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Sequence and functional similarities between pro-apoptotic Bid and plant lipid transfer proteins

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Abstract

Pro-apoptotic proteins of the Bcl-2 family are known to act on mitochondria and facilitate the release of cytochrome *c*, but the biochemical mechanism of this action is unknown. Association with mitochondrial membranes is likely to be important in determining the capacity of releasing cytochrome *c*. The present work provides new evidence suggesting that some pro-apoptotic proteins like Bid have an intrinsic capacity of binding and exchanging membrane lipids. Detailed analysis indicates a significant sequence similarity between a subset of Bcl-2 family proteins including Bid and Nix and plant lipid transfer proteins. The similar structural signatures could be related to common interactions with membrane lipids. Indeed, isolated Bid shows a lipid transfer activity that is even higher than that of plant lipid transfer proteins. To investigate the possible relevance of these structure–function correlations to the apoptotic action of Bid, cell free assays were established with isolated mitochondria, recombinant Bid and a variety of exogenous lipids. Micromolar concentrations of lysolipids such as lysophosphatidylcholine were found to change the association of Bid with mitochondria and also stimulate the release of cytochrome *c* promoted by Bid. The changes in mitochondrial association and cytochrome *c* release were enhanced by the presence of liposomes of lipid composition similar to that of mitochondrial membranes. Thus, a mixture of liposomes, mitochondria and key lysolipids could reproduce the conditions enabling Bid to transfer lipids between donor and acceptor membranes, and also change its reversible association with mitochondria. Bid was also found to enhance the incorporation of a fluorescent lysolipid, but not of a related fatty acid, into mitochondria. On the basis of the results presented here, it is hypothesised that Bid action may depend upon its capacity of exchanging lipids and lysolipids with mitochondrial membranes. The hypothesis is discussed in relation to current models for the integrated action of pro-apoptotic proteins of the Bcl-2 family.

Keywords: Mitochondria; Apoptosis; Bid; Bcl-2; Nix; Protein sequence similarity; Lipid transfer; Cytochrome *c*

Abbreviations: BH3, Bcl-2 homology domain 3; BODIPY-PA, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate; BODIPY-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; EST, expressed sequence tag; LIP, lipid interacting protein region; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LTP, lipid transfer protein; LUV, large unilamellar vesicles (liposomes); OG, octylglucoside; OM, outer mitochondrial membrane; PC, phosphatidylcholine; PDB, Protein Data Bank; PT, permeability transition

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1. Introduction

Recently, it has been established that many pathways of apoptosis involve alterations in mitochondrial function that revolve around the release of cytochrome *c* from mitochondria into the cytosol [1]. The release of cytochrome *c* can occur when the integrity of the mitochondrial outer membrane (OM) is disrupted, for instance due to swelling of the matrix consequent to the opening of a permeability transition (PT) pore in the inner membrane [1,2]. The concept that apoptosis signals induce PT opening with consequent bioenergetic impairment [2] seems to be contradicted by the emerging evidence that cytochrome *c* release induced by apoptosis promoters such as Bid and Bax occurs either before, or in the absence of, measurable changes in PT [3–6]. The early signalling of apoptosis is regulated by Bcl-2 family proteins which either suppress or promote the release of cytochrome *c* [1,7,8]. Anti-apoptotic proteins like Bcl-2 and Bcl-x_L normally reside on the OM, while pro-apoptotic proteins such as Bax and Bid are predominantly cytosolic, and translocate to mitochondria during apoptosis signalling [3,9–17]. To date, the structural determinants for the association of these proteins to mitochondria are not well defined.

Standard methods of sequence comparison have indicated that many of the apoptosis promoter proteins show sequence similarity with Bcl-2 only in the BH3 domain, an amphipathic helical region of about ten amino acids [7,9–11]. Mutational analysis has shown that this BH3 domain is crucial for the biological function of apoptosis promotion, and in part also for reciprocal binding among different members of the family [7,10–14]. Full-length Bid has a global structure resembling that of the anti-apoptotic Bcl-x_L, with the BH3 domains of both proteins forming a partially buried helix [18,19]. Thus, neither sequence nor structural results are able to explain why Bid and other proteins of the Bcl-2 family promote apoptosis by facilitating cytochrome *c* release, while the anti-apoptotic members of the same Bcl-2 family block the release of cytochrome *c*. Moreover, it is not clear whether different pro-apoptotic proteins of the 'BH3-only' subfamily have alternative modes of action that may be related to their divergent sequence.

