Perspective

Bim and the Pro-Survival Bcl-2 Proteins
Opposites Attract, ERK Repels

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ABSTRACT

Bim (Bcl-2-interacting mediator of cell death) is a BH3-only protein (BOP), a pro-apoptotic member of the Bcl-2 protein family. The Bim mRNA undergoes alternate splicing to give rise to the short, long and extra long protein variants (BimS, Bim, and BimEL). These proteins have distinct potency in promoting death and distinct modes of regulation conferred by their interaction with other proteins. Quite how Bim and other BOPs promote apoptosis has been the subject of some debate. Bim was isolated by its interaction with pro-survival proteins such as Bcl-2 and it has been suggested that this is key to the ability of Bim to induce apoptosis. However, an alternative model argues that some forms of Bim can bind directly to the pro-apoptotic Bax and Bak proteins to initiate apoptosis. A new study may finally put this debate to rest as it provides strong evidence to suggest that Bim and other BOPs act primarily by binding to pro-survival Bcl-2 proteins, thereby releasing Bax or Bak proteins to promote apoptosis. The importance of the interaction between Bim and the pro-survival Bcl-2 proteins is underlined by our demonstration that it is regulated by ERK1/2-dependent phosphorylation of BimEL. ERK1/2-dependent dissociation of BimEL from pro-survival proteins is the first step in a process by which the pro-survival ERK1/2 pathway promotes the destruction of this most abundant Bim splice variant. In this review we outline the significance of these new studies to our understanding of how BOPs such as Bim initiate apoptosis and how this process is regulated by growth factor-dependent signaling pathways.

THE Bcl-2 PROTEIN FAMILY

The Bcl-2 family of proteins are evolutionarily conserved regulators of the cell intrinsic apoptosis pathway, which act to regulate the integrity of the outer mitochondrial membrane.1,2 These proteins are sub-divided on the basis of structural and functional differences but all members contain at least one of four conserved Bcl-2 homology (BH) domains.1,2 The pro-survival Bcl-2 proteins (e.g., Bcl-2, Bcl-XL and Mcl-1) typically contain all four BH domains (BH1-BH4) whilst the multi-domain pro-apoptotic proteins, Bax and Bak, contain the BH1, BH2 and BH3 domains. A third class, termed the BH3-only proteins (BOPs), are a structurally diverse group of pro-apoptotic proteins sharing in common only the BH3 domain (e.g., Bad, Bid, Bim, Noxa and Puma).3 Bax and Bak are critical downstream effectors of the Bcl-2-regulated pathway of apoptosis since cells lacking both proteins are resistant to a wide variety of apoptotic insults.4,5 When activated, these proteins promote the release of cytochrome c through the outer mitochondrial membrane, leading to activation of caspases and cell death. In viable cells Bax and Bak are restrained by their physical interaction with the pro-survival Bcl-2 proteins and it is this ‘happy balance’ that must be overcome if a cell is to undergo apoptosis. A variety of studies indicate that it is the BOPs that are primarily responsible for disrupting the balance between Bax/Bak and the pro-survival Bcl-2 proteins in response to cellular stress.3,5 The BOPs are regulated by a variety of stress-induced signaling pathways and appear to serve as the primary link between stress-dependent signaling and the core Bcl-2 proteins (Bax/Bak and the pro-survival Bcl-2 proteins).3
MODELS FOR BOP-INDUCED ACTIVATION OF BAX AND BAK: SUPPORT FOR THE INDIRECT ACTIVATION MODEL

The role of BOPs as initiators of apoptosis is increasingly accepted and it is clear that BOPs require Bax and/or Bak to induce cell death; however, there has been considerable debate about precisely how the BOPs effect activation of Bax and Bak. On the one hand, an indirect activation model has been proposed in which BOPs activate Bax or Bak by binding to the pro-survival Bcl-2 proteins, neutralising them and thereby relieving their inhibition of Bax and Bak (Fig. 1A). Alternatively, a direct activation model postulates that certain BOPs (notably Bim and Bid) can bind directly to Bax or Bak and are known as "activators", whereas other BOPs act as "sensitizers", serving to displace Bim and Bid from the pro-survival proteins so that they can go on to bind to Bax or Bak (Fig. 1B). In recent years support for the direct activation model has waxed and waned but now a new study could sound its death knell.

