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Pro-apoptotic effect of maize lipid transfer protein on mammalian mitochondria

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Abstract

To assess the effect of lipids and lipid exchange in the pro-apoptotic release of cytochrome *c*, we investigated the ability of a plant lipid transfer protein (LTP) to initiate the apoptotic cascade at the mitochondrial level. The results show that maize LTP is able to induce cytochrome *c* release from the intermembrane space of mouse liver mitochondria without significant mitochondrial swelling, similarly to mouse full-length Bid. This effect is influenced by the presence of specific lipids, since addition of lysolipids like lysophosphatidylcholine strongly stimulates the LTP-induced release of cytochrome *c* while it is inhibited by removal of endogenous free lipids with a complete suppression of the LTP-induced release of cytochrome *c*. The results are discussed in light of the possible role of lipid exchange in apoptosis.

Keywords: Lipid transfer protein; Lysolipids; Mitochondria; Apoptosis; Cytochrome *c* release

Non-specific lipid transfer proteins (nsLTPs)[1] are ubiquitous plant proteins that have been shown to bind, in vitro, various amphiphilic molecules including lysolipids and glycolipids and to facilitate in vitro transfer of phospholipids between membranes [1–4]. The subfamily of LTP called nsLTP1 possess a molecular mass around 9 kDa; they are different in sequence but share a common structural fold made of four helices stabilized by four conserved disulfide bridges (eight cysteine residues). This structure creates an internal hydrophobic cavity that possesses high plasticity, conferring the ability to accommodate the binding of different lipid molecules and fatty acids [5,6]. Some of these LTP proteins exhibit a high lipid transfer capacity, as is the case of maize LTP [7]. nsLTPs have been shown to be involved in the assembly of extracellular hydrophobic polymers [2], in plant defence and signalling [8]. Besides their role in plant metabolism, these proteins are also known to be pan-allergens of plant-derived foods [9].

Recently, a sequence similarity between Bid, a mammalian pro-apoptotic protein of the Bcl-2 family, and plant LTPs has been observed [10]. Bid is a BH3-only cytosolic protein that connects the extrinsic with the intrinsic pathways of apoptosis and acts on mitochondrial membranes [11,12]. To different extents, both uncleaved Bid and tBid facilitate the release of apoptogenic factors like cytochrome *c* from the mitochondrial intermembrane space [10–12]. The similarity with plant LTP's is not limited to some amino acids in the sequence, but extends to functional activities. Indeed, Bid proteins possess an intrinsic capacity of binding and exchanging membrane lipids [10–12]. This property has been correlated with the pro-apoptotic action of full length Bid when lysolipids are present [11,12].

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[1] *Abbreviations used:* LTP, lipid transfer protein; LPC, lysophosphatidylcholine; LPC-C16, 1-palmitoyl-LPC; LPC-C10, 1-caproyl-LPC; LPE, lysophosphatidylethanolamine; MCL, monolysocardiolipin; LPG, lysophosphatidyl-glycerol; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; FCCP, carbonyl cyanide–*p*-trifluoromethoxyphenyl hydrazone.

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The aim of this work was to verify whether a well-known plant nsLTP is able to induce cytochrome *c* release from purified animal mitochondria and display other similarities with mammalian Bid. Such similarities could provide further insights into our understanding of the relevance of this lipid exchange mechanism to cell death signalling, especially at the level of mitochondria.

Materials and methods

Purification of maize nsLTP

To purify maize nsLTP we have used the method reported for wheat nsLTP [5] with the following modifications. Soluble proteins were extracted from maize flour in distilled water (49 g in 100 ml). After stirring for 2 h at room temperature, the slurry was filtered on a Buchner funnel and then centrifuged for 30 min at 9000*g*. The supernatant was filtered and dialysed with 50 times the sample volume of 25 mM Na-acetate, pH 4.2, under continuously stirring at room temperature. After dialysis, the extract was filtered on a 0.2 μm filter and the solution was then loaded on a cation exchange chromatography column (MonoS, Pharmacia) equilibrated with 25 mM Na-acetate, pH 4.2, and the fraction containing the LTP was eluted by applying a gradient from 0 to 0.25 M NaCl in acetate buffer, pH 4.2 for 15 min at a flow rate of 0.8 ml/min. The enriched fraction was further purified on a gel filtration column Superdex 75 equilibrated with 0.1 M ammonium acetate. The fraction containing the purified nsLTP was recovered and analysed with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 15% polyacrylamide gel. The purified protein was stored either frozen or lyophilized.