Pro-apoptotic proteins including Bax [4–6,20,21], Bid [3,6,21–24] and Nix/BNIP3L [25] interact with isolated mitochondria and facilitate the release of cytochrome *c*. Of these, Bid appears to be the most potent, acting at nanomolar levels especially after cleavage by caspase-8 [6,22,24,26]. The molecular reasons for the differential potency of pro-apoptotic proteins are unknown. However, it is likely that binding to mitochondrial phospholipids is important for the cytochrome *c*-releasing capacity of Bid, which shows a lipid-dependent distribution in intracellular organelles [24]. The evidence presented here expands this concept further. Bid and a few other 'BH3-only' proteins like Nix show local similarity, in a region which includes the BH3 domain but extends to several amino acids on either side of it, with plant proteins which transport lipids, the lipid transfer proteins (LTP). Similarity in sequence correlates with similarity in function, for Bid transfers lipids in a manner that resembles that of plant lipid transfer proteins. Like LTP, Bid also interacts with lysolipids, which enhance its capacity of releasing cytochrome *c* from isolated mitochondria. It is discussed how transfer of lysolipids could be important in the biochemical mechanism of cytochrome *c* release by pro-apoptotic proteins of the Bcl-2 family.

2. Materials and methods

2.1. Sequence analysis

Sequence similarities of the Bcl-2 family proteins were computed by considering a database of peptides centred at Gly-94 of human Bid that extended several residues at either side of the BH3 domain (Fig. 1A). To focus the analysis around the BH3 domain, the peptides examined were aligned to Ala-84 which initiates helix 2 in Bcl-x_L [27] (Fig. 1B). Sequences were taken from available databank entries, including some recently deposited EST such as those of chicken Bid. Nip3 and Nix, also called BNIP3L [25], were aligned using iterative sequence comparisons due to their weak sequence similarity to Bcl-2 family proteins. An optimised alignment was obtained with a shift of 7 positions towards the N-terminus with respect to the alignments reported previously [16,17,25], as shown in Fig. 1B. A 'BH3

database' was then compiled with 40 aligned BH3-containing peptides.

To perform a systematic sequence comparison, different methods were used, including an in house program for local sequence analysis in membrane proteins [28,29]. These algorithms are based on the matrix of Levin and Garnier [30], which has a less compressed spread of pair-wise scoring than the BLOSUM matrices [31] for rare residues such as cysteine – a characteristic of special significance in the present analysis because LTP proteins have several conserved cysteines [32]. The programs BLASTP [33] and ALIGN [34] were obtained from Web sources.

The 'LTP database' was constructed with peptides from 40 different LTP proteins aligned to residues Lys-51 to Lys-74 in wheat LTP (Fig. 1). To evaluate the statistical significance of sequence similarity with the LTP database, peptides from unrelated proteins – including those like drosomycin which show diverse similarities with LTP (e.g., a large proportion of basic residues, antimicrobial properties and prevalent α-helical structure) – were analysed using an in-house program [28]. Score values above 9, indicative of structural resemblance [30], had a low random frequency with the LTP data base (around 0.3%). Hence, a test peptide showing one or more scores above 9 could be considered to have a high probability of sequence similarity with the target region of LTP proteins.

2.2. Assays of lipid transfer

Assays of lipid transfer between donor and acceptor liposomes were carried out using protocols similar to those routinely used with plant lipid transfer proteins [24,35,36]. The fluorescent lipid probes, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-HPC) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate (BODIPY-PA), were used at a final concentrations of 20–40 nM in assay buffer (0.02 M K-HEPES, 0.12 M mannitol, 0.08 M KCl, 1 mM EDTA, pH 7.4) [24]. Donor liposomes were prepared by ethanol injection or sonication of 1:3 (w/w) mixtures of lipid probes and other phospholipids, composed of equal proportion of phosphatidylcholine and negatively charged lipids such as phosphatidylserine and phosphatidylinositol. Acceptor liposomes were usually prepared as large unilamellar vesicles (LUV) [37] with a lipid composition similar to that of the donor liposomes, but with the further addition of 10% of cardiolipin [24]. Measurements were undertaken with a Perkin Elmer LS50B fluorimeter with excitation at 490 nm and emission at 515 nm (with a 10 nm bandwidth). Fluorescence was calibrated for the quantum yield of the probe in the presence of lipids as described previously [36].

