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Letters to the Editor

Interactions of cardiolipin and lyso-cardiolipins with cytochrome *c* and tBid: conflict or assistance in apoptosis

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Dear Editor,

Bid, a BH3-only proapoptotic member of the Bcl-2 family, and the carboxyl terminus of its cleavage product, tBid, are essential regulators of early mitochondrial apoptotic events and release of pro-apoptotic factors into the cytosol.[1] Bid and tBid have a high-affinity binding domain for a mitochondria-specific phospholipid, cardiolipin (CL).[2–5] In normal cells, CL is predominantly localized in the inner membrane and contact sites. It plays a very important role in the assembly and maintenance of functionally active respiratory complexes (I, III and IV and F_0/F_1-ATPase) in the mitochondrial membrane and in normal operation of other mitochondrial multicomponent machines such as adenine nucleotide translocator, as well as tricarboxylate, pyruvate and phosphate carriers.[6–9] Decreased levels of CL caused by siRNA manipulations of CL synthase (CDP-diacylglycerol-phosphatidylglycerol phosphatidyltransferase) in cells were shown to result in disorganization of mitochondria and release of cytochrome *c* (cyt *c*) into the cytosol; these cells elicited enhanced sensitivity to proapoptotic stimuli.[10] Recently, interactions of tBid with CL have been implicated as major players in orchestrating apoptotic changes in mitochondrial electron transport as well as activation of pro-apoptotic proteins Bak and Bax.[5,11] During apoptosis, however, CL undergoes significant hydrolysis whose products – mono-lysoCL (MCL) and di-lyso-CL (DCL) – accumulate.[4,12]

Cyt *c* is an important contributor to redox reactions in the intermembrane space of mitochondria where it functions as (1) an electron shuttle between respiratory complexes III and IV and (2) a scavenger of superoxide anion radicals.[13] Recently, we have reported that CL avidly binds cyt *c*, partially unfolds the protein, and that the complex functions as a peroxidase, catalyzing CL peroxidation essential for the release of proapoptotic factors.[14,15] Notably, CL and MCL display comparable affinities for tBid.[4] Thus, two major components of proapoptotic machinery – tBid and cyt *c* – interact and possibly compete for the same phospholipid, CL, or its hydrolysis products, MCL and DCL, in apoptotic mitochondria. This potential competition may impede the execution of the apoptotic program. The affinities and role of MCL and DCL in proapoptotic activation of cyt *c* into a peroxidase and the interactions with tBid have not been characterized. Therefore, we studied MCL and DCL as activators of cyt *c* into a peroxidase compared to CL in the presence and absence of tBid. Tetraoleoyl-CL (TOCL) and its lyso-derivatives (MCL, DCL), obtained after hydrolysis of TOCL by porcine pancreatic phospholipase A_2 (PLA_2), were used. The purity of CL-derivatives was confirmed by electrospray ionization mass spectrometry (ESI-MS) (an MS-spectrometer MAT TSQ-700, Thermo Electron Co.) after their separation by two-dimensional high-performance thin-layer chromatography (2D-HPTLC). Characteristic signals with *m/z* values of 727.7, 595.9 and 463.4 for doubly charged ions of TOCL and its hydrolysis products, mono-lyso-tri-oleoyl-CL and di-lyso-dioleoyl-CL, respectively, were detected (data not shown).

To characterize the peroxidase reaction, we evaluated luminol oxidation monitored by the chemiluminescence response (Figure 1Aa). Expectedly, CL was very effective in activating cyt *c* into a peroxidase and caused a 25.0-fold higher response than cyt *c* alone or cyt *c* in the presence of dioleoyl-phosphatidylcholine (DOPC), which does not effectively bind cyt *c*.[16] MCL and DCL enhanced cyt *c* peroxidase activity less effectively than CL (by a factor of 6.7 and 3.7, respectively). tBid almost completely inhibited the peroxidase activity of CL/cyt *c* complex only at ratios exceeding 20 : 1 (*versus* cyt *c*) (Figure 1Ab). Similarly, MCL- and DCL-stimulated peroxidase activity of cyt *c* could be eliminated by a large excess (*versus* cyt *c*) of tBid (data not shown).

