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During Apoptosis Bcl-2 Changes Membrane Topology at Both the Endoplasmic Reticulum and Mitochondria

Short Article

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Summary

In healthy cells the antiapoptotic protein Bcl-2 adopts a topology typical of tail-anchored proteins with only the hydrophobic carboxyl terminus inserted into the membrane, as shown by labeling cell lysates with a membrane-impermeant sulfhydryl-specific reagent. Induction of apoptosis in cells triggered a change in the conformation of Bcl-2 such that cysteine 158 near the base of helix 5 inserted into the lipid bilayer of both endoplasmic reticulum and mitochondria where it was protected from labeling. Addition of a peptide corresponding to the BH3 domain of the proapoptotic protein Bim to cell lysates triggered a similar conformational change in Bcl-2, demonstrating that preexisting, membrane-bound Bcl-2 proteins change topology.

Introduction

Despite more than ten years of analysis and a plethora of experimentally tested models, the molecular details of the mechanism by which Bcl-2 prevents apoptosis remain elusive. One approach to probing the mechanism at the molecular level is to examine the subcellular localization and topology of Bcl-2. Such investigations led to the discovery that Bcl-2 is targeted to membranes by a carboxyl-terminal tail-anchor where it appears to adopt the same topology as other tail-anchored proteins (Chen-Levy and Cleary, 1990; Janiak et al., 1994). Thus, similar to tail-anchored proteins with a wide variety of cellular functions, only the hydrophobic sequence at the extreme carboxyl terminus (helix 9 of Bcl-2) is inserted into the bilayer of the membrane (Figure 1A). Furthermore, removal of helix 9 abolishes the targeting of Bcl-2 to membranes while substitution of this sequence with the hydrophobic carboxyl-terminal sequence from other tail-anchored proteins restores both Bcl-2 binding to membranes and inhibition of apoptosis (Zhu et al., 1996).

Unlike the vast majority of tail-anchored proteins which are targeted to a single membrane, Bcl-2 is targeted to both the endoplasmic reticulum (ER) and mitochondria (Janiak et al., 1994). Mutants of Bcl-2 targeted to endoplasmic reticulum or mitochondria prevent apoptosis, but the protein has overlapping rather than identical functions at each organelle (Zhu et al., 1996; Annis et al., 2001; Thomenius et al., 2003; Hacki et al., 2000). The recent discovery that specific cell death pathways converge at either the ER or mitochondria suggests that dual localization of Bcl-2 allows it to inhibit multiple pathways of apoptosis but has done little to shed light on the molecular mechanisms involved.

Structural data suggest that Bcl-2 may be an atypical tail-anchor protein. NMR and crystal structures of Bcl-2 and its close relative Bcl-XL revealed that the core of these proteins is similar to the pore-forming domain of diphtheria toxin with the relatively hydrophobic helices 5 and 6 protected from aqueous solution by surrounding amphipathic helices (Figure 1C). These structures and subsequent electrophysiology data led to a hypothetical topology for Bcl-2 (Figure 1B) in which helices 5 and 6 are transmembrane sequences (reviewed in Schendel et al., 1998). Similar to channel proteins but unlike other transmembrane sequences, helices 5 and 6 each contain charged residues (Figure 1D). However, a multitransmembrane conformation for Bcl-2 has never been experimentally demonstrated or investigated with physiologic membranes. There is no known precedent of a tail-anchored protein with multiple transmembrane domains. Moreover, based on what is known about the mechanism of targeting to and integration into membranes, multiple transmembrane domains are not predicted to occur in tail-anchored proteins (Wattenberg and Lithgow, 2001).

Currently, there is no experimental data that distinguishes the two proposed topologies (Figure 1). For example, in the multiple transmembrane topology the loop between helix 5 and 6 located on the lumenal side of the membrane is too small to be detected in proteolysis assays (Figure 1). Furthermore, dual localization of Bcl-2 may result in different topologies at ER and mitochondria.

To prevent apoptosis, Bcl-2 has been proposed to interact with and sequester the structurally similar but proapoptotic proteins Bax and Bak. Unlike Bcl-2 and Bak, Bax is a cytoplasmic protein that inserts into membranes only upon induction of apoptosis whereupon it and Bak form a pore large enough to release cytochrome c and other proteins from the intermembrane space (Hsu et al., 1997), consistent with Bax adopting a transmembrane topology. However, a multitransmembrane topology for Bax has not been experimentally demonstrated. Bax differs from Bcl-2 in that the hydrophobic carboxyl terminus of Bax does not function as a tail-anchor (Nechushtan et al., 1999). Thus, a conformational change during apoptosis has not been previously proposed for Bcl-2.