2.3. Cell-free assays of Bid distribution and cytochrome c release

Mouse liver mitochondria, prepared as described earlier [24], were suspended at a final concentration of 1 mg/ml in assay buffer supplemented with a cocktail of protease inhibitors, and co-incubated with 2–10 nM of recombinant mouse Bid (purchased from R&D Systems) for 15–20 min at room temperature (ca. 22°C). Various lipids and liposomes were added to the mixture, and mitochondria were then separated by centrifugation [24]. For each sample, an equivalent aliquot of mitochondrial pellet and supernatant was analysed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting using polyclonal anti-Bid antibodies and, when required, re-blotted with a monoclonal cytochrome *c* antibody (7H 8.2C12 Pharmingen) (cf. [24]).

2.4. Incorporation of BODIPY-lipids in mitochondria

Incorporation of lysolipids and fatty acids into mitochondria was evaluated using BODIPY-based fluorescent lipids (Molecular Probes). Bis-BODIPY-F5-PC, a phospholipid probe containing two acyl-BODIPY groups [38], was hydrolysed with pancreatic phospholipase A2 (Sigma) for 1 h in a mixture containing 1 mM $CaCl_2$. Subsequently, the mixture was supplemented with 5 mM EGTA and the phospholipase separated by gel chromatography. The fluorescent products were then incubated with mitochondria in the absence or presence of recombinant Bid (20–40 nM) and the mitochondria separated by centrifugation as in the cell free assay. Parallel experiments were conducted using 20 μM of C12-BOD-

IPY, a fatty acid probe. The mitochondrial pellet was dissolved in assay buffer containing 0.5% Triton-X-100 and diluted 5-fold in a cuvette to measure the fluorescence recovery of the BODIPY probe using the complete emission spectrum. Fluorescence was normalised to the level obtained in the absence of Bid after subtraction of the background fluorescence of a mitochondrial blank [24].

3. Results

3.1. The BH3 region of Bid shows sequence similarity with plant lipid transfer proteins

While an increasing number of proteins have been identified as Bcl-2 family members solely on the basis of sequence similarities with the BH3 domain, e.g., [39], it is maintained that pro-apoptotic proteins of the Bcl-2 family are unrelated to other types of proteins [7,10]. However, a standard BLASTP [33] search with the PDB database indicates that the sequence of human Bid apparently shows local similarity with a region of wheat lipid transfer protein (LTP) (Fig. 1A). This similarity encompasses the BH3 domain (Fig. 1B), which is crucial for the pro-apoptotic activity [3,5,9–11]. Sequence similarities such that presented in Fig. 1A may be fortuitous, given that both Bid [18,19] and LTP proteins [32,35] contain many amphipathic helices. A systematic analysis was thus undertaken to examine whether the local sequence similarity between Bid and wheat LTP had statistical significance. Databases were formed that contained the known sequence variation in a 21-residues region surrounding the BH3 domain of Bcl-2 proteins and the corresponding C-terminal region of LTP proteins (Fig. 1). This protein region was named LIP (Lipid Interacting Protein region, noted by the box in Fig. 1B) for it partially overlaps the central part of Bid which is involved in the binding to cardiolipin [40].

Table 1
Similarity scores in the LIP region of Bcl-2 family proteins with the LTP database

Protein and species		Number of scores above 9	Maximal score, with
Bid	Human	10	21, wheat NLTA
Bid	Cattle	5	15, wheat NLTA
Bid	Mouse	1	13, wheat NLTA
Bid	Chicken	3	11, maize NLTP
Nix	Human	26	20, maize NLTP
Nix	Zebrafish	14	16, maize NLTP
Nip3	Human	14	15, rice NLT1
Bax	Human	4	11, cotton NLT6
Bax	Zebrafish	None	–
Bak	Human	None	–
Bcl–x_L	Human	None	–

Table 1 summarises the results of the systematic sequence comparison of Bcl-2 family proteins with the LIP region of 40 LTP proteins. For reference, the average value of the similarity scores between the Bid peptides and those of Bcl-2 proteins was 9 (not shown). Not only Bid, but also Nix and its related Nip3 showed many scores higher than 9 with the LTP database (Table 1). Strikingly, the top scores (21 between human Bid and wheat LTP, and 20 between Nix and maize LTP) were higher than any score obtained with the BH3 database (results not shown). With the possible exception of human Bax, other pro-apoptotic proteins of the Bcl-2 family did not show equivalent scores of sequence similarity, while all the anti-apoptotic proteins of the Bcl-2 family exhibited no score above 7 (Table 1 and results not shown). Thus, the high scores of sequence similarity with plant LTP could reflect signatures peculiar to a subset of pro-apoptotic proteins including Bid, Nix and Nip-3. In further support for this possibility, the extension of the above analysis to all the Bax and Bak sequences which are now available, including *Drosophila* Drob-1/Dbcl, and also to Noxa [39] and Puma [41], revealed no significant similarity with the LTP database (Table 1 and data not shown).

