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EGL-1 BH3 mutants reveal the importance of protein levels and target affinity for cell-killing potency

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Studies of the cell death pathway in the nematode *Caenorhabditis elegans* provided the first evidence of the evolutionary conservation of apoptosis signalling. Here we show that the worm Bcl-2 homology domain-3 (BH3)-only protein EGL-1 binds mammalian pro-survival proteins very poorly, but can be converted into a high-affinity ligand for Bcl-2 and Bcl-x_L by subtle mutation of the cysteine residue at position 62 within the BH3 domain. A 100-fold increase in affinity was observed following a single atom change (cysteine to serine substitution), and a further 10-fold increase by replacement with glycine. The low affinity of wild-type EGL-1 for mammalian pro-survival proteins and its poor expression correlates with its weak killing activity in mammalian cells whereas the high-affinity C62G mutant is a very potent killer of cells lacking Mcl-1. Cell killing by the C62S mutant with intermediate affinity only occurs when this EGL-1 BH3 domain is placed in a more stable context, namely that of Bim_S, which allows higher expression, though the kinetics of cell death now vary depending on whether Mcl-1 is neutralized by Noxa or genetically deleted. These results demonstrate how levels of BH3-only proteins, target affinity and the spectrum of neutralization of pro-survival proteins all contribute to killing activity.
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Landmark studies in *Caenorhabditis elegans* revealed that four genes, *CED-9*, *CED-4*, *CED-3* and *EGL-1*, are essential for the programmed cell death (apoptosis) of 131 out of 1090 somatic cells during hermaphrodite nematode development.[1] Of these, *EGL-1*, *CED-3* and *CED-4* encode pro-apoptotic proteins whereas *CED-9* encodes a pro-survival protein. CED-9 is the nematode homologue of mammalian B-cell lymphoma-2 (Bcl-2) pro-survival proteins (including Bcl-2 itself as well as Bcl-x_L, Bcl-w, Mcl-1 and A1),[2] while CED-4 is similar to the mammalian adapter protein APAF-1[3] acting as a positive regulator of caspases, in particular, CED-3.[4] EGL-1 is the nematode Bcl-2 homology domain-3 (BH3)-only protein equivalent to mammalian BH3-only proteins such as Bim, Bad and Noxa.[5–7]

In nematode cells, CED-9 functions by sequestering CED-4 at the mitochondrial membrane.[8–10] Following a developmental cue, *EGL-1* is transcriptionally upregulated and the protein binds CED-9,[5–7] releasing CED-4, allowing its oligomerization.[11] These CED-4 oligomers then translocate to the perinuclear region of the cell[12] where they facilitate CED-3 activation by bringing these caspases into close proximity.[4,9] Biochemical and structural studies have revealed that binding of the EGL-1 BH3 domain into a hydrophobic groove on CED-9 causes a conformational change at the CED-4:CED-9 interface that results in CED-4 dissociation.[13–15] The BH3 domains of mammalian BH3-only proteins bind into similar grooves on mammalian pro-survival molecules, but a conformational change as seen in CED-9 has yet to be observed.[16–18]

The functional equivalence, and hence evolutionary conservation, of CED-9 and mammalian Bcl-2 was demonstrated in studies showing Bcl-2 could inhibit apoptosis in the nematode.[2,19] More recently, studies employing yeast-based functional and other biochemical assays determined that in the nematode studies described above, Bcl-2 is probably functioning by binding to EGL-1 thereby preventing its association with CED-9.[20] However, although CED-9 appears able to prevent cellular processes such as mitochondrial fission in mammalian cells (suggesting potential interactions with mammalian proteins),[21] EGL-1 seems ineffective at inducing apoptosis.[21] We have also shown that mammalian BH3-only proteins such as Bim and Bad, unlike EGL-1, cannot dissociate CED-4:CED-9 complexes.[22] This suggests that either these mammalian BH3-only proteins cannot engage CED-9 directly, or alternatively, they bind CED-9 but cannot induce the conformational change necessary for CED-4 release.[11]

These studies indicate that the molecular mechanisms, and evolutionary conservation, of nematode and mammalian apoptosis pathways are still not completely understood. Here we have performed detailed biochemical analyses of mammalian and nematode BH3 domain:pro-survival protein interactions. We demonstrate that certain mammalian BH3 domains cannot bind CED-9 with high affinity, and surprisingly, that EGL-1 is a weak ligand for mammalian pro-survival molecules, accounting for its weak/absent apoptotic activity in mammalian cells. Importantly, we also discovered that subtle mutations convert EGL-1BH3 into a tightly binding ligand for select mammalian Bcl-2 proteins. By examining the ability of these mutants to kill mammalian cells we determined that both their affinity and sequence context, which influenced

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Abbreviations: Bcl-2, B-cell lymphoma-2; BH3, Bcl-2 homology domain-3; ITC, isothermal titration microcalorimetry; MEF, mouse embryonic fibroblast
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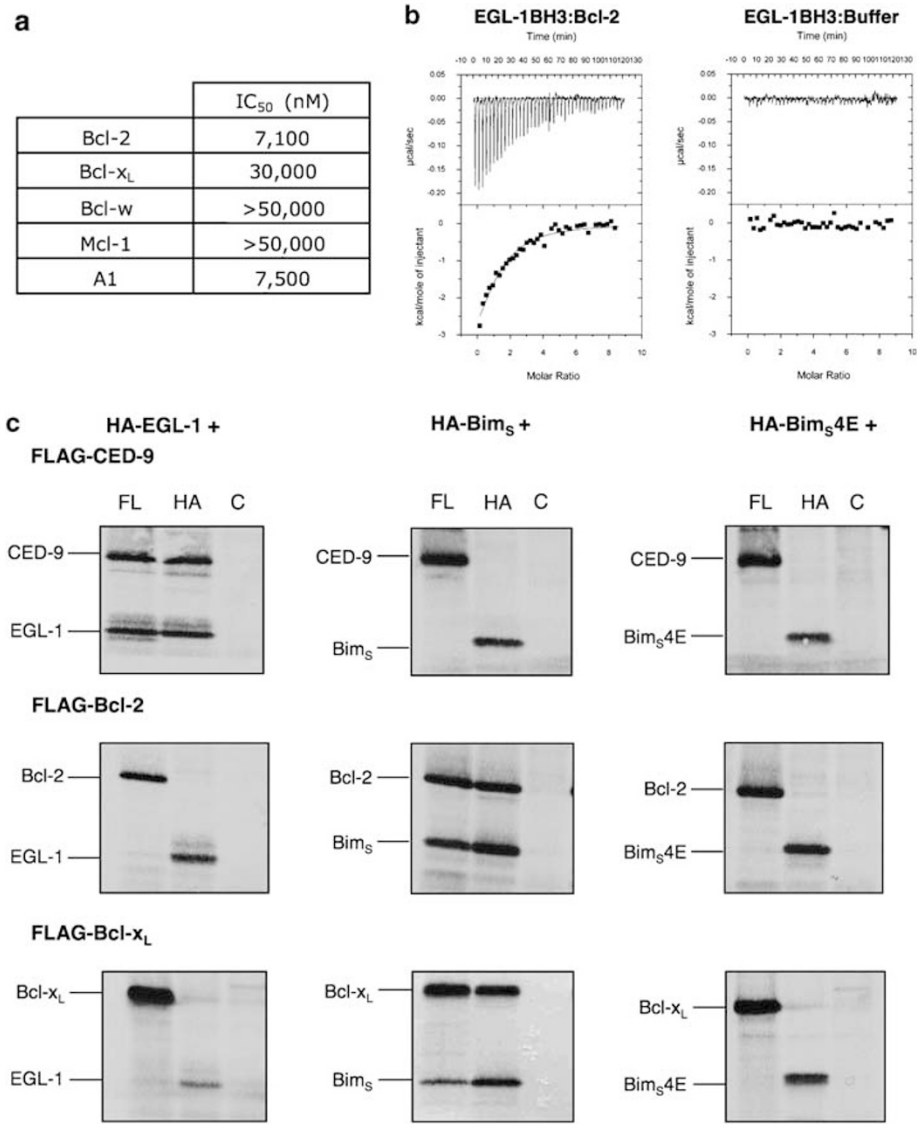