Recombinant Mouse Bid was obtained from R&D systems [10,11], or as kind gifts from Dr. J.C. Martinou, University of Geneva (cf. [12]). All other reagents and chemicals were from Sigma, Avanti Polar lipids or Bio-Rad.

Isolation of mitochondria

Mouse liver mitochondria were prepared essentially as described previously [11]. After being soaked with ice-cold PBS, mouse livers were cleaned of connective tissue, cut with scissors and suspended. The cleaned tissues were homogenized with a Teflon pestle homogenizer in isolation medium (10 mM K-Hepes, 0.25 M mannitol, 1 mM EGTA, 0.2% bovine serum albumin [BSA], pH 7.4), generally containing a cocktail of protease inhibitors (0.1% [vol/vol] of P3840 [Sigma]) and then centrifuged at 600*g* for 5 min. The supernatants were filtered to remove fats and centrifuged at 10,000*g* for 10–15 min at 4 °C. The pellet containing crude mitochondria was resuspended in isolation medium without BSA and protease inhibitors and subsequently re-centrifuged at 10,000*g* for 15 min. Finally, the resulting pellet was resuspended in a minimal volume of 20 mM K-Hepes pH 7.4, 0.12 M mannitol, 80 mM KCl, 1 mM EDTA (assay buffer) and utilised for the experiments. When necessary, purified mitochondria were resuspended in assay buffer containing 0.05% fatty acid free BSA (SIGMA) to remove endogenous lipids, incubated for 10 min in ice and then centrifuged at 10,000*g* for 15 min at 4 °C and the final pellet resuspended in a minimal volume of assay buffer (without BSA). The protein content was determined with Bio-Rad Bradford mini-assay in the presence of the non-ionic detergent Triton X-100. The detergent was added to a concentrated solution of mitochondria to solubilize completely the membrane proteins; the sample was then diluted for Bradford analysis (final Triton X-100 concentration <0.002%).

Cytochrome c release assay

Mitochondria (1 mg/ml in assay buffer) were supplemented with different lysolipids and proteins (Bid and maize LTP usually at a final concentration of 0.5 μM) and cell-free assay were undertaken as described earlier [10–12]. The mixtures were incubated at 25 °C for 15 min, and mitochondria were separated by centrifugation for 10 min at 12,000 rpm in a Eppendorf minicentrifuge refrigerated at 4 °C.

The levels of cytochrome *c* in the supernatants were routinely determined by immunoblotting as described previously [11,12]. No direct interaction between nsLTP and cytochrome *c* in solution was observed.

Supernatants were subjected to 15% SDS–PAGE and then transferred to PVDF membranes. Membranes were blocked with 5% defatted dried milk in PBS containing 0.2% Tween 20 (PBST) and probed with the primary antibody (monoclonal anti-cytochrome *c*, Clone 7H8.2C12, BD Pharmingen) in PBS containing 0.05% Tween 20 and 1% defatted dried milk (PBS-ab) at room temperature for 1 h. After PBST washes, the membranes were treated with the secondary antibody (Amersham sheep anti-mouse or Dako rabbit anti-mouse HRP-conjugated antibodies) in PBS-ab for 1 h. Blots were visualized on X-ray film by means of chemiluminescence (Amersham ECL Plus Detection Kit) and was quantitated using a Bio-Rad image software. All of Western blot analyses were repeated at least five times in independent experiments.

