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Review

From Cytosol to Mitochondria: The Bax Translocation Story

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The balance between life and death of a cell regulates essential developmental processes in multicellular organisms. Apoptotic cell death is a complex, stepwise program involving multiple protein components that trigger and execute the demise of the cell. Of the many triggers of apoptosis, most are not well understood, but some key components have been identified, such as those of the Bcl-2 family, which function as anti-apoptotic or pro-apoptotic factors. Bax, a pro-apoptotic member of this family, has been shown to serve as a component of many apoptotic triggering cascades and its mechanism of action is the focus of intense study. Herein we discuss current, differing ideas on the function of Bax and its structure, and suggest novel mechanisms for how this death protein targets mitochondria, triggering apoptosis.

Keywords: Apoptosis, Bax, Bcl-2, Cell death

The pro-apoptotic protein, Bax, was the first death-inducing member identified as part of the Bcl-2 family of proteins (Oltvai *et al.*, 1993). Like all Bcl-2 family members, Bax contains highly conserved regions, BH1-BH3, involved in dimerization as well as in the induction of death (Sedlak *et al.*, 1995; Yin *et al.*, 1995). Despite extensive functional and structural studies, Bax has remained enigmatic and controversial regarding its activities, its location and most recently, its structure. Herein we present current, differing ideas on the function of Bax and its structural framework, and suggest mechanisms for how this death protein targets mitochondria, triggering apoptosis.

Originally described as a protein that co-immunoprecipitated with Bcl-2, Bax was cloned, shown to promote cell death, and a model was developed demonstrating that the ratio of Bax to Bcl-2 controlled survival (Oltvai *et al.*, 1993). Many studies examined the physical association of Bax with Bcl-2, however these earlier conclusions were later challenged by the observation that some of the detergents used to produce cell lysates could artificially induce the dimerization of Bax with Bcl-2 (Hsu and Youle, 1997b). As a consequence, the nature of the opposing forces mediated by Bax versus Bcl-2 is far from being completely understood.

The first clues to the workings of the Bcl-2 proteins came with resolution of the crystal structure of an anti-apoptotic member, Bcl-X_L, which proved to be strikingly similar to the membrane translocation domain of bacterial toxins (Muchmore *et al.*, 1996). Homology modeling of Bax based on the Bcl-X_L structure suggested that it could insert in membranes (Aritomi *et al.*, 1997) and form ion-conducting channels (Schlesinger *et al.*, 1997). The ability of Bax to dimerize (and form channels) was studied further and observed to increase at acidic pH (pH 4), in comparison to neutral pH (pH 7-7.4) (Xie *et al.*, 1998). Therefore, acidic pH induced a conformational change in Bax allowing membrane insertion and channel formation.

Bax, like Bcl-2 and Bcl-X_L, has a hydrophobic segment at the C-terminus that could serve as membrane anchor, potentially localizing it to membranes. Because Bax was initially thought to associate with Bcl-2 and because Bcl-2 was mainly found in the outer mitochondrial membrane (Krajewski *et al.*, 1993), Bax was thought to primarily reside at the same mitochondrial site. It was therefore a surprise to find that, unlike the membrane-bound Bcl-2 or Bcl-X_L, in non-apoptotic cells, Bax was predominantly a cytosolic protein (Hsu *et al.*, 1997a; Hsu and Youle, 1997b). Different detergents could induce conformational changes in Bax, allowing various degrees of dimerization with Bcl-2 proteins that could be correlated to the exposure of the N-terminus (Hsu and Youle, 1998c). It was upon the induction of apoptosis that Bax redistributed its subcellular localization to membranes (Hsu *et al.*, 1997a), specifically, mitochondrial (Wolter *et al.*, 1997).

Removal of the C-terminus inhibited this mitochondrial translocation as well as Bax's killing function (Zha *et al.*, 1996; Wolter *et al.*, 1997). In contrast, deletion of the first nineteen amino acids at the N-terminus induced the

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movement from cytosol to membrane, suggesting that targeting to membranes required the C-terminus of Bax, but cytosolic retention required interactions between specific domains, with early studies suggesting involvement of the N-terminus (Goping *et al.*, 1998). Following integration of Bax into the membrane, there was disagreement over which regions spanned the membrane: the 9th alpha helix encompassing the hydrophobic C-terminus (Wolter *et al.*, 1997; Suzuki *et al.*, 2000) versus both the 5th and 6th alpha helices (Nouraini *et al.*, 2000).