	IC_{50} (nM)
Bcl-2	7,100
Bcl-x_L	30,000
Bcl-w	>50,000
Mcl-1	>50,000
A1	7,500

Figure 1 EGL-1 binds only certain mammalian pro-survival Bcl-2 proteins weakly. (**a**) IC_{50} values determined by solution competition assays using EGL-1BH3 peptide and mammalian pro-survival proteins. EGL-1 can only bind some of the mammalian Bcl-2 proteins very weakly in contrast to the much stronger affinity (low nM) for CED-9.[13,14,22] (**b**) The weak interaction between Bcl-2 and EGL-1 was confirmed by isothermal titration microcalorimetry (ITC) where a K_D of 10 μM was determined (left). The control titration of the peptide alone into buffer is also shown (right). (**c**) Confirmation of the weak *in vitro* binding. Interactions between FLAG (FL)-tagged CED-9, Bcl-x_L or Bcl-2 and HA-tagged EGL-1, Bim$_S$ (positive control) or a non-binding Bim$_S$ mutant (Bim$_S$4E; negative control), were assessed by co-immunoprecipitation. Equivalent ^{35}S-labelled lysates, harvested from transiently transfected 293T cells, were co-immunoprecipitated with antibodies to the FLAG (FL), HA or control (C) anti-glu tags

intracellular expression levels/stability, together with how Mcl-1 was inactivated, determined their cell-killing kinetics and potency.

Results

EGL-1BH3 binds mammalian pro-survival Bcl-2 proteins weakly. Although previous studies suggest that mammalian pro-survival proteins interact with EGL-1,[20] others demonstrated that EGL-1 is ineffective at killing mammalian cells.[6,21] To better understand this paradox, we measured the affinity of EGL-1BH3 for mammalian pro-survival proteins. The ability of the worm pro-survival protein CED-9 to bind to BimBH3, a 'promiscuous' mammalian BH3 domain, was also assessed.

Solution competition experiments using the Biacore optical biosensor demonstrated that an EGL-1BH3 26-mer synthetic peptide bound a subset of mammalian pro-survival proteins, but did so only relatively weakly with the highest affinities measured for Bcl-2 and A1 (IC_{50}, 7–8 μM; Figure 1a). Typical high-affinity interactions between mammalian BH3 peptides and pro-survival proteins give IC_{50} values in similarly performed assays in the low nanomolar range.[23] The affinity measured for Bcl-x_L was even weaker (IC_{50}, 30 μM), and undetectable for Mcl-1 or Bcl-w. We confirmed the binding interaction between Bcl-2 and EGL-1 using isothermal titration microcalorimetry (ITC), where a K_D of approximately 10 μM was determined (Figure 1b), consistent with the Biacore results.

As the Biacore assay was dependent on pro-survival proteins binding an immobilized BimBH3 peptide, CED-9

could not be assessed in this assay as no interaction was detected, suggesting that the affinity of CED-9 for BimBH3 is also very weak. This was confirmed by ITC where no interaction was detected (data not shown), consistent with previous results.[22]

To determine that the binding affinity data for the BH3 peptides accurately reflected the ability of full-length BH3-only proteins to interact with pro-survival proteins, we performed co-immunoprecipitation experiments using over-expressed full-length proteins in HEK293T cells (Figure 1c). As expected, a strong interaction was observed between wild-type EGL-1 and CED-9, but not with Bcl-2 or Bcl-x_L, consistent with the low affinity of the EGL-1BH3 peptide measured for these proteins (Figure 1a). Similarly, full-length Bim_S was unable to co-immunoprecipitate with CED-9, but a strong interaction was observed with Bcl-2 and Bcl-x_L. Finally, as a negative control, we showed that a Bim_S mutant (Bim_S4E) with BH3 domain mutations rendering it incapable of binding any (mammalian) pro-survival molecules[23] was unable to co-immunoprecipitate any FLAG-tagged proteins examined.

Together these results demonstrate that EGL-1 is a weak ligand for mammalian pro-survival proteins. Furthermore, Bim, which binds all mammalian pro-survival proteins with high affinity, is incapable of interacting with the worm pro-survival molecule, CED-9.

Subtle mutations of EGL-1BH3 convert it to a high-affinity ligand for some mammalian pro-survival proteins. The low affinity of EGL-1 for mammalian pro-survival proteins was unexpected as its BH3 domain shares most of the key features of mammalian BH3 domains (Figure 2a). In particular, the conserved hydrophobic residues (h1–h4 in Figure 2a) that project into the hydrophobic groove on pro-survival molecules[16–18,23] are similar to corresponding residues found on tightly binding mammalian BH3 domains. Similarly, the conserved aspartate in all mammalian BH3 domains is present in EGL-1BH3. One difference we noted in positions that are relatively highly conserved was in the residue immediately before the conserved aspartate (Figure 2a). In most mammalian BH3 domains this is either glycine or alanine, and in BadBH3, a serine. However, in EGL-1BH3 the corresponding residue is cysteine, not seen in any mammalian BH3 domains. We therefore examined the effect of replacing this residue in EGL-1BH3 with serine or glycine.