3.2. Bid has a lipid transfer activity comparable to that of plant lipid transfer proteins

Bid displays lipid transfer activity under conditions equivalent to those of the assay of cytochrome *c* release [24]. To extend these observations in relation to the sequence similarities presented here (Fig. 1), LTP activity was assayed with fluorescent lipids using the same protocols previously applied to the measurement of lipid transfer by plant LTP [36]. In the first protocol, the de-quenching of the concentrated BODIPY-lipid was monitored after addition of ex-

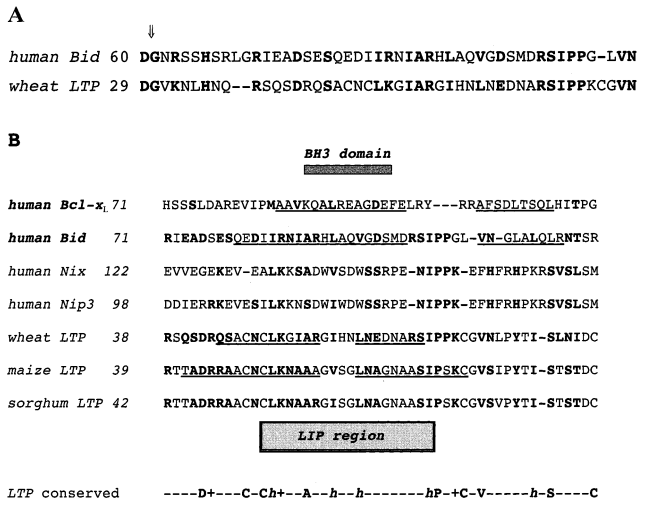

Fig. 1. Alignment of Bid and other pro-apoptotic proteins to plant LTP proteins. (A) BLASTP [33] results using human Bid sequence as a query against the Protein Data Bank (PDB). Residues in bold are either identical or very similar (i.e. basic, acidic or large and hydrophobic). The arrow indicates the major position at which caspase-8 cleaves Bid to produce tBid [3]. (B) Optimised alignment of Nip3 and Nix with Bid and Bcl-x_L, and with cereal LTP proteins. Underlined are the known α helices as determined by solution structure. Residues in bold are identical or show positive scores in the BLOSUM62 scale [31] when comparing pro-apoptotic and LTP proteins. *h* indicates hydrophobic residues conserved in all LTP proteins. The LIP region that has been used for the systematic sequence analysis (see Section 2 and Table 1) is noted by the box beneath the alignment.

cess of acceptor liposomes either in the absence or presence of Bid (cf. [24,36]). This protocol was most suitable when the spontaneous rate of lipid transfer was small in comparison to the Bid-catalysed rate, as shown in Fig. 2A. Alternatively, the lipid probe was equilibrated with acceptor liposomes first and subsequently Bid was added to promote further lipid transfer (Fig. 2B, cf. [36]). Fresh preparations of recombinant mouse Bid exhibited transfer rates ranging from 2.5 (with BODIPY-PA, Fig. 2A) to 7.2 (with BODIPY-PC, Fig. 2B) nmol/min per mg of protein. These rates are comparable to, and in some cases even higher than, those reported for various plant lipid transfer proteins [32,35,36].

3.3. *Bid association with mitochondria is influenced by lipids*

The noted similarities in local sequence (Fig. 1) and lipid transfer activity (Fig. 2) inevitably led to the question of what relevance could they bear to the biochemical action of Bid on mitochondria. While plant LTP may have multiple physiological roles, including defence mechanisms against plant pathogens (see [32] for a review), the biological function of Bid and related proteins appears to be clear. They sense apoptotic stimuli and then move to mitochondria for facilitating the release of cytochrome *c*. So, the simplest possibility would be that the structure and function similarities with plant LTP reflect com-

