Pro-apoptotic Bid induces membrane perturbation by inserting selected lysolipids into the bilayer

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Bid is a BH3-only member of the Bcl-2 family that regulates cell death at the level of mitochondrial membranes. Bid appears to link the mitochondrial pathway with the death receptor-mediated pathway of cell death. It is generally assumed that the f.l. (full-length) protein becomes activated after proteolytic cleavage, especially by apical caspases like caspase 8. The cleaved protein then relocates to mitochondria and promotes membrane permeabilization, presumably by interaction with mitochondrial lipids and other Bcl-2 proteins that facilitate the release of apoptogenic proteins like cytochrome *c*. Although the major action may reside in the C-terminus part, tBid (cleaved Bid), uncleaved Bid also has pro-apoptotic potential when ectopically expressed in cells or *in vitro*. This pro-apoptotic action of f.l. Bid

has remained unexplained, especially at the biochemical level. In the present study, we show that f.l. (full-length) Bid can insert specific lysolipids into the membrane surface, thereby priming mitochondria for the release of apoptogenic factors. This is most effective for lysophosphatidylcholine species that we report to accumulate in mitochondria during apoptosis induction. A Bid mutant that is not pro-apoptotic *in vivo* is defective in lysophosphatidylcholine-mediated membrane perturbation *in vitro*. Our results thus provide a biochemical explanation for the pro-apoptotic action of f.l. Bid.

Key words: apoptosis, Bcl-2, Bid, lysolipid, mitochondria, phospholipid.

INTRODUCTION

Proteins of the Bcl-2 family regulate most pathways of apoptosis [1,2]. Although various members of the family have diverse subcellular locations, their common action is primarily exerted at the level of mitochondrial membranes [1–4]. According to currently favoured views, BH3-only proteins like Bid act upstream of multidomain members, Bax or Bak, which then alter the integrity of the OM (outer mitochondrial membrane) [1–5]. Anti-apoptotic members of the family, like Bcl-2 itself, block this membrane alteration and prevent the escape of mitochondrial apoptogenic factors in the cytosol [1–3]. It is generally considered that Bid ‘activates’ Bax or Bak by stimulating its oligomerization in the OM [4–6], but the biochemical details of this process remain unclear.

Bax and Bid were reported to exhibit channel-like properties in lipid membranes [7–10], which reflected a perturbation of the lipid bilayer rather than a transmembrane channel activity [8–12]. Similar to some bacterial toxins, this perturbation requires oligomerization of monomeric Bax (accompanied by large structural changes) into complexes that effectively produce lipid pores [8,10–13]. Intriguingly, micelles of some detergents and liposomes of defined composition induce oligomerization of Bax in the absence of other proteins [7,8,14,15]. This raises the question of how Bid produces a similar change in the quaternary structure of Bax, or its close homologue Bak, at the mitochondrial surface.

Another major question surrounding Bid is the biological role of its f.l. (full-length) forms within cells. Although the pro-apoptotic capacity of the protein is generally considered to be cryptic

until proteolytic cleavage occurs, most specifically by caspase 8 [2,16,17], ectopic expression of f.l. Bid induces cell death [5,18–21], even in the absence of the major site of caspase cleavage [21]. The pro-apoptotic role of f.l. Bid is consistent with *in vitro* evidence indicating that the uncleaved protein, albeit less potent than tBid, is at least as efficient as Bax in releasing cytochrome *c* from mitochondria [7–10,16,21–23]. Moreover, it has been reported that f.l. Bid accumulates in mitochondria early after apoptosis induction with a variety of stimuli [22–28].

The biochemical aspects of the pro-apoptotic action of f.l. Bid have not been explored in detail. In the present study, we analyse the membrane-perturbing action of f.l. Bid, which relies on interaction with specific lipids like LPC (lysophosphatidylcholine) and could facilitate the transmembrane alteration brought about by other Bcl-2 proteins. Our results provide a new and comprehensive explanation of the biochemical action of f.l. Bid in mitochondria and cells.

MATERIALS AND METHODS

Biological systems

Mouse liver from male mice (usually MF strains) were treated with Jo-2 anti-Fas agonist and processed as described previously [22,27]. Liver mitochondria and other subfractions were prepared as described earlier [22] and, if not used in cell-free assays, stored at –80 °C in assay buffer (0.12 M mannitol, 0.08 M KCl, 1 mM EDTA and 20 mM K-Hepes, pH 7.4), containing a cocktail of protease inhibitors (#P3840; Sigma).

Abbreviations used: tBid, cleaved bid; CL, cardiolipin; DCL, di-lysocardiolipin; FasL, Fas ligand; f.l., full-length; LPC, lysophosphatidylcholine; LPE, 1-oleoyl-lysophosphatidylethanolamine; LPG, lysophosphatidyl-glycerol; MCL, monolysocardiolipin; OM, outer mitochondrial membrane; PC, phosphatidylcholine; PLA, phospholipase A; Q-TOF, quadrupole-time-of-flight; SM, sphingomyelin; wt, wild-type.

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Subcellular fractions were isolated from human Jurkat T cells essentially as reported previously [28]. Cells grown as described earlier [22,28] were left untreated (controls) or treated with recombinant human FasL (Fas ligand, 100 ng/ml, usually for 1 h). After a wash in PBS, cells were suspended in cold isolation buffer (0.25 M mannitol, 1 mM EDTA, 10 mM K-Hepes and 0.2 % BSA, pH 7.4), collected by centrifugation and then frozen. After thawing, they were homogenized in 0.8–1.3 ml of isolation buffer and briefly centrifuged at 600 *g* in the cold. The pellet and supernatant were combined, rehomogenized and then centrifuged at 800 *g* for 10 min at 4 °C. The supernatant was further centrifuged at 10 000 *g* for 10 min at 4 °C. The pellets were suspended in assay buffer (0.12 M mannitol, 0.08 M KCl, 1 mM EDTA and 20 mM K-Hepes, pH 7.4, containing a cocktail of protease inhibitors) after two centrifugation washings in the same buffer (compare [22,28]). Protein was measured with the BioRad assay [22,23].

Cell-free assays

Freshly isolated mitochondria from mouse liver were resuspended at 1 mg/ml in assay buffer and incubated at room temperature (22 °C) for 20 min with either recombinant mouse Bid (obtained from R & D Systems (Minneapolis, MN, U.S.A.) [22,23] and as described previously [8]) or native Bid isolated from mouse kidney [22]. Ethanol-dissolved lysolipids (from either Sigma or Avanti Polar Lipids, Alabaster, AL, U.S.A.) were added to the incubation mixture; subsequently, mitochondria were separated by centrifugation and the release of cytochrome *c* was measured in the supernatant by immunoblotting as described earlier [22,23].