Surprisingly, substitution of the cysteine for serine, representing a single atom change (oxygen for sulphur on the side chain) resulted in significant affinity gain for Bcl-2 and Bcl-x_L, and to a slightly lesser extent A1 (Figure 2b). Furthermore, much greater increases in affinity were observed for all pro-survival proteins following substitution of the cysteine with glycine (Figure 2b).

To determine the effect of the corresponding substitutions in full-length EGL-1 we performed co-immunoprecipitation experiments (Figure 2c). As anticipated, both EGL-1 mutants were capable of strongly co-immunoprecipitating with Bcl-x_L and Bcl-2, and no negative effect was seen for the interaction with CED-9. This high affinity for CED-9 was confirmed by ITC where the EGL-1BH3-C62G peptide bound with a K_D of 14 nM (Figure 2d), similar to wild-type EGL-1BH3.[13,22]

Finally, as CED-4:CED-9 complex dissociation is dependent upon a conformational change in CED-9 following EGL-1 binding,[13] we also determined the effect of substitution of the cysteine residue in EGL-1BH3 peptide on its ability to dissociate recombinant CED-4:CED-9 complex (Figure 2e). Both mutant peptides were able to dissociate the complex as with wild-type EGL-1BH3, but as previously, no dissociation was observed with the BimBH3 peptide.[22]

These results demonstrate the exquisite sensitivity of pro-survival protein:BH3 domain interactions and suggest BH3 domain-binding profiles can be readily manipulated.

EGL-1 is poorly expressed and unstable in mammalian cells. Previous studies indicated that EGL-1 is unstable in mammalian cells,[6] hence experiments examining its killing activity may be affected by low intracellular concentrations. To explore this possibility we examined the relative levels of HA-tagged EGL-1 and mutants compared to HA-tagged Bim_S expressed in *bax*$^{-/-}$/*bak*$^{-/-}$ mouse embryonic fibroblasts (MEFs) from retroviruses where expression was linked to green fluorescent protein (GFP) by an internal ribosome entry site. In pools of infected cells (where $>85\%$ of cells expressed GFP; Figure 3a), Bim was basally expressed at markedly higher levels than EGL-1 or C62S and C62G mutants (Figure 3b). As this low-level expression in MEFs might influence biological activity, we created Bim_S chimeric constructs where the Bim_S BH3 domain was replaced with EGL-1BH3 or EGL-1BH3 mutants. Such chimeras possess the pro-survival protein-binding and cell-killing characteristics of the newly integrated BH3 sequence.[23,24] As a consequence we now see much higher basal expression of these chimeras compared to the EGL-1 constructs, though less than parental wild-type Bim_S (Figure 3b). In stability studies where protein synthesis was inhibited with cycloheximide, the C62G mutant of either EGL-1 or Bim_SEGL-1BH3 persists longer than the wild-type counterpart (Figure 3c), perhaps due to their ability to better associate with pro-survival proteins (Figures 1c and 2c), hence shielding them from proteolytic attack. For example, wild-type EGL-1 has a half-life of less than 15 min whereas EGL-1-C62G persists for 2–4 h (Figure 3c).

Wild-type EGL-1 is a poor killer of mammalian cells. We and others have established that diverse pro-survival proteins must be neutralized by BH3-only proteins for apoptosis to proceed, though the exact mechanism of Bak/Bax activation remains controversial.[24–28] It has been proposed that for Bak-mediated killing, BH3-only proteins must engage both Mcl-1 and Bcl-x_L[24] whereas for Bax, a larger range of pro-survival proteins must be neutralized.[25] As EGL-1BH3 binds all mammalian pro-survival proteins very weakly, it is not surprising that previous studies have shown EGL-1 to only weakly kill mammalian cells.[21] We confirmed this in short- (24 h) and long-term (6 days – clonogenic) survival assays using wild-type MEFs infected with retroviruses expressing full-length EGL-1 (Figure 4a and b).

mcl-1$^{-/-}$ or *bcl-x*$^{-/-}$ MEFs are sensitive to BH3-only proteins that engage the complementary pro-survival protein.[29] Hence, although Bad or Noxa are ineffective killers of