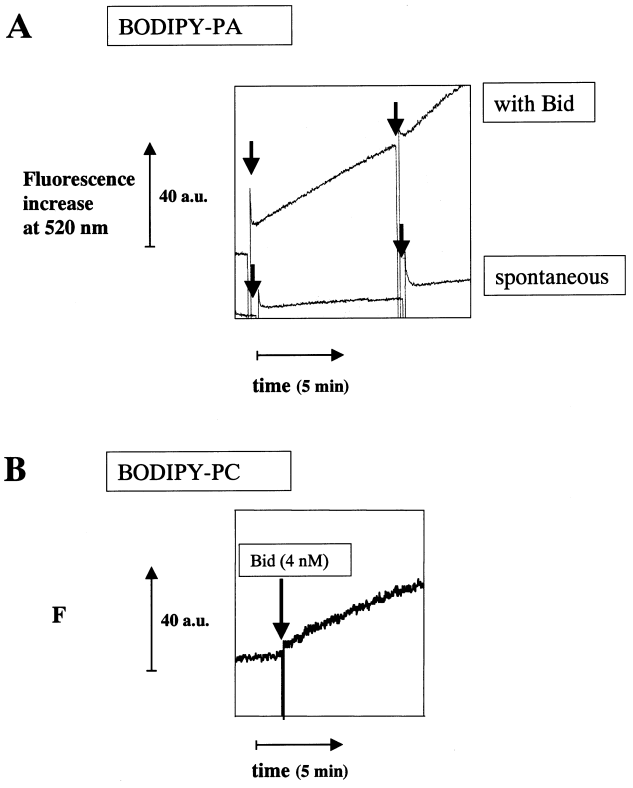

Fig. 2. Lipid transfer activity of Bid with different lipid probes. (A) Donor liposomes (20 nM BODIPY-PA) were equilibrated in the absence (bottom trace) and presence (top trace) of 10 nM recombinant mouse Bid. Subsequently, the fluorescence changes were recorded following two consecutive (arrows) additions of 1 μg/ml of acceptor liposomes. After the second addition, the initial rate of Bid-stimulated lipid transfer was calculated to be 2.5 nmol/min per mg protein. (B) Lipid transfer activity of Bid was evaluated with BODIPY-PC (40 nM) using an alternative protocol (see text and [36]), in which donor and acceptor liposomes (LUV) were equilibrated together for 20 min to allow completion of spontaneous transfer. Subsequently, 4 nM recombinant mouse Bid was added to stimulate further transfer of the lipid probe.