Here we demonstrate that the topology of Bcl-2 in healthy cells is similar to that of other tail-anchor proteins by using chemical labeling in cell lysates of the two cysteine residues native to Bcl-2. Remarkably, a variety of apoptotic signals cause the tail-anchor topology of Bcl-2 to change such that cysteine 158 near the

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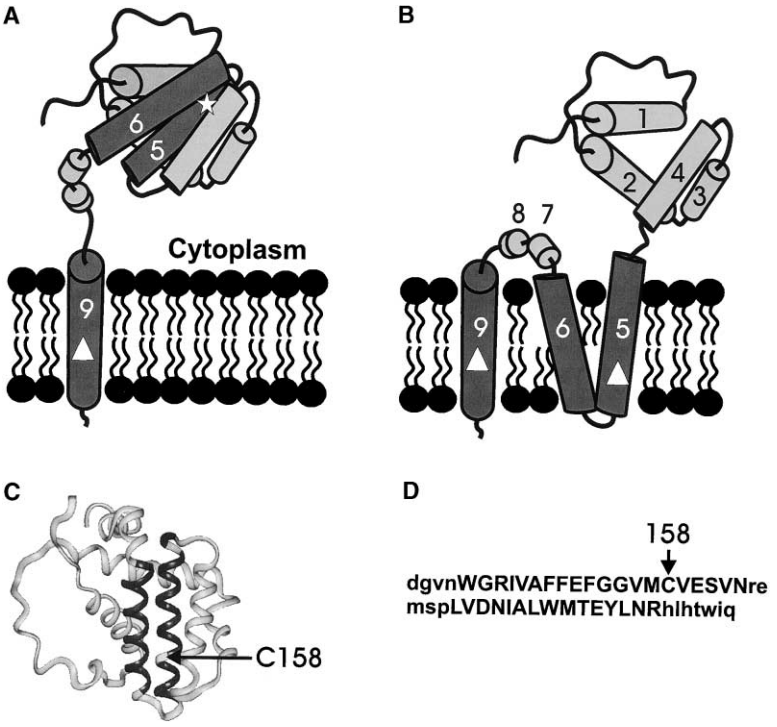

Figure 1. Models for the Topology of Membrane-Bound Bcl-2

(A) Tail-anchored topology. Helix 9, the carboxyl-terminal hydrophobic sequence, functions both as a targeting signal and transmembrane anchor (cylinder labeled 9). The cysteine in helix nine (position 229) is protected from IASD by the bilayer (triangle). The amino terminus of the protein including helices 5 and 6 is located in the cytoplasm; therefore, cysteine 158 in helix 5 can be labeled by IASD (*).

(B) Multispanning transmembrane topology. The central hydrophobic helices (5 and 6) are inserted into the lipid bilayer, and helix 9 is transmembrane. Therefore, both cysteines are protected from IASD (triangles).

(C) Model of the NMR structure of Bcl-2 highlighting helices 5 and 6 (black) and residue C158 (arrow).

(D) Sequence of Bcl-2 residues 140–190, with helices 5 and 6 (capitalized) indicating the location of C158. Adjacent residues and the loop joining the two helices are indicated in lowercase.

base of helix 5 moves from the cytoplasm into the lipid bilayer of both the ER and mitochondria.

Results and Discussion

Bcl-2 Adopts a Typical Tail-Anchor Topology In Vitro

Previous examinations of the topology of Bcl-2 have established that Bcl-2 is accessible to proteases added to the cytoplasmic side of the membrane (Janiak et al., 1994). The carboxyl-terminal hydrophobic sequence of Bcl-2 (helix 9) functions as a tail-anchor and therefore spans the membrane. However, the tail-anchor of Bcl-2 does not insert into liposomes, suggesting that in cells additional proteins are involved (Kim et al., 1997). To further examine the topology of Bcl-2 in cells, we have used the membrane impermeant sulfhydryl-reactive reagent IASD (4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid) to label cysteine residues that are not protected by the lipid bilayer (Krishnasastry et al., 1994). Labeling by this reagent can be detected as a shift in migration of the protein on SDS-PAGE. Bcl-2 contains two cysteine residues. Cysteine 229 is located within the tail-anchor and should be protected from IASD labeling in either of the proposed topologies (Figure 1). The cysteine residue near the base of helix 5 at position 158 (Figure 1D) is predicted to be accessible to IASD in the tail-anchored topology (Figure 1A) but protected by the bilayer if the protein adopts a multispanning transmembrane topology (Figure 1B).