Measurement of lysolipids and phospholipases

Binding of Bid to lysolipids was measured in assay buffer following the fluorescence quenching of the fatty acid-sensitive probe ADIFAB (Molecular Probes, Eugene, OR, U.S.A.). ADIFAB is a fatty acid-binding protein with a fluorescent reporter, which responds with high sensitivity to lipids like lysolipids [29]. The fluorescence of ADIFAB, which progressively decreases on increasing the concentration of LPC and other lysolipids (compare [27]), is maximally quenched by saturation with the specific ligand oleate (compare Figure 2A and [27,29]). When Bid proteins are added to the system, ADIFAB fluorescence becomes less sensitive to LPC concentration, reflecting competition of the two proteins for the same ligand [27]. To evaluate quantitatively the affinity of Bid for a lysolipid, we first derived the K_d of the same lysolipid for ADIFAB alone by numerically solving the equations of Richieri et al. [29]. The observed quenching in the presence of Bid was then simulated by computing the reduced levels of free lysolipid resulting from its binding to Bid, which was independently verified by the changes in the intrinsic fluorescence of the protein [27].

PLA (phospholipase A) activity of mitochondria and other fractions was assayed in assay buffer using bis-BODIPY-F5-PC [30] or following the quenching of the ADIFAB probe by endogenous fatty acids [29].

Membrane perturbation measurements

Assays of lipid transfer were performed as described previously [22,23] and adapted to measure perturbation of lipid membrane and surface insertion of molecules [31] by following the enhanced fluorescence of the lipid probe, BODIPY-PC [32]. The probe was added at a 1:2 ratio to a lipid mixture, normally containing 50 % PC, 30 % phosphatidylinositol and 20 % phosphatidylserine, and diluted with ethanol to a final concentration of 0.2 mM;

unilamellar vesicles were then produced by rapid injection in assay buffer [22,32]. Emission fluorescence at 516 nm (10 nm bandwidth) was recorded with excitation at 490 nm (5 nm bandwidth); maximal dequenching, indicative of total membrane rupture, was obtained with excess detergents, e.g. 0.1 % Triton X-100 [22,33]. Overall, the assay was fundamentally similar to those used to monitor membrane fusion [12,33,34].

Lipid extraction and analysis by MS

Extraction of lipids from subcellular fractions was undertaken with chloroform/methanol as described previously [27,28,35]. Analysis of all the lipids extracted from subcellular fractions was performed by nanospray Q-TOF (quadrupole–time-of-flight) MS essentially as described earlier [27,28,35,36]. Analyses were performed using a Micromass instrument (Manchester, U.K.), as described previously [27,28]; for Jurkat cell extracts, we have also used a QSTAR® XL Q-TOF mass spectrometer (AB/MDS, Sciex, Toronto, Canada), equipped with a nanospray ion source (Protana, Odense, Denmark). In this instrument, collision-induced decomposition in MS/MS mode was achieved using nitrogen as the collision gas at a pressure of $3\text{--}4 \times 10^{-5}$ Torr (1 Torr $\equiv$ 133.322 Pa). The MS and MS/MS data were acquired in continuum mode over the range of 100–2000 *m/z*; for MS/MS the range depended on the *m/z* of the precursor ion.

For quantitative evaluation of lipid species, we used a variety of reference lipid ions, selected on the basis of their relative proportion and absolute peak intensity within each nano-electrospray acquisition (compare [27,28]). Some of these ions remained substantially unchanged during Fas-mediated death, and could then be used as internal standards for evaluating specific changes induced by apoptosis (compare [37]). They included a major SM (sphingomyelin) species present in mouse liver mitochondria, C_{16:0}-SM at 703.6 *m/z* in its protonated form [36], and ceramide species like C_{24:0}, which was detected as a CI-adduct in both positive-ion mode (686.4 *m/z*) and negative-ion mode (684.4 *m/z*, compare [37,38]). Fragmentation signatures of sodiated lysolipids were recognized according to the literature [35,36,39]. Data were acquired and processed using MassLynx v.3.4 or Analyst QS (AB/MDS Sciex) software.

RESULTS AND DISCUSSION

LPC rapidly accumulates in mitochondria during apoptosis

Previous studies indicated that Bid action is modulated by interaction with some phospholipids [13,22,23,27,40]. Although tBid (cleaved Bid) has been shown to be selective for CL (cardiolipin) and its metabolites, in particular MCL (monolysocardiolipin) [13,27], lysolipids like LPC have been reported to potentiate the action of f.l. Bid on isolated mitochondria [13,23]. To verify whether the effects of LPC had relevance to the physiological changes occurring in mitochondrial lipids during apoptosis, we refined our MS analysis, focusing on the mass region of natural lysolipids (Figure 1, compare [35,39]). As in previous studies [27,28], we used Fas-mediated cell death as a model of physiological apoptosis requiring the involvement of Bid [1,2,17].

Treatment of mouse liver with the Fas-agonist Jo-2 antibody induced an early increase in lysolipids corresponding to major LPC species with palmitoyl (C_{16:0}) and stearoyl (C_{18:0}) groups (Figure 1A). These LPC species are normally present at basal levels in healthy cells and mitochondria [41,42]; they are also secreted by apoptotic cells to attract engulfing macrophages [43]. The basal level of palmitoyl- and stearoyl-LPC (and their stable Na adducts) increased after 1–2 h treatment with Jo-2, during which time

Figure 1 Lysolipids accumulate in apoptotic mitochondria

(A) Electrospray Q-TOF MS of mitochondrial lipid extracts analysed in positive-ion mode. The identity of the observed lysophospholipid ions was established using product-ion scanning and comparison with previously reported m/z values and fragmentation patterns ([35], compare [39]). The intensity of the observed ions was normalized to the same content of dominant PC lipids [44]. (B) Quantitative evaluation of two major LPC species from the spectra in (A), normalized to the average level of palmitoyl, C_{16:0} LPC in untreated liver, was performed using various internal references such as SM (see Materials and methods section, compare [27,28]). Histograms represent means $\pm$ S.D. ($n=4$). The bottom panel shows the Western blotting of endogenous f.l. Bid in the same mitochondria, obtained with identical protein loads as described previously [22,27]. (C) The top panels show the MS profile of lipid extracts from control (left) and FasL-treated (right) Jurkat T cells. Cells were incubated first with 100 μ M Z-VAD-FMK and then treated for 1 h with 100 ng/ml of human recombinant FasL, harvested and subjected to subcellular fractionation as described in the Materials and methods section. Equivalent concentrations of lipid extracts (also documented by phosphorus analysis) were analysed by nanospray ionization Q-TOF MS in positive-ion mode as described earlier (compare [27,28]). Note that the dominant ion at 485 m/z corresponds to a stable sodiated adduct of 1-oleoyl-LPC [39]. The bottom panels show an expansion of a selected region of the same spectra, which was recorded according to the absolute intensity of the signals to provide a quantitative estimate of the internal standard, C_{24:0}-CI (see text, compare [37]). Complementary MS analysis was also performed in negative-ion mode (results not shown, compare [38]). Similar results were obtained in the absence of Z-VAD-FMK. (D) The MS/MS fragmentation profile of the precursor ion at 485.3 m/z in the lipid extract of control cells (top left panel in C) was obtained using a collision energy of 40 eV, compare [39,63]. The fragmentation yielding the major ions at 204 and 282 m/z is shown; other ions such as those at 187 and 265 m/z also corresponded to previously reported fragments of LPC [39,63].