The peroxidase activity of CL/cyt *c* complex is associated with a partial unfolding of cyt *c* as evidenced by the appearance of Trp_{59} fluorescence completely quenched by the heme moiety in the native protein.[17] MCL and DCL were much less potent than CL in stimulating the Trp_{59} fluorescence of cyt *c* (Figure 1Ac). To determine the extent to which this could be due to different binding of CL and lyso-CLs with cyt *c*, we studied electrophoretic mobility of CL/cyt *c* complexes in native agarose gels. As expected, positively charged cyt *c* molecules moved to the cathode (Figure 1B, a, first panel, first line), and anionic CL/cyt *c* complexes migrated to the anode. Coomassie blue staining was used for the detection of free and CL-bound cyt *c*. The apparent mobility of cyt *c* decreased with increase of CL/cyt *c* ratio. Isoelectric points (zero electrophoretic mobility) were achieved for CL/cyt *c*, MCL/cyt *c* and DCL/cyt *c* complexes at ratios of 5 : 1, 9 : 1 and 10 : 1, respectively. Because binding of 10-nonyl acridine orange (NAO) to CL has been quantitatively characterized in simple model systems,[14,15,18] we further used it as a competitive CL-binding agent (Figure 1Bb). Assessments of

binding constants revealed that MCL and DCL were approximately fivefold weaker ligands for cyt *c* than CL. Thus only much higher concentrations of MCL and DCL, if formed in mitochondria during apoptosis, could out-compete CL as a mitochondrial switch initiating the peroxidase activity of cyt *c*. In fact, the contents of MCL and DCL in mitochondria of apoptotic HL-60 cells do not exceed 6 and 1 mol%, respectively (*versus* nonhydrolyzed CL).[19] Therefore, it is highly unlikely that MCL or DCL can exert an inhibitory effect on activation of cyt *c* into a peroxidase during apoptosis. In line with this, treatment of mitochondria with MCL or DCL (in contrast to CL oxidation products) caused no release of cyt *c* (data not shown).

Mitochondrial contact sites are dynamic structures formed by specific interactions of the inner and outer membranes.[6,8,20] Although the content of CL in the outer