Cytochrome *c* levels were also determined quantitatively by HPLC using a 5 μm C4 reverse-phase column (150 × 4.6 mm) on an Beckman HPLC system Gold equipped with the diode array function, using commercial cytochrome *c* standards as described earlier [13]. Horse heart cytochrome *c* (1–100 pmol) was injected in 100 μL aliquots and the linearity of the response was derived from integrating the chromatographic peak obtained.

A gradient of 20% acetonitrile in water with trifluoroacetic acid (0.1% vol/vol) to 60% acetonitrile in water with trifluoroacetic acid (0.1% vol/vol) with a flow rate of 1.0 ml/min was used. Mitochondria were pelleted by centrifugation and the resulting supernatants were injected onto the HPLC for the quantitative analysis following the absorp-

tion spectra with a diode array (maximum for cytochrome *c* in buffer containing 0.1% trifluoroacetic acid at 393 nm).

Statistics

All the quantitative data (densitometric analysis and HPLC measurements) were subjected to statistical evaluation (Student's *t* test). Probability values $P < 0.05$ have been defined statistically significant.

Intrinsic fluorescence analysis

Purified maize nsLTP was dissolved in 25 mM Na-acetate, pH 4.2, 1 mM DTT, at a final concentration of 85 μM. Changes in fluorescence intensity were measured at 25 °C with a Jasco FP-777 spectrofluorimeter using excitation wavelength at 275 nm and recording emission between 280 and 340 nm. Lysolipids dissolved in ethanol were added in a stepwise manner and fluorescence changes were measured after 2 min equilibration. Emissions were corrected for the effect of dilution and of lipids (in the absence of the protein). The intrinsic fluorescence of Bid (0.5–1 μM in assay buffer) was measured as reported previously [14].

Results

Intrinsic fluorescence studies

The lipid transfer activity of both nsLTP and Bid implies discrete, reversible binding to lipids in solution. Such binding appears to be particularly efficient with lysolipids, presumably because of their water solubility and amphipatic character [15,16]. Maize nsLTP possesses two tyrosine residues in its amino acid sequence. The fluorescence of these aromatic side chains is sensitive to lipid binding [15,16] and has been utilized here as a sensitive method to verify the functional binding of lipids to the purified protein, necessary for the subsequent cell-free assays.

When the purified maize LTP (Fig. 1 shows the maize flour extract and the final pure chromatographic fraction of nsLTP) was incubated in the presence of lysolipids like C16-LPC, the intensity of tyrosine fluorescence emission increased as a function of LPC concentration (Fig. 2A), reaching apparent saturation levels that approximately corresponded to stoichiometric concentrations with respect to the protein (Fig. 2B). These results were compared with data obtained with Bid to verify similar LPC-dependent changes in intrinsic fluorescence that would be indicative of lipid binding. Mammalian Bid contains a single tryptophan residue, the fluorescence of which dominates the intrinsic fluorescence spectrum of the protein that includes a discernable contribution by a few tyrosines, especially around 300 nm (Fig. 2C, cf. [14]). Incubation with selected lysolipids increased the fluorescence intensity of Bid, which in the case of C16-LPC was especially evident in the region of tyrosine emission (Fig. 2C). LPC-induced changes in the emission of Bid aromatic residues resembled those seen with nsLTP and the same lipid ligand (Figs. 2A and C left panel). Interestingly, the spectral changes associated with LPC binding were significantly lower in a Bid mutant lacking a functional BH3 domain (cf. [12]) than in the wild type protein, in particular at low concentration of the lipid ligand (Fig. 2C, right panel).

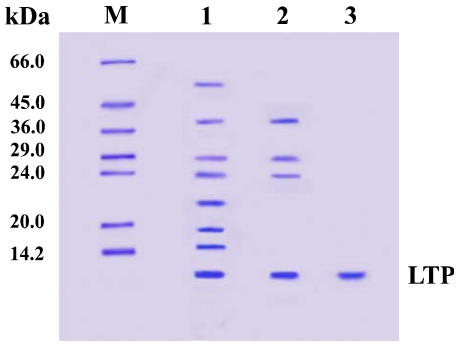

Fig. 1. SDS–polyacrylamide gel electrophoresis of maize nsLTP. From left to right: lane M, molecular mass standards; lane 1, maize flour extract; lane 2, fraction containing nsLTP from cation exchange chromatography; lane 3, pure nsLTP obtained after gel filtration chromatography.