there was no significant increase in the activity of mitochondrial phospholipases (results not shown, compare [7,28]). The increase in LPC levels was then followed by the appearance or increase in oleoyl-LPG (lysophosphatidyl-glycerol at 532 m/z ; Figure 1A, bottom panel) and other lysolipids. Although local activation of PLA₂ could be excluded (compare [28]), we were able to detect a small increase in the activity of cytosolic, PC-specific lipases after stimulation with FasL. The increase appeared to be calcium-independent but also insensitive to bromoenol lactone, a classical inhibitor of cytosolic PLA₂ (iPLA₂), (E. S. Mundy and M. D. Esposti, unpublished work). This enhanced lipase activity may be responsible, at least in part, for the increased lysolipid levels observed in mitochondria (Figure 1), but further studies are required to pinpoint the enzyme(s) involved.

LPC accumulates also in mitochondria of Jurkat cells

Cell death signalling brings about a variety of cellular alterations, the manifestation of which often depends on the given biological system under study. This is particularly the case for lipid-degrading enzymes [37,38,44]. To verify common trends in mitochondrial lipids during Fas-mediated cell death, we expanded our research on mouse liver to human cell lines that are exquisitely sensitive to Fas activation. To this end, a cell clone was selected from the commonly used Jurkat T cell that displayed comparable sensitivity to FasL, and other death ligands like TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand), without the requirement of protein synthesis inhibition (F. Sandra, M. D. Esposti and R. Khosravi-Far, unpublished work). The cultured

cells were stimulated with 100 ng/ml of human FasL inducing overt activation of caspases after 2–3 h (results not shown). Even before this activation, treatment with FasL induced significant alteration of various lipids of the mitochondrial fraction (Figure 1C). These changes persisted or were even exacerbated when Jurkat cells had been treated with saturating concentrations of the pan-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethane) before Fas stimulation (Figures 1C and 1D, results not shown).

For the purpose of this study, we concentrated our analysis on Fas-induced changes in lipids related to PC (phosphatidylcholine) and its metabolites like LPC. Mitochondria from Jurkat cells, similar to those from most other cells including mouse hepatocytes, contain a large complement of PC, which constitutes the dominant phospholipid of intracellular membranes [36,42,44]. In part owing to the limited purification of mitochondria from cultured cells, lipid extracts from Jurkat mitochondria contained abundant lipid metabolites related to PC (Figure 1, compare [44]). A by-product ion at 204 m/z (most probably the sodiated adduct of glyceryl-dimethylene-phosphate, compare [36,39]) appeared to be the most intense ion in the MS spectra obtained from the Q-TOF MS analysis (Figure 1C). After 1 h of FasL treatment, the intensity of this ion remained high, but other lipid species became comparatively stronger, in particular the ion at 485.3 m/z (Figure 1C). This ion could be assigned to a stable fragment of sodiated oleoyl-LPC ($C_{18:1}$ LPC) that is commonly observed in electrospray spectra of lipid extracts containing NaCl, since Na facilitates the neutral loss of trimethylamine (59 atomic mass units) from PC or LPC [39]. Although weak signatures of this LPC moiety were detected in mouse liver mitochondria, they remained at least one-order-of-magnitude less abundant than stearoyl-LPC (546 m/z , Figure 1A, results not shown). The identity of the 485.3 m/z ion was confirmed by product-ion scanning in positive-ion mode (Figure 1D, compare [39]).

One main reason for the accumulation of different LPC species in mitochondria of mouse liver and Jurkat T cells is the differential degradation of specific PC molecules. In mitochondria from the human cell line, di-oleoyl-PC (786.6 m/z , an abundant phospholipid in cultured cells [36]) undergoes a significant decrease after 1 h of Fas activation (results not shown). This PC species is present at very low levels in mouse liver mitochondria, which instead contain abundant levels of palmitoyl-linoleoyl-PC (758.6 m/z), a species virtually absent in Jurkat cells (compare [37] and [44]). In addition, major CL species of human cell line cells contain a greater proportion of oleoyl groups than in mouse liver [27,28,45]. Given the changes in CL remodelling that occur during Fas-induced apoptosis [27,28,45] and the relevance of PC species in this remodelling [44,46,47], the increase in oleoyl-LPC observed in Jurkat mitochondria may relate to alteration in CL remodelling as well [44]. Indeed, LPC and LPG accumulated in parallel to a progressive degradation of mitochondrial CL (results not shown, compare [27,28]).

To attempt a quantitative evaluation of LPC changes in apoptotic mitochondria, we used various reference ions and some internal standards. Of value was the identification of a peculiar ceramide lipid, $C_{24:0}$, which is abundant in Jurkat as well as other cultured cell lines [37,38] and invariably associated with mitochondria. Stable CI-adducts of this lipid were clearly detected in both positive-ion mode (686.4 m/z , Figure 1D) and negative-ion mode (results not shown, compare [38,45]), and have been previously shown to remain unchanged during Fas-mediated apoptosis [37]. Once normalized to the intensity of the $C_{24:0}$ ceramide adduct, the m/z 485.3 peak of oleoyl-LPC showed a substantial increase in cells treated with FasL for 1 h. This increase was independent of caspase activation, since Z-VAD-FMK treatment

Figure 2 Binding of lysolipids in solution

(A) Measurements of LPC binding were conducted with 0.2 μ M ADIFAB and Bid [27]. The left panel shows a titration with 1-oleoyl-LPC that was performed in the absence of Bid; addition of 6 μ M oleate (dotted spectrum) produced maximal quenching [29]. Bid-induced reduction in fluorescence quenching was computed as described earlier [27] to evaluate solution binding to LPC. a.u., arbitrary units. (B) Immunoblots of cytochrome *c* released from intact mouse liver mitochondria after incubation with 10 nM native Bid isolated from mouse kidney [22] (left) or 10 nM recombinant mouse Bid (right). Samples were incubated for 20 min in the absence or presence of 2 μ M LPC, which was a mixture of natural stearoyl and palmitoyl analogues (left) or synthetic 1-oleoyl-LPC (right). Control experiments conducted with the same LPC concentrations but in the absence of Bid showed no significant increase in the basal release of cytochrome *c* (see below Figure 3C, compare [23]). Equal protein loading was verified by subsequent blotting with porin and India ink staining.

of the cells before Fas stimulation did not significantly affect its level (Figure 1C and results not shown). Of note, the LPC increase was accompanied by a clear increase in the observed ions corresponding to the fatty acid fragments, oleoyl-H (282.3 m/z) and oleoyl-Na (304.3 m/z , Figure 1C).

