Membrane promotes tBID interaction with BCL_{XL}

Ana J. García-Sáez[1], Jonas Ries[1], Mar Orzáez[2], Enrique Pérez-Payà[2,3] and Petra Schwille[1]

[1] Biophysics Group, BIOTEC, TU Dresden, Tatzberg 47-51, D-01307 Dresden, Germany.
[2] Department of Medicinal Chemistry, Centro de Investigación Príncipe Felipe, Avda. Autopista del Saler 16, E-46013 Valencia, Spain.
[3] Instituto de Biomedicina de Valencia, IBV-CSIC, Jaume Roig 11, E-46010 Valencia, Spain

Correspondence should be addressed to P.S. (petra.schwille@biotec.tu-dresden.de) and A.J.G.S. (ana.garcia@biotec.tu-dresden.de).

Summary

Two important questions on the molecular mechanism of the BCL2 proteins involve the interaction network between pro- and anti-apoptotic members and the role of their translocation to the mitochondrial membrane during apoptosis. By quantifying the molecular interactions of BID and tBID with $BCL_{XL}\Delta Ct$ in solution and in membranes by fluorescence correlation spectroscopy, we found that: 1) only the active form tBID binds to $BCL_{XL}\Delta Ct$, and 2) the membrane strongly promotes binding between them. Particularly, a BH3 peptide from BID disrupts the tBID/BCL_{XL} complex in solution, but only partially in lipid bilayers. These data indicate that tBID/BCL_{XL} interactions in solution and lipid membranes are distinct, and support a model in which BCL_{XL} inhibition of tBID takes place predominantly at the membrane. Our findings imply an active role of the membrane in modulating the interactions between BCL2 proteins that has been underestimated.

Introduction

The proteins of the BCL2 family are key regulators of apoptosis that control the permeability of the mitochondrial outer membrane to cytochrome c (cyt c) and other apoptotic factors. They have an important role in the formation of tumors and in cellular responses to anticancer treatments[1-3]. The members of the family are classified into three subgroups: i) antiapoptotic proteins, like BCL2 or BCL_{XL}, that inhibit apoptosis by binding the proapoptotic members; ii) proapoptotic proteins like BAX or BAK, that are believed to participate directly in permeabilization of the mitochondrial outer membrane (MOM); and iii) BH3-only proteins, which are further classified as direct activators and sensitizers/derepressors. Direct activators, like BID, activate BAX and BAK for MOM permeabilization[4], while sensitizer/derepressor BH3-only proteins induce apoptosis by binding to and neutralizing anti-apoptotic Bcl-2 members[5]. Under normal conditions BID is inactive in the cytosol, but in the presence of apoptotic stimuli it is proteolytically cleaved to give an active truncated form called tBID that induces cyt c release[6].

Despite intense research, the molecular mechanisms of action of the proteins of the BCL2 family remain controversial. At the center of the debate, two questions seem to be crucial. First, the translocation to the MOM that most members of the family experience during apoptosis activation. Second, the interactions between them, which determine the final outcome of whether or not apoptosis is induced. Three models have been proposed to explain how the BCL2 proteins regulate apoptosis[7]. The "indirect model" states that the antiapoptotic members are bound to intrinsically active BAX and BAK, thus blocking their action and ensuring cell survival. During apoptosis, the BH3-only proteins are stimulated and bind to the antiapoptotic proteins, thus liberating BAX and BAK to induce cyt c release[8-10]. The "direct activation model" states that BAX and BAK are normally inactive and during apoptosis they are activated by the BH3-only proteins to promote MOM permeabilization[11, 12]. In this scenario the antiapoptotic proteins block cell death by binding and neutralizing BH3-only proteins. Recent data support a third alternative, the "embedded together" model, which assumes that insertion into the MOM during apoptosis affects the affinities between the different BCL2 proteins and exposes new interaction surfaces[4, 13]. Since the role of the membrane has been underestimated for a

long time, there is a lack of experimental data for the interaction of these proteins in membranes and, as a result, their nature and relative importance remain unknown.
To establish the importance of the MOM, it is essential to examine the strength of the interactions between BCL2 proteins in aqueous and lipid environments. For this purpose, a chemically defined system that avoids unwanted effects of unknown factors is required. Cell-free systems have been shown to reproduce satisfactorily the features of the BCL2 proteins observed during apoptosis[4, 14, 15]. We performed a comparative and quantitative analysis of the nature and strength of the interactions between human BID or tBID and human $BCL_{XL}\Delta Ct$ in aqueous solution and in the membrane using reconstituted systems. We used a new variant of fluorescence cross-correlation spectroscopy (FCCS) to demonstrate that active tBID, but not full length BID, binds to $BCL_{XL}\Delta Ct$ in solution, and that such interaction is strongly enhanced in the membrane. This finding sheds light on a key event in MOM permeabilization that is the contribution of the membrane environment to the modulation of interactions between BCL2 members. Altogether, our data suggest that BCL_{XL} neutralizes tBID mainly at the membrane and support a model in which membrane association represents an additional regulatory stage for the control of MOM permeabilization.

Results

tBID and $BCL_{XL}\Delta Ct$ induce each other's binding to membranes

Because chemically defined systems are necessary to quantify the interactions between tBID and BCL_{XL} under controlled conditions, we used a cell-free reconstituted system with highly purified proteins and model membranes. We selectively mutated recombinant tBID and $BCL_{XL}\Delta Ct$ to allow fluorescent labeling at single cysteines with Alexa647 and Alexa488, respectively. The mutations introduced and the positions of labeling in the proteins are highlighted in their structures shown in Figures 1a and b. To ensure that our system mimics as faithfully as possible the natural process, we checked that the labeled proteins, namely $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$, retained their ability to induce or inhibit cyt c release from isolated mitochondria at concentrations comparable to the unlabeled wild type versions (**Fig. 1c,d**).
We investigated the membrane binding properties of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ in giant unilamellar vesicles (GUVs). These are model membrane systems that offer the advantage of free-standing bilayers with sizes ranging from a few to hundreds of micrometers, therefore being suitable for optical microscopy and fluorescence correlation spectroscopy (FCS).
$tBID_{red}$ did not bind to vesicles containing phosphatidylglycerol (**Fig. 2a**), but showed specific binding to GUVs containing cardiolipin (CL) (**Fig. 2b**), in accordance with previous reports on the lipid-mediated selectivity of tBID binding to lipid membranes[16-21]. Interestingly, $tBID_{red}$ associated to GUVs containing tethered $BCL_{XL}\Delta Ct$ (see below), even in the absence of CL (**Fig 2c**) (see control without $BCL_{XL}\Delta Ct$ in **Supplementary Fig. 1**).The active, cleaved form tBID was required for membrane binding. Full length BID labeled with Bodipy did not show binding to CL containing vesicles under similar conditions (**Fig. 2d**).
Next we analyzed membrane binding of $BCL_{XL}\Delta Ct_{green}$. At neutral pH, the protein remained in the aqueous phase (**Fig. 2e**), but bound to anionic vesicles at acidic pH (**Fig.**