To validate the labeling system and identify the labeling characteristics of each of the two cysteine residues in Bcl-2, we conducted experiments using protein synthesized in reticulocyte lysate. The results of these experiments are consistent with the protein adopting the tail-anchored topology at both ER (Figure 2A) and mitochondria (Figure 2B). Labeling of Bcl-2 without added membranes resulted in two discrete shifts; the uppermost results from labeling at both cysteine residues (Figure 2A, **) while the lower one is due to labeling of one cysteine (*). In the absence of membranes, single labeling identifies a small subfraction of Bcl-2 molecules for which the labeling reaction did not go to completion. When Bcl-2 was incubated with ER membranes, and membranes then isolated and labeled with IASD, the migration of Bcl-2 in SDS-PAGE changed (Figure 2A, *), indicating a single cysteine residue was labeled. The difference in band intensities between reactions with and without added membranes reflects the efficiency of Bcl-2 targeting to membranes in vitro because the membranes were separated from nontargeted proteins by centrifugation prior to labeling. Masking of the cysteine by protein folding or by Bcl-2 binding to another protein is unlikely to account for single labeling because the reaction is carried out in 4M urea. Furthermore, extended incubation in IASD did not increase labeling (data not shown). For a cysteine residue to be protected, it must be inserted into the bilayer of a membrane since IASD crosses intracellular membranes and thereby labels cysteine residues on either side.

To unambiguously demonstrate that cysteine 158 is labeled and cysteine 229 is protected by the membrane, we examined Bcl-2 mutants containing only one of the native cysteine residues. Bcl2-Cb5 is a functional version of Bcl-2 in which the tail-anchor (containing cysteine 229) has been exchanged with that of the ER-specific isoform of cytochrome b5 that does not contain cysteine (Zhu et al., 1996). In reactions with or without ER membranes, labeling of Bcl2-Cb5 results in a single shift on SDS-PAGE (Figure 2A, *) demonstrating that

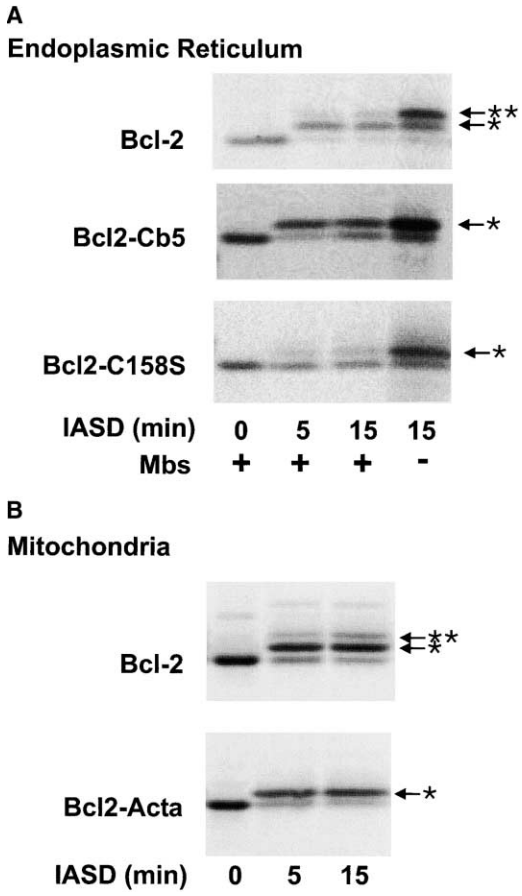

Figure 2. IASD Labeling Suggests that Bcl-2 Adopts a Typical Tail-Anchored Topology In Vitro at Both the Endoplasmic Reticulum and Mitochondria

Bcl-2, Bcl2-Acta, and Bcl2-cb5 were synthesized in reticulocyte lysate, and then (A) ER microsomes (Mbs) or (B) mitochondria were added to the indicated reactions. The membranes were collected by centrifugation, and IASD was added for the times indicated below the lanes. IASD labeling of either cysteine 158 or 229 resulted in a shift in the migration of the 35S methionine-labeled protein on SDS-PAGE detected by phosphorimaging (*). In the absence of membranes the centrifugation step was omitted, and both cysteine 158 and cysteine 229 were labeled, resulting in a double shift in migration (**). The only cysteine in Bcl2-cb5 (targeted to ER) and Bcl2-Acta (targeted to mitochondria), at position 158, was labeled in reactions with and without membranes (*). The single cysteine at position 229 in Bcl2-C158S was protected from IASD by insertion in the lipid bilayer and therefore was labeled only in the absence of membranes (*).