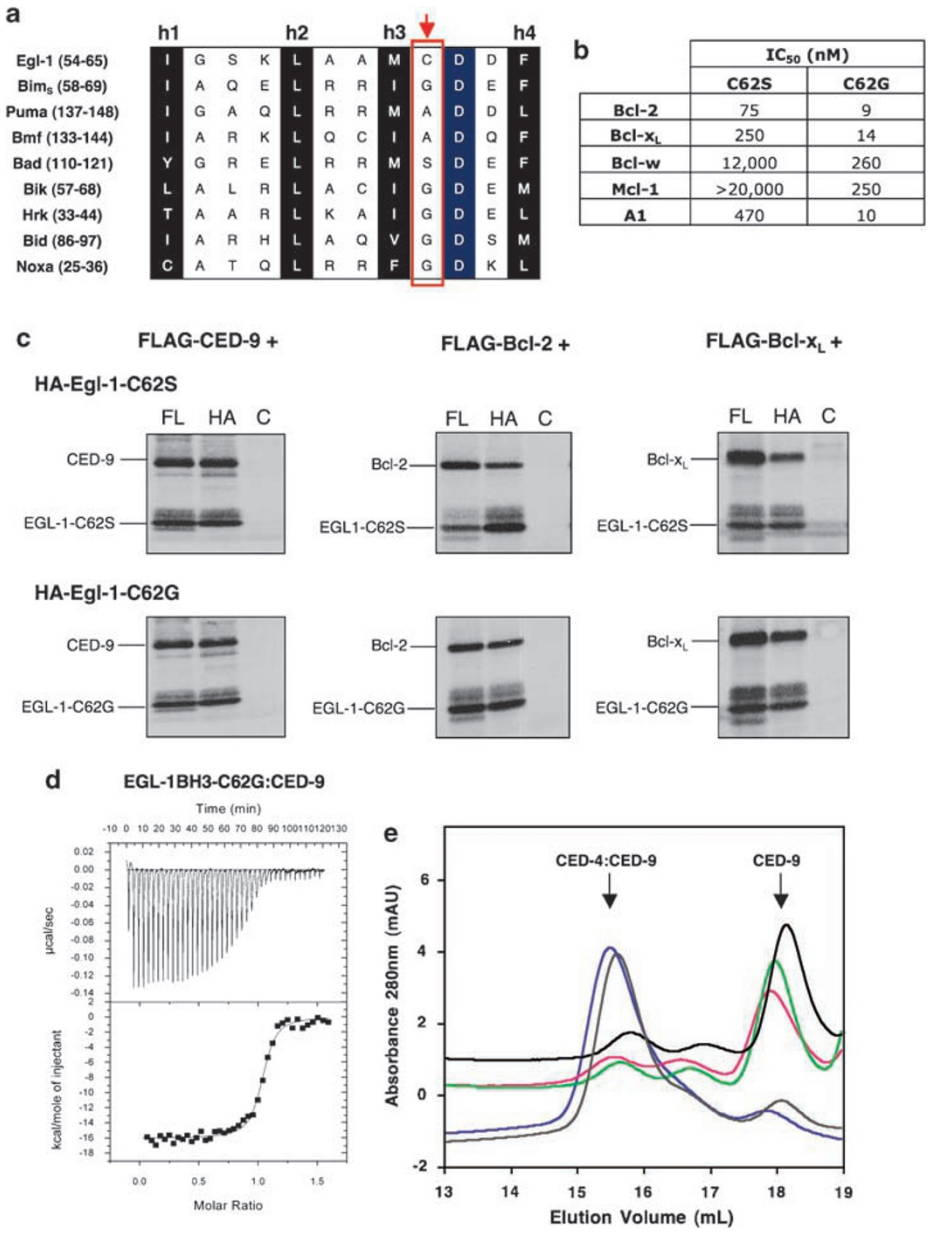

Figure 2 EGL-1 can be converted into a high-affinity ligand for mammalian pro-survival proteins. (**a**) Sequence comparison of the BH3 domains of EGL-1 and a subset of mammalian BH3 domains. EGL-1 has the key features of mammalian BH3 sequences including the conserved hydrophobic residues (h1–h4) (shaded black) and the conserved aspartate (shaded blue). Although, the residue immediately preceding the aspartate is usually a small amino acid such as glycine or alanine, it is uniquely a cysteine in EGL-1. (**b**) IC_{50} values for the binding of EGL-1BH3 mutants to mammalian pro-survival proteins as determined in solution competition assays. Mutation of the cysteine residue at position 62 of EGL-1 to serine or glycine results in significant increases in binding affinity, particularly to Bcl-2 and Bcl-x_L. (**c**) Unlike wild-type EGL-1, full-length EGL-1 bearing these mutations can detectably bind to Bcl-2 and Bcl-x_L in co-immunoprecipitation experiments performed as in Figure 1c. (**d**) The C62G mutation does not affect binding to CED-9. Isothermal titration microcalorimetry (ITC) analysis of EGL-1BH3-C62G mutant peptide binding to CED-9. The affinity (14 nM) is equivalent to the wild-type EGL-1BH3 peptide.[13,14,22] (**e**) Wild-type and mutant EGL-1BH3 mutant peptides, but not Bim BH3, can dissociate CED-4:CED-9 complexes as effectively as wild-type EGL-1BH3. The disappearance of the earlier eluting peak (CED-4:CED-9) and concomitant appearance of a later eluting one (CED-9) is indicative of the CED-4:CED-9 complex dissociation.[22] Dissociated free CED-4 is not observed in the profile as it precipitates upon dissociation from CED-9 and is removed by centrifugation prior to chromatography, as previously described.[22] Grey, no peptide treatment; blue, Bim BH3; red, wild-type EGL-1BH3; green, EGL-1BH3-C62S; black, EGL-1BH3-C62G

wild-type MEFs due to their limited binding profiles (Bad targets Bcl-x_L, Bcl-2 and Bcl-w; Noxa targets Mcl-1), they are able to kill *mcl-1*$^{-/-}$ and *bcl-x*$^{-/-}$ MEFs respectively (Figure 4c and d). However, EGL-1 was unable to kill either cell line in short- or long-term assays (Figure 4c and d). As killing activity may have been influenced by poor expression (Figure 3b), we similarly examined the Bim$_S$EGL-1 BH3 chimera. No killing was observed in any cell line despite relatively higher expression levels (Figure 4c and d).

We next determined whether our EGL-1 mutants (C62S and C62G), which bind mammalian pro-survival proteins with higher affinity than the wild-type sequence (Figure 2b), would