2f)[22]. Moreover, we tethered $BCL_{XL}\Delta Ct_{green}$ to GUVs at neutral pH via its histidine tag when small amounts of the lipid derivative DOGS-NTA-Ni (Ni-lipid) were incorporated into GUVs (**Fig. 2g**). This non-covalent association with the lipid moiety mimics the membrane anchored state of full length BCL_{XL} via its C-terminal transmembrane helix[23]. In addition, tBID induced $BCL_{XL}\Delta Ct_{green}$ association to CL containing GUVs at neutral pH (**Fig 2h**). This indicates that hydrophobic fragments of BCL_{XL} besides the absent C-terminus also participate in the tBID-induced membrane interaction. These results are in accordance with those that previously suggested a role for BCL_{XL} helices 5 and 6 in the membrane binding process[24]. The fluorescence spectrum of $BCL_{XL}\Delta Ct$ in presence of tBID and lipid vesicles is in support of this observation (**Supplementary Fig. 2**). Since tBID has no tryptophan residues and its contribution to the spectrum is negligible, the spectral blue shift obtained for $BCL_{XL}\Delta Ct$ in the presence of tBID and membranes is indicative of a transition of $BCL_{XL}\Delta Ct$ to a more hydrophobic environment, likely accompanied by a conformational change. The effect of tBID is supported by the absence of spectral changes for $BCL_{XL}\Delta Ct$ alone in the presence of LUVs.

tBID and $BCL_{XL}\Delta Ct$ cooperate in membrane permeabilization

tBID has been shown to induce a conformational change to the membrane-bound form of the BCL_{XL} homolog BCL2 that is related to the formation of small pores in the lipid membrane[25]. To investigate if BCL_{XL} exhibits a similar behavior, we used the GUV approach. We added Alexa543 free dye to the outside solution of the GUV samples containing $tBID_{red}$ and/or $BCL_{XL}\Delta Ct_{green}$. Membrane permeabilization was monitored following the incorporation of the Alexa543 dye into the initially empty interior of the giant liposomes. The advantage of this strategy is that protein binding, membrane permeabilization and vesicle integrity can be simultaneously accessed. Moreover, additional information, for example about heterogeneities in the permeabilization process, is obtained in comparison to bulk measurements[26, 27]. Since membrane permeabilization is followed at the level of individual vesicles, the permeabilization kinetics of single GUVs can be measured and compared to other vesicles and to the kinetics of ensemble sample permeabilization.

We imaged the effect of $tBID_{red}$ and/or $BCL_{XL}\Delta Ct_{green}$ on the permeability of GUVs for 4h at room temperature (**Fig. 3a and Supplementary Movie 1**). Over that time, most of the vesicles were permeabilized in the samples containing both $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$. Single GUV permeabilization was complete in less than 2 min, indicating fast kinetics. We analyzed the pore forming activity of the proteins by quantifying the permeabilized GUVs. Binding of $tBID_{red}$ or $BCL_{XL}\Delta Ct_{green}$ individually to the lipid membranes caused substantial permeabilization of the vesicles when compared to control samples or to non-binding conditions (**Fig. 3b**). However, the percentage of permeabilized liposomes in presence of both $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ reaches 60% for PC:CL (8:2) and 85% for PC:Ni-lipid (99:1). This is nearly two times higher than the sum of the activities of the individual proteins for those lipid compositions and supports a synergistic effect of tBID and $BCL_{XL}\Delta Ct$ on their membrane binding and/or permeabilizing activities. The increase in the percentage of permeabilized vesicles with time and the synergistic effect at several protein concentrations are shown in **Figure 3c** and **d**, respectively.

Binding between tBID and $BCL_{XL}\Delta Ct$ is enhanced in membranes

The autocorrelation of the FCS data obtained for $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ allows quantification of their concentrations and diffusion properties. Besides, the cross-correlation (CC) analysis of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ diffusion through the focal volume of the microscope provides quantitative information about protein/protein binding and can be used for measurements both in solution and in lipid membranes[28, 29]. A scheme of the experimental setup used is depicted in **Figure 4a**.

Total $tBID_{red}$ or $BCL_{XL}\Delta Ct_{green}$, comprising free protein and protein in the complex give rise to the corresponding autocorrelation curves (green and red lines in **Fig. 4b,c**). But only when $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ interact and diffuse as a complex, the fluorescence fluctuations are similar in both channels and contribute to the amplitude of the cross-correlation curve (blue line in **Fig. 4b,c**). As a result, the larger the cross-correlation amplitude, the higher the concentration of tBID/$BCL_{XL}\Delta Ct$ complexes is in the sample (See **Supplementary Methods**).

At concentrations of 100 nM of each protein in PBS, we measured ~15 % CC (**Fig.4b**), which indicates the formation of tBID/$BCL_{XL}\Delta Ct$ complexes in solution. Interestingly, we did not observe any cross-correlation for full length BID_{red} and $BCL_{XL}\Delta Ct_{green}$ at concentrations up to 330 nM (**Supplementary Fig. 3**). These results strongly suggest that activation of BID by proteolytic cleavage is necessary to promote binding to BCL_{XL}.

To overcome the technical difficulties associated with the quantification of complex formation in lipid membranes, we used a novel variant of FCS named two-color two-focus scanning FCS[30] (see **Methods**). $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ were added to a suspension of GUVs composed of PC:CL (8:2) and incubated 2h at room temperature to ensure equilibrium conditions before measuring scanning FCS across the membrane plane of giant liposomes containing the bound proteins (**Fig. 4c**). The positive amplitude of the cross-correlation curve demonstrated the interaction between tBID and $BCL_{XL}\Delta Ct$ in lipid membranes. Compared to solution, addition of 2 nM $tBID_{red}$ and 16 nM $BCL_{XL}\Delta Ct_{green}$ (lower than used in solution) to the GUVs suspension provided 40% CC (higher than detected in solution), which showed that the interaction between the two proteins is enhanced by the presence of lipid membranes.

