Cell Death and Differentiation (2006) 13, 1277–1280

www.nature.com/cdd

News and Commentary

Regulated targeting of Bax and Bak to intracellular membranes during apoptosis

HM Heath-Engel[1] and GC Shore*,[1]

[1] Department of Biochemistry, McGill University, Montreal, Quebec, Canada
* Corresponding author: GC Shore, McGill University, Department of Biochemistry, McIntyre Medical Sciences Building, Room 906B, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6. Tel: +514 398 7282; Fax: +514 398 7384; E-mail: gordon.shore@mcgill.ca

Cell Death and Differentiation (2006) **13,** 1277–1280.
doi:10.1038/sj.cdd.4401961; published online 19 May 2006

As the result of a paper that one of us (GCS, together with Jian-Ming Li) had published in 1992 in *Science* on the regulation of the orientation of transmembrane (TM) protein segments in the mitochondrial outer membrane (MOM), a series of discussions and collaborations was initiated with Stan Korsmeyer on the question of targeting and insertion of Bcl-2 into the MOM. During one such conversation, Stan (enthusiastically, as always) described how his lab had made the totally unanticipated discovery of a Bcl-2 binding partner, which was related to Bcl-2 but exhibited pro-apoptotic activity. The protein, of course, was Bax, and its description by Havai *et al.* ushered in the 'rheostat' model for regulation of apoptosis by opposing pro-survival and pro-death proteins.[1] Thousands of papers later, Bax and the related protein Bak have emerged as the critical death effectors among a triumvirate of Bcl-2 subfamilies, and a hotly debated relationship between these subfamilies has emerged to explain, at least in part, the regulation of the core mitochondrial apoptosis pathway.[2]

Here, we have focused primarily on just one aspect of the complex regulation of the Bax and Bak death effectors: that of their regulated insertion into the MOM, as a pre-condition for their subsequent oligomerization and permeabilization of the outer membrane, which is necessary for the release of apoptotic factors such as cytochrome *c*. Our own interest in this question again resulted in a collaboration with Stan, which was initiated by a discussion with Stan and Atan Gross outside the cafeteria at Cold Spring Harbor in 1997. We later decided to join forces to address our observation that insertion of Bax into the outer membrane was regulated by a short 20-amino-acid sequence at the extreme NH_2-terminus of the protein, which seemed to repress the TM segment. Atan, meanwhile, had discovered that insertion was accompanied by a corresponding conformational alteration, requiring the activation of caspases in response to withdrawal of a growth factor, IL-3. As always, Stan was remarkably open to interactions and discussions.

It is now known, of course, that the three subfamilies of the Bcl-2 family are involved in a dynamic interaction. The pro-apoptotic BH3 only subfamily (so-called because these members contain only one of the four possible Bcl-2 homology domains, BH1–4, that define the Bcl-2 family) represents a diverse group of proteins, which are either activated (if they pre-exist in the cell) or their synthesis is induced by specific apoptosis-initiating stress signals. Once activated, the BH3 only proteins then regulate the downstream activity of the two multi-BH domain-containing subfamilies. All BH3 only members seem to bind to and antagonize one or more of the pro-survival multidomain proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1, which contain all four BH domains). Some (for example, truncated Bid and Bim) transiently interact with and activate the multidomain death effectors Bax and Bak, which, in contrast to the pro-survival members, lack the BH4 domain.

In excess, the pro-survival Bcl-2 proteins can bind to and prevent the oligomerization of Bax and/or Bak, either through constitutive interactions (as suggested for Mcl-1 and Bcl-X_L) or following activating conformational changes in Bax/Bak (as suggested for Bcl-2). Thus, a complex three-way relationship based on binary interactions exists between the multiple members of the Bcl-2 subfamilies. Such complexity has presumably evolved in order to construct a Bcl-2 network that can interpret, regulate, and execute the myriad signaling pathways that can ultimately trigger the mitochondrial death machinery. As if this were not enough, studies over the past several years have identified a plethora of 'outsiders' that may interact with and therefore overlay the Bcl-2 network, adding additional complexity to its regulation (see Lucken-Ardjomande and Martinou[3] for a recent review). But at the heart of all this is whether or not Bax and/or Bak become oligomerized within the MOM in response to a death stimulus, unleashing proteins from within the mitochondrial intermembrane space, and thereby activating cytoplasmic caspases and causing fragmentation of chromatin. In addition to their role in regulating the mitochondrial apoptosis program, however, Bcl-2 family proteins also function at the endoplasmic reticulum (ER) to regulate, among other things, the homeostasis and release of ER Ca^{2+}, by mechanisms that are as yet unclear.[4]