The observed similarity in lipid binding properties prompted us to investigate whether nsLTP could mimic the pro-apoptotic action of Bid with isolated mitochondria, which has been shown to be modulated by LPC and other lysolipids [11,12,14].

Induction of cytochrome c release

Isolated intact mitochondria were utilised as the standard model for comparing the pro-apoptotic action of Bid and maize nsLTP. The release of cytochrome *c* was studied with these proteins, either alone or in the additional presence of exogenous lipids. In particular, lysolipids chemically or metabolically related to phosphatidylcholine (PC) were studied, since it has been demonstrated that Bid can show a specificity in binding to LPC species [11,17], while nsLTPs are also reported to bind to the same lysolipids (Fig. 2, cf. [15,16]).

In the first set of experiments, we determined whether maize nsLTP could promote the release of cytochrome *c* from mouse liver mitochondria. Purified maize nsLTP was found to produce a significant increase in the basal level of cytochrome *c* that leaks out of isolated mitochondria (Fig. 3A). Subsequently, we compared the pro-apoptotic activity of the nsLTP with that of various preparation of mouse Bid, including tBid, which is the most active form of the protein (Fig. 3B). The results showed that maize nsLTP appear to be more efficient than the full length Bid at the same final concentration, but clearly less active than tBid (in the absence of exogenous lipids). Of note, results obtained by immunoblotting (analysed quantitatively by densitometry, Figs. 3C and D) were independently confirmed by the HPLC method of detecting cytochrome *c* (Fig. 3E, cf. [13]).