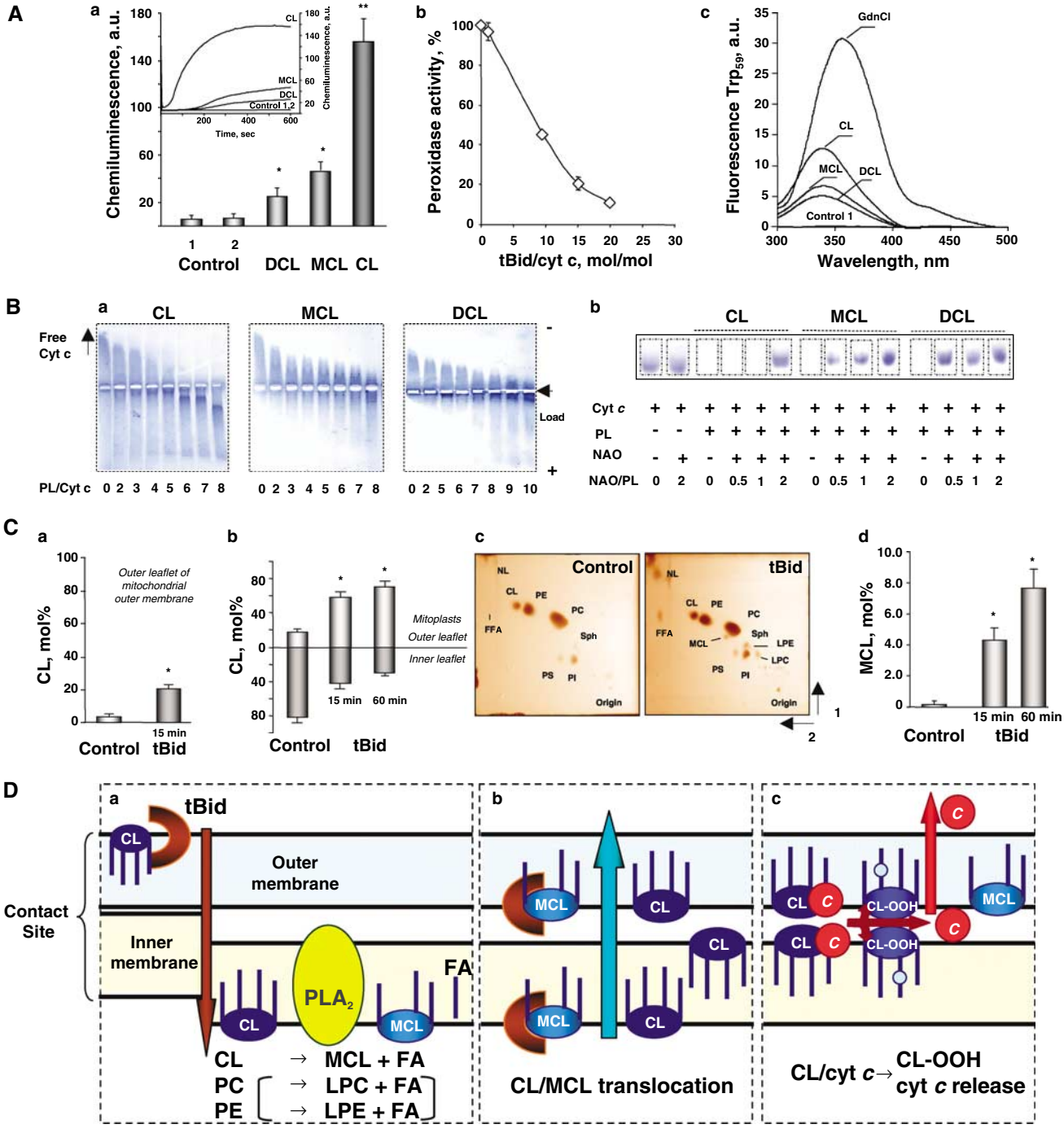

Figure 1

membrane is very low (4 mol% of phospholipid content), the contact sites are believed to be highly enriched in CL – up to 20% of total phospholipids.[6,8] Thus the CL/phospholipids ratio within the contact sites is essentially the same as in the inner mitochondrial membrane. Recently, it has been shown that tBid induces destabilization of the mitochondrial bioenergetic homeostasis; the inhibitory effect on respiration was not observed in CL-deficient mitochondria.[5] tBid alone can disrupt phospholipid bilayers by promoting the formation of highly curved CL-containing nonlamellar (hexagonal) phases.[21] In line with this membrane-perturbing capacity, we found that incubation of mitochondria with tBid resulted in an almost fivefold increase (up to 24 mol%) of CL in the outer membrane compared to only 5 mol% in controls (as evidenced by the availability of CL to exogenous PLA_2) (Figure 1Ca). The CL distribution between the matrix and intermembrane surfaces of the inner mitochondrial membrane was also drastically changed in tBid-stimulated mitochondria: from 20 mol% of CL in the outer monolayer in the control up to more than 70 mol% after the treatment (Figure 1Cb).

Reportedly, tBid plus Bax causes release of cyt *c*, increased production of reactive oxygen species and activation of Ca-independent PLA_2 in brain mitochondria.[22] We performed direct and quantitative 2D-HPTLC analysis of major phospholipids of control mitochondria and mitochondria treated with tBid (Figure 1Cc). Phosphatidylcholine (PC, $49.1 \pm 0.6\%$) and phosphatidylethanolamine (PE, $30.1 \pm 0.4\%$) represented the two major fractions of the total pool of phospholipids in mouse liver mitochondria. CL was the next most prominent phospholipid ($11.6 \pm 0.9\%$); its concentrations