One prerequisite of the direct activation model is that Bim and Bid should bind directly to Bax and Bak. In the case of Bim, which exists as three common splice variants, Bimα, Bimβ and Bimγ, only the short form, Bimα, could bind directly to Bax. This was an attractive observation since Bimα is the most effective of the three splice variants in promoting cell death. However, other reports have failed to demonstrate binding of any Bim isoforms to Bax or Bak and even the isolated Bim BH3 peptide binds very poorly to Bax whereas the same peptide binds to the full repertoire of pro-survival Bcl-2 proteins with nanomolar affinity. Cell-based studies also argue against the direct activation model; for example, variants of Bim or Bid carrying mutations in the BH3 domain that inhibit binding to Bax/Bak retain their ability to bind to the pro-survival proteins and are fully competent to promote apoptosis. Additionally, the suggestion that sensitizer BOPs (such as Bad or Noxa) promote the release of activator BOPs (Bim and Bid) from pro-survival proteins so that they bind to Bax and Bak, is difficult to rationalize with the binding affinities of the different BH3 domain peptides of the BOPs. For example, the Bim BH3 peptide binds with high affinity to all the pro-survival proteins so it is difficult to imagine how it could be effectively displaced by Noxa, which only exhibits significant binding to a subset of the pro-survival proteins. The direct model also predicts that death induced either by stress or the overexpression of sensitizer BOPs should require Bim and Bid. However, MEFs derived from mice lacking Bim and Bad undergo cell death in response to stresses or expression of sensitizer BOPs such as Bad or Noxa. In contrast, Bax/Bak double knock-out MEFs are resistant to death under these conditions.

The simplest conclusion from these and related studies is that BOPs are fully competent to initiate apoptosis without binding to Bax or Bak. Rather, an increasing body of evidence supports the indirect activation model. This includes the high affinity binding of BOP BH3 domain peptides to pro-survival Bcl-2 proteins in vitro and the fact that variants of Bim or Bid carrying mutations in the BH3 domain that inhibit their binding to pro-survival Bcl-2 proteins are defective in cell killing assays. In addition, the binding of BOPs to pro-survival Bcl-2 proteins is readily detectable in cell extracts and actually increases when cells are exposed to appropriate cellular stresses.

For example, Bim expression is required for apoptosis following serum withdrawal from fibroblasts and this treatment increases the abundance of Bim and its binding to Bcl-xL and Mcl-1, concomitant with a decrease in the amount of Bax bound to pro-survival Bcl-2 proteins. Finally, the greater potency of certain BOPs, notably Bim and Puma, correlates well with their ability to bind and neutralise all the pro-survival Bcl-2 proteins, whereas the weaker BOPs only target a sub-set of survival proteins. Taken together these latest results provide strong support for the indirect activation model in which BOPs act primarily by binding to pro-survival Bcl-2 proteins, thereby promoting the de-repression and activation of Bax and Bak.