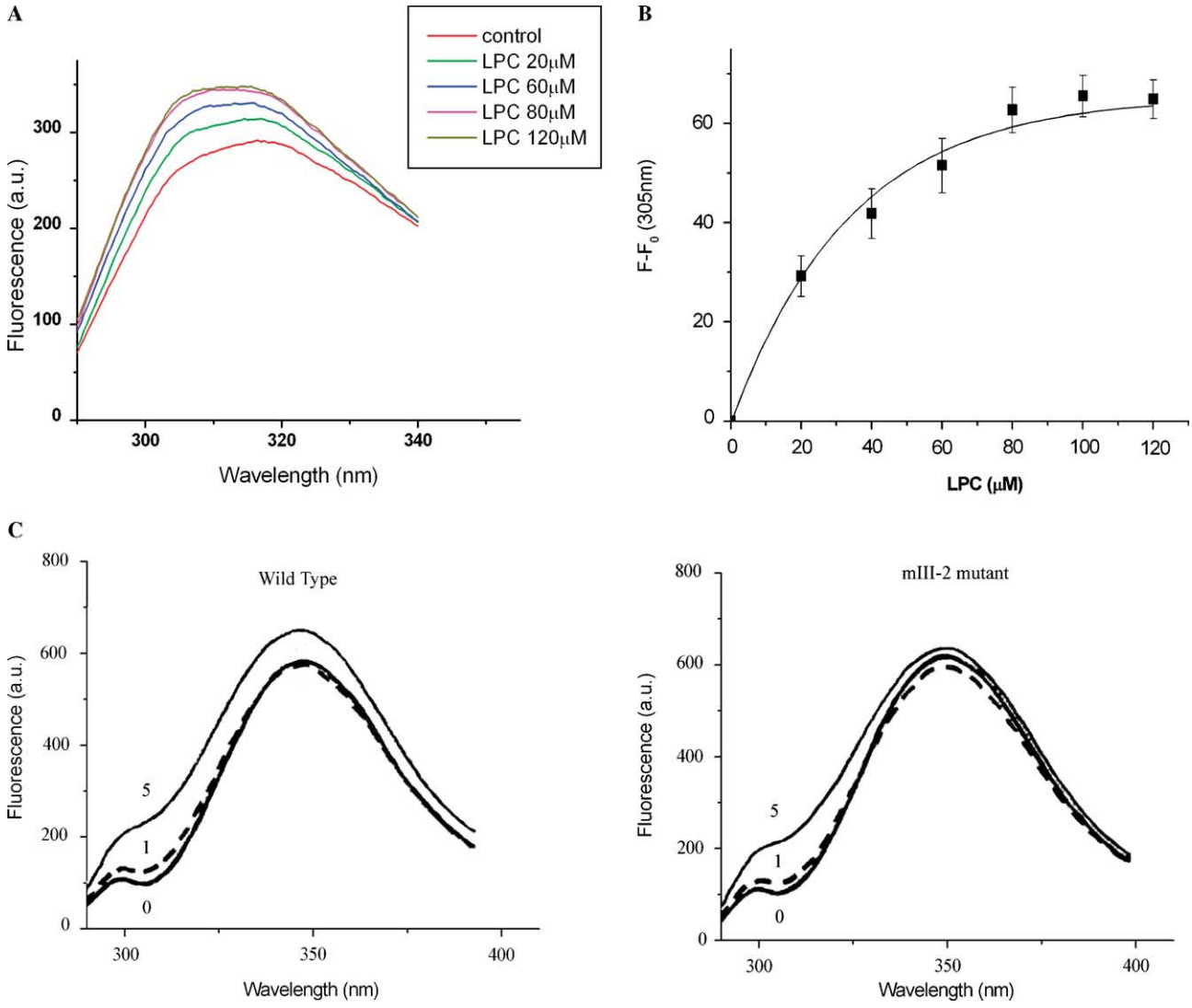

Fig. 2. Binding analysis of lysophospholipids (LPC-C16) monitored through tyrosine and thryptophan fluorescence changes. (A) Intrinsic tyrosine fluorescence spectra of maize nsLTP (85 μM) in the presence of different concentration of LPC-C16. LPC was added from a concentrated solution in ethanol to 1 ml cuvette; after binding equilibration was reached, the fluorescence spectra were recorded. Data were corrected for dilution. Results are representative of five independent measurements. (B) Variation of nsLTP fluorescence intensity as a function of the lysolipid concentration. Mean normalized $F - F_0$ values ± SD are shown (for five independent experiments). (C) Intrinsic fluorescence of Bid, either wild type (left panel) or mutated (having a non-functional BH3 domain, [12]; right panel). LPC-C16 concentrations are indicated by the numerals above the spectra; the spectrum obtained in the presence of 1 μM lysolipid is dashed.

Studies were then conducted in the presence of diverse exogenous lysolipids. With LPC C16, nsLTP was more effective in promoting cytochrome *c* release than in the absence of lipids (Fig. 4A). In this case, maize nsLTP was at least twofold more effective than full length Bid (as measured by the relative intensity of the cytochrome blots). Expanding our studies to other lysolipids, we found that the release of cytochrome *c* in the presence of lysophosphatidylethanolamine (LPE) (a natural mixture with acyl chains ranging from C14 to C18) was relatively high with Bid but negligible, if any, with nsLTP (Fig. 4B). The plant protein thus exhibited more selectivity than Bid in its lysolipid-dependent capacity of releasing cytochrome *c*, as further sustained by data obtained with MCL and LPG (not shown).

To study the role of the acyl chain in the pro-apoptotic activity mediated by nsLTP, we combined the plant protein with LPC species with different acyl length, e.g., palmytoyl (C16) and caproyl (C10) acyl chains. The results (Fig. 4C) showed that the extent of nsLTP-stimulated release of cytochrome *c* was comparable with these different LPC species. Of note, at the concentrations used the added LPC species had no significant effect on the basal level of cytochrome *c* release (i.e., negative controls in Fig. 4, cf. [10,12]).