cysteine 158 can be labeled even when Bcl-2 is bound to ER. In contrast, significant labeling of a Bcl-2 mutant in which cysteine 158 was replaced by serine (Bcl2-C158S) was observed only in the absence of membranes, demonstrating that cysteine 229 is protected from IASD when membranes are present and is therefore buried in the bilayer (Figure 2A). Similar results were obtained when rat liver mitochondria were used instead of ER (Figure 2B). In experiments with mitochondria, labeling of Bcl-2 and Bcl2-Acta (a mutant targeted to and functional at mitochondria that contains only cysteine 158 [Zhu et al., 1996]) were assayed (Figure 2B). Similar to the results with ER, only cysteine 229 was protected from IASD by mitochondria. The origin of the small fraction of Bcl-2 labeled on both cysteine residues (**) was not investigated but probably results from protein bound to the outside of the mitochondria or membrane degradation during the assay. Thus, cysteine labeling demonstrates that, in vitro, Bcl-2 adopts a typical tail-anchored topology at both ER and mitochondria. Furthermore, protection of residue 229 from labeling serves as an internal control for membrane integrity that may be compromised by prolonged exposure to urea or proteases in cell lysates.

Bcl-2 Changes Topology upon Induction of Apoptosis

The topology adopted by Bcl-2 during apoptosis may depend on either the pathway activated or the organelle at which Bcl-2 is located. Therefore, we exposed cells expressing Bcl-2 to either etoposide or ceramide, two drugs that induce apoptosis via different pathways (Annis et al., 2001). The cell lines were derived from a Rat-1 cell line (Rat-1MycER^{TAM}), in which activity of the c-*myc* protooncogene can be induced by adding tamoxifen to sensitize the cells to a wide variety of apoptotic signals (Juin et al., 1999). To assay apoptosis in drug-treated cells, we examined both the nuclear morphology of cells stained with Hoescht 33342 and exposure of phosphatidylserine at the plasma membrane with Annexin V conjugated to AlexaFluor 488. In vector-transfected cells, about half of the cells are undergoing apoptosis after 12 hr exposure to etoposide (Figure 3A) while only a small fraction of the cells expressing Bcl-2 exhibit signs of apoptosis. Even after 18 hr exposure to etoposide, Bcl-2 provides some protection to etoposide-treated cells (Figure 3A).

In response to ceramide treatment, apoptosis progresses more slowly than for cells treated with etoposide, but Bcl-2 clearly delays this apoptosis pathway too (Figure 3A). Similar results were obtained for cells treated by either drug when apoptosis was assayed by examining cleavage of the caspase substrate poly (ADP-ribose) polymerase (data not shown). Thus, both drugs efficiently induced apoptosis inhibited by Bcl-2.

Prior to the addition of drug (Figure 3B, top panel, 0 hr), one of the two cysteine residues in Bcl-2 was protected from labeling while the other cysteine was fully labeled. Labeling was examined by immunoblotting for Bcl-2 after 5, 10, and 15 min exposure of cell membranes to IASD. Increasing either the concentration of IASD or the incubation time did not increase labeling (data not shown). The small amount of double labeling detected in Bcl-2 immunoblots probably represents a small fraction of protein that did not anchor into membranes or is due to membrane degradation during incubation. Experiments described below using Bcl2-Cb5 and Bcl2-Acta demonstrated that in healthy cells cysteine 158 in helix 5 was labeled while cysteine 229 in the tail-anchor was protected. Thus, in healthy cells Bcl-2 adopts the tail-anchored topology (Figure 1A).

In response to the induction of apoptosis, most Bcl-2 molecules become fully protected from labeling (18 and 48 hr for etoposide and ceramide, respectively), indicating that cysteine 158 becomes embedded in membranes (Figure 3B, arrows) as quantified by densitometry (Figure 3C). Protection from labeling is not due solely to a con-