REGULATION OF THE INTERACTION BETWEEN BIMα AND PRO-SURVIVAL BCL-2 PROTEINS BY THE ERK1/2 PATHWAY

The new results from Huang and co-workers highlight the importance of the interaction between BOPs, such as Bim, and the pro-survival proteins in the initiation of cell death. Given their importance, such interactions are likely to be subject to regulation by stress- or survival-induced signaling events, but whilst it is true that the binding of BOPs to pro-survival Bcl-2 proteins increases in response to appropriate stresses, this is often simply due to an increase in the abundance of the BOPs concerned. For example, following DNA damage and p53 activation, expression of Noxa and Puma increases and they bind to pro-survival proteins. However, there is no suggestion that the intrinsic binding properties of Noxa or Puma change under these conditions; there is simply more protein and therefore more of it is bound to the pro-survival proteins. To date there have been only two examples in which the binding properties of BOPs have been shown to be regulated at the post-translational level. Caspase-dependent cleavage of Bid generates a C-terminal fragment (tBid) which exhibits greater binding to Bcl-xL than the native form of Bid, whilst phosphorylation of Bad regulates its interaction with Bcl-xL.
Our own studies have now shown that the interaction between the major form of Bim, Bim<sub>EL</sub>, and pro-survival Bcl-2 proteins is subject to dynamic, post-translational regulation due to phosphorylation of Bim<sub>EL</sub>.<sup>13</sup> These observations arose from the study of fibroblast cell death following withdrawal of serum survival factors. Bim is required for caspase activation under these conditions and contributes significantly to cell death.<sup>13</sup> Serum withdrawal promotes a rapid increase in expression of Bim<sub>EL</sub> (and to a lesser extent Bim<sub>subcrit</sub>),<sup>13,20</sup> but the addition of defined growth factors, such as thrombin, at the time of serum withdrawal prevents Bim expression and cell death.<sup>21</sup> Since thrombin prevented Bim<sub>EL</sub> expression it also prevented the binding of Bim<sub>EL</sub> to its target pro-survival proteins, Mcl-1 and Bcl-x<sub>L</sub>. The surprise came when we serum starved cells for six hours to promote the assembly of Bim<sub>EL</sub>:Mcl-1 complexes. When such cells were restimulated with thrombin for as little as 15 minutes the amount of Bim<sub>EL</sub> recovered in Mcl-1 immunoprecipitates was substantially reduced suggesting that thrombin was actually promoting the dissociation of the pre-formed Bim<sub>EL</sub>/Mcl-1 complex.<sup>13</sup>

Growth factor stimulation has previously been shown to promote the proteasomal degradation of Bim<sub>EL</sub><sup>22-25</sup> so a simple explanation for this rapid dissociation of Bim<sub>EL</sub> from Mcl-1 was that it resulted from the destruction of Bim<sub>EL</sub>. However, several lines of evidence argue against this. First, the short 15 min stimulation time had no impact on Bim<sub>EL</sub> degradation and did not affect the total expression of Bim<sub>EL</sub>. Second, dissociation was not prevented by pharmacological inhibition of the proteasome, and was not compromised when we used a mutant version of Bim<sub>EL</sub> that could not be ubiquitylated.<sup>26</sup> Finally, since dissociation proceeded in the presence proteasome inhibitors and protein synthesis inhibitors, this indicated that growth factor stimulation promoted dissociation of pre-existing Bim<sub>EL</sub>/Mcl-1 complexes rather than reducing the stability of newly expressed Bim<sub>EL</sub>. Growth factor stimulation also promoted the rapid dissociation of Bim<sub>EL</sub> from another pro-survival Bcl-2 family member, Bcl-x<sub>L</sub>, raising the possibility that this is a common mode of regulation for all Bim<sub>EL</sub>/pro-survival Bcl-2 protein complexes, although this needs to be formally confirmed.

The dissociation of Bim<sub>EL</sub> from Mcl-1 was rapid, reversible and completely dependent upon the phosphorylation status of Bim<sub>EL</sub>. Through the use of pharmacological inhibitors and the conditional kinase ARAF-1:ER<sup>+</sup> it was determined that activation of the MEK1/2-ERK1/2 pathway was both necessary and sufficient to promote dissociation of Bim<sub>EL</sub> from Mcl-1 and although all three major Bim splice variants could interact with Mcl-1, only Bim<sub>EL</sub> dissociated following activation of ERK1/2. Subsequent experiments defined Serine 65 (Ser65, Ser69 in human Bim), a site unique to Bim<sub>EL</sub>, as the major ERK1/2 phosphorylation site that promoted dissociation of Bim<sub>EL</sub> from Mcl-1. These results define a new mechanism by which ERK1/2 can antagonize Bim-dependent cell death; namely, by preventing the interaction of Bim<sub>EL</sub> with its target pro-survival Bcl-2 proteins.