Figure 1 (**A**) Peroxidase activity and partial unfolding of cyt *c* upon its interaction with TOCL, MCL and DCL in the presence and absence of tBid. H_2O_2-dependent chemiluminescence responses of luminol oxidation induced by cyt *c* complexes with TOCL, MCL and DCL; the cyt *c*:CL ratios were 1 : 20. CL, MCL and DCL were delivered as liposomes with the DOPC:CL ratio of 1 : 1. (a) Peroxidase activity of cyt *c*/CL complex is significantly higher than that of cyt *c*/lyso-CLs/DOPC and cyt *c*/DOPC complexes. Control 1 – 0.05 μM cyt *c*/100 μM H_2O_2; control 2 – cyt *c*/DOPC/H_2O_2; DCL – cyt *c*/DOPC/DCL/H_2O_2; MCL – cyt *c*/DOPC/MCL/H_2O_2; CL – cyt *c*/DOPC/CL/H_2O_2. (Inset) Typical chemiluminescence responses of luminol (100 μM) oxidation induced by cyt *c*/H_2O_2 in the absence and presence of CL and lyso-CL complexes. $^*P < 0.05$ *versus* control 1 or control 2; $^{**}P < 0.01$ *versus* DCL or MCL, $n = 3$. (b) tBid inhibits the peroxidase activity of CL/cyt *c* and lyso-CLs/cyt *c* complexes. CL – cyt *c*/TOCL/DOPC/H_2O_2/tBid. (c) Peroxidase activity of CL/cyt *c* complexes is associated with a partial unfolding of cyt *c* as evidenced by the appearance of Trp59 fluorescence completely quenched by the heme moiety in the native protein. Control – 1, – native state of cyt *c*, cyt *c* plus DOPC liposomes; DCL – DCL/DOPC (1 : 1) liposomes incubated for 15 min with 5 μM cyt *c* (at 50 : 1 molar ratio) in 25 mM phosphate buffer, pH 7.4; MCL – MCL/DOPC (1 : 1) liposomes plus cyt *c*; CL – CL/DOPC (1 : 1) liposomes plus cyt *c*; GdnCl (guanidine hydrochloride) – unfolded state of cyt *c*, after addition of 6.0 M guanidine hydrochloride. MCL and DCL were much less potent than CL in stimulating the Trp_{59} fluorescence of cyt *c*. (**B**) Assessments of TOCL and lyso-CLs competitive binding with cyt *c*. (a) Native gel electrophoresis confirmed weaker interactions of cyt *c* with MCL and DCL compared to CL. Assessments of CL and lyso-CLs competitive binding with cyt *c* were performed by probing electrophoretic mobility of CL/cyt *c* complexes in native agarose gels. Coomassie blue staining R-250 was used for detection of free and CL-bound cyt *c*. Note that addition of TOCL changed the migration profile of cyt *c* and disappearance of its monomeric form (the complex migrates to the cathode). MCL and DCL displayed lower efficiencies in changing the mobility of cyt *c*. (b) Assessments of CL and lyso-CLs competitive binding with cyt *c*. After incubation of 2 mM DOPC/TOCL, DOPC/MCL or DOPC/DCL (1 : 1) liposomes with 40 μM cyt *c* in 25 mM phosphate buffer at pH 7.0, samples were applied to a 7.5% PAGE. Typical PAGE gels stained for cyt *c* with Coomassie blue R-250 showed that, in the absence of CLs, cyt *c* was readily detectable. Addition of CL resulted in the disappearance of the monomeric band of cyt *c*. Titration with different amounts of NAO (2–12 mM) caused the reappearance of cyt *c* bands owing to competitive binding of CL with NAO. Using a known binding constant of NAO with CL,[18] we estimated that cyt *c* has a fivefold lower affinity for lyso-CLs compared to CL (3×10^8 for MCL and 3.2×10^8 for DCL compared to $1.6 \times 10^9\ M^{-1}$ for CL). (**C**). tBid-induced hydrolysis and redistribution of CL in the outer membrane and mitoplasts of C57BL/6J mouse liver mitochondria. tBid-induced CL redistribution between the inner and outer membranes of mitochondria (a) and between the matrix leaflet and the intermembrane leaflet of the inner membrane (b). $^*P < 0.05$ *versus* control; $n = 3$. The membrane distribution of CL in mouse liver mitochondria was determined by monitoring CL hydrolysis after treatment with porcine pancreatic PLA_2. Mitochondria and mitoplasts were isolated from C57BL/6J mouse liver as described previously.[25] Mitochondria were incubated with 18.5 nM tBiD (R&D System Inc.) in buffer containing 20 mM HEPES, pH 7.4, 250 mM sucrose, 10 mM KCl, 3 mM KH_2PO_4, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.5 mg/ml BSA at 37°C during 15 and 60 min. After incubation, mitochondria were spun down, resuspended and incubated with PLA_2 (0.2 U/mg protein) in the presence of 220 mM manitol, 70 mM sucrose, 20 mg/ml BSA (free fatty acid), 0.1 mM $CaCl_2$, 10 mM HEPES, pH 7.4. The reaction was stopped by the addition of EDTA (5 mM). The presence of albumin was absolutely necessary to block detergent-like effects of lysophospholipids and FFA and to avoid nonspecific interaction of PLA_2 with CL.