In summary, our detailed MS analysis indicates that Fas stimulation induces early alteration of PC-related metabolites in mitochondria, with a strong increase in LPC species and their fragments that may differ in different cell systems, as they reflect the different fatty acid compositions of major phospholipids.

Bid binding to LPC in solution

Having found that LPC species consistently increase in mitochondria early after Fas stimulation, we next investigated whether Bid had a particular affinity for LPC. Using the ADIFAB approach [27,29], we determined that Bid had more affinity for LPC than LPG species with the same acyl group, e.g. oleoyl (Figure 2A, compare [27]). The estimated K_d values for oleoyl-LPC was 0.05 μ M, i.e. approx. 10-fold lower than that of the ADIFAB protein for the same lipid (Figure 2A). Of note, Bid did not

significantly bind oleate (indeed, ADIFAB quenching by oleate was not affected by the addition of Bid, Figure 2A) nor did it bind to di-acyl species of PC. Interestingly, caspase cleavage of Bid had little effect on its binding to LPC, the K_d values for oleoyl-LPC remaining at approx. $0.05 \mu\text{M}$ as for the uncleaved protein (Figure 2A and results not shown). This contrasts with previous findings that caspase cleavage strongly enhances the affinity of Bid for CL [48] and MCL [27]. Consequently, f.l. Bid appears to be comparatively more selective than caspase-cleaved Bid (tBid) for soluble lysolipids like LPC, which are physiologically present in mitochondria and increase during cell death (Figure 1).

The binding data (Figure 2A), combined with previous results indicating that LPC could modify mitochondrial association of f.l. Bid [23], suggested the hypothesis that Bid may have dual specificity for chemically different lysolipids. In its f.l. form, Bid may preferentially bind to LPC, and possibly transport it between different membranes [22,23]. In contrast, after caspase cleavage (t)Bid may avidly associate with MCL and other CLs [27,28,44,48]. Indirect support for this hypothesis emerged from our unpublished findings that caspase-cleaved Bid did not show the tightly bound lysolipids of bacterial origin that are normally associated with the f.l. protein (compare [27]). To obtain more direct evidence, we tested the capacity of mouse Bid to release cytochrome *c* from mouse liver mitochondria.

Our preparation of native Bid [22] did not appear to contain tightly bound lipids (results not shown) and hardly affected the basal leak of cytochrome *c* from intact mitochondria (Figure 2B, left). However, the addition of natural lysolipids, especially a mixture of palmitoyl- and stearoyl-LPC, strongly enhanced the release of the apoptogenic protein with respect to either native Bid or LPC alone (Figure 2B and [23]). At the same nanomolar concentrations, recombinant mouse Bid was significantly more effective than the native protein preparation in the absence of lysolipids (Figure 2B). Nevertheless, LPC potentiation of cytochrome *c* release was also observed with recombinant Bid, especially with 1-oleoyl-LPC at concentrations below its micellar aggregation [41] (Figure 2B, see also [23]). In comparison, equivalent concentrations of di-acyl PC, CL, and other lysolipids like LPG (as well as detergents), did not produce a significant increase in cytochrome *c* release in the presence of f.l. Bid (results not shown, compare [23,27]). Complementing previous studies [22,23,27], these results suggested a peculiar interaction of f.l. Bid with LPC species of physiological relevance. This interaction may derive from binding specificity, leading to selective transport to membranes containing other lipids like CL, which may facilitate the docking and release of bound lysolipids as for fatty acid binding proteins [49].

LPC stimulates the membrane-perturbing action of Bid

Following the observation that LPC enhances the cytochrome *c* releasing capacity of f.l. Bid (Figure 2 and [23,27]), and of Bax too [11,12,14], we investigated the potential role of lysolipids in the membrane perturbation induced by Bid in model lipid membranes. Since the first stage of membrane perturbation invariably requires surface interaction leading to discontinuity in the external lipid layer of the membrane [31,34], we specifically measured perturbations in the external layer of membranes using the fluorescent phospholipid BODIPY-PC [32]. In principle, these measurements were very similar to established assays of membrane lipid fusion [33,34]. In the context of this work, the use of a PC analogue was particularly pertinent, given the substantial changes in PC and its metabolites occurring in mitochondria after apoptosis induction (Figure 1). Membrane perturbation was detected as a transient increase in liposome fluorescence due to

insertion of lipophilic molecules into the membrane, which diluted the constituent lipids and partially relieved the self-quenching of the concentrated probe [31,34]. Since external lysolipids incorporate rapidly on the external layer, but then move very slowly on to the internal layer of the membrane [31,32], the measured fluorescence changes (Figure 3) essentially reflected transient modification of the lipid surface with little trans-bilayer alteration [31]. Maximal changes were then evaluated by the full dispersion of the liposomes with micellar detergents (Figure 3A, right).

Although nanomolar concentrations of f.l. Bid did not affect the stability of lipid membranes of composition similar to the OM (Figure 3B, left), the subsequent addition of specific LPC species produced a substantial increase in fluorescence (Figures 3A and 3B). At the concentrations $0.5\text{--}2 \mu\text{M}$ used, which were comparable with the lysolipid levels present in mitochondria (Figure 1 compare [41,42]) LPC alone had some destabilizing, yet limited effect (Figure 3B). Hence, our results suggested that Bid could enhance the insertion of LPC in the surface layer of the lipid membrane, with consequent perturbation of the physical packing of the phospholipids but no gross alteration of trans-bilayer continuity (no significant increase in the release of trapped fluorophores was observed, in agreement with earlier studies [10,31]). Bid-facilitated insertion of lysolipids showed chemical specificity, since it was negligible with the short chain 1-dodecyl-LPC or the chemically different LPE (1-oleoyl-lysophosphatidylethanolamine), whereas it was pronounced with 1-oleoyl-LPC (Figure 3B). Indeed, the intensity of membrane perturbation produced by Bid appeared to be proportional to the strength of its binding to lysolipids. As shown later (Figure 6), the perturbation effect of Bid with 1-oleoyl-LPG was much greater than that with 1-oleoyl-LPA, reflecting a 5-fold difference in affinity for the two lipids [27].

To establish whether LPC-enhanced membrane perturbation of f.l. Bid (Figures 3A and 3B) had relevance to the biological action of releasing cytochrome *c*, cell-free assays were performed under the same conditions. In these experiments, the release of cytochrome *c* promoted by exogenous f.l. Bid was directly compared with that produced by a cytosolic extract of Fas-stimulated liver (Figure 3C, top panel, compare [17,22]). The enhancement of apoptogenic alteration was comparable with that observed with 1-oleoyl-LPC (Figure 3C, top panel, right). The same physiological LPC species increased cytochrome *c* release by f.l. Bid more significantly than short-chain LPC species (bottom panel in Figure 3C). Consequently, results of cell-free assays were consistent with the liposome data of membrane perturbation (Figure 3B compare Figure 3C).