The amount of particles forming tBID/$BCL_{XL}\Delta Ct$ complexes can be calculated from the ratio of cross-correlation and autocorrelation amplitudes, allowing the estimation of effective dissociation constants. From the FCCS data measured at different protein concentrations (**Fig. 4d**), we obtained a K_d of 200±20 nM (120±10 molec μm^{-3}) (s.d.) for complex formation in solution (see **Supplementary Fig. 4** and **Supplementary Methods**). This value is in the same order of magnitude as the K_d reported in the literature for BH3 peptides[8, 31, 32]. However, in the presence of lipid membranes, we obtained maximum cross-correlation (which is reduced to 45% due to partial protein labeling) within the statistical variance at all concentrations measured (**Fig. 4e**), which suggests that the affinity between tBID and $BCL_{XL}\Delta Ct$ is so high that we were in saturation under the conditions tested. This allows us to infer that the K_d in the membrane will be lower than 10 molec μm^{-2} and demonstrates a higher affinity between tBID and $BCL_{XL}\Delta Ct$ embedded in lipid membranes when compared with the complex in solution.

From the respective autocorrelation curves, we calculated that tBID in solution diffused at 143±9 $\mu m^2\ s^{-1}$ (s.d.), while $BCL_{XL}\Delta Ct$ had a diffusion coefficient of 78±10 $\mu m^2\ s^{-1}$

(s.d.), in agreement with their respective molecular weights (focal volume calibrated with Atto655[33] and Alexa488[34]). Under conditions of complex formation with a cross-correlation of 30%, their average mobility decreased to 53±3 $\mu m^2 s^{-1}$ (s.d.) for tBID and to 58±6 (s.d.) $\mu m^2 s^{-1}$ for $BCL_{XL}\Delta Ct$. As expected, we deduced values from one to two orders of magnitude slower from the experiments performed in the presence of lipid membranes with two-focus scanning FCS. For the individual proteins, we obtained diffusion coefficients of 5.0±0.3 $\mu m^2 s^{-1}$ and 4.8±0.7 $\mu m^2 s^{-1}$ (s.d.) for tBID and $BCL_{XL}\Delta Ct$, respectively. When tBID and $BCL_{XL}\Delta Ct$ formed complexes in the lipid bilayer, these values decreased to 4.4±0.6 $\mu m^2 s^{-1}$ (s.d.). Since the diffusion coefficient of a particle is related to its hydrodynamic radius[35], the decrease in mobility observed in the presence of tBID/$BCL_{XL}\Delta Ct$ complexes is in good agreement with the size increase associated to complex formation. However, in the case of 2D diffusion in membranes, the diffusion coefficient scales with the logarithm of the hydrodynamic radius[36], which accounts for the weaker effect observed.

Inhibition of tBID/$BCL_{XL}\Delta Ct$ complexes in membranes

The heterodimerization of tBID with BCL_{XL} in solution is believed to happen via insertion of the BH3 domain of tBID into the hydrophobic cleft formed by the BH1, BH2 and BH3 regions of BCL_{XL}. This interaction can be reproduced with peptides derived from the BH3 region of BH3-only proteins that bind to the hydrophobic cleft of BCL_{XL} and other BCL2 homologs[37, 38]. As a negative control for our cross-correlation experiments and to explore whether the protein-protein interaction surfaces implicated in tBID/BCL_{XL} complex formation are similar in solution and in lipid membranes, we evaluated the ability of a BH3-derivative peptide (BidBH3) to compete for such interactions. In FCCS experiments in solution, addition of an excess of non-fluorescent BidBH3 peptide to the complex tBID/$BCL_{XL}\Delta Ct$ lead to a decrease in the %CC to values close to zero, indicating competitive disruption of the complex (**Fig. 5a**). Similarly, unlabeled, wild-type tBID at ~1μM concentration can also totally displace the interactions between $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ after 3h (**Fig. 5b**). The kinetics of inhibition was similar to that of complex formation (**Fig. 5c**). Due to solubility problems, we could not exceed ~1μM unlabeled tBID in these experiments, which decreased the inhibition rate when compared to the peptide. However, when we assayed BidBH3 also at ~1μM, the extent and rate of inhibition was comparable to unlabeled tBID (data not shown).

In contrast, when we added a mixture of $tBID_{red}$, $BCL_{XL}\Delta Ct_{green}$ and an excess of BidBH3 to a suspension of GUVs, CC values in the membrane were similar to control samples without the BH3 peptide (**Fig. 5d**). This resistance to BidBH3 indicates that even in the presence of the peptide, tBID and $BCL_{XL}\Delta Ct$ bind to the membrane and form a stable complex. The inability of BidBH3 to compete for the complex in membranes could be related to its low membrane affinity or to the different nature of tBID/BCL_{XL} interactions. To distinguish between these possibilities, we used a version of the BidBH3 peptide containing a 6-His-tag on the C-terminus (BidBH3His) that can be efficiently targeted to membranes containing Ni-lipid. This peptide has been previously shown to potently activate BAX[39]. Interestingly, when we added BidBH3His in a large excess to GUVs containing interacting tBID and $BCL_{XL}\Delta Ct$, the percentage of complexes remained

unaltered. Only when it was added simultaneously with tBID and $BCL_{XL}\Delta Ct$ to the GUVs suspension, the BidBH3His peptide could partially disrupt the complex between the proteins (**Fig. 5e**). A mutant peptide that cannot interact with BCL_{XL} ($BidBH3His_{MUT}$) did not affect complex formation under the same conditions (**Fig. 5e**). These results indicate that the interactions between tBID and BCL_{XL} in solution and within lipid membranes are not equivalent.

To check if complex formation in the membrane could be totally disrupted, we used again unlabeled tBID. We incubated a suspension of GUVs containing $tBID_{red}/BCL_{XL}\Delta Ct_{green}$ complexes with a molar excess of unlabeled tBID (**Fig. 5f, g**). The cross-correlation amplitude became almost zero (**Fig. 5g**). These observations indicate that unlabeled tBID can bind to $BCL_{XL}\Delta Ct_{green}$ and displace $tBID_{red}$ from the complex, which also means that complex formation in the membrane is reversible.

Estimation of the number of particles in the membrane focal area showed that the excess of unlabeled tBID decreased the number of dual-color fluorescent $tBID/BCL_{XL}\Delta Ct$ particles, but also influenced the membrane binding of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ (**Fig. 5h**). The number of $tBID_{red}$ molecules associated to the membrane decreased, indicating that tBID binding to lipid bilayers is reversible and only a finite number of binding sites is allowed. The increase in the total number of $BCL_{XL}\Delta Ct_{green}$ particles bound to the membrane indicates that tBID induces $BCL_{XL}\Delta Ct$ association to lipid membranes.

