($m/z$ 279), or fragmentation of the phosphate-glycerol bond (Fig. 1A). The same fragmentation experiment was performed on the corresponding sample from the WT yeast (parent ion $m/z$ 1399.4), and an analogous fragmentation pattern emerged. The fragments of $m/z$ 253 and 281 correspond to the loss of acyl-linked fatty acids with the carbon:unsaturation index of 16:1 and 18:1, respectively. There were two more peaks in the TIC of the WT sample, but fragmentation data were not obtained, likely due to their lower amounts. However, the latest eluting peak (20 min) had an $m/z$ of 1427, which could correspond to a CL species with a fatty acid composition of (16:1)$_1$(18:1)$_3$. Therefore the MS-MS experiments unambiguously show that the major fatty acid compositions of bovine heart and WT yeast CL are (18:2)$_4$ and (16:1)$_2$(18:1)$_2$, respectively.

After identifying the masses of the major yeast CL species, we confirmed their absence in the ∆CRD1 mutants by the more sensitive and specific LC-MS method (Fig. 2, A and C). The low $m/z$ range was used to analyze the phospholipid mixtures from each strain of yeast, and the similar intensities of the IS peaks demonstrated that there was similar recovery of the phospholipids from the extraction procedure (Fig. 2A). The masses corresponding to the major CL species with $z = 2$ were extracted from each chromatogram in Fig. 2A, and the results are shown in Fig. 2C. Although the WT yeast mitochondria contain these major CL species, there is a complete absence in the ∆CRD1 mitochondria. The peak at $\sim$5 min that is present in both types of mitochondria is most likely a phospholipid with $z = 1$ that shares the same $m/z$ as one of the CL species. A similar mass extraction of $m/z$ values corresponding to different yeast PG species was done (Fig. 2B). When compared with the peak area of the IS, the ∆CRD1 yeast had over 800-fold more PG than WT mitochondria. The virtually absent level of PG in WT mitochondria is consistent with PG being a biosynthetic precursor to CL (1). Whereas in CL-synthase-lacking ∆CRD1 mitochondria, there appeared to be a build-up of PG.

Furthermore, to ensure that our preparations of mitochondria yielded viable, respiring species, we verified that growth on a non-fermentable carbon source and ambient temperatures, ∆CRD1 mitochondria respire and maintain their membrane potential (∆Ψ_m) in a manner comparable to their WT counterparts (Fig. 3). Addition of ADP to mitochondria resulted in a temporary decrease of the ∆Ψ_m followed by its restoration when phosphorylation of added ADP was complete. There was no difference in phosphorylation time between ∆CRD1 and WT mitochondria (Fig. 3A). Measurements of mitochondrial respiration using an oxygen electrode also yielded similar results in both strains of mitochondria (Fig. 3B). Hence, the absence of cardiolipin does not dramatically affect the functional parameters of these mitochondria grown and isolated under our conditions. It has been demonstrated that PG, the precursor of CL, can partially substitute for CL (29). Indeed, the LC-MS analyses of the mitochondrial phospholipids showed high PG levels in the ∆CRD1, whereas the WT had a trace amount. To complete the characterization of ∆CRD1 and WT mitochondria for the purposes of this study, we demonstrated by Western blot
that both WT and ∆CRD1 mitochondria contained similar amounts of cytochrome c per milligram of protein (Fig. 4, pellets).

Spontaneous Cytochrome c Release from ∆CRD1 and WT Mitochondria—Previously, our laboratory showed that the release of cytochrome c from rat liver mitochondria depended not only on permeabilization of the outer mitochondrial membrane by Bax but also on the dissociation of cytochrome c from the inner mitochondrial membrane (16). Preliminary studies with isolated mitochondria from both yeast strains yielded analogous results, however, the ∆CRD1 mutants released more cytochrome c than the WT (Fig. 4). The mitochondria were incubated in either LIS or HIS buffers and spontaneous release of cytochrome c was monitored after 0, 15, and 30 min of incubation (Fig. 4). There was no visible amount of cytochrome c in the supernatant fractions when both WT and ∆CRD1 mitochondria were incubated in LIS buffer, which is also isoosmotic for yeast (30). However, when the ionic strength was increased (i.e. KCl replacing sorbitol) there was a time-dependent release of cytochrome c from the ∆CRD1 mitochondria, which could not be accounted for by mitochondrial swelling (data not shown). In general, the WT mitochondria were more resistant to spontaneous cytochrome c release, even in the HIS buffer.