The translocation of Bax to mitochondria was established not only for apoptotic stimuli, such as staurosporine treatment, but also for hematopoietic cell lines following growth factor withdrawal (Goping *et al.*, 1998; Khaled *et al.*, 1999a). But the elusive trigger that caused this movement of Bax from the cytosol to the mitochondria upon cell death remained unknown. Multiple mechanisms have been proposed. 1) Dimerization of Bax was suggested as a means for promoting changes in subcellular localization (Gross *et al.*, 1998), but the soluble, cytosolic form of Bax was confirmed to be monomeric (Hsu and Youle, 1998c; Khaled *et al.*, 1999a), and no cytosolic partners have ever been identified. 2) Bid, another Bcl-2 family member, induced a conformational change in Bax. A single Bid molecule appeared to catalyze unfolding of multiple Bax targets. This mechanism occurred following Bid activation by Fas and caspase 8 (Desagher *et al.*, 1999; Ruffolo *et al.*, 2000). However Bax translocation is known to be caspase independent in that use of caspase inhibitors did not block the process (Hsu *et al.*, 1997a; Khaled *et al.*, 1999a), and no other mechanism, other than caspase 8 cleavage, is known to activate Bid. 3) Detergents induced a conformational change in Bax *in vitro* (Hsu and Youle, 1997b; Suzuki *et al.*, 2000). However, an *in vivo* counterpart to detergent that is activated during apoptosis has not been identified (Khaled *et al.*, 1999a). 4). Phosphorylation has been theorized to change Bax folding. Bax has multiple serines and threonines interspersed with hydrophobic residues that could be phosphorylated to regulate translocation. However changes in Bax phosphorylation have not been detected during apoptosis (Khaled *et al.*, 1999a; Nechushtan *et al.*, 1999). 5) Calpain cleavage can generate a potent Bax fragment (Wood *et al.*, 1998), but this cleavage does not occur following trophic factor withdrawal (Khaled *et al.*, 1999a). Therefore, despite intensive study, the mechanism for the mitochondrial translocation of Bax remains elusive.

The reported structural similarities between bacterial toxins and the Bcl-2 family members opened a novel direction for evaluation of protein movement (Muchmore *et al.*, 1996). In particular, diphtheria toxin translocates to membranes in response to a change in pH. This movement is initiated by charged amino acids in the T domain, which are protonated at acidic pH rendering them neutral and facilitating membrane insertion (Lanzrein *et al.*, 1996; Falnes and Sandvig, 2000). Likewise, apoptotic cells undergo a change in cytosolic pH-a transient intracellular alkalinization is produced by various apoptotic stimuli (Tsao and Lei, 1996; Dai *et al.*, 1998; Belaud-Rotureau *et al.*, 2000), including trophic factor withdrawal (Khaled *et al.*, 1999a; Khaled *et al.*, 2001c). We and others have shown that this rise in pH activates Bax causing its mitochondrial translocation (Khaled *et al.*, 1999a; Belaud-Rotureau *et al.*, 2000).

How the shift to alkaline pH causes Bax to translocate, and whether this is a direct or indirect effect, is still unclear. Taking a cue from the bacterial toxin studies, we proposed a possible means through the neutralization of charged residues in the termini of Bax. These regions were shown in previous studies with epitope-specific antibodies (Hsu and Youle, 1997b; Hsu *et al.*, 1997a; Hsu and Youle, 1998c), deletion studies (Goping *et al.*, 1998) and Fas-activation (Murphy *et al.*, 2000) to be essential for Bax's movement and cell killing. Substitution of specific residues (Lys189, Lys190) in the C-terminus or (Asp2, Glu6) in the N-terminus with non-charged amino acids reproduced the protease-sensitivity of membrane-integrated (or unfolded) Bax in a manner similar to that observed under alkaline conditions, independent of an apoptotic stimuli (Khaled *et al.*, 1999a). Inducible expression of a Bax mutant (in which Lys189, Lys190 were substituted for neutral amino acids) showed constitutive mitochondrial integration of Bax (A. Khaled and S. Durum, unpublished observation). Such results suggested the model that Bax is a pH-sensitive protein. Thus, neutralization of charged amino acids (at alkaline pH) would promote insertion of Bax into membranes. This insertion would unfold the protein, producing a conformational change measured by increased protease-sensitivity.