[26] Phospholipid extraction and separation was performed as described previously.[15] Digestion of CL resulted in the appearance of MCL and DCL. Determination of the extent of digestion by a decrease of the parent CL and by an increase of lyso-CLs yielded quantitatively identical results. In control mitoplasts, CL distribution between the matrix and the intermembrane monolayer of the inner mitochondrial membrane was 80 : 20, in agreement with published results.[26,27] (c) Typical 2D-HPTLC of total lipids obtained before and after incubation of liver mitochondria with tBid. The phospholipid classes in the extracts were separated by 2D-HPTLC on silica G plates (5×5 cm, Whatman, USA). The plates were first developed with a solvent system consisting of chloroform : methanol : 28% ammonium hydroxide (65 : 25 : 5 v/v). After the plate was dried with a forced N_2 blower to remove the solvent, the plates were developed in the second dimension with a solvent system consisting of chloroform : acetone : methanol : glacial acetic acid : water (50 : 20 : 10 : 10 : 5 v/v). The phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic phospholipid standards. The spots identified by iodine staining were scraped and transferred to tubes. Lipid phosphorus was determined as described.[15] Abbreviations: NL, neutral lipids; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; FFA, free fatty acids; MCL, mono-lysoCL; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine. (d) tBid caused accumulation of MCL (likely owing to activation of mitochondrial PLA_2). (**D**) Participation of tBid in PLA_2-catalyzed hydrolysis and transmembrane redistribution of CL and lyso-CL in mitochondria during apoptosis. During apoptosis, tBid binds to mitochondria at the contact sites[8,22,24] and causes activation of PLA_2; this induces hydrolysis of CL and other major mitochondrial phospholipids, PC and PE yielding FFA and lyso-phospholipids – MCL, LPC and LPE (a). It is possible that accumulation of membrane-perturbing lyso-phospholipids and fatty acids contributes to trans-bilayer redistribution of CL (b). Consequently, the CL content in the outer mitochondrial membrane remarkably increases. The CL level in the outer leaflet of the inner membrane is also augmented. Thus two membrane locations – the inner monolayer of the outer membrane and the outer monolayer of the inner membrane – during apoptosis become enriched in CL available for interactions with cyt *c* that is abundant in the intermembrane space (c). These interactions result in the formation of cyt *c*/CL complexes with peroxidase activity.[14,15,28,29] The latter is induced by partial unfolding of the cyt *c* globule resulting in facilitated access of the heme catalytic site to H_2O_2.[14] The complex acts as a CL- specific oxygenase and causes accumulation of CL oxidation products (e.g., CL hydroperoxides, CL-OOH) which participate in the mitochondrial permeabilization.[28] Notably, tBid – as a much weaker binder of CL than cyt *c* – does not interfere with the cyt *c*'s capacity to bind CL and form the peroxidase complex. Moreover, because the affinities of MCL and DCL for cyt *c* are much lower than that of CL, the products of CL hydrolysis do not effectively form the peroxidase complexes with cyt *c*. Experiments with CL-sufficient and CL-deficient mitochondria indicate that CL provides specificity for targeting tBid to mitochondria[2] resulting in mitochondrial destabilization during apoptosis.[5] Further studies are necessary to refine topography and specific interactions of tBid with CL on the mitochondrial surface. However, the absence of cyt *c* outside of mitochondria at this early stage of apoptosis may be essential for effective binding of tBid with CL at the contact sites and its penetration into mitochondria[8,24]