Again, in this brief News and Commentary, we have focused on a defined step of the Bax and Bak activation pathway: the question of how these proteins are targeted to and inserted into the bilayer of the MOM, before the formation of oligomeric structures that contribute to membrane-permeabilizing pores. The induction of higher order oligomeric structures of Bax and Bak, their role in permeabilizing the MOM and releasing proteins, including cytochrome *c*, from mitochondria, as well as their possible relationship with the mitochondrial fission–fusion machinery have been extensively reviewed in the recent literature (and elsewhere in this issue of *Cell Death and Differentiation*). Finally, we comment on the emerging question of regulated targeting of Bax and Bak to the membrane of the ER.

Initiation

Bax contains a COOH-terminal TM segment, referred to as a signal-anchor or tail-anchor sequence, which is responsible for the initial targeting and integration of the protein into the lipid bilayer of the MOM.[5,6] However, in contrast to Bak, which is constitutively integrated in the MOM in viable cells, Bax remains, in the absence of an apoptotic stimulus, either as a soluble entity in the cytoplasm or loosely associated with membrane surfaces.[5–7] Dissection of Bax by domain deletion and domain swapping revealed that the TM is functionally repressed by an NH_2-terminal segment termed the apoptosis regulation of Bax targeting (ART) domain, which precedes helix 1 in the Bax structure.[6] Moreover, several recent studies have indicated that the active/inactive conformations of Bax are controlled by a specific residue within the ART domain (Pro13) and by Pro168, which precedes the TM domain.[8,9] In particular, Pro168 has been proposed to mediate communication between the ART domain and the TM.[8] Although other domains within the protein might also affect Bax targeting,[9] the key appears to be de-repression of the TM region, which then likely serves to target, and seed the insertion of, multiple helices into the MOM bilayer.

Elucidation of the three-dimensional structure of Bax, derived following cleavage of a recombinant fusion protein, revealed that the TM segment (helix 9) occupies a hydrophobic groove in the protein formed by the BH1–3 domains, thereby sequestering the TM region and preventing membrane targeting.[10] Moreover, by occupying this groove, the TM domain may occlude interactions with a BH3-only protein such as Bid. Interestingly, the NH_2-terminus of Bax is unstructured and solvent exposed in this recombinant construct, contrary to the finding that in native Bax, the NH_2-terminus is strongly resistant to exogenous protease before a death stimulus.[6] Whether this means that the NH_2-terminus is shielded by another protein[3,11] or that the NH_2-terminus adopts a different orientation during the processive $NH_2 \rightarrow$ COOH synthesis of the Bax polypeptide *in situ* as compared to the bacterial fusion protein remains unresolved. If shielded by another protein, this protein must be a component of reticulocyte lysate in the absence of mitochondria, as the NH_2-terminus remains inaccessible following Bax translation *in vitro*.[6] Nevertheless, the combined structural and domain mutagenesis information has revealed, not surprisingly, that the specificity for the initial stages of Bax activation and mitochondrial targeting during apoptosis is encoded within the primary sequence.

The signature event associated with the transition of Bax that results in its insertion into the MOM is the attendant (and dramatic) change in protein conformation. But what stimulates this change? Perhaps the best understood scenario is the activation of Bax through its interaction with Bid,[7] but there is the potential for many other mechanisms at play. For instance, proteins proposed to sequester cytosolic Bax may be inactivated/degraded,[3,11] and changes in the intracellular environment such as acidification, alkalinization, increased production of reactive oxygen species, and heat shock have also been linked to Bax activation.[12–14] One possibility is that these stresses act directly on Bax to elicit conformational transformations. Such processes may function alone or in combination, are likely dependent on the nature of the apoptotic stimulus, and almost certainly do not represent the full spectrum of potential mechanisms.