Discussion

The physiological relevance of the effect of BH3-only proteins on pro-survival BCL2 members during apoptosis has been described earlier[40], although the molecular mechanism has not been clarified yet. Our imaging and FCS results show that tBID and $BCL_{XL}\Delta Ct$ enhance each other's binding to lipid membranes. One of the most relevant findings of this study is that tBID promotes BCL_{XL} binding to CL containing vesicles, in agreement with recent biochemical experiments[41] and providing a molecular basis for the translocation of BCL_{XL} to the MOM during apoptosis[42]. We found that the C-terminal transmembrane region of BCL_{XL} is not strictly necessary for membrane binding, though it most likely contributes to it. According to our observations, tBID would induce a conformational change in BCL_{XL} in the presence of lipid membranes that favors the exposure of BCL_{XL} hydrophobic regions important for membrane insertion. These probably include the C-terminus but also the central hairpin formed by helices 5 and 6[43], as already reported for BAX and BCL2[44, 45].

Moreover, tBID also associated to GUVs containing tethered BCL_{XL} even in the absence of CL. This may have physiological implications, since a portion of pro-survival BCL2 members are located in cellular membranes other than the MOM. It is reasonable to postulate that this could be used by cells in order to decrease free tBID molecules that could reach the MOM and induce apoptosis[46]. It could also happen that tBID binding to pro-survival BCL2 proteins in the endoplasmic reticulum inactivates their protective action and contributes to apoptosis induction[47]. Moreover, our quantitative analysis reveals that tBID binding to CL containing membranes is reversible and probably reaches saturation. This suggests that CL acts as a mitochondrial receptor for tBID. However, the interaction does not seem stoichiometric one-to-one and other factors, like increased membrane tension, may impair further protein insertion into the lipid bilayer. What is more interesting is that this implies that tBID membrane binding could be blocked by CL

binding competitors. Future work will be required to check if similar behavior can be observed *in vivo* and if it affects apoptosis, hence constituting a new therapeutic target for apoptosis regulation.

Imaging single vesicle permeabilization induced by tBID and $BCL_{XL}\Delta Ct$ showed that membrane integrity is maintained and that the process follows fast kinetics once it starts. The cooperative effect observed is in agreement with the high affinity between the two proteins in lipid membranes measured by FCCS. This implies that complex formation alters membrane permeability, as reported for tBID and BCL2[48]. This activity is difficult to reconcile with the antiapoptotic function of BCL_{XL} and BCL2, but in the context of the "embedded together" model, pro-survival members behave as defective, dominant-negative BAX molecules, able to insert and alter the membrane permeability, yet unable to oligomerize and release cyt c[13, 41].

In this study, we employed a quantitative approach to examine binding of BID and tBID to $BCL_{XL}\Delta Ct$ in a biochemically well-defined model system. A novel FCCS strategy allowed us to evaluate dynamic protein/protein interactions in lipid bilayers and, for the first time, we estimated effective dissociation constants for tBID/$BCL_{XL}\Delta Ct$ complexes in solution and in the membrane. Our results show that tBID and BCL_{XL} interact in both environments, and importantly, they reveal the dramatic effect of the membrane on their effective affinity, which had been underestimated. The molecular details behind this effect are related to the reduction of dimensionality in the system from a 3D solution to the 2D plane of the membrane, which increases the collision probability, and the translocation into an anisotropic, hydrophobic environment, which induces structural reorganizations and modifies the thermodynamics of tBID/$BCL_{XL}\Delta Ct$ interactions. Our observations have novel implications for the biological function of tBID and BCL_{XL}, since they strongly suggest that the most relevant interaction between these proteins, and probably between other homolog proteins of the family, occurs in the membrane, as recently reported for BAX and tBID[4]. Similar membrane-based mechanisms have been proposed in other signaling processes, and indeed this could be a general strategy used by cells to increase interactions between proteins and to modulate downstream cellular responses[49-51]. The strong effect of the membrane on tBID/BCL_{XL} complex formation begs other questions, like the role of lipids in the function of the BCL2 proteins, the structures of these proteins in the membrane-bound state or the nature of the interaction surfaces in the lipidic environment.

The absence of interactions between full length BID and $BCL_{XL}\Delta Ct$ under the conditions investigated links the activation of BID and its role as an apoptosis inducer to the promotion of its interactions with BCL_{XL} and probably other members of the BCL2 family.

A BH3-derived Bid peptide (BidBH3) induced dissociation of the tBID/$BCL_{XL}\Delta Ct$ complex in solution, but not when the complex was stabilized in lipid membranes. Even when BidBH3 efficiently shifts the equilibrium in solution, tBID and $BCL_{XL}\Delta Ct$ translocated to the lipid bilayer and formed complexes. To ensure accessibility of the peptide to the membrane, we used a His-tagged version (BidBH3His) which can be tethered to membranes containing Ni-lipid and has been shown to efficiently activate BAX[52]. However, membrane targeted BidBH3His could not disrupt previously formed tBID/$BCL_{XL}\Delta Ct$ complexes within membranes and was only able to block complex formation partially when added simultaneously with tBID and $BCL_{XL}\Delta Ct$, this is, prior to

complex formation. These findings are extremely interesting because they indicate the existence of two populations of tBID and $BCL_{XL}\Delta Ct$ with different susceptibility to the BH3 peptide in membranes. Based on the different membrane-bound states proposed for BCL2 proteins[53], it is tempting to speculate that BidBH3His could bind to membrane-associated $BCL_{XL}\Delta Ct$ but not to the membrane inserted form, which in turn is still able to interact with tBID. This could be explained in terms of the low hydrophobicity of BidBH3His that would not be able to insert into the lipid bilayer and displace the more hydrophobic tBID from the complex with BCL_{XL}. But also, the existence of alternative binding sites for the membrane inserted proteins that further stabilize the complex cannot be ruled out. The differences observed with BAX activation by BidBH3His could be based on the induction of BAX association with the membrane by the peptide. In such a situation, BAX would then insert into the membrane and form pores, for which the peptide is not necessary anymore.

Given the importance of BCL2 targeting in cancer therapy, these findings have important consequences for the design of inhibitors of BCL2 pro-survival proteins[54]. Membrane inserted tBID/BCL_{XL} appear as an additional drug target that cannot be inhibited with BH3 mimetics and related molecules, but probably with more lipophilic compounds. Indeed, the small molecule ABT-737 and PUMA induced apoptosis by releasing tBID from complexes with prosurvival BCL2 members within mitochondria, which was associated with a "direct activator:prosurvival BCL2 protein:sensitizer/derepressor" network[55]. Also, the proapoptotic effect of the small molecule obatoclax has been linked to its ability to block MCL1 in the membrane[56]. Importantly, the experimental approach shown here constitutes a powerful method to investigate the molecular mechanism of BCL_{XL} inhibitors quantitatively.