An apoptosis-defective Bid mutant shows altered interaction with LPC

Following these *in vitro* results, we addressed the crucial question: does the pro-apoptotic action of f.l. Bid *in vivo* [18,21] arise from interaction with LPC leading to enhanced membrane perturbation? It is notoriously difficult to extrapolate biochemical results obtained with model systems to the complex situation within cells. However, apoptosis-defective mutants of Bid have been documented in cellular studies [18]. If such mutated proteins were found to display distinctive biochemical properties, explanations for the pro-apoptotic action *in vivo* could be reasonably argued from biochemical data. We thus compared the properties of wt (wild-type) f.l. Bid with those of the mIII-2 mutant, which has a highly modified BH3 domain ($1^{93}\text{GDE}^{96} \rightarrow \text{AAAA}$ [18]) and is severely defective in inducing apoptosis [16–18]. Previously, the mutated protein has been studied only after caspase cleavage [8,10].

Figure 3 Membrane destabilization induced by Bid and lysolipids

To measure perturbation of the lipid membrane, we followed the principles of assays routinely applied to measure membrane fusion [33,34]. The lipid probe BODIPY-PC [32] was added at a 1:2 ratio to a lipid mixture consistent with the composition of the OM (compare [27]), before the production of vesicles by rapid ethanol injection [22,32]. **(A)** LPC induced a transient increase in fluorescence due to its rapid incorporation into the membrane that was balanced by progressive reabsorption into the medium [31]. Subsequent addition of 0.002 % Tween 20R strongly enhanced the probe fluorescence due to disruption of the lipid membrane; maximal dequenching was then obtained with excess of the detergent 0.1 % Triton X-100. The panel on the right shows the effect of preincubation of 20 nM f.l. Bid on the transient change elicited by palmitoyl-LPC (note the 2.5-fold expansion of the ordinate scale with respect to the left panel). **(B)** Chemically related lysolipids (all added at a final concentration of 1.2 μ M) showed different effects in the absence (left) and presence (right) of 4 nM recombinant f.l. Bid. The dotted trace on the left was obtained after the injection of Bid in the absence of lysolipids. The abbreviations of the various lysolipid analogues are as follows: LPC-C16, 1-palmitoyl-LPC; LPC-C18, 1-oleoyl-LPC; LPC-C12, 1-dodecyl-LPC. **(C)** Representative data are shown of cell-free assays with mouse liver mitochondria, which were conducted under the same conditions as for the membrane perturbation measurements; lipids were added at a final concentration of 2 μ M. Cytochrome *c* was immunoblotted in the supernatant after 20 min incubation in the presence (compare Figure 2B) or absence of 8 nM recombinant f.l. Bid. The release of cytochrome *c* could be compared with that promoted by incubation with cytosol (1 mg/ml) of Jo-2-treated liver (lane 2, top panel).

Considering the increase in 1-palmitoyl-LPC in a mouse model of apoptosis (Figure 1A) and its efficacy in enhancing the membrane perturbing action of f.l. Bid (Figure 3A), we first studied this lysolipid when combined with the mIII-2 mutant protein, comparing the results with those obtained with a wt protein prepared with exactly the same procedure (compare [8]). With low LPC concentrations minimizing membrane perturbation by the lipid alone,

the mutant Bid protein was clearly less efficient than wt Bid in enhancing membrane perturbation with LPC (Figure 4). The difference between mutant and wt Bid progressively diminished by increasing the final concentration of the protein in the assay (Figure 4). This result was important because it was consistent with cellular data showing that the pro-apoptotic defect of mIII-2 Bid progressively diminished by increasing its expression levels

Figure 4 Apoptosis-defective mutant of Bid has different membrane-perturbation capacity

Membrane perturbation was evaluated under the same experimental conditions as in the experiment of Figure 3(A), using $0.5 \mu\text{M}$ palmitoyl-LPC (synthetic, from Avanti Polar Lipids) and conditions that minimized the effects of the lipid alone. Both wt and mIII-2 Bid were obtained as His-tagged f.l. proteins as described earlier [8]. The inset in the bottom of the figure shows the results obtained with LPE ($1 \mu\text{M}$), which could be considered as a negative control because it did not induce significant membrane perturbation with or without Bid proteins (see also Figure 3B).

[18]. Moreover, it also provided an explanation for the similar transmembrane perturbation induced by mIII-2 and wt Bid proteins when used at concentrations above 10 nM [8]. The lipid specificity of the different levels of perturbation induced by wt and mIII-2 Bid was confirmed by the similar (lack of) effects with other lysolipids, including oleoyl-LPE that could be used as a 'negative control' (Figures 3B and 4, bottom panel; see also [11,12]).

The results in Figure 4 provide the first biochemical evidence for a dynamic difference in lipid interactions of Bid due to mutations in the BH3 domain. In the reported NMR-deduced structures of Bid, the BH3 domain is either buried [50] or partially surface exposed (especially in the presence of detergents [51,52]). In any case, the BH3-domain is essential for reciprocal interaction of Bid with other Bcl-2 proteins and apoptosis induction [4,5,17,18], but not for binding to CL and mitochondria [48,52–54]. If f.l. Bid has a dual specificity for LPC and CLs as hypothesized here, then it is possible that its BH3 domain confers some specificity to modulate the differential binding (and release) of LPC in the presence of CLs at the membrane surface.

To verify this possibility, we modified the lipid composition of the liposomes by adding MCL and repeated the membrane perturbation experiments also with a physiological mixture of LPC (Figure 5). As shown in Figure 5(A), the addition of LPC produced significant perturbation with a longer time course in the presence of wt Bid than in its absence. The protein effectively prolonged the perturbation effect, presumably by facilitating membrane reabsorption of LPC from the aqueous phase (compare [31]). The lipid transferase activity of Bid could account for such an effect, because this activity would enhance the forward reaction in the equilibrium between LPC adsorbed in the membrane (surface) and

Figure 5 Apoptosis-defective mutant of Bid does not respond to LPC in the presence of MCL

Membrane perturbation measurements were performed as in the experiments of Figure 4 using $1 \mu\text{M}$ LPC (producing an enhanced perturbation) and, with the exception of the data in the inset, liposomes containing an additional 10% (mol/mol) fraction of MCL, which was then part of the membrane structure. The insertion of MCL produced liposomes with limited stability due to the intrinsic non-bilayer properties of this lipid. Nevertheless, membrane perturbation measurements could still be performed with freshly prepared liposomes. (A) Data were obtained with wt Bid (5 nM). Note the sudden increase in fluorescence occurring when the protein was added after LPC had equilibrated with the liposomes. The inset shows that this rapid destabilization was less pronounced when MCL is not present in the composition of the liposome membrane. (B) Data were obtained with the same preparation of mIII-2 Bid mutant as that used in Figure 4. Note the very limited destabilization induced by Bid addition after LPC had equilibrated with the liposomes. The limited effect was very similar to that observed in the presence of LPE (compare Figures 3B and 4, inset).