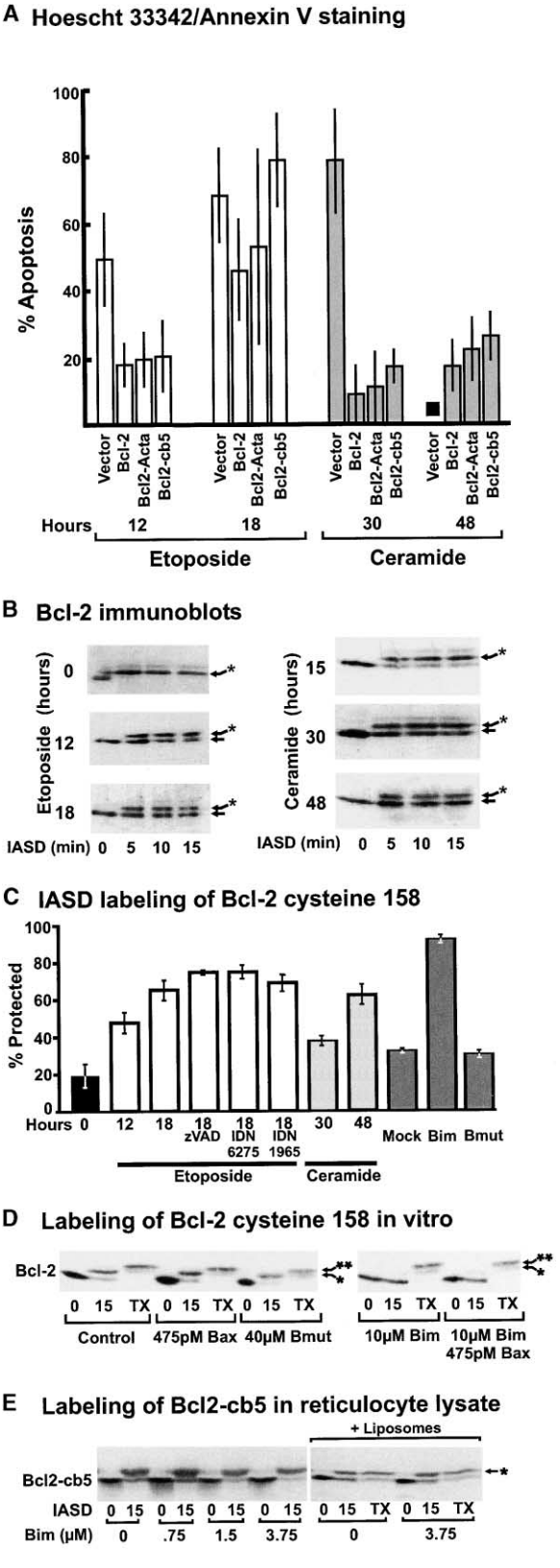

Figure 3. Bcl-2 Changes Topology upon Induction of Apoptosis

(A) Apoptosis assayed by staining cells with Annexin V and Hoescht 33342. Cells were scored as apoptotic if they stained by Annexin V and exhibited a condensed nucleus. The total number of cells was determined by counting all nuclei. The solid square indicates most of the cells had died and too few cells were left on the slide to count.

(B) Bcl-2-expressing cells were treated with etoposide or ceramide for the times indicated to the left of the panels. Following drug treatment, membrane fractions were labeled by incubation with IASD for the times indicated. The samples were separated by SDS-PAGE, and protected (arrows) and labeled Bcl-2 molecules (*) were identified by immunoblotting.

(C) The percentage of Bcl-2 cysteine 158 protected from IASD labeling for 15 min was determined by densitometry of data obtained from three independent experiments; error bars indicate one standard deviation. In some reactions caspase inhibitors (zVADfmk [zVAD], IDN-6275, or IDN-1965, as indicated) were added throughout treatment with etoposide. The changes characteristic of apoptosis were induced directly in mitochondria by incubating whole-cell lysates with Bim peptide (Bim) for 30 min prior to IASD labeling. As controls cell lysates were mock treated (Mock) or treated with a Bim mutant peptide (Bmut).

(D) Bim peptide triggers protection of Bcl-2 cysteine 158 from IASD labeling on isolated mitochondria. * indicates Bcl-2 molecules labeled at cysteine 158. ** indicates labeling of both cysteine 158 and 229 in control reactions containing 1% Triton X-100 (TX).

(E) In reactions containing >100-fold excess Bim peptide, cysteine 158 of Bcl2-cb5 synthesized in reticulocyte lysate is labeled (*) after incubation with IASD for 15 min with and without liposomes.

formational change in the cytoplasmic domain of Bcl-2 or to binding to another protein as the labeling reaction is performed in 4M urea (to unfold Bcl-2 and efficiently label residue 158), yet the protected residue can be labeled when either CHAPS or Triton X-100 is added to solubilize the membranes (Figure 3D and data not shown). Cysteine 158 is located within a hydrophobic region of Bcl-2 (helix 5–6) that is similar to the pore-forming domain of diphtheria toxin. Thus, it is very likely that protection of cysteine 158 is due to insertion of both helix 5 and 6 into the membrane as transmembrane sequences. These observations suggest two remarkable and unexpected results. First, proteins targeted to membranes by tail-anchor sequences can adopt complex topologies including multiple transmembrane domains. Second, Bcl-2 membrane topology is not fixed during or immediately after biosynthesis but undergoes a major structural transformation upon induction of apoptosis.