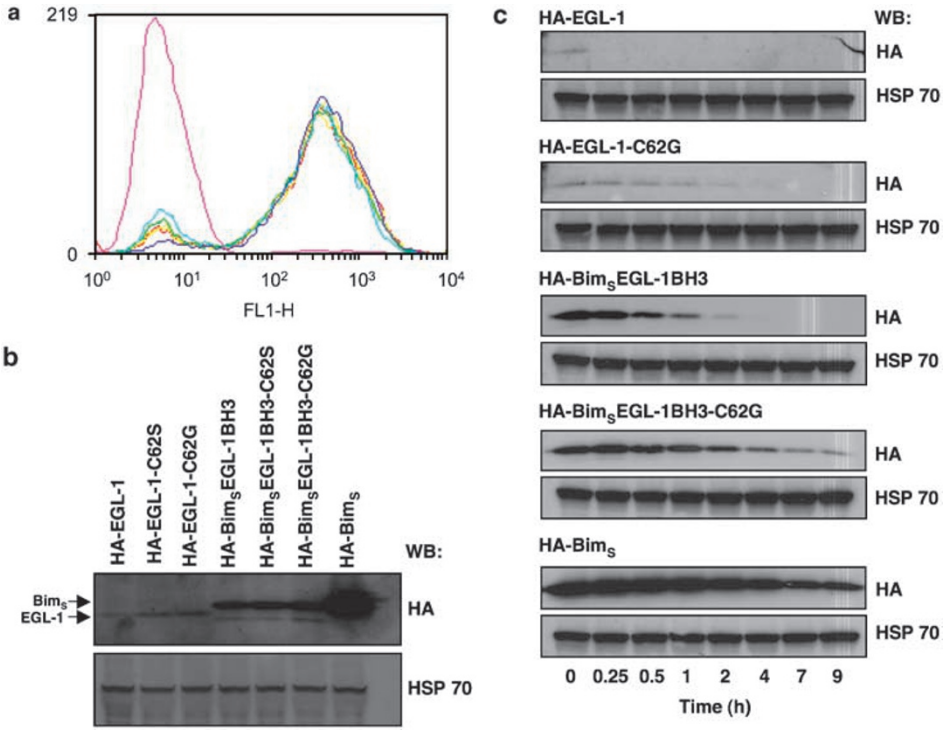

Figure 3 Expression and stability of EGL-1 or its mutants in mammalian cells. (**a**) FACS plot overlay showing green fluorescent protein (GFP) expression (FL1-H) of mouse embryonic fibroblast (MEF) cell lines where EGL-1 or Bim$_S$EGL-1BH3 chimera expression was linked to GFP following retroviral infection of *bax*$^{-/-}$/*bak*$^{-/-}$ MEFs. EGL-1 (black), EGL-1-C62S (red), EGL-1-C62G (orange), Bim$_S$EGL-1BH3 (yellow), Bim$_S$EGL-1BH3-C62S (green), Bim$_S$EGL-1BH3-C62G (light blue), Bim$_S$ (navy blue), untransfected (pink). (**b**) Western blot showing basal-level expression of HA-tagged EGL-1 and Bim$_S$EGL-1 chimeras from equivalent cell lysates derived from lines shown in (**a**). Blots were re-probed with anti-HSP 70 as a loading control. Note the strikingly low levels of wild-type or mutant EGL-1. (**c**) Cells from (**a**) were either left untreated or exposed to 50 μg/ml cycloheximide for the indicated times after which they were analysed by western blotting using the anti-HA antibody. The C62G mutants of EGL-1BH3 (compare second with fourth panel) appeared more stable especially in the context of Bim$_S$, potentially because they can associate with endogenous pro-survival proteins such as Bcl-2. Blots were re-probed for anti-HSP 70 as a loading control

be able to kill the same cell lines. As these mutants displayed enhanced affinity for Bcl-2 and Bcl-x$_L$ in particular, we predicted they would behave similarly to Bad, only killing *mcl-1*$^{-/-}$ cells, but not wild-type or *bcl-x*$^{-/-}$ MEFs. Indeed, neither mutant was able to kill the wild-type or *bcl-x*$^{-/-}$ MEFs in the short- or long-term assays (Figure 4a–d). Importantly, unlike the wild-type EGL-1BH3 domain, both EGL-1BH3 mutants displayed potent killing of *mcl-1*$^{-/-}$ MEFs in the context of the Bim$_S$ backbone in short- and long-term assays. Moreover, although the C62G mutant was also an effective killer in the parental EGL-1 context, the C62S mutant did not kill in either assay (Figure 4a–d). Hence our data demonstrate that a combination of expression levels and binding affinity dictates killing activity.

EGL-1BH3 mutants can complement Noxa for cell killing. Co-expression of BH3-only proteins such as Bad and Noxa possessing restricted binding profiles kills cells such as MEFs, though expression of either alone does not.[23] Both EGL-1-C62S and C62G possess restricted binding profiles similar to Bad, and as such were unable to kill wild-type MEFs regardless of their context (i.e. in the EGL-1 or Bim$_S$ backbone). We next examined their ability to complement Noxa for cell killing. Again wild-type EGL-1 and Bim$_S$EGL-1 were ineffective, unable to kill Noxa-expressing wild-type MEFs in short- or long-term assays (Figure 5a and b), while Bim$_S$EGL-1BH3-C62S was only effective in the long-term assay (Figure 5a and b). EGL-1-C62G also only killed Noxa-expressing MEFs in long-term assays whereas the Bim$_S$EGL-1BH3-C62G variant was a potent killer in short- and long-term assays. No constructs were able to kill Bad-expressing MEFs, though these were readily killed by Noxa in short-term assays, supporting the specificity of the EGL-1 constructs.

As the release of cytochrome *c* from mitochondria is a hallmark of apoptosis, we examined the ability of EGL-1BH3 synthetic peptides to induce cytochrome *c* release from permeabilized cells expressing Noxa or Bad. Consistent with the killing results, wild-type EGL-1BH3 was unable to release cytochrome *c* in either cell line, although significant release was observed with EGL-1BH3-C62G in the Noxa-expressing cells, but not those expressing Bad (Figure 5c).

EGL-1BH3-binding kinetics. Although Biacore competition assays provide a relative measure of binding affinities, allowing comparison between proteins, they do not provide affinity dissociation constants (K_D), nor do they provide information about binding kinetics. We established an assay using an S51 Biacore biosensor that allows direct measurement of both these parameters for peptides, though only have robust assays for Mcl-1 and Bcl-x$_L$. Importantly, the K_Ds show the same trend as in the