LPC returning in the medium (water) after transient incorporation in the membrane. Whereas the addition of Bid alone had no significant perturbing effect on the membrane surface (Figure 5A, left, compare Figure 3B), when LPC was equilibrated first with liposomes, the addition of Bid usually induced some surface perturbation (Figure 5A, inset and results not shown).

Interestingly, when MCL was present in the composition of the lipid membrane, the perturbation induced by wt Bid after LPC equilibration was much stronger than when MCL was absent (Figure 5A, inset). Other negatively charged lipids did not show the same effect (see also Figure 6). Crucially, mIII-2 Bid did not induce any significant perturbation when added after LPC equilibration (Figure 5B). Hence, the Bid mutant not only displayed a reduced efficiency in enhancing membrane perturbation with LPC (Figure 4), but it was also ineffective in facilitating LPC incorporation into membranes containing MCL (Figure 5). These results bear relevance to physiological situations during apoptosis, since MCL is the CL metabolite that increases most rapidly after Fas activation and death signalling [27,28], whereas mitochondria accumulate LPC species (Figure 1) and f.l. Bid (Figure 1B, bottom panel, compare [27,28]).

Figure 6 Membrane destabilization with CL metabolites and Bid

Experiments were conducted with wt f.l. Bid from R & D Systems as in Figures 2 and 3. The concentration of the protein was 4 nM in the experiments of (A, B), but 20 nM in the experiment of (C). All lipids were added to standard liposomes to a final concentration of 1 μ M, except for MCL in (C), which was used at 0.3 μ M to limit its strong perturbation capacity. In all panels, the dotted traces were obtained without Bid. For DCL, Bid was preincubated with a 20-fold concentrated solution of the lipid for 30 min to allow oligomer formation (compare [27]). Note the very limited perturbation enhancement by Bid with lyso-bis-phosphatidic acid (A) or LPA (B), and the opposite effect, of inhibition of membrane perturbation, with CL (B) and MCL (C). Results are representative of multiple separate experiments.

CL metabolites and Bid-mediated membrane perturbation

Given the observations above, we completed the picture of Bid-mediated membrane perturbation by investigating the effects of all lysolipids related to CL metabolism. Once added to the medium, only LPG showed some Bid-mediated enhancement of membrane perturbation (Figures 6A and 6B). CL and its lyso-metabolites, including DCL (di-lysocardiolipin), produced strong alteration of the membrane. In contrast, an equivalent concentration of lyso-bis-phosphatidic acid, another negatively charged lipid that is structurally similar to DCL, had very limited effects (Figure 6). Hence, the membrane perturbation by external CLs was due to properties other than negative charge or mere hydrophobicity, presumably reflecting a propensity to form non-bilayer structures [12,55].

When DCL was preincubated with Bid before addition to the liposome system, there was only a limited change in the strong perturbation produced by DCL alone (Figure 6A). This was due to avid interaction between Bid and the excess of DCL that induced nearly complete oligomerization of the protein (results not shown, compare [27]), effectively neutralizing any dynamic action of Bid. In this respect, DCL acted like a surrogate inhibitor of Bid action, consistent with recent results [14]. However, with either CL or MCL, Bid significantly decreased the extent of incorporation and/or perturbation of the external lipid layer (Figures 6B and 6C). This effect was the opposite of that obtained with LPC (Figure 3), reflecting a modification of the physical absorption of the exogenous lipids on to the membrane surface, presumably mediated by tight binding to Bid that competed with membrane insertion

(compare [27]). Indeed, the highly destabilizing capacity of MCL was progressively reduced by increasing concentrations of pre-equilibrated Bid (Figure 6C and results not shown).

In summary, the analysis of the membrane perturbing effects of Bid suggests a selective action depending on specific lipids. Dynamic binding to lysolipids like 1-palmitoyl-LPC enables Bid to facilitate their incorporation into the external layer of the membrane, with consequent surface alteration of the phospholipid membrane, which could facilitate subsequent loss of trans-bilayer integrity (Figures 4 and 5). In contrast, interaction of Bid with CL and MCL reduces the intrinsic membrane destabilizing properties of these lipids, whereas it facilitates the docking of the protein to the membrane and the release of bound LPC (Figure 5B, compare [27]), similar to some fatty acid transporters [49].

Conclusion

Although the mechanism of pro-apoptotic proteins of the Bcl-2 family has remained elusive, it is now established that relocation to mitochondrial membranes constitutes the common feature of their action [1–3]. Accumulating evidence indicates that mutual interaction between cytosolic and OM-resident members of the family and association with membrane lipids are involved in this relocation. Previously, we reported that Fas-mediated apoptosis induces progressive degradation of CL with accumulation of MCL [27,28]. In the present study, we have shown that apoptotic mitochondria also accumulate defined LPC species early after Fas stimulation (Figure 1). These LPC molecules may be transported from other cellular compartments like the cytosol, in which enhanced activity of lipases follows Fas activation [43,56]. As discussed recently [44], the level of mitochondrial LPC may also increase as a consequence of enhanced remodelling of mitochondrial lipids, PC and CL in particular [27,28,46,47,57]. The fact that the major LPC species that increase in mitochondria reflect the abundance of the CL species that are present in the same cells (Figure 1 and results not shown, compare [44–47]), suggests that remodelling reactions significantly contribute to LPC elevation during Fas-mediated apoptosis.

Bid probably plays a role in LPC accumulation, owing to its lipid transfer capacity [22,23] and dynamic specificity for physiological LPC species (Figures 2–6). The hypothesis that emerges from this work is that f.l. Bid may have a dual specificity for diverse lysolipids. Bid may first ‘sense’ changes in the cellular levels of major LPC species. Once bound to LPC, Bid can then transport it to intracellular membranes, especially those that contain CL or MCL (Figure 5), towards which the protein has a ‘static’ selectivity of binding [14,27,28,48]. Perhaps this occurs at basal levels in all cells. Alternatively, other reactions mediated by death signalling may promote the transport capacity of f.l. Bid, even before activation of caspases occurs. Once docked to the OM, Bid could release bound LPC of extra-mitochondrial origin or prevent the escape of locally produced LPC from mitochondria.

In the former case, Bid might effectively induce a lipid exchange with MCL or other surface-exposed lipids. The consequent elevation of LPC will change the physical state of the OM surface, leading to the formation of local microdomains rich in LPC that may cause a positive change in membrane curvature [11,58]. This physical change in the state of the external lipid layer would ‘prime’ mitochondrial OM [27,59] to the action of other proteins like Bak and Bax [5,60], thereby producing coordinate damage, perhaps with the formation of lipid or lipid/protein pores [11,58], which then promote the release cytochrome *c* and other apoptogenic factors [3,44,60].