in mitochondria and mitoplasts were 39.3 ± 2.9 and 70.4 ± 3.9 nmol/mg protein, respectively. Other phospholipids in the order of their abundance were phosphatidylinositol (PI, $7.1 \pm 0.2\%$), sphingomyelin (Sph, $1.4 \pm 0.2\%$) and phosphatidylserine (PS, $0.8 \pm 0.2\%$). We established that incubation of mouse liver mitochondria with tBid induced activation of PLA_2, yielding the accumulation of phospholipid hydrolysis products (MCL, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, free fatty acids (FFA)). The molar concentrations of MCL after 15 and 60 min incubation of mitochondria with tBid were as high as 4.6 ± 1.4 and 7.7 ± 1.1 mol% (of the CL molar concentration), respectively, compared to less than 0.2 mol% in control mitochondria (Figure 1Cd). Although other phospholipids also underwent PLA_2-catalyzed hydrolysis, the relative molar amounts of their hydrolysis products after 60 min incubation were lower and constituted 3.0 and 2.5 mol% for lyso-phosphatidylcholine (LPC) and lyso-phosphatidylethanolamine (LPE), respectively, suggesting that tBid activation of PLA_2 was CL-specific.

Several studies employed isolated mitochondria exposed to tBid to prove specific interactions between the wild-type and mutated forms of the protein and CL.[5] Obviously, utilization of NAO and its effects in these experiments cannot be unequivocally interpreted in terms of tBid binding with CL due to the lack of absolute specificity of NAO towards CL. The results of this work, however, indicate that even direct assessments of these interactions by ESI-MS[8] should be interpreted more cautiously at least for two reasons: (1) semiquantitative character of signal intensities in MS and (2) induction of endogenous catalytically competent PLA_2 followed by hydrolysis of CL, resulting in decreased amounts of its parental form and accumulation of MCL/DCL and FFA. Similarly, interpretation of physico-chemical measurements of anisotropy of fluorescence probes in tBid-treated mitochondria[6] should take into consideration well-known strong membrane-perturbing effects[23] of accumulating FFA and lyso-PLs.

As mentioned above, Gonzalvez *et al*.[5] analyzed the consequences of tBid interaction with mitochondrial contact sites, and the mechanisms by which tBid alters the bioenergetic properties of mitochondria. They demonstrated that tBid caused CL-dependent inhibition of state-3 respiration which did not require the tBid's BH3 domain but rather was mediated by the reorganization of CL within the mitochondrial membranes. The authors speculated that the polymorphic phase behavior of CL was important for its access to the outer mitochondrial membrane. At the contact sites, tBid interacted with CL and facilitated the reorganization of phospholipids, especially CL, into microdomains. These results are consonant with previous findings of Scorrano *et al*.[24] on tBid-initiated remodeling of mitochondrial structure with mobilization of the cyt *c* stores via fusion of individual cristae and opening of the junctions between the cristae and the intermembrane space. Notably, the remodeling process was found to be independent of the tBid's BH3 domain. Our results indicate that tBid-triggered activation of PLA_2, accumulation of lyso-CLs, and their binding with tBid may contribute to destabilization of mitochondrial membranes and bioenergetics as well as to cyt *c* release during apoptosis.

As schematically summarized in Figure 1D, it is also possible that tBid – through activation of PLA_2, hydrolysis of CL and binding with MCL and DCL – acts as a transmembrane transporter of lyso-CLs in mitochondria and participates in remodeling of CL taking place in endoplasmic reticulum membranes.[4] Competition between tBid and cyt *c* for CL may represent a potential conflict for the effectiveness of their functions, particularly in the area of contact sites in mitochondria. It is likely, however, that tBid's interaction with mitochondria precedes massive cyt *c* release into the cytosol,[8,24] which may also require activation of caspases.[1,11] This temporal separation may be essential for preventing potential interference in binding of the two proteins with CL.

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