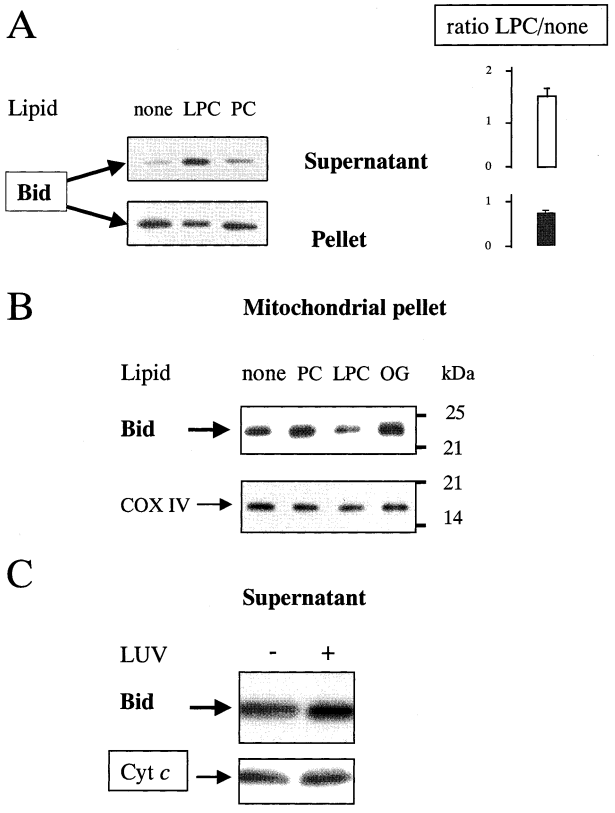

Fig. 3. Screening of lipids that change the mitochondrial association of Bid. Cell-free assays were carried out with 1 mg/ml of mouse liver mitochondria incubated for 20 min with recombinant mouse Bid (2 nM in A,B, and 7 nM in C); after centrifugation at $10\,000\times g$ for 15 min, equivalent amounts of the supernatant and mitochondrial pellet were analyzed by SDS–PAGE and blotted for Bid as described earlier [24]. LPC, PC (both from egg yolk) and OG were added at 2 μM from ethanol solutions (equivalent volumes of ethanol had no effect, see also Fig. 4B). (A) Bid was blotted in either supernatant (top panel) or pellet (bottom panel) after incubation with the indicated compounds. The histograms on the right of the panel represent the quantitative evaluation of the relative intensity of the blots as obtained by densitometric analysis with the AIDA software (Raytest, Germany). The blots obtained with LPC showed a statistically significant change in both the supernatant and pellet with respect to the control blots ($P<0.02$, $n=5$). (B) The level of recombinant Bid recovered in the pellets (top panel) was estimated in comparison with the detection of subunit IV of cytochrome oxidase (COX IV, bottom panel), a standard mitochondrial marker that was blotted with monoclonal antibodies (Molecular Probes). The position of molecular size markers is noted on the right of the panels. (C) Bid was incubated either in the absence or presence of 1 mg/ml of large unilamellar vesicles (LUV) and its level remaining in the supernatant was evaluated as in A (top panel). The blotting membrane was then re-probed with a cytochrome *c* antibody (bottom panel).

mon lipid interactions underlying the mitochondrial association of Bid.

To verify this possibility, a screening system was developed from the standard cell-free assay of cytochrome *c* release (cf. [6,24]). Several lipid compounds were tested for their capacity to change the association of recombinant mouse Bid with mouse liver mitochondria. Under the conditions of the assay, Bid was largely recovered in the mitochondrial pellet and most lipids had little effect on this recovery (Fig. 3A and data not shown). However, micromolar concentrations of lysophosphatidylcholine (LPC) promoted

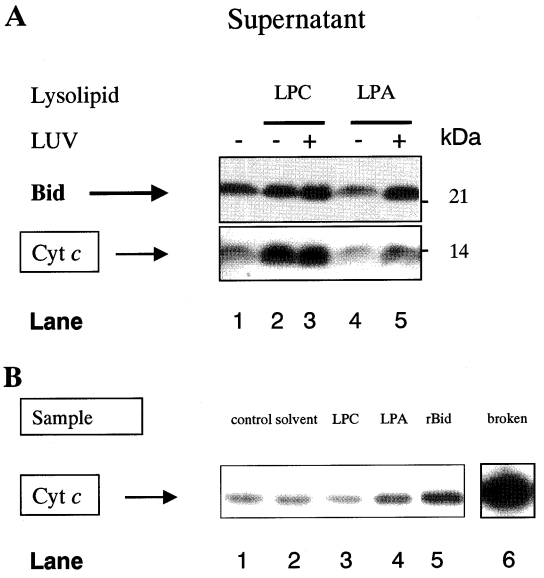

Fig. 4. Lysolipids enhance Bid-induced release of cytochrome *c*. Mitochondria were incubated with 7 nM recombinant Bid as in the experiment in Fig. 3C in the absence or presence of 2 μM lysolipids and, when indicated, of 1 mg/ml LUV. (A) The top panel shows the blot of Bid remaining in the supernatant; the relative increase or decrease of the protein band was mirrored by complementary changes in the blots of the mitochondrial pellet, as in Fig. 3A. The bottom panel shows the levels of cytochrome *c* released in the supernatant of control mitochondria (lane 1), in the presence of 1-oleyl-LPC or 1-oleyl-LPA alone (lanes 2 and 4, respectively) or together with LUV (cf. Fig. 3C, lanes 3 and 5). The results are representative of four replicate experiments and the average values obtained with LPC showed differences of high statistical significance (not shown). (B) Basal levels of cytochrome *c* release were evaluated with no addition (lane 1), after addition of an equivalent volume of the solvent carrier (lane 2), with either 2 μM LPC (lane 3) or LPA (lane 4) as in A, and finally with recombinant Bid (cf. A, lane 5). Lane 6 contained a positive control for cytochrome *c* release, which was obtained by breaking the OM with a freeze–thaw cycle.

a substantial redistribution of Bid from the pellet to the supernatant (Fig. 3A). The decrease in mitochondrial association was specifically produced by LPC, since identical concentrations of either diacyl PC or the synthetic detergent octyl-glucoside (OG) (used to mimic the amphipathic character of LPC) induced no significant change in the association of Bid to mitochondria (Fig. 3B).

It was previously found that some phospholipids, including PC, were able to increase the distribution of endogenous Bid from ER to mitochondria when added at concentrations comparable to the content of mitochondrial membrane lipids in the assay, e.g., around 0.2 mM [24]. These concentrations were much higher than those enabling LPC to decrease the association of recombinant Bid to mitochondria (Fig. 3A,B). To determine whether these differences derived from a different mitochondrial association of recombinant and endogenous (native) Bid, experiments like those of Fig. 3 were repeated using Bid isolated from mouse kidney (as described previously [24]). The results showed similar effects of LPC with either isolated native or recombinant Bid, despite quantitative differences in the extent of mitochondrial association of the two types of proteins (data not shown).