The above discussion regards primarily f.l. Bid. Cleavage by apical caspases (or other proteases) irreversibly enhances Bid

affinity for MCL and other CLs [27,48], thus increasing the association with mitochondria (and other membranes where CL metabolites may accumulate [28]). The molecular reasons for this change in lipid affinity are unknown, but may include some inhibitory action of the N-terminus domain on the CL-binding capacity of tBid (compare [8]). In any case, the increase in affinity of tBid for CL and MCL will exacerbate membrane alteration, which may propagate from the OM to the inner membrane through contact sites. Because of the crucial role of CL in membrane structure and plasticity of mitochondria [55], tBid-mediated alteration of CL homeostasis would be responsible for the structural and functional changes observed *in vitro* and *in vivo* (compare [61]). In particular, tight binding of tBid to MCL would sequester this lipid from other reactions, thus removing it as a product inhibitor of the transacylase process of CL remodelling [47], as well as of mitochondrial PLA2 [62]. Further studies are required to verify the biochemical details of the predicted effects of tBid on mitochondria.

In conclusion, the present study has identified selective interaction with LPC as the fundamental event underlying the proapoptotic action of f.l. Bid, *in vitro* as well as *in vivo*.

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REFERENCES

- Martinou, J.-C. and Green, D. R. (2001) Breaking the mitochondrial barrier. *Nature Reviews Mol. Cell Biol.* **2**, 63–67
- Wang, X. (2001) The expanding role of mitochondria in apoptosis. *Genes Dev.* **15**, 2922–2933
- Newmeyer, D. D. and Ferguson-Miller, S. (2003) Mitochondria, releasing power for life and unleashing the machineries of death. *Cell (Cambridge, Mass.)* **112**, 481–490
- Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. and Korsmeyer, S. J. (2001) Proapoptotic BAX and BAK, a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 624–626
- Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T. and Korsmeyer, S. J. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell* **8**, 705–711
- Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J.-C. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J. Cell Biol.* **144**, 891–901
- Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J.-C. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem. J.* **345**, 271–278
- Kudla, G., Montessuit, S., Eskes, R., Berrier, C., Martinou, J.-C., Ghazi, A. and Antonsson, B. (2000) The destabilisation of lipid membranes induced by the C-terminal fragment of caspase 8-cleaved Bid is inhibited by the N-terminal fragment. *J. Biol. Chem.* **275**, 22713–22718
- Kluck, R. M., Esposito, M. D., Perkins, G., Renken, C., Kuwana, T., Bossy-Wetzel, E., Goldberg, M., Allen, T., Barber, M. J., Green, D. R. et al. (1999) The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J. Cell Biol.* **147**, 809–822
- Roucou, X., Rostovtseva, T., Montessuit, S., Martinou, J.-C. and Antonsson, B. (2002) Bid induces cytochrome c-impermeable Bax channels in liposomes. *Biochem. J.* **363**, 547–552
- Basanez, G., Sharpe, J. C., Galanis, J., Brandt, T. B., Hardwick, J. M. and Zimmerberg, J. (2002) Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. *J. Biol. Chem.* **277**, 49360–49365
- Eband, R. F., Martinou, J.-C., Fornallaz-Mulhauser, M., Hughes, D. W. and Eband, R. M. (2002) tBid promotes leakage by altering membrane curvature. *J. Biol. Chem.* **277**, 32632–32639
- Eband, R. F., Martinou, J.-C., Montessuit, S. and Eband, R. M. (2004) Fatty acids enhance membrane permeabilization by proapoptotic Bax. *Biochem. J.* **377**, 509–516
- Hsu, Y. T. and Youle, R. J. (1997) Nonionic detergents induces dimerization among members of the Bcl-2 family. *J. Biol. Chem.* **272**, 13829–13834
- Yethon, J. A., Eband, R. F., Leber, B., Eband, R. M. and Andrews, D. W. (2003) Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. *J. Biol. Chem.* **278**, 48935–48941
- Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell death receptors. *Cell (Cambridge, Mass.)* **94**, 481–490
- Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Millman, C., Erdjument-Bromage, H., Tempst, P. and Korsmeyer, S. J. (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release while BCL-XL prevents this release but not tumor necrosis factor death. *J. Biol. Chem.* **274**, 1156–1163
- Wang, K., Yin, X. M., Chao, D. T., Millman, C. L. and Korsmeyer, S. J. (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev.* **10**, 2859–2869
- Yi, X., Yin, X. M. and Dong, Z. (2003) Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed. *J. Biol. Chem.* **278**, 16992–16999
- Renshaw, S. A., Dempsey, C. E., Barnes, F. A., Bagstaff, S. M., Dower, S. K., Bingle, C. D. and Whyte, M. K. (2004) Three novel Bid proteins generated by alternative splicing of the human Bid gene. *J. Biol. Chem.* **279**, 2846–2855
- Sarig, R., Zaltsman, Y., Marcellus, R. C., Flavell, R., Mak, T. W. and Gross, A. (2003) BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. *J. Biol. Chem.* **278**, 10707–10715
- Degli Esposti, M., Erler, E. T., Hickman, A. J. and Dive, C. (2001) Bid, a widely expressed pro-apoptotic protein of the Bcl-2 family, displays lipid transfer activity. *Mol. Cell. Biol.* **21**, 7268–7276
- Degli Esposti, M. (2002) Sequence and functional similarities between pro-apoptotic Bid and plant lipid transfer proteins. *Biochim. Biophys. Acta* **1553**, 331–340
- Nechushtan, A., Smith, C. L., Lamensdorf, I., Yoon, S. H. and Youle, R. J. (2001) Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J. Cell Biol.* **153**, 1265–1276
- Tafani, M., Karpnich, N. O., Hurster, K. A., Pastorino, J. G., Schneider, T., Russo, M. A. and Farber, J. L. (2002) Cytochrome c release upon Fas receptor activation depends on translocation of full-length bid and the induction of the mitochondrial permeability transition. *J. Biol. Chem.* **277**, 10073–10082
- Deng, Y., Ren, X., Yang, L., Lin, Y. and Wu, X. (2003) A JNK-dependent pathway is required for TNF α -induced apoptosis. *Cell (Cambridge, Mass.)* **115**, 61–70
- Degli Esposti, M., Cristea, I. M., Gaskell, S. J., Nakao, Y. and Dive, C. (2003) Pro-apoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. *Cell Death Differ.* **10**, 1300–1309
- Sorice, M., Circella, A., Cristea, I. M., Garofalo, T., Di Rienzo, L., Alessandri, C., Valesini, G. and Degli Esposti, M. (2004) Cardiolipin and its metabolites move from mitochondria to other cellular membranes during death receptor-mediated apoptosis. *Cell Death Differ.* **11**, 1133–1145
- Richieri, G. V., Ogata, R. T. and Kleinfeld, A. M. (1992) A fluorescently labeled intestinal fatty acid protein. *J. Biol. Chem.* **267**, 23495–23501
- Hendrickson, H. S., Hendrickson, E. K., Johnson, I. D. and Farber, S. A. (1999) Intramolecularly quenched BODIPY-labeled phospholipid analogs in phospholipase A2 and platelet-activating factor acetylhydrolase assays and *in vivo* fluorescence imaging. *Anal. Biochem.* **276**, 27–35
- Zhelev, D. V. (1996) Exchange of monooleylphosphatidylcholine with single egg phosphatidylcholine vesicle membrane. *Biophys. J.* **71**, 257–273
- Bai, J. and Pagano, R. E. (1997) Measurement of spontaneous transfer and transbilayer movement of BODIPY-labeled lipids in lipid vesicles. *Biochemistry* **36**, 8840–8848
- Gunther-Ausborn, S., Praetor, A. and Stegmann, T. (1995) Inhibition of influenza-induced membrane fusion by lysophosphatidylcholine. *J. Biol. Chem.* **270**, 29279–29285
- Gaudin, Y. (2000) Rabies virus-induced membrane fusion. *J. Cell Biol.* **150**, 601–611
- Petkovic, M., Schiller, J., Muller, M., Bernard, S., Reichl, S., Arnold, K. and Arnold, J. (2001) Detection of individual phospholipids in lipid mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Biochem.* **289**, 202–216
- Pulfer, M. and Murphy, R. C. (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev.* **22**, 332–364
- Watts, J. D., Gu, M., Polverino, A. J., Patterson, S. D. and Aebersold, R. (1997) Fas-induced apoptosis of T cells occurs independently of ceramide generation. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7292–7296
- Nam, S. Y., Amoscatto, A. A. and Lee, Y. J. (2002) Low glucose-enhanced TRAIL cytotoxicity is mediated through the ceramide-Akt-FLIP pathway. *Oncogene* **21**, 337–346
- Han, X. and Gross, R. W. (1996) Structural determination of lysophospholipid regioisomers by electrospray ionization tandem mass spectrometry. *J. Am. Chem. Soc.* **118**, 451–457