Cytochrome c Binding to LUVs—Because the spontaneous and rapid release of cytochrome c from ∆CRD1 mitochondria in HIS was not due to mitochondrial swelling or PTP (i.e. incubations were done in the presence of EGTA), and to further test our recently proposed two-step hypothesis of cytochrome c release, we studied the binding of cytochrome c to large unilamellar vesicles (LUVs) of varying phospholipid constructs. In addition, we studied the effect of ionic strength on binding. Cytochrome c required the presence of CL or PG in the liposomal preparation for binding in a low ionic strength buffer (25 mM KCl), whereas there was substantially less binding of the hemoprotein to LUVs composed of only PC (Fig. 5A). In a buffer of higher ionic strength (150 mM KCl), cytochrome c was bound only to LUVs containing CL (Fig. 5A). Furthermore, it was clear that quantitatively more cytochrome c was bound to LUVs in incubations containing 25 mM KCl compared with 150 mM KCl. Separation of the liposomes from supernatant after ultracentrifugation was verified by LC-MS (Fig. 5B) and less than 5% of the total phospholipids were detected in the supernatant fractions after ultracentrifugation.

Bax-induced Cytochrome c Release from WT and ∆CRD1 Mitochondria—To test if Bax-mediated cytochrome c release depends on the presence of CL, isolated mitochondria from WT and ∆CRD1 yeast were incubated with 800 ng of Bax in HIS buffer. After a 10-min incubation period followed by a 5-min centrifugation, the supernatants and pellets were analyzed for cytochrome c content by Western blotting (Fig. 6A). WT mitochondria did not show any spontaneous release of cytochrome c at this time point (control incubation), whereas some release could be detected in the supernatant fraction of the Bax-treated incubations. Although a portion of the total pool of cytochrome c was released spontaneously from ∆CRD1 mitochondria, this portion was significantly increased in the presence of Bax (Fig. 6, A and B). Furthermore, full-length Bax inserted into the outer membrane of both WT and ∆CRD1 mitochondria, and in all experiments (n = 3) there were no striking differences in the degree of full-length Bax insertion in both types (Fig. 6C).
DISCUSSION

In the present study, we have chosen a model of CL-deficient and WT yeast mitochondria, which ought to clearly delineate the requirement for CL in the mechanism of Bax-mediated cytochrome c release from mitochondria. Yeast do not constitutively express bcl-2 genes. However, these cells are easily transfected with human genes and, hence, have been used to study mechanisms of apoptosis mediated by Bcl-2 family proteins. Human Bax expression in yeast cells inhibited their proliferative capacity and caused the release of cytochrome c from their mitochondria (31, 32). Furthermore, exogenous Bax treatment of isolated yeast mitochondria resulted in the release of cytochrome c that was shown to be independent of the voltage-dependent anion channel (20). Unlike mammalian models, yeast mitochondria do not require tBid for Bax oligomerization in the outer mitochondrial membrane. As such, both full-length and oligomerized Bax can similarly mediate the release of cytochrome c from yeast mitochondria (20). Our Bax preparation was reconstituted in 1% octylglucoside and had been used previously in studies using isolated rat liver mitochondria. In the present study, we found this Bax preparation to mediate cytochrome c release from yeast mitochondria and we also detected full-length Bax that had inserted in the mitochondrial membrane (Fig. 6). Other lines of evidence have also argued for the use of yeast to study the regulators of apoptosis in higher eukaryotes (33).

There is no evidence to suggest that different fatty acid moieties of CL will affect its function. On the contrary, like in mammalian cells, CL in yeast has been shown to be essential for supercomplex formation in the inner mitochondrial membrane (3), as well as optimizing the osmotic stability (30), oxidative phosphorylation (34), and the general mitochondrial function of these cells (28). Furthermore, in the present study, we have demonstrated the importance of yeast CL in binding cytochrome c to the inner mitochondrial membrane. Hence, despite the different chemical structures of yeast and mammalian CL, it appears as though this yeast model can adequately define the role of CL in mitochondrial function in that the unique CL species of yeast supports similar functions as its mammalian counterpart.