In continuing studies of the hydrophobic C-terminal end of Bax, mutational analysis of the last ten residues of the C-terminus suggested that Ser184 was a critical regulator of Bax's activity (Nechushtan *et al.*, 1999). Deletion of this residue produced a form of the protein that constitutively targeted to mitochondria, while substitution at the same site for charged amino acids yielded a cytosolic form (Nechushtan *et al.*, 1999). Was Ser184 the regulatory site on Bax targeted during apoptosis? The answer was not obvious. The Bax constructs studied were fused to the bulky GFP group, a construct capable of inducing apoptosis upon overexpression even without an additional death stimulus (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999), and mitochondrial translocation was measured by confocal microscopy, overlapping GFP fluorescence with a mitochondrial dye on a single-cell basis. Therefore no determinations were made as to whether the Ser184-deleted Bax actually integrated into mitochondria membranes; an alternative explanation is that this mutein was peripherally associated with mitochondrial membranes, rather than inserted, and induced death by binding to and inhibiting other anti-apoptotic proteins. It was shown that Ser184 was not a target of phosphorylation (Nechushtan *et al.*, 1999), and no alternative mechanism for its alteration during apoptosis was proposed.

To resolve the question of how Bax changes conformation

and translocates to membranes, the solution structure of this protein was recently determined. Bax was expressed in bacteria and a water-soluble form of the protein was purified. NMR analysis of this form revealed a structure of 9α helices, with helices α1 to α8 resembling Bcl-X_L (Suzuki *et al.*, 2000). Furthermore, the C-terminus (the α9 helix) rests in a hydrophobic pocket, consistent with the conformation of a water-soluble, monomeric form of Bax (Suzuki *et al.*, 2000). Under cell-free conditions, no observed changes in the solution structure of this form of Bax occurred between the pH range of 6 to 8, suggesting to the authors no direct link between intracellular pH changes and Bax translocation into the mitochondria. However, if changes in pH serve to neutralize charged amino acids in the termini of Bax, facilitating membrane insertion and translocation, then the pH-sensitivity of Bax would only be observed in the presence of mitochondrial membranes and not in a cell-free solution. The authors did not perform such an experiment, but proceeded to demonstrate that detergent treatments induced further aggregation of Bax and showed that oligomerization produces structural changes in the protein. The role of the N-terminus remained unclear (no interactions were observed with the C-terminus) and alterations in helical packing were proposed to be the primary means for aggregation (Suzuki *et al.*, 2000). Therefore, the NMR structure of a water-soluble form of Bax showed that the hydrophobic C-terminus was constrained to a hydrophobic pocket, offering one explanation of how the molecule could reside in the cytosol, however the (presumed) process of inserting this C-terminus into membranes during apoptosis remains to be explained.

Hence, discerning the nature of Bax and its death-promoting activity remains to date an elusive goal. Resolving the structures of native, eukaryotic-expressed Bax in its cytosolic and membrane-integrated forms would be formidable. One approach that could help evaluate the functional components of Bax would be to mutate critical residues (i.e. pH sensitive amino acids in the termini, or serine 184) and express these using an inducible expression system, thereby minimizing the stress produced by transient transfection procedures. Such Bax constructs could made with small epitope tags (rather than the bulky GFP tag that may alter Bax conformation) and analysis should include multiple biochemical approaches by which to study large populations of cells in addition to single cell analysis previously used. Lastly, efforts should be made to determine the regions within Bax which interact and are exposed or hidden after apoptotic stimuli (perhaps through the use of a variety of epitope-specific antibodies). Such directions should advance the study of Bax and could answer the persisting question-how does Bax deliver the sting of death?

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