At present, it is unclear how phosphorylation of Bim<sub>EL</sub> at Ser65 directly induces its dissociation from Mcl-1. Ser65 lies outside the BH3 domain and therefore phosphorylation of this residue may not directly disrupt binding to pro-survivals proteins: indeed, access to the Bim BH3 domain might be expected to be limited in pre-assembled complexes. However, since the crystal structure of Bim<sub>EL</sub> has not been solved it is possible that in the 3-dimensional structure Ser65 may lie close to the interface between Bim<sub>EL</sub> and pro-survival proteins, allowing phosphorylation of this residue to directly interfere with the interaction. Furthermore, there is evidence suggesting that Bim<sub>EL</sub> can be phosphorylated in a hierarchical fashion, with phosphorylation at Ser65 directing phosphorylation of different sites.<sup>27</sup> At least three ERK1/2-dependent phosphorylation sites, including Ser65, occur in the region of Bim that is unique to Bim<sub>EL</sub>,<sup>13,27,28</sup> so it may be this multi-site phosphorylation within a discrete domain that interferes with binding to Mcl-1. Alternatively, phosphorylation of Ser65 and other sites may allow binding of accessory proteins that act to promote the dissociation of Bim<sub>EL</sub>/pro-survival complexes, in a similar mechanism to the regulation Bad/Bcl-x<sub>L</sub> complexes.

Phosphorylation of Bad at Ser136<sup>17</sup> and Ser112<sup>18</sup> is required to recruit 14-3-3 proteins and although this does not directly disrupt the Bad/Bcl-x<sub>L</sub> complex it is thought to increase the accessibility of Bad to kinases which phosphorylate Ser155<sup>19</sup> a residue lying within the BH3 domain, thereby destabilizing the complex. Although the Bim<sub>EL</sub> BH3 domain does not contain a serine residue corresponding to Ser155 in the Bad BH3 domain, there are several serine/threonine residues in the BH3 containing helix that could possibly perform a similar function. Interestingly, it has also recently been reported that Bim<sub>EL</sub> interacts with 14-3-3 proteins<sup>29</sup> and although this was in a PKB-dependent manner it would be interesting to determine if 14-3-3 proteins play a role in the disassembly of Bim<sub>EL</sub>-containing complexes.

This new mechanism by which ERK1/2 can antagonize Bim-dependent cell death, by preventing it binding to its target pro-survival Bcl-2 proteins, is observed in a number of different cell types including fibroblasts and epithelial cells suggesting that it may be a common mechanism of regulation. It may also provide an explanation for two recent observations in the literature. Whilst the majority of previous studies have shown that ERK1/2-dependent phosphorylation targets Bim<sub>EL</sub> for degradation, at least two notable studies have demonstrated that the ERK1/2 dependent phosphorylation of Bim<sub>EL</sub> attenuated its apoptotic activity independently of affects on protein stability. Wang et al demonstrated that dephosphorylation of Bim alone was sufficient to induce caspase activation in mouse mammary epithelial cells<sup>30</sup> whilst Reginato et al. have proposed that mammary epithelial outer-matrix acini cells are protected from apoptosis by maintaining Bim<sub>EL</sub> in a phosphorylated state without changing its abundance.<sup>31</sup> In light of our results it would be interesting to examine whether ERK1/2-dependent disruption of Bim<sub>EL</sub> binding to pro-survival proteins could account for the antagonism of Bim<sub>EL</sub> in these model systems.

**ERK1/2-DEPENDENT DISSOCIATION OF Bim<sub>EL</sub> FROM Bcl-2 PROTEINS: A ROLE IN ERK1/2-DEPENDENT DESTRUCTION OF Bim<sub>EL</sub>?**