The contribution of the membrane to the modulation of the interactions between BCL2 proteins showed here support the "embedded together" hypothesis. It constitutes an additional regulatory step of apoptosis and reveals a more complex scenario with multiple parallel equilibriums that affect the final event of MOM permeabilization. This is consistent with our observations that BidBH3His cannot dissociate totally the tBID/$BCL_{XL}\Delta Ct$ complex stabilized in lipid membranes, indicating that the protein-protein interactions in solution and in the membrane are not equivalent. Understanding the molecular mechanisms of the BCL2 proteins involves detailed knowledge of their interaction networks. Therefore, introduction of BAX and derepressor/sensitizer BH3-only proteins in the system, and quantification of their relative affinities in solution and in lipid membranes will help to identify which interactions within the family are favored and thus, crucial for MOM permeabilization regulation.

Altogether, the data reported here support a new model in which BID cleavage acts as a switch that allows its interaction with BCL_{XL} and probably other multi-domain BCL2 proteins (**Fig. 6**). The active fragment tBID is then capable of binding to BCL_{XL} in solution and induce its translocation into the MOM, which is accompanied by conformational changes and alteration of the membrane permeability. tBID also binds BCL_{XL} in the membrane, where their effective affinity is greatly enhanced so that BCL_{XL} can inhibit tBID more efficiently. As a consequence, neutralization of tBID action by BCL_{XL} happens mainly within the membrane. Such scenario also implies that tBID promotes BCL_{XL} inhibitory activity, which may constitute a control mechanism to avoid

unwanted MOM permeabilization while ensuring rapid induction of apoptosis once tBID is displaced by derepressor BH3-only proteins.

In summary, our findings show the active role of the membrane in modulating the interactions between tBID and BCL_{XL}. This has important consequences for the current understanding of apoptosis regulation, as we have introduced in a new model for BCL_{XL} inhibition of tBID. We found unanticipated strong interactions between tBID and BCL_{XL} in lipid membranes, as compared to the interactions in solution. This was possible thanks to unprecedented quantitative binding studies with full-length BCL2 proteins in both environments. Our results link the cleavage of BID to a change in its affinity for BCL_{XL} that allows complex formation. The findings that tBID and BCL_{XL} induce each other's binding to membranes and that they cooperate to induce membrane permeabilization are in good agreement with the formation of a stable complex within the membrane, where, as a consequence, the main inhibition of tBID by BCL_{XL} takes place. Importantly, the distinct susceptibility to BH3 peptides in solution and tethered to membranes demonstrates the existence of different tBID/BCL_{XL} complex conformations in both environments. Our novel experimental approach offers new perspectives on the molecular mechanisms of interactions at interfaces. The quantitative analysis based on FCS constitutes a promising strategy for a better understanding of the regulation of BCL2 proteins and for the investigation of protein/protein interactions within membranes in general, an issue that has proven extremely challenging so far.

Acknowledgments

We thank J. Salgado, E. Petrov and S. Chiantia for useful discussions and J. Suckale for careful reading of the manuscript. We are grateful to Francisco Pan-Montojo (MPI-CBG and TU Dresden, Dresden, Germany) for supplying the mouse livers and Susana Rubio and Anke Borrmann for technical assistance in protein mutagenesis and purification. This work was supported by a Marie Curie Intra-European Fellowship (A.J.G.S.) within the 6th European Framework Program. Work in EPP laboratory is supported by the Spanish Ministry of Science and Innovation grants BIO2007-60066 and CSD2008-00005.

Author contributions

A.J.G.S. proposed the project, prepared materials, performed the experiments and analyzed the data; J.R. developed the method for FCS data analysis and helped with data analysis; M.O. and E.P.P. prepared materials; P.S. provided the infrastructure and expertise in FCS and model membranes; A.J.G.S. wrote the manuscript with the help of all other authors.

FIGURE LEGENDS

Figure 1 Fluorescently labeled tBID and $BCL_{XL}\Delta Ct$ retain their apoptotic activity. (**a**) and (**b**) Structures of soluble BID and $BCL_{XL}\Delta Ct$ respectively, obtained from pdb models (PBD 2BID[57] and PDB 1LXL[58]). Mutation of Cys to Ser is shown in yellow, mutation of Ser to Cys is shown in red, and the cleavage site of BID by caspase 8 is depicted in green. The stars indicate the single Cys sites at which the fluorophores are attached. (**c**) Cytochrome c (cyt c) release from mitochondria at different concentrations of unlabeled tBID (lanes 2 and 3) or $tBID_{red}$ (lanes 4 and 5). Released cytc is detected in the supernatant fraction, while cyt c associated to mitochondria remains in the pellet fraction. (**d**) $BCL_{XL}\Delta Ct_{green}$ blocks cyt c release from mitochondria induced by tBID in a concentration dependent manner.

Figure 2 Binding of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ to giant unilamellar vesicles. Pictures on the right and left panels depict the same sample imaged on different channels of the microscope. On the left, giant unilamellar vesicles (GUVs) are labeled with 0.05% of a hydrophobic dye, DiO shown in green, or DiD shown in red. The panels on the right show protein binding to the same GUVs. $tBID_{red}$ (red) did not associate to GUVs composed of PC:PG (8:2) (**a-b**), but showed binding to GUVs containing cardiolipin (PC:CL, 8:2, panels **c-d**). $BCL_{XL}\Delta Ct$ tethered to GUVs via DOGS-NTA-Ni (PC:DOGS-NTA-Ni, 99:1) promoted $tBID_{red}$ binding to GUVs, even in the absence of cardiolipin (**e-f**). As a control, full length BID_{green} (green) did not bind to PC:CL (8:2) vesicles (**g-h**). In the case of $BCL_{XL}\Delta Ct_{green}$ (green), it did not associate to anionic membranes (PC:CL, 8:2) at pH 7 (**i-j**), but bound at pH5 (**k-l**). Panels (**m-n**) show $BCL_{XL}\Delta Ct_{green}$ tethering to membranes containing DOGS-NTA-Ni (PC:DOGS-NTA-Ni, 99:1). (**o-p**) tBID induced $BCL_{XL}\Delta Ct_{green}$ binding to membranes containing cardiolipin (PC:CL, 8:2). Scale bar 15 μm.

Figure 3 $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ cooperate on membrane permeabilization. (**a**) Kinetics of GUV permeabilization in the presence of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$, monitored by the internalization of free Alexa543 externally added. Permeabilized vesicles pointed with white arrows. Time in minutes. Scale bar 10 μm. (**b**) Percentage of permeabilized vesicles after addition of 16 nM $BCL_{XL}\Delta Ct_{green}$ and/or 9 nM $tBID_{red}$. Error bars are s.d. (**c**) Increase of the percentage of permeabilized vesicles with time after addition of 32 nM $BCL_{XL}\Delta Ct_{green}$ and/or 18 nM $tBID_{red}$. The white circles correspond to a control experiment without proteins added. (**d**) Percentage of vesicle permeabilization at several concentrations of $BCL_{XL}\Delta Ct_{green}$ and/or $tBID_{red}$. In (c) and (d) GUVs are composed of PC:CL (8:2). Error bars correspond to s.d.