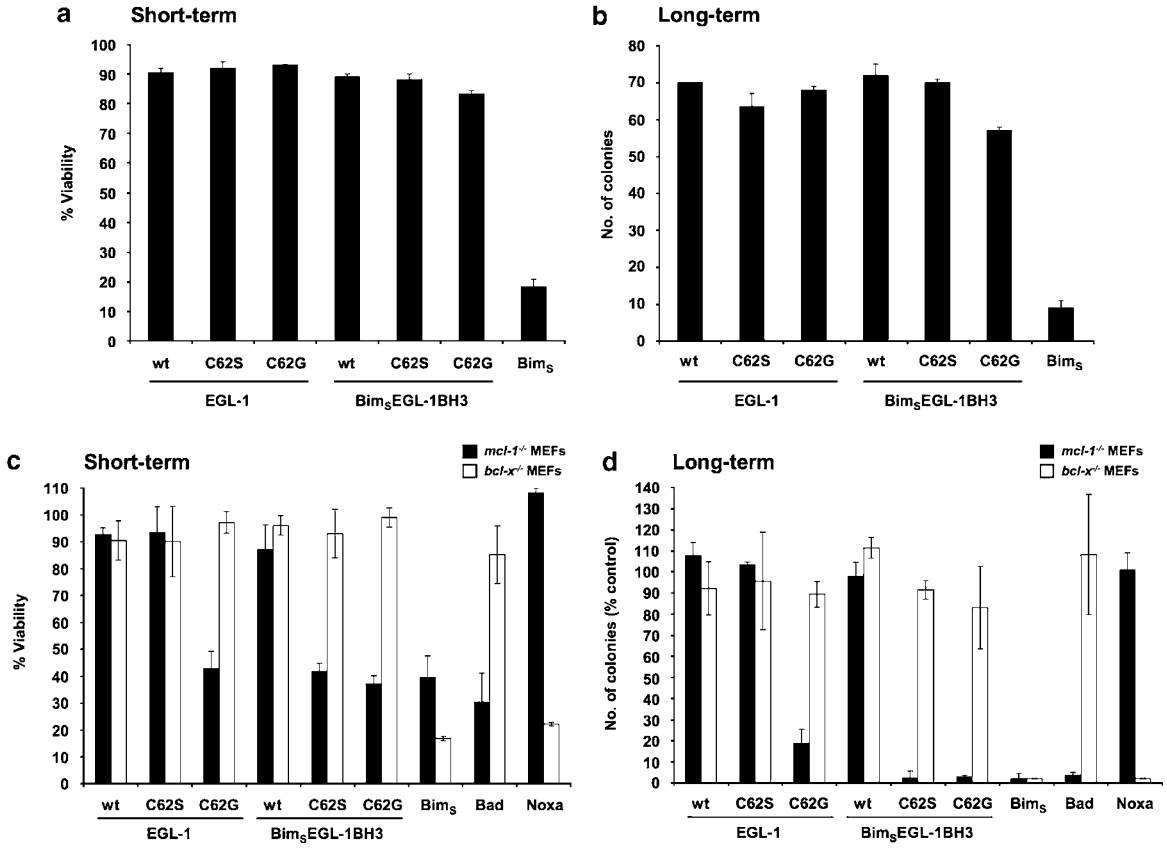

Figure 4 Wild-type EGL-1 is a poor killer of mouse embryonic fibroblasts (MEFs). To evaluate the cytotoxic effect of EGL-1 over-expression, retroviruses expressing either Bim_S, wild-type EGL-1BH3 or EGL-1BH3 mutants, both in the EGL-1 or Bim_S backbones, were used to infect wild-type MEFs (**a**, **b**). Cell viability was assessed either (**a**) 24 h after infection by propidium iodide exclusion analysed by flow cytometry, or (**b**) 6 days after infection when colonies were scored. Similar assays were also performed in (**c**) *mcl-1*$^{-/-}$ (black bars) or (**d**) *bcl-x*$^{-/-}$ (white bars) MEFs. Note that although all EGL-1-based constructs are unable to kill the wild-type cells, significant cell death is observed in *mcl-1*$^{-/-}$ cells but not *bcl-x*$^{-/-}$ with the higher affinity ligands. Representative assays are shown where error bars indicate the standard deviation from the mean for duplicate or triplicate data points. All constructs have been tested two or more times in each assay

competition assays, although the data are shifted towards higher affinities (Table 1). The C62G peptide bound almost as tightly to Bcl-x_L as BimBH3. The C62S mutant with intermediate affinity has a similar on-rate to BimBH3 but the complex dissociates nearly 200-fold more rapidly than the C62G mutant. Essentially no binding was detected for wild-type EGL-1BH3 peptide to Bcl-x_L or Mcl-1. Only the C62G mutation provided a significant affinity gain for Mcl-1, again consistent with the competition data.

Discussion

BH3-only protein concentrations and affinities determine apoptotic thresholds. Here we show that EGL-1 binds all mammalian pro-survival proteins weakly and is a poor killer of mammalian cells. However, by manipulating its binding affinity and stability/intracellular concentration, we demonstrated how different combinations of these key biophysical parameters, together with how pro-survival proteins are 'neutralized', determine apoptotic thresholds.

The combination of weak affinity, poor expression and low stability presumably all contribute to wild-type EGL-1 being an inefficient killer of mammalian cells. However, the threshold required to trigger apoptosis cannot be overcome by simply increasing protein levels through the placement of the EGL-1 BH3 sequence in a more stable context (i.e. Bim_S), probably because its affinity for pro-survival proteins is still too weak. Nevertheless, incremental increases in killing potency occurred upon manipulation of the above-mentioned parameters (see Table 2 for a summary of killing data).

Probably the single change that had the greatest impact on cell fate was the increase in affinity for Bcl-x_L and Bcl-2 from the μM to low-sub-nM range, following the C62G substitution in EGL-1BH3. Provided Mcl-1 was inactivated (either through Noxa expression or genetic deletion), this mutant was able to kill cells, even when expressed at relatively low levels, as occurs in the parent EGL-1 sequence context.

The EGL-1-C62S mutant that had intermediate affinity for Bcl-x_L and Bcl-2 was less potent in killing assays than C62G even though it expressed at similar levels. Indeed, EGL-1-C62S was unable to kill MEFs, even when Mcl-1 was also 'neutralized', except when placed in the context of Bim_S where the protein was present at higher levels. The Bim_S context may also allow the protein to target the mitochondria more efficiently, hence the improved potency may not be just a consequence of increased levels.