Membrane Recognition and Insertion

Although multiple domains and domain interactions within Bax may aid in mitochondrial delivery of the protein, the cardinal requirement of the TM segment for targeting and membrane insertion is well established.[5,6,10] The fact that the Bax TM region efficiently delivers and inserts a monomeric cytosolic reporter protein, dihydrofolate reductase, into the MOM, argues, on thermodynamic grounds, that simple accessibility of this segment in the context of monomeric Bax should suffice. The initial suggestion that Bax dimerizes to insert into the MOM bilayer[15] was based on the enforced dimerization of FKBP-Bax by the bivalent ligand FK1012. Such forced dimerization at the NH_2-terminus of Bax could have conformationally altered the NH_2-terminal domain, indirectly de-repressing the TM region. Under physiological conditions, de-repression is likely a property of monomeric Bax. Nevertheless, de-repressed Bax is presumably recognized by a 'receptor' at the MOM, and, in one case, activated tBid might act as a combined de-repressor and receptor, either alone or in combination with other MOM proteins. An interaction between tBid and cardiolipin reportedly mediates recruitment of this protein to sites where the outer and inner mitochondrial membranes are in close contact,[16] and this event could potentially be the initial stimulus required for Bax translocation.

In addition to membrane insertion of the Bax TM domain, the *functional* insertion of Bax involves additional integration of helices 5 and 6 into the MOM lipid bilayer. This process, in the context of Bax oligomers, is thought to contribute to/mediate pore formation, which in turn may allow the release of intermembrane space proteins such as cytochrome *c*.[2] This model is based in part on the structural similarities between Bax and the pore-forming domains of diphtheria toxin and colicin, ideas that have contributed to the notion that Bax oligomerization occurs prior to or concomitant with insertion of helices 5 and 6 into the bilayer. This is in contrast, however, to the finding that Bax can achieve stimulus-induced alkaline-resistant insertion into the MOM bilayer without mediating release of cytochrome *c* to the cytosol.[17] More recently, an elegant and informative analysis of the sequence of events involved in Bax insertion and oligomerization has been conducted by Annis *et al.* This study employed a genetic block of Bax oligomerization at the MOM, combined with the use of a chemical probe, to assess domain insertion into the bilayer.[18] In this way, events before Bax oligomerization could be interrogated.

Dissecting Bax Insertion into the MOM Bilayer

Annis *et al.*[18] have exploited the observation that a rat fibroblast cell line deleted of c-*myc* is defective in releasing cytochrome *c* from mitochondria in response to DNA damage, and yet appears to support Bax migration to the organelle. To

elucidate the defective step along the Bax pathway for cytochrome *c* release, mitochondria isolated from Myc+ and Myc−/− cells were incubated with recombinant Bax and tBid. The results of this experiment indicated that the defect in Myc−/− cells resides at the level of their mitochondria. Moreover, the defect was correlated with an inability of these mitochondria to support oligomerization of Bax, as assayed by standard chemical crosslinking and gel filtration chromatography. Nevertheless, Bax migrated to mitochondria in Myc−/− cells and inserted into the MOM in response to a stress stimulus (etoposide), probably as a monomer.

The events associated with membrane insertion of pore-forming helices 5 and 6, and the TM helix 9, were probed following expression of Bax mutants harboring cysteine residues introduced at specific sites within the sequence. Labeling of these cysteine residues with the membrane bilayer-impermeant chemical 4-acetamido-4′-((iodoacetyl) amino) stilbene-2,2′-disulfonic acid (IASD) was then monitored in mitochondria isolated from etoposide-treated or control Myc−/− cells, providing an assay for membrane insertion of sequences tagged with the reactive cysteine residue. Collectively, the results of this experiment indicated that all three helices inserted in the bilayer in the absence of Bax oligomerization. These findings argue that, unlike other pore-forming proteins, Bax forms membrane-integrated multi-spanning monomers that subsequently oligomerize within the bilayer, as opposed to insertion of the pore domain of preformed oligomers.[18]

Although it remains formally possible that the Myc−/− block interfered with Bax oligomerization at the mitochondria before membrane insertion, and that the authors were actually measuring a fall-back pathway, this seems unlikely in view of the above-discussed mechanisms of Bax targeting and membrane insertion. Furthermore, Bax insertion into the MOM of Myc−/− cells appeared to be efficient, which would not be expected for a fall-back pathway. It remains possible, however, that the pre-oligomerization pathway that was manifested by the Myc−/− block may not extend to all pro-apoptotic stimuli. Nevertheless, the study described by Annis *et al.*[18] is significant because it clearly demonstrates that insertion of the Bax TM region and of the pore-forming helices 5 and 6 can be distinct from the follow-on mechanism of Bax oligomerization, which therefore must occur within the bilayer. A logical extrapolation of this finding is that oligomerization may drive pore formation, either autonomously or in combination with other component(s) of the MOM. Furthermore, it is reasonable to expect that additional mitochondria-associated proteins at least regulate this follow-on oligomerization, because Myc−/− cells presumably either overexpress an inhibitor or have lost an activator of this process. Such a concept is consistent with previous studies on the activation of Bax by tBid, which indicated that the oligomerization step was dependent not only on tBid but also on another (unidentified) mitochondrial protein.[19]