Protection of cysteine 158 from labeling occurs after drug treatment, suggesting that caspase activation may indirectly trigger this conformational change. However, inhibition of caspases using three different inhibitors (zVADfmk, IDN-6275, and IDN-1965) at concentrations that completely inhibit PARP cleavage (data not shown) neither prevented nor delayed insertion of cysteine 158 into membranes (Figure 3C). This suggests that insertion of cysteine 158 into the bilayer begins prior to, or is independent of, activation of caspases.

In Response to BH3 Peptides, Bcl-2 Changes Conformation in Isolated Mitochondria

To determine whether the change in conformation occurs in preexisting Bcl-2 or Bcl-2 newly synthesized in response to drug treatment, we tested whether protection of cysteine 158 could be triggered in cell lysates. We used a peptide corresponding to the BH3 domain of the proapoptotic BH3-only protein Bim (Coultas et al., 2002), known to bind to and antagonize the antiapoptotic activity of Bcl-2. Addition of Bim BH3 peptide to

lysates from Rat-1mycER^{TAM} cells led to protection of cysteine 158 of Bcl-2 from IASD labeling, consistent with insertion into membranes (Figure 3C, Bim). Protection from labeling was not observed in a mock reaction or with a control peptide containing an inactivating Leu to Ala mutation corresponding to position 94 in Bim (Figure 3C, Bmut).

To determine whether cytosolic proteins are required to trigger the conformational change in Bcl-2, we added Bim peptide to isolated membrane fractions. When Bcl-2 topology was examined using membrane fractions isolated from Bcl-2-expressing cells, cysteine 158 was labeled irrespective of whether or not Bax or the Bim mutant peptide (Bmut) were added (Figure 3D, *). However, addition of Bim peptide triggered insertion into membranes confirming that cytosolic proteins are not required for the conformation change (Figure 3D). Furthermore, when the membranes were solubilized by 1% Triton X-100 (Figure 3D,**), protection of the cysteine was abolished confirming protection is afforded by the bilayer. This concentration of detergent does not interfere with the binding of proapoptotic family members to Bcl-2 (Hsu and Youle, 1997) demonstrating that interactions with these proteins do not protect cysteine 158 from labeling.

Thus, the interaction of the Bim peptide (but not the Bmut peptide) with Bcl-2 and/or membrane proteins provokes a conformational change in preexisting Bcl-2 molecules that results in the insertion of cysteine 158 into the lipid bilayer of ER, mitochondria, or both membranes.

Bcl-2 will not target to liposomes. Therefore, to determine whether there is any role for membrane proteins in the conformational change, we targeted Bcl2-cb5 synthesized in reticulocyte lysate to liposomes and then added Bim peptide. The cytochrome b5 tail-anchor has been well established to insert spontaneously into liposomes. In control experiments without liposomes, cysteine 158 of Bcl2-cb5 incubated with a large excess of Bim peptide ($>$100-fold) remained accessible to IASD (Figure 3E). Although the protein efficiently inserted into liposomes (data not shown), only a small fraction inserted in a topology in which cysteine 158 was protected, and that fraction was not increased by addition of Bim peptide. Thus, in response to Bim peptide, cysteine 158 of Bcl2-cb5 did not insert into liposome membranes, suggesting that additional proteins may be required.

Bcl-2 Inserts Cysteine 158 into ER and Mitochondria

To determine whether the Bcl-2 conformational change occurs at both ER and mitochondria, Rat-1MycER^{TAM} cell lines were constructed that stably express Bcl2-Acta (Figure 4A) or Bcl2-Cb5 (Figure 4B), and targeting was confirmed by immunofluorescence microscopy (Figure 4, top panels). Hoescht and Annexin V staining (Figure 3A) suggest that these cell lines are more resistant to etoposide and ceramide than vector-transfected controls. IASD labeling of cell lysates confirmed that protection of cysteine 158 occurs only after drug treatment (Figure 4, bottom panels). Taken together our results demonstrate that the drug-induced conformational change occurs at both mitochondria and ER.

To extend our results to human cells and a clinically relevant anticancer drug, we investigated conformational changes for Bcl-2 expressed in the MCF-7 breast cancer cell line treated with doxorubicin. In untransfected cells treated with 10 micromolar doxorubicin, PARP is all cleaved after 24 hr. However, in Bcl-2-expressing cells treated with 80 micromolar doxorubicin, PARP cleavage was less than 50% at 24 hr and does not approach completion until after 48 hr. Consistent with the results obtained using rat fibroblasts, protection of cysteine 158 from IASD labeling was observed 24 and 48 hr after adding doxorubicin to cells expressing Bcl-2 (data not shown).