Figure 4 Measurement of tBID/$BCL_{XL}\Delta Ct$ interactions by fluorescence cross-correlation spectroscopy. (**a**) The optical setup is based on an inverted laser scanning microscope. The laser light from 488 and 633 nm lines is focused onto the sample. Diffusion and binding of labeled proteins in solution are measured with fluorescence correlation spectroscopy (FCS) using a fixed focal volume. For measurements in the membrane,

scanning FCS is used and the focal volume is scanned perpendicularly through the membrane plane. The photons emitted are spectrally split by a dichroic mirror and detected with avalanche photo diodes coupled to an online autocorrelator. Data are analyzed with a computer using custom software. (**b**) Auto- and cross-correlation (CC) analysis of measurements in solution. The fitted autocorrelation curves of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ are shown in orange and green, respectively. The amplitude of the fitted CC curve (blue) indicates formation of $tBID/BCL_{XL}\Delta Ct$ complexes. (**c**) Auto- and CC global analysis of two-focus scanning FCCS measurements in the membrane of GUVs. The orange and green lines correspond to the fitted autocorrelation curves of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$, respectively. The light blue and yellow lines show the two-focus analysis for $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$, respectively. Eq.(8) (see Supplementary Methods) was used for the global fitting. Interactions between tBID and $BCL_{XL}\Delta Ct$ in the membrane are demonstrated by the amplitude of the CC curve (dark blue line). GUVs composed of PC:CL (8:2). Thin black lines depict raw data. (**d**) and (**e**) Quantification of $tBID/BCL_{XL}\Delta Ct$ complexes, expressed as %CC, as a function of tBID concentration in solution (**d**) and in the membrane (**e**).

Figure 5 Inhibition of $tBID/BCL_{XL}\Delta Ct$ interactions in solution and in the membrane. (**a**) $tBID/BCL_{XL}\Delta Ct$ complex in solution can be disrupted by addition of BidBH3, as shown by the neutralization of the CC curve (solid blue) after incubation with 1,7 μM of BidBH3 (dashed blue). Concentrations of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ were ~220 nM and ~160 nM, respectively. (**b**) Similarly, unlabeled tBID (1 μM) can also disrupt the complex between $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ after 3h incubation. Concentrations of $tBID_{red}$ and $BCL_{XL}\Delta Ct_{green}$ were ~200 nM and ~180 nM, respectively. (**c**) Time course of the experiments in (**a**), (,), and (**b**), (,). %CC, related to complex concentration, was calculated with respect to $tBID_{red}$. Error bars are averaged absolute errors from the FCCS fittings. (**d**) Addition of BidBH3 excess does not inhibit $tBID/BCL_{XL}\Delta Ct$ interactions in lipid membranes (PC:CL, 8:2). The amplitude of the CC curves (blue) is similar in absence (solid line) and presence (dashed line) of ~400 pmol BidBH3. (**e**) The membrane targeted peptide BidBH3His (~1.13 μmol) can partially disrupt the $tBID/BCL_{XL}\Delta Ct$ complex in the membrane if added simultaneously (0') with the proteins, but not after they have formed the complex in the membrane (2 h). GUVs composed PC:CL:Ni-lipid, 75:20:5. Error bars are s.d. (**f**) and (**g**) $tBID_{red}/BCL_{XL}\Delta Ct_{green}$ complex in GUVs (PC:CL, 8:2) can be totally disrupted by unlabeled tBID. The amplitude of the CC curve (blue) in (**f**) decreases and after addition of ~400 pmol unlabeled tBID to GUVs (**g**). GUVs samples contain ~9 pmol $BCL_{XL}\Delta Ct_{green}$ and ~3 pmol $tBID_{red}$. In all graphs, green and orange lines correspond to the autocorrelation of $BCL_{XL}\Delta Ct_{green}$ and $tBID_{red}$, respectively. Raw data shown in black. Eq.(8) (see Supplementary Methods) was used for the global fitting. (**h**) Number of particles in the membrane focal area before and after addition of unlabeled tBID. Excess of unlabeled tBID increases total $BCL_{XL}\Delta Ct_{green}$ bound to the membrane, but decreases total $tBID_{red}$ and $tBID/BCL_{XL}\Delta Ct$ complex. Error bars are s.d.

Figure 6 Proposed model for the interactions between tBID and BCL_{XL}. (**a**) Initially, BCL_{XL} and BID do not interact. (**b**) Upon cleavage during apoptosis, tBID is able to bind BCL_{XL}. tBID and BCL_{XL} promote each other's binding to the membrane, where they

have a higher affinity for each other (indicated by the thickness of the two-headed arrows). (**c**) As a consequence, BCL_{XL} inhibition of tBID happens mainly at the membrane.

Methods

Protein preparation. We prepared fluorescently labeled human tBID as described[59], from BID cDNA in pET15b with a single Cys at position 64 in the tBID fragment and labeled it with Alexa647 maleimide (Invitrogen). We purified full length BID with the same protocol as tBID, but from a cDNA with C15S and C28S mutations, and labeled it with Bodipy or Alexa647 maleimide.

We introduced mutations S2C and C151S in human BCL_{XL}^{1-209} ($BCL_{XL}\Delta Ct$) in a modified pET15b vector. We overexpressed the protein in *E. coli*. For purification, we used in a His-trap HP column (Amersham Biosciences) with an imidazole gradient in 50 mM Na_2HPO_4, 300 mM NaCl 1 mM DTT, pH 7. Then, we dialyzed the protein against 50 mM Na_2HPO_4, 300 mM NaCl, 1 mM DTT, pH 7 and labeled it with Alexa488 maleimide.

Release of cytochrome c from isolated mitochondria. We isolated mouse liver mitochondria as described[60] (for details, see **Supplementary Methods**). We performed experiments of cyt c release with 60 μg of isolated mitochondria in AT buffer supplemented with 5 mM succinate, 2 mM ATP, 10 μM phosphocreatine and 10 μg ml^{-1} creatine kinase. We incubated the indicated proteins for 1 h at 37°C in a volume of 30 μL. We centrifuged the samples at 5,500 g for 10 min at 4°C. We checked the presence of cyt c in the pellets and supernatants by western blot with a cyt c antibody (BD Biosciences).

Preparation of giant unilamellar vesicles. All lipids were from Avanti Polar Lipids. We prepared GUVs by the electroformation method. We spread 5 μL of lipid mixture at 1 mg ml^{-1} in chloroform on two Pt wire electrodes. We immersed the lipid films in a chamber containing 300 mM sucrose solution and connected the Pt wires to a power generator. We performed electroformation at 2.3 V and 10 Hz for 1h at room temperature. We released the GUVs from the electrodes by changing the frequency to 2 Hz for 30 min.