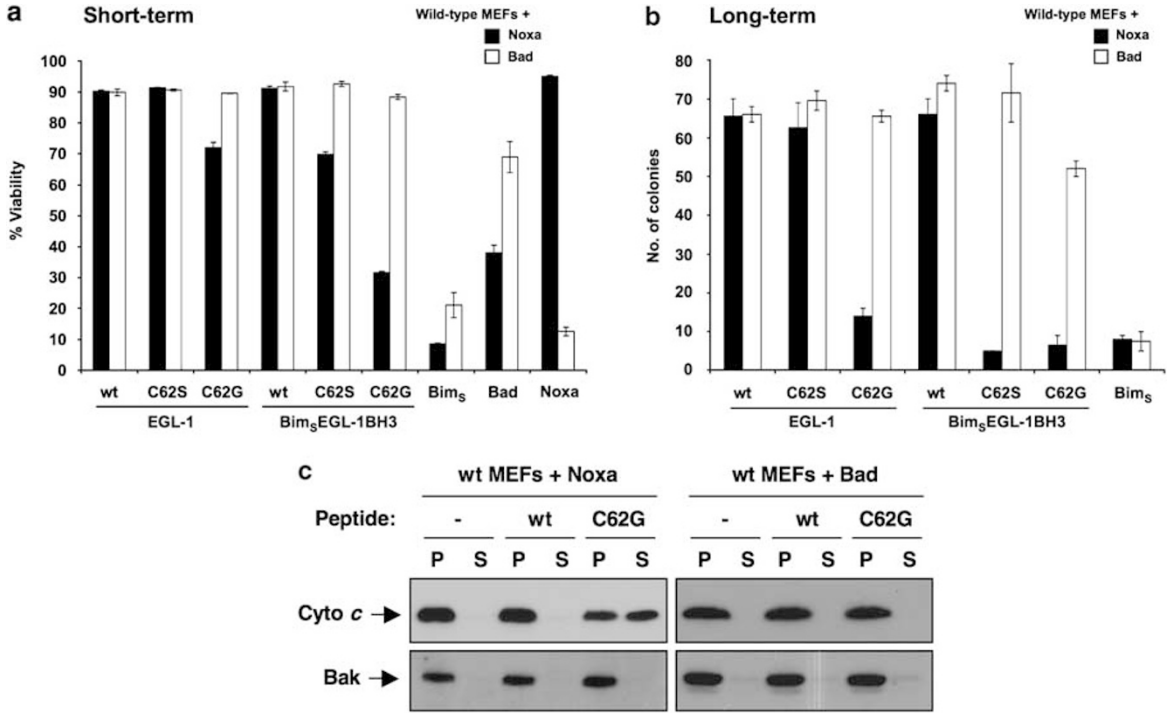

Figure 5 EGL-1 mutants can combine with Noxa to kill mouse embryonic fibroblasts (MEFs). MEFs stably expressing Noxa (black bars) or Bad (white bars) were infected with retroviruses expressing either Bim_S, wild-type EGL-1BH3 or EGL-1BH3 mutants, both in the EGL-1 or Bim_S backbones. Cell viability was assessed either (**a**) 24 h after infection by propidium iodide exclusion analysed by flow cytometry, or (**b**) 6 days after infection when colonies were scored. EGL-1 mutants with higher affinities kill Noxa-expressing cells, though the kinetics of cell death depends on the sequence context. Representative assays are shown where the error bars indicate the standard deviation from the mean for duplicate data points. All constructs have been tested two or more times in each assay. (**c**) EGL-1BH3 peptides were assessed for their ability to release cytochrome *c* from crude mitochondrial lysates prepared from MEFs expressing either Noxa or Bad. Following incubation with or without (−) indicated peptides (wt, wild-type EGL-1BH3, C62G mutant), lysates were separated into mitochondria-containing pellet (P) or soluble (S) fractions. Only the EGL-1-C62G peptide was able to cause release of cytochrome *c* from the mitochondria from the Noxa-expressing cells. Bak was present only in the pellet fractions of all samples

Table 1 Kinetics of BH3 domain peptide binding to Bcl-x_L and Mcl-1

Binding to	**Bcl-x_L**			**Mcl-1**		
Peptide ligand	**K_D (nM)**	**k_d (1/s)**	**k_a (1/Ms)**	**K_D (nM)**	**k_d (1/s)**	**k_a (1/Ms)**
EGL-1BH3	>5000	ND	ND	>5000	ND	ND
EGL-1BH3-C62S	29	0.1	3.5×10^6	>2000	ND	ND
EGL-1BH3-C62G	0.35	6.3×10^{-4}	1.8×10^6	11	0.03	2.6×10^6
BimBH3	0.14	5.3×10^{-4}	3.7×10^6	0.12	3.5×10^{-4}	2.9×10^6

Affinity and kinetic measurements (K_D, equilibrium dissociation constant; k_a, rate constant of association; k_d, rate constant of dissociation) were made using a Biacore S51 biosensor where the indicated peptides were injected across a chip in which either Bcl-x_L or Mcl-1 was immobilized. ND, not determined as binding was too weak.

We also observed interesting differences in killing kinetics where certain mutants (Bim_SEGL-1BH3-C62S and EGL-1-C62G) were only active in the long-term assay but not in the short-term assay. This difference, though, was only apparent in Noxa-expressing cells. Hence, as these mutants exhibited potent killing in both short- and long-term assays when over-expressed in Mcl-1-deficient cells, it suggests that another event that delays the onset of apoptosis, presumably involving Mcl-1 inactivation, occurs in the Noxa-expressing cells.

One possibility is that there is still some 'functional' Mcl-1 available in the Noxa-expressing cells capable of inhibiting Bak/Bax. We propose that for Mcl-1 to be inactivated, BH3-only proteins such as Bim or Bid, which engage Mcl-1 with high affinity[23,26] (or perhaps bind directly to Bak or Bax[26–28,30]), need to be released from pro-survival protein sequestration. As this displacement involves competition for the same binding site, higher affinity ligands will be more effective as their slower off-rate will mean each binding site is occupied for longer. Alternatively, higher ligand concentrations also favour binding, hence the binding kinetics will translate to the killing kinetics. The slower kinetics of EGL-1-C62G killing in Noxa-expressing cells compared to in the *mcl-1*$^{-/-}$ cells likely correlates with its low expression/concentration, whereas in the case of the Bim_SEGL-1BH3-C62S mutant this probably relates to its affinity for pro-survival proteins such as Bcl-x_L. Furthermore, as EGL-1-C62G (which can engage Mcl-1) could not kill Bcl-x_L-deficient or Bad-expressing cells, this suggests that the threshold affinity for Mcl-1 was not reached. As the affinity of this ligand was similar to EGL-1-C62S for Bcl-x_L, it appears that the threshold for

Table 2 Summary of killing activity of EGL-1 and Bim_SEGL-1 chimeric constructs

Ligand	Cells	Assay	
		Short-term	Long-term
EGL-1	Noxa-expressing	Live	Live
	mcl-1$^{-/-}$	Live	Live
Bim_SEGL-1BH3	Noxa-expressing	Live	Live
	mcl-1$^{-/-}$	Live	Live
EGL-1-C62S	Noxa-expressing	Live	Live
	mcl-1$^{-/-}$	Live	Live
Bim_SEGL-1BH3-C62S	Noxa-expressing	Live	Die
	mcl-1$^{-/-}$	Die	Die
EGL-1-C62G	Noxa-expressing	Live	Die
	mcl-1$^{-/-}$	Die	Die
Bim_SEGL-1BH3-C62G	Noxa-expressing	Die	Die
	mcl-1$^{-/-}$	Die	Die

Mcl-1 neutralization is higher. This difference possibly just reflects a higher intracellular concentration of it compared to Bcl-x_L. Alternatively, the relative affinities of Bak for Mcl-1 and Bcl-x_L need to be considered. Indeed, the higher affinity of the Bak BH3:Mcl-1 interaction compared with Bak BH3:Bcl-x_L (IC_{50} 10 *versus* 50 nM) is entirely consistent with the higher threshold required for Mcl-1 neutralization.[24]

Hence this model involves elements of both 'indirect' and 'direct' models of apoptotic activation whereby pro-survival proteins act as sinks for BH3-only proteins, though these need not necessarily be 'activators' in the sense proposed previously, rather they exert their pro-apoptotic effect by engaging pro-survival molecules available to inhibit Bax/Bak.