The conformational change in Bcl-2 may be triggered by an activated BH3 protein directly (Bim peptide) or indirectly via an interaction with activated Bax/Bak. Nevertheless, the coordination between insertion of cysteine 158 and the induction of apoptosis suggests three potential roles for the conformational change. (1) It may be integral to the antiapoptotic activity of Bcl-2. (2) It may convert Bcl-2 from anti- to proapoptotic as has been reported for caspase-dependent cleavage of Bcl-2 (Cheng et al., 1997). (3) It may be a consequence of apoptosis rather than involved in regulation (Wilson-Annan et al., 2003).

We do not see an acceleration in the rate of apoptosis once the membrane-inserted form is detected, a result inconsistent with model 2. Although our data do not distinguish models 1 and 3, we favor model 1 because it suggests a mechanism of action for Bcl-2 that explains why Bcl-2 delays rather than abolishes apoptosis. In this model Bcl-2 functions as a suicide inhibitor during apoptosis by inserting into the membrane and binding to Bax/Bak, thereby promoting their assembly in dead end complexes. If BH3 protein binding to Bcl-2 and Bax/Bak is competitive, then according to this scenario the rate of cell death would be determined by the expression levels and the relative affinities of binding of BH3-only proteins to Bcl-2 versus Bax/Bak. Since Bax does not need to bind to other proteins in order to form pores in membranes, model 1 is also the most consistent with the observation that Bcl-2 binds to Bak (and presumably Bax) only after induction of apoptosis (Cheng et al., 2003). However, it remains to be determined which conformation of Bcl-2 binds to Bax/Bak. Moreover, in some cell types Bcl-2 prevents Bax from translocating to mitochondria, suggesting that Bcl-2 may also regulate apoptosis independent of binding to Bax/Bak. Therefore, the conformational change may play an active role in regulating apoptosis at only one organelle (ER or mitochondria) or in response to only a subset of apoptotic agonists. It will be of great interest to determine how the different conformations of Bcl-2 contribute to these seemingly disparate regulatory processes.

Our observation that preexisting Bcl-2 molecules anchored to membrane in the tail-anchored topology can be triggered to undergo a change in membrane topology demonstrates an entirely novel method for generating a polytopic membrane protein at the ER. Because the ER is the source of membrane for a wide variety of intracellular organelles, it is possible that initial targeting via tail-anchor sequences could result in proteins that later adopt complex topologies at many locations within the cell. A regulated topology change would be an at-