Next, it was investigated whether liposomes with composition similar to that of mitochondrial membranes could affect the mitochondrial association of Bid or modify the effects of LPC. LUV liposomes, when added at concentrations comparable to that of mitochondrial lipids, had no significant effect on the extent of cytochrome *c* release, either basal or stimulated by recombinant Bid (Fig. 3C and data not shown). However, the proportion of Bid remaining in the supernatant after separating the mitochondria increased in the presence of LUV (Fig. 3C, lane 2). This suggested that liposomes interacted with Bid in competition with mitochondrial lipids. Indeed, by increasing the concentration of liposomes much above the lipid content in the assay, strong inhibition of Bid-induced release of cytochrome *c* was observed (data not shown, and R. Kluck, personal communication).

Interestingly, after incubation with a mixture LUV, mitochondria and lysolipids, Bid changed not only its association with mitochondria, but also its capacity of releasing cytochrome *c* (Fig. 4). LPC strongly stimulated the Bid-induced release of cytochrome *c* even in the presence of liposomes that decreased the mitochondrial association of Bid (Fig. 4A, cf. Fig. 3C). Of note, LPC alone had little effect

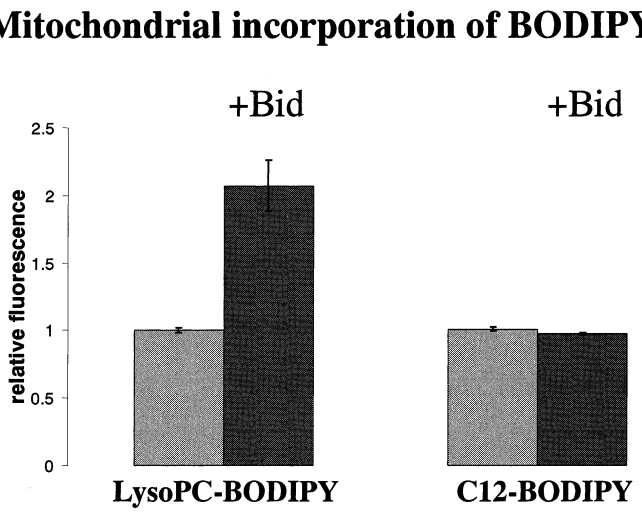

Fig. 5. Effect of Bid on the incorporation of fluorescent lipids into mitochondria. Mitochondria were incubated with the products of phospholipase hydrolysis of Bis-BODIPY-F5-PC (at a final concentration of ca. 0.5 μM BODIPY) or C12-BODIPY fatty acid (20 μM) either in the absence (light grey histograms) or presence (dark grey histograms) of recombinant Bid. The fluorescence recovery of the probe in mitochondria was estimated from the emission spectrum as detailed in Section 2. Data were normalised to the values obtained in the absence of Bid, with the error bar representing the standard deviation in the results ($n = 5$).

on the basal release of cytochrome *c* (Fig. 4B). The enhanced release of cytochrome *c* appeared to be specific for LPC, since it was not reproduced by other lysolipids such as lysophosphatidic acid (LPA) (Fig. 4) or detergents like OG (data not shown).

The results in Fig. 4A indicated that also LPA had some effects on Bid, since it clearly decreased the amount of the protein remaining in the supernatant (Fig. 4A, lane 4) and produced a small increase in the release of cytochrome *c* in the presence of LUV (Fig. 4A, lane 5). Taken together, the effects of LPA on Bid interactions with mitochondria resembled those previously seen with negatively charged lipids added at higher concentrations [24].

3.4. *Bid enhances the incorporation of a lysolipid probe in mitochondria*

The novel effects of LPC on the mitochondrial association of Bid (Fig. 3) and cytochrome *c* release (Fig. 4) suggested that Bid could promote the transport of lysolipids to mitochondria. To test this possibility, a fluorescent lysolipid was prepared from the phospholipase substrate Bis-BODIPY-F5-PC and incubated with mitochondria in the presence and absence of recombinant Bid. Its incorporation in mitochondrial membranes was then evaluated from the fluorescence recovery of the BODIPY probe. Because phospholipase treatment of Bis-BODIPY-F5-PC produced also a fluorescent fatty acid, parallel experiments were conducted using the fatty acid probe C12-BODIPY, which also distributed to mitochondria. Bid did not significantly affect the distribution of this fatty acid probe in mitochondria, but increased over 2-fold the incorporation of the lysolipid product of Bis-BODIPY-F5-PC (Fig. 5). Hence, Bid is able to specifically enhance the incorporation of a fluorescent analogue of LPC into mitochondrial membranes.