Finally, the findings of Annis *et al.*[18] are potentially informative with respect to the mechanism of Bak activation, which involves the transition of previously integrated membrane monomers to oligomers following a stimulus such as tBid.[20] What has not been determined for Bak, however, is the orientation of (predicted) helices 5 and 6 relative to the bilayer in unstimulated monomers that are anchored via the TM. Interestingly, these membrane-integrated but inactive monomers of Bak share with cytosolic Bax an NH_2-terminal domain that becomes exposed following a stimulus initiating intramembrane oligomerization of the protein.[20,21] Also of note, inactive Bak monomers appear to be sequestered by the voltage-dependent anion channel 2 (VDAC2), and this interaction likely stabilizes Bak in an inactive conformation.[22] Mcl-1 and Bcl-X_L might perform a similar function.[11] Whether or not any of these factors contribute to the targeting of newly synthesized Bak as it leaves the ribosome and diffuses to mitochondria has not yet been explored.

Based on the above-described studies, we can propose a basic model for Bax/Bak membrane targeting and insertion, as shown in Figure 1. This model, although incomplete, provides a basic mechanistic framework that can be expanded and refined as appropriate, based on future and ongoing studies.

Bax/Bak at the Endoplasmic Reticulum

Compared to their role at the mitochondria, our understanding of the role of Bcl-2 family members at the membrane of the ER

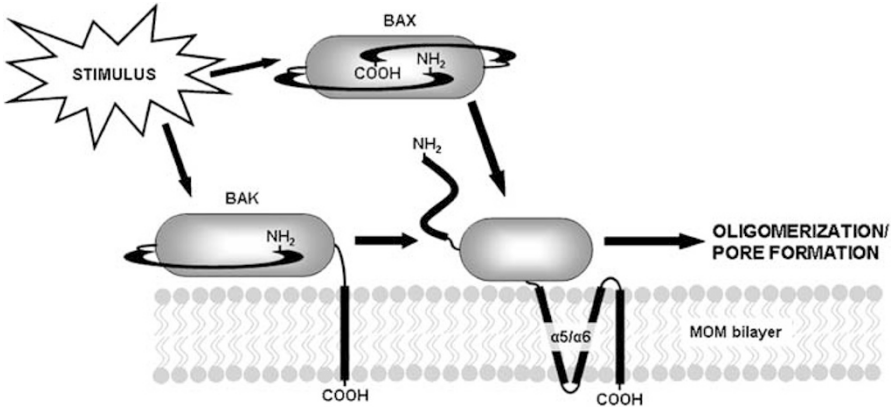

Figure 1 Working model for the activation and membrane insertion of Bax and Bak. In response to a pro-apoptotic stimulus, Bax and Bak undergo an activating conformational change, leading to exposure of a previously hidden NH_2-terminal segment and membrane insertion of the COOH-terminal TM domain (Bax) and the pore-forming region (helices 5 and 6) (Bax and presumably Bak). Subsequent oligomerization and pore formation events then follow. See text for further details

is in its infancy. Analyses of MEFS, derived from the *Bax,Bak* double-knockout (DKO) mouse, however, clearly establish a role for Bax and Bak in the regulation of ER Ca^{2+} stores as a control point for downstream events in apoptosis.[23] As Scorrano *et al.*[23] also reported that about 15% of cellular Bax in healthy wild-type cells is associated with the ER, the question of targeting and insertion becomes relevant. For example, is this ER population of Bax/Bak in unstimulated wild-type cells integrated in the membrane or is it peripherally associated? If the latter, is it inactive or is it perhaps associated with one or more ER proteins, and are these proteins regulated by Bax and Bak?