It is important to observe that maize nsLTP was able to induce cytochrome *c* release even in the absence of exogenous lipids (Figs. 3A and B); this could be due to the presence of endogenous lipids within the mitochondrial preparation, perhaps released from endocellular membranes. To verify this possibility, we repeated the

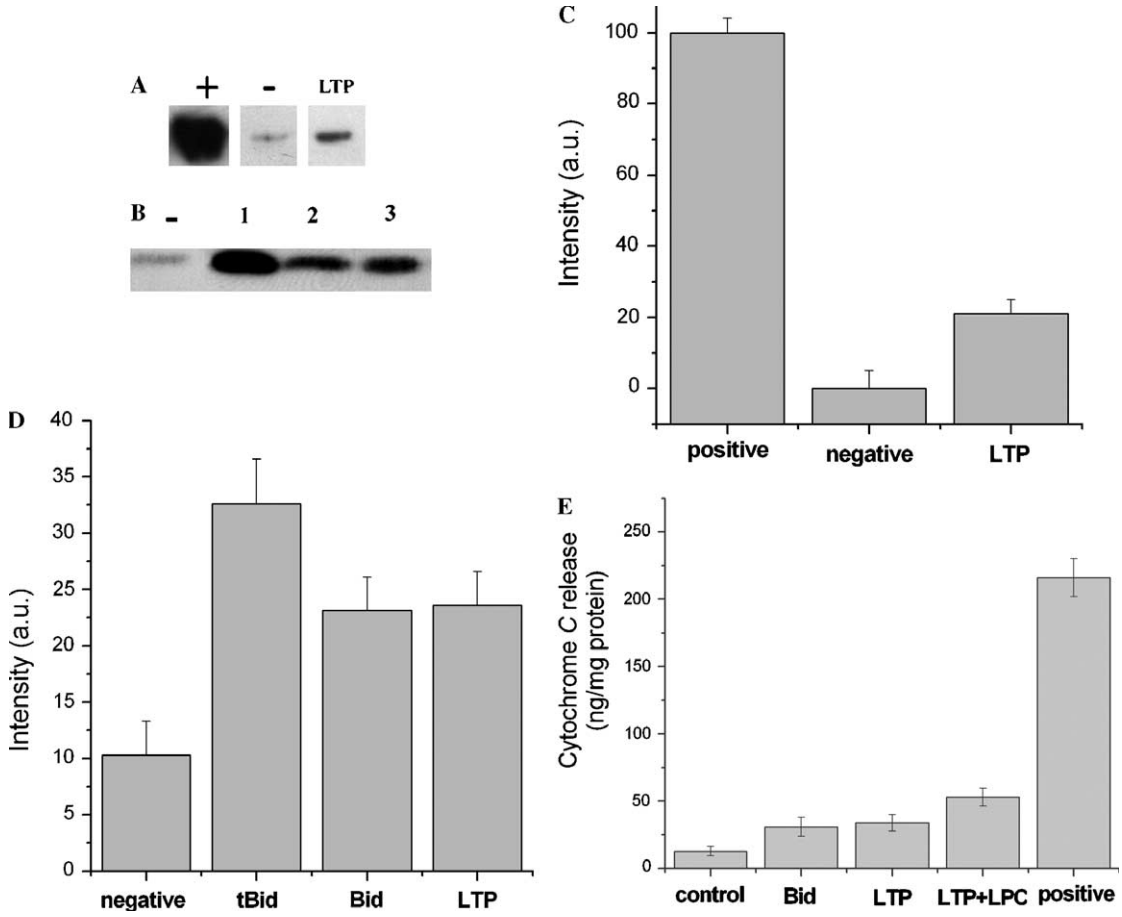

Fig. 3. Analysis of cytochrome *c* release from mitochondria (1 mg/ml) induced by purified maize nsLTP and by mouse recombinant Bid at a final concentration of 0.5 μM. (A) Pure maize nsLTP enhances cytochrome *c* release. (+) control mitochondria solubilized with 5% SDS; (−) mitochondria, negative control; (LTP) mitochondria incubated with purified maize nsLTP. (B) Comparison of the effect induced by nsLTP, tBid and Bid. (−) mitochondria negative control; (1) tBid; (2) full length Bid; (3) maize nsLTP. (C and D) Histograms report the densitometric analysis of a set of blots as shown in (A) and (B), respectively; data are the means ± SD for five independent experiments and statistical evaluation (with *t* test) indicated significant differences with respect to the negative control ($P < 0.05$). (E) HPLC detection of cytochrome *c* release from intact mitochondria in the presence of nsLTP or Bid. After incubation, mitochondria were centrifuged and the supernatant was withdrawn, filtered, then injected into the HPLC instrument for determination of cytochrome *c* levels. Control samples as in (A) and (B). Each value represents the means ± SD of the cytochrome *c* measurements of four independent determinations; statistical analysis (*t* test) indicated significant differences vs. the control for all samples ($P < 0.05$).

experiments in Fig. 3 using mitochondria washed with BSA to remove free lipids and fatty acids. No significant LTP-induced release of cytochrome *c* was observed when lipids were removed by this procedure (Fig. 5, lane 4); addition of exogenous lipids cause again cytochrome *c* release only when the LTP protein is present (Fig. 5, lanes 3 and 5). The BSA protein alone or with exogenous lysolipids did not cause any change in the basal level of cytochrome *c* (not shown). These results thus indicated that the effects of nsLTP were unlikely to derive from non-specific perturbation of mitochondrial membranes but depended upon presence of (lyso)lipids.