4. Discussion

The 'BH3-only' subfamily was originally defined for Bik [9] and Bid [10] and now includes an increasing number of proteins [1,16,17,39,41,42]. To date, there has been no systematic analysis of the sequence similarity in the BH3 region to evaluate a possible structural hierarchy within the 'BH3-only' subfamily [42]. This work reports that a group of 'BH-3-only' proteins including Bid and Nix show sequence similarities with plant LTP proteins in the region surrounding the BH3 domain (Fig. 1 and Table 1). Other BH3-only proteins, as well as all the anti-apoptotic proteins of the Bcl-2 family, do not show equivalent similarities with LTP proteins (Table 1 and results not shown). Thus, a subset of pro-apoptotic proteins could be defined on the basis of a local structural similarity with plant LTP that can be correlated to a common function of lipid interaction. In fact, the sequence similarity between Bid and LTP corresponds to a comparable activity of lipid transfer between membranes (Fig. 2, cf. [24]).

By investigating the specificity of this activity in relation to the mitochondrial action of Bid, it was found that lysolipids such as LPC modified both the mitochondrial association (Fig. 3) and the cytochrome *c* releasing capacity of Bid (Fig. 4). Notably, some plant LTP bind LPC more strongly that diacyllipids [35]. Other similarities between pro-apoptotic proteins of the Bcl-2 family and LTP proteins include the strong thermal stability (see [43] for LTP and [3,19] for Bid), the possible involvement in the intracellular traffic of lipids [24,32], and the comparable anti-microbial activities of some LTP proteins [32,33,44] and Bax [5,45]. Ultimately, all these similarities may stem from common structural properties in protein regions such as LIP (Fig. 1) which participate to the binding or exchange of membrane lipids.

Previous reports suggested that Bid binds to negatively-charged lipids [24,40,46], but did not verify whether this binding reflected additional or underlying interactions with lysolipids. The effects of LPC and LPA reported here (Figs. 3 and 4) were obtained with concentrations two orders of magnitude lower than those previously used for lipids [24,40,46]. This raises the possibility that low levels of lysolipid contaminants, which are usually present in commercial phospholipids, might have contributed to the observed interactions of Bid with lipids.

Current models consider that Bid acts by activating multidomain proteins of the Bcl-2 family, Bax and Bak, which are tethered to the OM and believed to be instrumental for cytochrome *c* release [26,47,48]. However, the association of Bid with ei-

ther Bax or Bak is transient [26,47,48] and unlikely to be responsible for the effects of lysolipids observed here (Figs. 3 and 4). The mouse mitochondria used had a small complement of Bak, and apparently no Bax (data not shown). If the interaction of recombinant Bid with mitochondria were mediated by binding to endogenous Bak, one would not expect that low concentrations of exogenous lysolipids, which predominantly incorporate in the membrane (cf. Fig. 5), reduce the mitochondrial association of Bid (Fig. 3). Indeed, detergents like lysolipids enhance, rather than inhibit, the reciprocal binding between proteins of the Bcl-2 family [15,49].

By considering that detergents promote the oligomerisation of Bax [49,50], lysolipids stimulate Bid-induced release of cytochrome *c* (Fig. 4), and Bid enhances the incorporation of lysolipids into mitochondria (Fig. 5), it is proposed that Bid affects the structural state of multidomain Bcl-2 proteins resident in the OM also by changing the lipid environment in which these proteins are immersed. Bid action would then be indirect, mediated by its transport activity that accumulates lysolipids and exchange them with constituent membrane lipids (Fig. 5), thereby producing perturbations in the OM [6,24,51,52]. This hypothesis would reconcile present and previous evidence for a direct interaction of Bid with lipids [24,40,43,51,52] with the evidence that Bid can promote the oligomerisation of Bax [47] and Bak [26,48], an event considered crucial for the release of mitochondrial cytochrome *c* [26,48,50]. While further studies are required to test such a comprehensive hypothesis, it is increasingly clear that lipid interactions are fundamental in the biochemical mechanism of pro-apoptotic proteins like Bid.

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