- 40 Zhai, D., Miao, Q., Xin, X. and Yang, F. (2001) Leakage and aggregation of phospholipid vesicles induced by the BH3-only Bcl-2-family member, BID. *Eur. J. Biochem.* **268**, 48–55
- 41 Stafford, R. E., Fanni, T. and Dennis, E. A. (1989) Interfacial properties and critical micelle concentration of lysophospholipids. *Biochemistry* **28**, 5113–5120
- 42 De Kroon, A. I. P. M., Dolis, D., Mayer, A., Lill, R. and De Kruijff, B. (1997) Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the outer mitochondrial membrane? *Biochim. Biophys. Acta* **1325**, 108–116
- 43 Lauber, K., Bohn, E., Krober, S. M., Xiao, Y. J., Blumenthal, S. G., Lindemann, R. K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S. et al. (2003) Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell (Cambridge, Mass.)* **113**, 717–730
- 44 Cristea, I. M. and Degli Esposti, M. (2004) Membrane lipids and cell death: an overview. *Chem. Phys. Lipids* **129**, 133–160
- 45 Matsko, C. M., Hunter, O. C., Rabinowich, H., Lotze, M. T. and Amoscato, A. A. (2001) *Biochem. Biophys. Res. Commun.* **287**, 1112–1120
- 46 Schlame, M. and Rustow, B. (1990) Lysocardiolipin formation and reacylation in isolated rat liver mitochondria. *Biochem. J.* **272**, 589–595
- 47 Xu, Y., Kelley, R. I., Blanck, T. J. and Schlame, M. (2003) Remodeling of cardiolipin by phospholipid transacylation. *J. Biol. Chem.* **278**, 51380–51385
- 48 Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X. and Wang, X. (2000) Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat. Cell Biol.* **2**, 754–756
- 49 Davies, J. K., Thumser, A. E. and Wilton, D. C. (1999) Binding of recombinant rat liver fatty acid-binding protein to small anionic phospholipid vesicles results in ligand release, a model for interfacial binding and fatty acid targeting. *Biochemistry* **38**, 16932–16940
- 50 Chou, J. J., Li, H., Salvesen, G. S., Yuan, J. and Wagner, G. (1999) Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell (Cambridge, Mass.)* **96**, 615–624
- 51 McDonnell, J. M., Fushman, D., Milliman, C. L., Korsmeyer, S. J. and Cowburn, D. (1999) *Cell (Cambridge, Mass.)* **96**, 625–634
- 52 Degli Esposti, M. (2002) The roles of Bid. *Apoptosis* **7**, 433–440
- 53 Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B. and Korsmeyer, S. J. (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* **14**, 2060–2071
- 54 Hu, X., Han, Z., Wyche, J. H. and Hendrickson, E. A. (2003) Helix 6 of tBid is necessary but not sufficient for mitochondrial binding activity. *Apoptosis* **8**, 277–289
- 55 Schlame, M., Rua, D. and Greenberg, M. L. (2000) The biosynthesis and functional role of cardiolipin. *Prog. Lipid Res.* **39**, 257–288
- 56 Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M. and Kudo, I. (1998) Fas-induced arachidonic acid release is mediated by Ca^{2+} -independent phospholipase A2 but not cytosolic phospholipase A2, which undergoes proteolytic inactivation. *J. Biol. Chem.* **273**, 13870–13877
- 57 George, P., Ardail, D., Rey, C., Louisot, P. and Levrat, C. (2001) Phospholipids reacylation and palmitoylCoA control tumor necrosis factor- α sensitivity. *Cytokine* **13**, 257–263
- 58 Terrones, O., Antonsson, B., Yamaguchi, H., Wang, H. G., Liu, J., Lee, R. M., Herrmann, A. and Basanez, G. (2004) Lipidic pore formation by the concerted action of proapoptotic BAX and tBID. *J. Biol. Chem.* **279**, 30081–30091
- 59 Duan, S., Hajek, P., Lin, C., Kyung-Shin, S., Attardi, G. and Chomyn, A. (2003) Mitochondrial outer membrane permeability change and hypersensitivity to digitonin early in staurosporine-induced apoptosis. *J. Biol. Chem.* **278**, 1346–1353
- 60 Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., Green, D. R. and Newmeyer, D. D. (2002) Bid, bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell (Cambridge, Mass.)* **111**, 331–342
- 61 Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S. A., Mannella, C. A. and Korsmeyer, S. J. (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev. Cell* **2**, 55–67
- 62 Reers, M. and Pfeiffer, D. R. (1987) Inhibition of mitochondrial phospholipase A2 by mono- and dilysocardiolipin. *Biochemistry* **26**, 8038–8041
- 63 Hsu, F. F. and Turk, J. (2003) Electrospray ionization/tandem quadrupole mass spectrometric studies on phosphatidylcholines: the fragmentation processes. *J. Am. Soc. Mass Spectrom.* **14**, 352–363

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