Protein binding and membrane permeabilization assays. We sedimented 50 μL of GUVs electroformed in 300 mM sucrose in 500 μL PBS containing the corresponding proteins. We added 16 nM of $BCL_{XL}\Delta Ct_{green}$, 9 nM of $tBID_{red}$ and/or 290 nM unlabeled tBID, as indicated. After ~2h incubation r.t., we measured protein binding to the GUVs by confocal microscopy. We used an LSM 510 Meta microscope (Zeiss) with a 40x 1.2 NA C-Apochromat water objective (Zeiss) in the multi-track modus. We used UV/488/543/633 and 545 nm filters as main and secondary dichroics. The green channel consisted of an Ar laser with excitation wavelength at 488 nm and a 505–530 nm band pass filter, while we configured the red channel with a 633 nm excitation laser and a 650 nm long pass filter.

For the GUVs permeabilization experiments, we added 5 μL of 50 μM Alexa 543 free dye to GUVs samples containing 16 nM $BCL_{XL}\Delta Ct_{green}$ and/or 9 nM $tBID_{red}$ and measured dye entrance into the GUVs with time. We imaged it in a different track with a 543 nm excitation laser and a 560–615 nm band pass filter. After 4 h incubation at r.t., we

acquired images over a large area of the sample and counted the permeabilized and non-permeabilized GUVs. We repeated all experiments at least three times and measured several hundreds of vesicles per experiment.

Fluorescence correlation spectroscopy. We performed FCS and FCCS measurements at 22°C on an laser scanning microscope (LSM) Meta 510 system (Carl Zeiss, Jena, Germany) using a 40x NA 1.2 UV-VIS-IR C Apochromat water-immersion objective, the 488 nm line of an Argon-ion laser (25 μW), the 633 nm HeNe laser (15 μW) and a home-built detection unit at the fiber output channel. We used a dichroic mirror and band-pass filters (D555, HQ520/40 and HQ700/75, AHF Analyse Technik, Tuebingen, Germany) behind a collimating achromat to split the emission for the dual color detection and to reject residual laser and background light. We then used achromats (LINOS Photonics, Goettingen, Germany) to image the internal pinhole onto the apertures of the fibers connected to the avalanche photodiodes (APD, PerkinElmer, Boston, MA). We recorded the photon arrival times in the photon mode of the hardware correlator Flex 02-01D (Correlator.com, Bridgewater, NJ).

For scanning FCS[30], we repeatedly scanned the detection volume perpendicularly through the equator of a GUV. We controlled its movement directly with the Zeiss LSM operation software. We used the frame mode with *Nx2* pixels to scan the two parallel lines. We measured their distance *d* by repeatedly scanning over a film of dried fluorophores and measuring the distance between the bleached traces in a high resolution LSM-image.

We performed data analysis with software written in MATLAB (MathWorks, Natick, MA). For scanning FCS, we binned the photon stream in bins of 2 μs and arranged it as a matrix such that every row corresponded to one line scan. We corrected for movements of the membrane by calculating the position of the maximum of a running average over several hundred line scans and shifting it to the same column. We fitted an average over all rows with a Gaussian and we added only the elements of each row between -2.5σ and 2.5σ to construct the intensity trace. We computed the auto- and cross-correlation curves of the resulting intensity traces with a multiple tau correlation algorithm and fitted them with a nonlinear least squares fitting algorithm. In all FCS data processing, we excluded from further analysis irregular curves resulting from major instabilities and identified by distortions of the curves and a systematic change in the intensity trace. We fitted the resulting auto- and cross-correlation functions to analytical models as described in **Supplementary Methods**. We checked that the FCS analysis was not affected by dye quenching due to complex formation or oligomerization by FIDA in solution and by analyzing the counts per molecule of the fluorophores in membranes (See **Supplementary Methods** and **Supplementary Fig. 5**). For details on the calculation of the K_d values from the FCS and FCCS data see **Supplementary Methods**.

Reference List

1. Kroemer,G., Galluzzi,L., & Brenner,C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev.* **87**, 99-163 (2007).

2. Danial,N.N. BCL-2 family proteins: critical checkpoints of apoptotic cell death. *Clin. Cancer Res.* **13**, 7254-7263 (2007).

3. Youle,R.J. & Strasser,A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev. Mol. Cell Biol* **9**, 47-59 (2008).

4. Lovell,J.F. *et al.* Membrane Binding by tBid Initiates an Ordered Series of Events Culminating in Membrane Permeabilization by Bax. *Cell* **135**, 1074-1084 (2008).

5. Chipuk,J.E. *et al.* Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. *Proc. Natl. Acad. Sci. U. S. A*(2008).

6. Gross,A. *et al.* Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* **274**, 1156-1163 (1999).

7. Chipuk,J.E. & Green,D.R. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18**, 157-164 (2008).

8. Chen,L. *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol. Cell* **17**, 393-403 (2005).

9. Willis,S.N. *et al.* Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev.* **19**, 1294-1305 (2005).

10. Willis,S.N. *et al.* Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315**, 856-859 (2007).

11. Kuwana,T. *et al.* Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331-342 (2002).

12. Wei,M.C. *et al.* tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* **14**, 2060-2071 (2000).

13. Leber,B., Lin,J., & Andrews,D.W. Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes. *Apoptosis.* **12**, 897-911 (2007).

14. Kuwana,T. *et al.* Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331-342 (2002).

15. Terrones,O. *et al.* Lipidic pore formation by the concerted action of proapoptotic BAX and tBID. *J. Biol. Chem.* **279**, 30081-30091 (2004).

16. Epand,R.F., Martinou,J.C., Fornallaz-Mulhauser,M., Hughes,D.W., & Epand,R.M. The apoptotic protein tBid promotes leakage by altering membrane curvature. *J. Biol. Chem.* **277**, 32632-32639 (2002).

17. Esposti,M.D., Cristea,I.M., Gaskell,S.J., Nakao,Y., & Dive,C. Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. *Cell Death. Differ.* **10**, 1300-1309 (2003).

18. Gonzalvez,F., Bessoule,J.J., Rocchiccioli,F., Manon,S., & Petit,P.X. Role of cardiolipin on tBid and tBid/Bax synergistic effects on yeast mitochondria. *Cell Death. Differ.* **12**, 659-667 (2005).

19. Kim,T.H. *et al.* Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. *Mol. Biol. Cell* **15**, 3061-3072 (2004).

20. Liu,J., Weiss,A., Durrant,D., Chi,N.W., & Lee,R.M. The cardiolipin-binding domain of Bid affects mitochondrial respiration and enhances cytochrome c release. *Apoptosis.* **9**, 533-541 (2004).