The demonstration that ERK1/2-dependent phosphorylation of Bim<sub>EL</sub> promotes its dissociation from pro-survival proteins also has important implications for the mechanism by which Bim<sub>EL</sub> is targeted for degradation by the proteasome. To date, the only signaling pathway implicated in targeting Bim<sub>EL</sub> for ubiquitination and degradation is the ERK1/2-pathway; however, the specific details of how this is achieved are not known. The sequential action of the E1, E2 and E3 enzymes in the ubiquitin-proteasome pathway is well established,<sup>32</sup> but whilst the details of the activation and conjugation of ubiquitin are now well understood, our knowledge...
of how E3 ligases target and interact with their substrate is only now emerging.\textsuperscript{33,34} In the case of substrates such as Bim\textsubscript{EL}, which are targeted for destruction by phosphorylation, the simplest explanation would be that phosphorylated Bim\textsubscript{EL} is recognised by an E3 ligase that has a phosho-specific recognition domain (Fig. 2, Pathway A). There is precedent for this in the degradation of phosphorylated IkB, which is recognised and ubiquitinated by an SCF (Skp1-Cullin-Fbox) E3 ligase in which the F-box protein \(\beta\text{TrCP}\) binds to the phosphorylated recognition sequence on IkB.\textsuperscript{35,36} Other phospho-specific F-box proteins are responsible for the degradation of c-Jun, c-Myc and cyclin E.\textsuperscript{33,34} However, the demonstration that ERK1/2-catalysed phosphorylation of Bim\textsubscript{EL} causes it to dissociate from pro-survival proteins suggests an alternative model in which phosphorylation serves only to cause Bim\textsubscript{EL} dissociation, thereby revealing an otherwise occluded and phosphorylation-independent E3 ligase binding site (Fig. 2, Pathway B).

Whilst Pathway A might appear the simplest, there is to date slightly more evidence to support the alternative Pathway B. For example, dissociation\textsuperscript{13} and degradation\textsuperscript{22,23} of Bim\textsubscript{EL} both require phosphorylation of Ser65 by ERK1/2. In addition, mutations in the Bim\textsubscript{EL} BH3 domain that greatly reduce binding to the pro-survival proteins cause accelerated turnover of the mutant protein compared to the wild type protein, even in serum free conditions when ERK1/2 activity should be low.\textsuperscript{33} This result suggests that dissociation of Bim\textsubscript{EL} from pro-survival proteins is sufficient to accelerate its turnover, providing some support for the idea that the E3 Ub ligase recognises a site on Bim\textsubscript{EL} that is normally occluded when Bim\textsubscript{EL} is bound to pro-survival proteins. One such site is of course the BH3 domain itself, but the fact that mutations in this domain actually accelerate Bim\textsubscript{EL} turnover perhaps argues against it being the binding site for the E3 ligase. Additionally, Bim\textsubscript{EL} has been proposed to be an intrinsically unstructured protein (IUP), a class of proteins that lack a precise tertiary structure and exhibit conformational flexibility upon interacting with their binding partners.\textsuperscript{37} In fact, when Bim\textsubscript{EL} binds to the pro-survival proteins, only the BH3 domain becomes structured into an \(\alpha\)-helical configuration, whilst the majority of the protein remains disordered, with functional domains arrayed as ‘beads on a string’. This may explain how ERK1/2 is able to bind and phosphorylate Bim\textsubscript{EL} even when it is bound to a pro-survival protein.

More importantly, ERK1/2-dependent phosphorylation and subsequent dissociation from pro-survival proteins may serve to destabilise Bim\textsubscript{EL}, rendering it more susceptible to proteolysis and degradation via the proteasome. These studies do not provide definitive support for the Pathway B model, but raise it as a credible alternative that is worthy of further investigation.

Interestingly, dissociation of Bim\textsubscript{EL} may also have consequences for the stability of Mcl-1 since it may regulate the interaction between Mcl-1 and its E3 Ub ligase, Mule.\textsuperscript{38} Mule contains a BH3 domain through which it can interact with Mcl-1 but not Bcl-2 or Bcl-xL.\textsuperscript{49} However, binding of Mule to Mcl-1 is differentially regulated by the binding of certain BOPs; Bim binding prevents Mule binding and thereby stabilizes Mcl-1, whereas binding of Noxa actually promotes the turnover of Mcl-1.\textsuperscript{40} Comparisons of the structure of Mcl-1 bound to either the Bim or Noxa BH3 peptides reveals no significant differences in the structure of Mcl-1 itself, suggesting that it is a structure formed in the Noxa:Mcl-1 complex (but not the Bim:Mcl-1 complex) which serves as the recognition motif for an E3 ligase or other adaptor protein involved in Mcl-1 turnover. This study together with the regulated dissociation of Bim\textsubscript{EL} by the ERK1/2 pathway underscore the importance of regulated protein-protein interactions in the control of protein degradation and cell death.