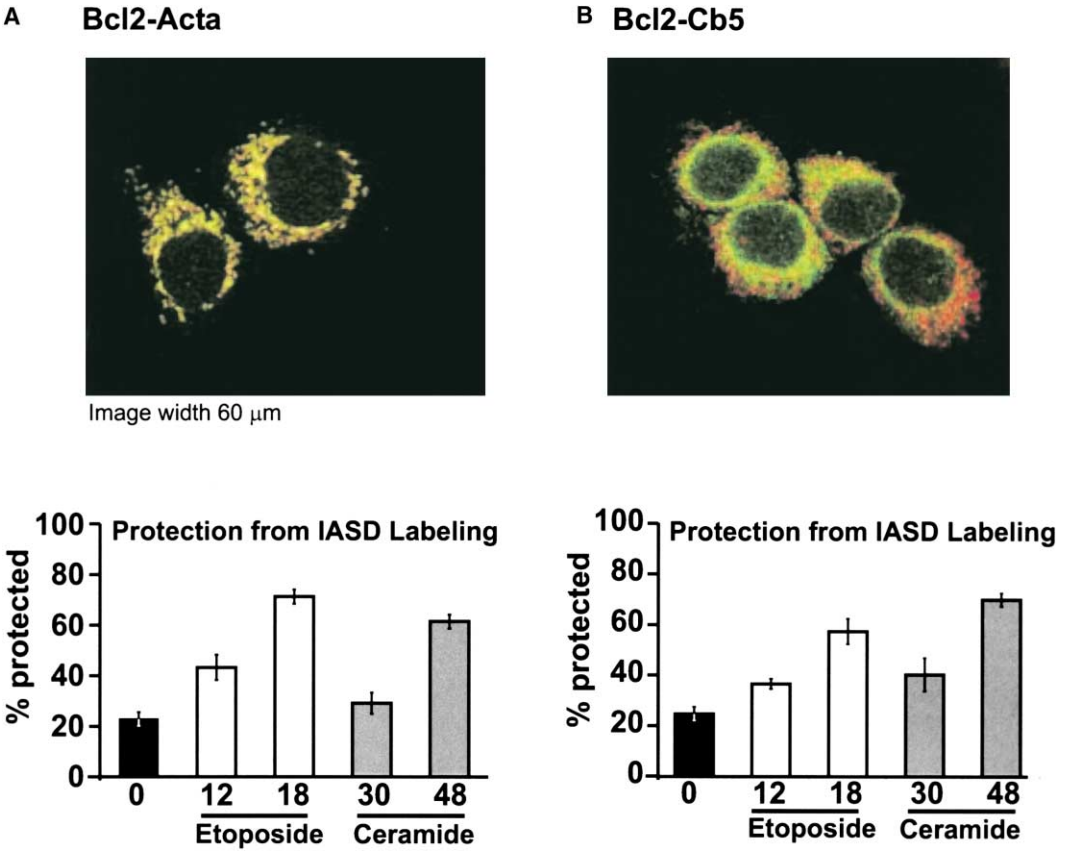

Figure 4. After Induction of Apoptosis, Bcl-2 Cysteine 158 Becomes Protected from IASD Labeling at Both Mitochondria and ER

Cells expressing (A) Bcl2-Acta at mitochondria or (B) Bcl2-Cb5 at the ER were examined by immunofluorescence microscopy (top panels) or treated with etoposide or ceramide for the times indicated. As expected, Bcl2-Acta (green) colocalized with mitochondrial Hsp60 (red) whereas Bcl2-cb5 (green) did not. Following drug treatment, membrane fractions were labeled by incubation with IASD for 15 min. After SDS-PAGE, immunoblotting, and quantification by densitometry, the percentage of protein protected from labeling was determined (bottom panels). Error bars indicate one standard deviation from data obtained from three independent experiments.

tractive novel method for ensuring that membrane proteins that might have a deleterious function at the ER achieve their correct subcellular localization prior to insertion into the membrane and activation.

Experimental Procedures

Plasmid Construction

The expression constructs encoding Bcl-2, Bcl2-Cb5, and Bcl2-Acta and retroviral infection of cell lines have been described (Zhu et al., 1996). To construct plasmids encoding Bcl2-C158S the codon for cysteine 158 was changed to a codon for serine using the QuikChange method (Stratagene).

Induction and Assesment of Apoptosis

To induce apoptosis, Rat1-MycER^{TAM} cells were treated with 100 nM tamoxifen and either 6 μM etoposide or 50 μM ceramide for the indicated times. Caspase inhibitors (IDN-6275, IDN-1965 [Wu and Fritz, 1999], provided by Idun Pharmaceuticals; or zVADfmk, from Enzyme System Products) were added at time zero (and for zVADfmk additionally every 6 hr) at 20, 40, and 20 μM final concentrations, respectively. Hoescht 33342 and AlexaFluor 488 AnnexinV (Molecular Probes) were used as recommended.

Subcellular Fractionation

Cells were disrupted using nitrogen cavitation as described (Annis et al., 2001). Lysate was subjected to centrifugation in a TL-100 table top centrifuge (rotor TLA100.2, Beckman) at 1000 × g for 25 min to yield supernatant (S^1) and pelleted (P^1) fractions. The S^1 fraction was further separated into S^2 and P^2 fractions by centrifugation at 213 000 × g for 30 min. Both pellets were resuspended in cell buffer (Annis et al., 2001) prior to labeling. Labeling of Bcl2-Acta and Bcl2-Cb5 is shown for the P^1 and P^2 fractions, respectively, as they contained the bulk of these proteins.

For cell free assays, heavy membrane fractions enriched in mitochondria were prepared as described (Desagher et al., 1999) except that cells were lysed by nitrogen cavitation (as above). Heavy membranes (50 μg of protein in 25 μl) were combined with Bim (Ac-MRPEIWIAQELRRIGDEFNA-amide) or Bmut (Ac-MRPEIWIAQEAR RIGDEFNA-amide) peptides and/or recombinant human full-length Bax (purified as described [Suzuki et al., 2000]) and incubated for 1 hr at 30°C. Membranes were separated from the reactions by centrifugation at 13,000 × g for 5 min, and the pellets were labeled with IASD.

Chemical Labeling of Cysteine Residues in Bcl-2

The chemical labeling gel-shift assay (see the Supplemental Data at http://www.molecule.org/cgi/content/full/14/4/523/DC1) was adapted from that described (Krishnasastry et al., 1994) in which cysteine residues were modified by incubation with IASD (4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid) (Molecular Probes). Samples were adjusted to 4M urea, and IASD was added to a final concentration of 16 mM; aliquots were removed at the indicated labeling times and mixed with 5 μl of 1M DTT to quench the reaction. The radioactive polypeptides were visualized and quantified using a phosphorimager (Molecular Dynamics). Proteins

expressed in Rat-1MycER^{TAM} cells were visualized by immunoblotting, developed using enhanced chemiluminescence as above, and quantified using a densitometer.

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