Our model of CL-deficient (ΔCRD1) and WT yeast mitochondria supports the view that CL is required for the attachment of cytochrome c to the inner mitochondrial membrane and hence serves to prevent the release of this hemoprotein from mitochondria. This view is supported by two observations: First, the ΔCRD1 mitochondria spontaneously released significantly more cytochrome c compared with their WT counterparts. This is consistent with numerous studies reporting the tight physical association between CL and cytochrome c in mammalian and in vitro systems, including our own, which demonstrated an increased solubilization of cytochrome c in isolated rat liver mitochondria when the ionic strength of the incubation buffer was increased (16). However, there was not a complete release of cytochrome c from the ΔCRD1 mitochondria in the yeast model, which may be explained by the relatively high amounts of PG measured in these membranes. PG, the biosynthetic precursor of CL, has been demonstrated to partially substitute for CL in many functions and could loosely bind cytochrome c to the inner mitochondrial membrane through electrostatic interactions. Indeed, our liposome experiments verified that cytochrome c binds exclusively to LUVs containing CL at a more physiologic ionic strength. Whereas, when the ionic strength of the medium is decreased, cytochrome c also binds to LUVs containing PG, thus suggesting the possibility of some degree of electrostatic interaction between cytochrome c and PG. Hence, once the electrostatic interactions between the anionic phospholipids and cytochrome c are breached, for instance by the buffer used in our incubations, more cytochrome c may be solubilized and eventually released from the ΔCRD1 mitochondria compared with the WT.

Second, we directly challenged the recent observation by Kuwana et al. (21) that CL is required for Bax-mediated pore
Cardiolipin Is Not Required for Cytochrome c Release

model of CL-deficient yeast mitochondria, which, relative to models of reconstituted systems such as membrane-derived and commercially derived liposomes, likely better defines the role of CL in the mechanism of cytochrome c release.

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formation in liposomes. Bax both inserted into the outer membrane and enhanced the release of cytochrome c above control levels from both ΔCRD1 and WT yeast mitochondria. The equivalent degree of Bax insertion in both types of mitochondria suggested that the formation of a pore in the outer mitochondrial membrane is independent of the presence of CL. This is not surprising if one considers native CL to be located exclusively in the inner mitochondrial membrane. Furthermore, the amplitude of cytochrome c release did not correlate with the amount of Bax inserted into the outer mitochondrial membrane. Cytochrome c release from both types of mitochondria occurred in the high ionic strength buffer, but not in the low ionic strength buffer. These results are in agreement with our previously demonstrated two-step model of cytochrome c release (16): First, cytochrome c must become detached from the inner mitochondrial membrane and this may be achieved by breaching its electrostatic and/or hydrophobic interactions with the inner mitochondrial membrane. Second, permeabilization of the outer mitochondrial membrane must occur in order for the solubilized cytochrome c to be released. In the present study, we show that CL is important for mitochondrial membrane integrity and for binding cytochrome c to the inner mitochondrial membrane. Importantly, we demonstrated that CL is not required for Bax insertion in, and permeabilization of, the yeast outer mitochondrial membrane.

At first glance, the paradigm imposed by our two-step model of cytochrome c release would suggest that cytochrome c should not be spontaneously released from mitochondria, WT or CL-deficient, in the absence of swelling or pore formation. However, we observed some spontaneous release of cytochrome c from ΔCRD1 mitochondria. This is not surprising, if one considers the importance of CL to both the binding of cytochrome c and the integrity of the mitochondrial membrane. If there is a deficiency of CL, more cytochrome c will be soluble in the intermembrane space and the membranes will be more fragile, rendering them more sensitive to buffer conditions and perhaps even centrifugation. Furthermore, spontaneous release of cytochrome c may also be dependent upon cell type. Indeed, many laboratories have reported cytochrome c in the supernatants of control incubations of mammalian (16, 17, 35–37) and yeast (20) cells/mitochondria, so it is not surprising that more cytochrome c is spontaneously released from CL-deficient mitochondria. The same argument applies to explain the enhanced release of cytochrome c from ΔCRD1 compared with WT mitochondria when both types of mitochondria are treated with Bax. Specifically, more cytochrome c will be solubilized if the mitochondrial membrane lacks CL hence, more cytochrome c will be released when a pore is formed. Given that there was little or absent spontaneous cytochrome c release from WT mitochondria under the same buffer conditions, these data support the importance of CL for cytochrome c binding rather than supporting a shortcoming of the model.

In summary, we show that CL is not an absolute requirement for Bax-mediated cytochrome c release. We selected a