No discussion of the E3 ligase responsible for Bim\textsubscript{EL} degradation is complete without considering the role of Cbl, the product of the \(c\text{-Cbl}\) proto-oncogene. Akiyama et al. demonstrated that osteoclasts deprived of macrophage colony-stimulating factor (MCSF) increased the expression of Bim and underwent Bim-dependent apoptosis.\textsuperscript{26} The increase in Bim expression was in part due to stabilization of Bim\textsubscript{EL} and they suggested Cbl as a possible candidate for the Bim\textsubscript{EL} E3 ligase. They argued that the MCSF-dependent degradation of Bim\textsubscript{EL} was impaired in osteoclasts from \(c\text{-Cbl}\)\textsuperscript{-/-} mice, and were able to co-immunoprecipitate Bim\textsubscript{EL} and Cbl from these cells. However, others have reported findings that contradict this suggestion; for example, whilst Bim\textsubscript{EL} levels are elevated in the testis of Cbl\textsuperscript{-/-} mice there was no interaction between Bim\textsubscript{EL} and Cbl suggesting that the effect of Cbl on Bim\textsubscript{EL} was indirect.\textsuperscript{41} Furthermore, the majority of proteins targeted for ubiquitylation by Cbl are tyrosine phospho-proteins that interact with Cbl at its phosphotyrosine specific tyrosine kinase binding domain (TKB).\textsuperscript{42} However, to date there has been no report that Bim\textsubscript{EL} is a phospho-tyrosine containing protein, suggesting that any putative interaction with Cbl is indirect or via a novel mechanism. Finally, it is apparent from the original study that loss of Cbl causes only a minor reduction in turnover of Bim\textsubscript{EL}\textsuperscript{26} suggesting that if Cbl is indeed an E3 ligase for Bim\textsubscript{EL} then it is certainly not the only one and other E3 ubiquitin ligases must exist that are responsible for the ERK1/2-dependent proteasomal degradation of Bim\textsubscript{EL}. Clearly further work is require to clarify the role of Cbl in regulation of Bim\textsubscript{EL}.

![Figure 2](https://www.landesbioscience.com(Cell Cycle 2239)
It is apparent that there is a prominent role for Bim in the initiation of apoptosis following withdrawal of survival factors, acting alone or in combination with other BOPs depending on the cell type. We are now starting to develop a more detailed knowledge of how Bim is regulated and the course of events during this process. In cells maintained in the presence of survival factors the ERK1/2 and PKB pathways will be active and expression of Bim will be low due to low levels of transcription, reduced message stability and proteasome-dependent turnover of BimEL. Following withdrawal of survival factors the decline in ERK1/2 activity will result in rapid de-phosphorylation of existing BimEL, its binding to pro-survival proteins (but not Bax) and its stabilization; indeed, stabilization may be a consequence of binding of BimEL to pro-survival proteins. Loss of Ras-dependent signals may also promote stability of the Bim mRNA but the selective stabilisation of the BimEL protein isoform may account for the fact that BimEL is very frequently the first isoform to increase in expression. Finally, increases in transcription may account for the somewhat slower increase in Bim1 (and perhaps BimC) protein, which tend to lag behind the increase in BimEL.

Growth and survival factors will act to antagonise Bim activity and expression by preventing BimEL binding to pro-survival proteins, promoting BimEL destruction, reducing Bim mRNA stability and repressing Bim transcription. All of these growth factor-dependent events are regulated by the Ras-dependent ERK1/2 or PKB pathways raising the possibility that repression of Bim is deregulated in tumors with mutations in components of these pathways. This together with the demonstration that the Bim locus is disrupted in some tumors suggests that Bim is a tumor suppressor that may serve as part of a barrier to growth factor-independent survival.

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