21. Lutter,M., Perkins,G.A., & Wang,X. The pro-apoptotic Bcl-2 family member tBid localizes to mitochondrial contact sites. *BMC. Cell Biol.* **2**, 22 (2001).

22. Thuduppathy,G.R., Terrones,O., Craig,J.W., Basanez,G., & Hill,R.B. The N-Terminal Domain of Bcl-xL Reversibly Binds Membranes in a pH-Dependent Manner. *Biochemistry* **45**, 14533-14542 (2006).

23. Peng,J. *et al.* tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation. *J. Biol. Chem.* **281**, 35802-35811 (2006).

24. Garcia-Saez,A.J., Mingarro,I., Perez-Paya,E., & Salgado,J. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. *Biochemistry* **43**, 10930-10943 (2004).

25. Peng,J. *et al.* tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation. *J. Biol. Chem.* **281**, 35802-35811 (2006).

26. Schon,P. *et al.* Equinatoxin II permeabilizing activity depends on the presence of sphingomyelin and lipid phase coexistence. *Biophysical Journal* **95**, 691-698 (2008).

27. Tamba,Y. & Yamazaki,M. Single giant unilamellar vesicle method reveals effect of antimicrobial peptide magainin 2 on membrane permeability. *Biochemistry* **44**, 15823-15833 (2005).

28. Bacia,K., Kim,S.A., & Schwille,P. Fluorescence cross-correlation spectroscopy in living cells. *Nat. Methods* **3**, 83-89 (2006).

29. Haustein,E. & Schwille,P. Single-molecule spectroscopic methods. *Curr. Opin. Struct. Biol.* **14**, 531-540 (2004).

30. Ries,J. & Schwille,P. Studying slow membrane dynamics with continuous wave scanning fluorescence correlation spectroscopy. *Biophys. J.* **91**, 1915-1924 (2006).

31. Certo,M. *et al.* Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9**, 351-365 (2006).

32. Letai,A. *et al.* Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183-192 (2002).

33. Dertinger,T. *et al.* Two-focus fluorescence correlation spectroscopy: a new tool for accurate and absolute diffusion measurements. *Chemphyschem.* **8**, 433-443 (2007).

34. Petrasek,Z. & Schwille,P. Precise measurement of diffusion coefficients using scanning fluorescence correlation spectroscopy. *Biophysical Journal* **94**, 1437-1448 (2008).

35. Saffman,P.G. & Delbruck,M. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. U. S. A* **72**, 3111-3113 (1975).

36. Kahya,N. & Schwille,P. Fluorescence correlation studies of lipid domains in model membranes. *Mol. Membr. Biol.* **23**, 29-39 (2006).

37. Petros,A.M. *et al.* Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Sci* **9**, 2528-2534 (2000).

38. Sattler,M. *et al.* Structure of Bcl-xL-Bak Peptide Complex: Recognition Between Regulators of Apoptosis. *Science* **275**, 983-986 (1997).

39. Oh,K.J. *et al.* A Membrane-targeted BID BCL-2 Homology 3 Peptide Is Sufficient for High Potency Activation of BAX in Vitro. *J. Biol. Chem.* **281**, 36999-37008 (2006).

40. Willis,S.N. *et al.* Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev.* **19**, 1294-1305 (2005).

41. Billen,L.P., Kokoski,C.L., Lovell,J.F., Leber,B., & Andrews,D.W. Bcl-XL inhibits membrane permeabilization by competing with Bax. *PLoS. Biol* **6**, e147 (2008).

42. Hsu,Y.T., Wolter,K.G., & Youle,R.J. Cytosol-to-membrane redistribution of Bax and Bcl-XL duringΓÇëapoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3668-3672 (1997).

43. Garcia-Saez,A.J., Mingarro,I., Perez-Paya,E., & Salgado,J. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. *Biochemistry* **43**, 10930-10943 (2004).

44. Desagher,S. *et al.* Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J. Cell Biol.* **144**, 891-901 (1999).

45. Peng,J. *et al.* tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation. *J. Biol. Chem.* **281**, 35802-35811 (2006).

46. Yano,M., Terada,K., Gotoh,T., & Mori,M. In vitro analysis of Bcl-2 proteins in mitochondria and endoplasmic reticulum: Similarities in anti-apoptotic functions and differences in regulation. *Experimental Cell Research* **313**, 3767-3778 (2007).

47. White,C. *et al.* The endoplasmic reticulum gateway to apoptosis by Bcl-X(L) modulation of the InsP3R. *Nat Cell Biol* **7**, 1021-1028 (2005).

48. Peng,J. *et al.* tBid Elicits a Conformational Alteration in Membrane-bound Bcl-2 Such That It Inhibits Bax Pore Formation. *J. Biol. Chem.* **281**, 35802-35811 (2006).

49. Gureasko,J. *et al.* Membrane-dependent signal integration by the Ras activator Son of sevenless. *Nat Struct Mol Biol* **15**, 452-461 (2008).

50. Ye,J. & Esmon,C.T. Factor Xa-Factor Va Complex Assembles in Two Dimensions with Unexpectedly High Affinity: An Experimental and Theoretical Approach. *Biochemistry* **34**, 6448-6453 (1995).

51. Melo,E. & Martins,J. Kinetics of bimolecular reactions in model bilayers and biological membranes. A critical review. *Biophysical Chemistry* **123**, 77-94 (2006).

52. Oh,K.J. *et al.* A Membrane-targeted BID BCL-2 Homology 3 Peptide Is Sufficient for High Potency Activation of BAX in Vitro. *J. Biol. Chem.* **281**, 36999-37008 (2006).

53. Garcia-Saez,A.J., Mingarro,I., Perez-Paya,E., & Salgado,J. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. *Biochemistry* **43**, 10930-10943 (2004).

54. Rajendran,L. *et al.* Efficient Inhibition of the Alzheimer's Disease {beta}-Secretase by Membrane Targeting. *Science* **320**, 520-523 (2008).

55. Chipuk,J.E. *et al.* Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. *Proc. Natl. Acad. Sci. U. S. A*(2008).

56. Nguyen,M. *et al.* Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proceedings of the National Academy of Sciences* **104**, 19512-19517 (2007).

57. Chou,J.J., Li,H., Salvesen,G.S., Yuan,J., & Wagner,G. Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell* **96**, 615-624 (1999).

58. Muchmore,S.W. *et al.* X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* **381**, 335-341 (1996).

59. Zha,J., Weiler,S., Oh,K.J., Wei,M.C., & Korsmeyer,S.J. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science* **290**, 1761-1765 (2000).

60. Yamaguchi,R. *et al.* Mitochondria frozen with trehalose retain a number of biological functions and preserve outer membrane integrity. *Cell Death and Differentiation* **14**, 616-624 (2007).