It should be pointed out that BSA is necessary during the tissue homogenisation process to protect the cytoplasmic organelles from membrane damage due to the high concentration of free lipids coming from the tissue, as well as to reduce the extent of proteolysis by lysosomal enzymes liberated by cell disruption. We cannot exclude the possibility that the routine addition of BSA in the first step of mitochondria preparation may also reduce the pro-apoptotic effect of nsLTP.

Non-specific modification of the mitochondrial membrane permeability induced by maize nsLTP was also excluded as no mitochondrial matrix swelling was observed (by monitoring the decrease in light scattering of the mitochondrial suspension) when mitochondria were treated in the same conditions utilized for the study of the efflux of cytochrome *c* (results not shown).

Discussion

Uniquely among Bcl-2 proteins, pro-apoptotic Bid displays an intrinsic capacity of binding and exchanging membrane lipids [10–14,17,18]. One of the mechanisms that have been proposed for the action of Bid on mitochondria involves this capacity [10,12]. The aim of this work was to verify whether other proteins with lipid transfer activity share the pro-apoptotic capacity of

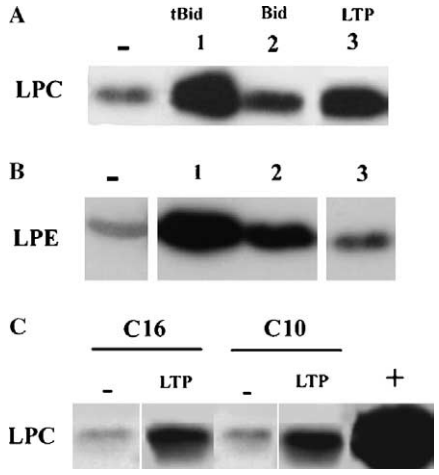

Fig. 4. Cytochrome *c* release induced by Bid, tBid, and purified maize nsLTP in presence of different lysolipids. Results are representative of five independent experiments. Mitochondria were incubated with 0.5 μM purified maize nsLTP in the absence or in the presence of 1 μM lysolipids: lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), 1-palmitoyl-LPC (16) or 1-caproyl-LPC (10). (A and B) (1) recombinant tBID; (2) full length recombinant Bid; (3) maize nsLTP (LTP). (C) comparison between the action of LPC-C16 and LPC-C10 in the presence of maize nsLTP, or without (−); (+) positive control as in Fig. 3.