In addition to their constitutive presence at the ER, stimulus-induced translocation and/or oligomerization of both Bax and Bak have been reported at this location, and correlate with Ca^{2+} redistribution.[24,25] Little, however, is known about the functional relevance of these oligomers. *A priori*, is there reason to suspect that the concepts that have emerged for regulated targeting, insertion, and control of these proteins at the MOM should not pertain to the ER? Is it simply that the 'client' (i.e., the ER) and not the underlying process is different? Or are there significant surprises in store?

Elucidation of the recruitment, insertion, and role of Bax and Bak in the MOM was propelled largely by the ability to reconstitute both the targeting and functional activities of these proteins in isolated mitochondria (and liposomes) *in vitro*. Whether such *in vitro* reconstitution techniques using ER-derived vesicles will prove useful and/or feasible seems far less certain, especially if Ca^{2+} is the main currency of the Bcl-2-regulated ER pathway in apoptosis. Novel approaches to the investigation of protein complexes at the ER that either contain or are influenced by Bax/Bak will likely be needed, as will development of new strategies to dissect the role of Bax and Bak at the ER in intact cells.

Of particular interest will be the relationship and potential crosstalk between Bax/Bak at the ER and Bax/Bak at the mitochondria, and whether the relative distribution of these proteins between the two organelles represents a potential regulatory strategy. In at least one case, selective targeting of a BH3-only protein to the ER can set in motion a Ca^{2+}-mediated signaling pathway, which is transmitted to mitochondria and leads to remodeling of inner membrane cristae. This then results in mobilization of intra-cristae stores of cytochrome *c*, allowing large-scale egress of cytochrome *c* to the cytosol following Bax/Bak-mediated MOM permeabilization.[26] Although such crosstalk between the two organelles provides at least one rationale for a role of the Bcl-2 family at the ER, future research will no doubt uncover many more. Unfortunately, however, as the quest to understand the role of Bcl-2 members at the ER unfolds, we will no longer benefit from the keen insights that Stan Korsmeyer so often provided in the past.

Acknowledgements

HMH-E is the recipient of a National Cancer Institute of Canada Terry Fox Foundation Studentship.

1. Oltvai ZN *et al.* (1993) Cell 74: 609–619.
2. Danial NN and Korsmeyer SJ (2004) Cell 116: 205–219.
3. Lucken-Ardjomande S and Martinou JC (2005) J. Cell Sci. 118: 473–483.
4. Distelhorst CW and Shore GC (2004) Oncogene 23: 2875–2880.
5. Wolter KG *et al.* (1997) J. Cell Biol. 139: 1281–1292.
6. Goping IS *et al.* (1998) J. Cell Biol. 143: 207–215.
7. Desagher S *et al.* (1999) J. Cell Biol. 144: 891–901.
8. Schinzel A *et al.* (2004) J. Cell Biol. 164: 1021–1032.
9. Cartron PF *et al.* (2005) J. Biol. Chem. 280: 10587–10598.
10. Suzuki M *et al.* (2000) Cell 103: 645–654.
11. Willis SN and Adams JM (2005) Curr. Opin. Cell Biol. 17: 617–625.
12. Cartron PF *et al.* (2004) FEBS Lett. 578: 41–46.
13. D'Alessio M *et al.* (2005) FASEB J. 19: 1504–1506.
14. Pagliari LJ *et al.* (2005) Proc. Natl. Acad. Sci. USA 102: 17975–17980.
15. Gross A *et al.* (1998) EMBO J. 17: 3878–3885.
16. Lutter M *et al.* (2000) Nat. Cell Biol. 2: 754–761.
17. Ruffolo SC *et al.* (2000) Cell Death Differ. 7: 1101–1108.
18. Annis MG *et al.* (2005) EMBO J. 24: 2096–2103.
19. Roucou X *et al.* (2002) Biochem J. 368: 915–921.
20. Ruffolo SC and Shore GC (2003) J. Biol. Chem. 278: 25039–25045.
21. Griffiths GJ *et al.* (2001) Oncogene 20: 7668–7676.
22. Cheng EH *et al.* (2003) Science 301: 513–517.
23. Scorrano L *et al.* (2003) Science 300: 135–139.
24. Zong WX *et al.* (2003) J. Cell Biol. 162: 59–69.
25. Mathai JP, Germain M and Shore GC (2005) J. Biol. Chem. 280: 23829–23836.
26. Germain M *et al.* (2005) EMBO J. 24: 1546–1556.