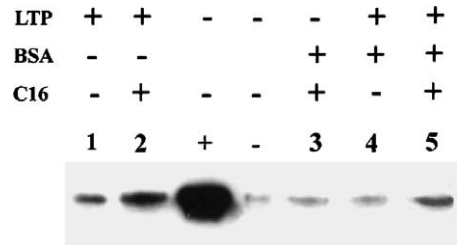

Fig. 5. Analysis of nsLTP induced cytochrome *c* release from control (lanes 1 and 2) and BSA-washed mitochondria (lanes 3–5) with or without the addition of exogenous LPC-C16 (C16). Positive (+) and negative (−) controls as in Fig. 3. Results are representative of five independent experiments.

releasing cytochrome *c* from mitochondria. To this end, we have used a plant lipid transfer protein, not involved in plant cell death, which is known to efficiently bind and exchange (lyso)lipids in vitro [15,16]. A sequence similarity was previously noted between this protein and Bid [10].

We did find that purified maize nsLTP induces the release of cytochrome *c* in the presence of different lipids, alike Bid (Figs. 3 and 4). To our knowledge, this is the first demonstration that plant nsLTP can have a membrane perturbing action equivalent to the pro-apoptotic capacity of established death proteins like Bid. This activity is dependent on the presence of specific lipids. Addition of lysolipids, like LPC, strongly stimulated the LTP-induced release of cytochrome *c*, with some similarities, as well as differences with Bid in regard to the chemical specificity of the effects (Fig. 4 cf. [10,12]). Conversely, removal of endogenous lipids with BSA completely suppresses LTP-induced release of cytochrome *c* (Fig. 5). Of note, the pro-apoptotic capacity of nsLTP to promote the release of cytochrome *c* did not derive from non specific perturbation induced by the plant protein on target membranes. Indeed, nsLTP did not induce any significant swelling of mitochondria when compared with mitochondria treated with the uncoupling agent FCCP, nor cytotoxic effect on human sarcoma cells under the conditions promoting the release of cytochrome *c* (results not shown).

On the other hand, we note that the three dimensional structures and the folding pattern of Bid and maize nsLTP [16–18] are rather different; while the regions showing sequence similarity [10] cannot be superimposed. This suggests that the similar action of these two proteins on mitochondria may depend more on their biochemical activity of lipid transfer than on common structural determinants underlying lipid binding.

How lipid transfer can enhance the permeability of the outer mitochondrial membrane and the consequent release of apoptogenic factors like cytochrome *c* is not known in detail. The specific binding to LPC with consequent enhancement of the pro-apoptotic activity that is common to both Bid and nsLTP (Figs. 2 and 4) suggests that any significant increase in the cellular levels of LPC (for example, due to increased activity of cytosolic lipases, cf. [22]) will lead to a Bid-mediated accumulation of this potentially toxic lipid in the outer mitochondrial membrane [10,12,17–21]. Local LPC elevation may perturb membrane integrity, thus contributing to a mitochondrial 'priming' for the membrane permeabilization promoted by pro-apoptotic proteins like Bax and Bak [10,23]. In several models of cell death activation of phospholipases has been documented (e.g. [22]; see [23,24] for a review). Because PC is the major phospholipid in mammalian cells, its degradation by apoptosis-stimulated lipases will inevitably lead to increased levels of cytosolic LPC, thereby providing the substrate for the transfer activity of Bid (and possibly other lipid shutting proteins) directed to mitochondria. In this scenario, plant LTP's could play a similar role, if expressed within mammalian cells—an intriguing possibility we will explore in the future.

In any case, the present results further highlight the concept that the dynamic capacity of exchanging lipids, which is intrinsic to diverse lipid transfer proteins, could alter the surface composition of the mitochondrial lipid membrane. This may represent a common biochemical mechanism of promoting pro-apoptotic reactions relevant to diverse forms of mitochondria-dependent death.

Acknowledgments

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