Implications for evolutionary conservation of pro-survival protein function. Our data also highlight how the interconnections between binding affinities and protein levels must be considered when interpreting the classical worm *bcl-2* transgene experiments.[2,19] The low affinity of EGL-1 for most mammalian pro-survival proteins, shown in the present study, is interesting in light of a recent report using a yeast model system.[20] Their data suggest Bcl-2 does not directly inhibit CED-4, as CED-9 does, but rather acts as a 'sink' to sequester EGL-1, preventing it from engaging CED-9. Our binding data would support this hypothesis provided the levels of Bcl-2 are sufficient to overcome its relatively low affinity ($K_D \sim 10\,\mu$M) for EGL-1. Hence, micromolar concentrations of Bcl-2 would be required for a significant proportion of EGL-1 to be sequestered. This is conceivable when Bcl-2 is over-expressed, such as in both the worm and yeast studies. Moreover, as Bcl-2 is predominantly located on mitochondrial membranes, it may have an even higher local concentration. Interestingly, of the mammalian pro-survival proteins, Bcl-2 (together with A1) has the highest affinity for EGL-1 (Figure 1a). Hence, in the light of our binding data, it would be predicted that if Mcl-1 was used instead of Bcl-2 in the classic worm transgene studies,[2,19] far less, if any pro-survival activity would be observed.

Subtle mutations influence BH3 domain affinity and specificity. One key finding of this study was that mammalian pro-survival proteins are exquisitely sensitive to small alterations in BH3 ligand sequences. Remarkably, we demonstrated that a single atom change (oxygen for the sulphur in C62) was sufficient to increase the affinity of EGL-1 for Bcl-2 and Bcl-x_L by two orders of magnitude (Figure 2b). A further order of magnitude (or greater) increase in affinity for most mammalian pro-survival proteins was achieved by reducing the size of the side chain of C62 further by replacing it with glycine. This EGL-1BH3-C62G mutant engages Bcl-2 and Bcl-x_L with an affinity approaching the highest affinity mammalian BH3 ligands for these pro-survival proteins.[23]

The requirement for a small residue at position 62 in EGL-1 reflects the sterically constrained nature of the binding groove in mammalian pro-survival proteins where this residue is inserted.[16–18,23] Indeed, a recent study demonstrated that the serine in the corresponding position in BadBH3 becomes phosphorylated and loses significant affinity for pro-survival proteins.[31] We also showed that substitution of the glycine in

Bim at the same position to glutamate is detrimental to binding pro-survival proteins, rendering it inactive.[29] Interestingly, this region of EGL-1 is adjacent to where the large conformational change occurs to CED-9 required for dissociation of CED-4.[13,15] However, we also showed that a larger residue is not essential to induce this conformational change as both serine- and glycine-substituted EGL-1 peptides dissociated the CED-4:CED-9 complex (Figure 2e). Moreover, as Bcl-x_L and Mcl-1 show less plasticity compared to CED-9 in the corresponding region,[16–18,32] this may explain the intolerance of these molecules for the cysteine in the EGL-1BH3 ligand. This finding has important implications for BH3 mimetic drug design as it demonstrates that minute changes to ligands can have significant consequences on binding affinities.

Materials and Methods

Recombinant proteins and synthetic peptides. Expression and purification of human Bcl-$x_L\Delta$C25, mouse/human Mcl-1 or N-terminal His_6-tagged human Bcl-2ΔC22 and human Bcl-wΔC29 (C29S, A129E), CED-9(68–251) and CED4:CED-9 complex have been previously described.[22,23,32] Synthetic peptides were synthesized by Mimotopes (Victoria, Australia) and purified by reverse-phase high-performance liquid chromatography to 70–90% purity.

Solution competition assays. Solution competition assays using the Biacore optical biosensor were performed essentially as described previously.[23] Pro-survival proteins (10 nM) were incubated with varying concentrations of synthetic peptides for 2 h prior to injection onto a CM-5 sensor chip with either wild-type BimBH3 or the inert BimBH3-4E mutant peptide immobilized.[23] The specific response was determined by subtracting the background signal on the BimBH3-4E mutant channel from the specific signal on the wild-type BimBH3 channel.

Isothermal titration microcalorimetry. ITC studies were performed using a MicroCal VP-ITC instrument. Proteins diluted to 5 μM (CED-9) or 10 μM (Bcl-2) in Tris-buffered saline (TBS) and peptides (prepared in the same buffer from 2 mM stocks) were injected from a 40 μM (EGL-1BH3 C62G) or 400 μM (wild-type EGL-1BH3) solution at 25°C. Data analysis was performed using the MicroCal Origin software.

Direct binding assays. Direct binding assays were performed at room temperature using a Biacore S51 biosensor with 10 mM NaH_2PO_4, 40 mM Na_2HPO_4, 150 mM NaCl, 1 mM EDTA, 0.03% (v/v) Tween 20, 5% (v/v) DMSO, pH 7.4 as the running buffer. Anti-GST was immobilized on a CM5 sensorchip using amine-coupling chemistry. Recombinant GST-tagged Bcl-x_L or Mcl-1 (100 μg/ml) were then injected at the flow rate of 10 μl/min and captured by the tag. All BH3 domain peptides were prepared in running buffer. Several concentrations of peptide around that peptide's K_D were injected at a flow rate of 90 μl/min. Weaker binding ligands ($K_D > 10$ nM) were allowed to associate with the protein for 60 s and dissociation was monitored for 60 s, whereas for tighter ligands the association time was 90 s and the dissociation time was 270 s. All sensorgrams were generated using double referencing by subtracting the binding response from a reference spot, followed by corrections for solvent bulk shifts and subtraction of an average of the running buffer bank injections over the immobilized spot. For K_D calculation, corrected response data were fitted using a 1 : 1 binding site model including mass transport limitations.

Co-immunoprecipitation of pro-survival proteins and Bim_S or EGL-1. FLAG-tagged mammalian expression vectors for human Bcl-2, mouse Bcl-x_L and HA-tagged human Bim_S, Bim_S4E, EGL-1 or its mutants subcloned into pEF PGKpuro have been previously described.[23,24,33,34] The maintenance, transfection and metabolic labelling of HEK293T cells with ^{35}S-methionine/cysteine, as well as co-immunoprecipitation experiments have been also described previously.[33–35] Cell lysates were immunoprecipitated with $\sim$5 μg antibody (anti-HA HA.11, Covance Research Products; anti-FLAG M2, Sigma) and control anti-Glu-Glu (anti-EE; Covance Research Products) as previously described.[24] The proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and immunoprecipitated proteins were detected by fluorography using Amplify (Amersham Biosciences).

CED-4/CED-9 complex dissociation assays. Dissociation of the recombinant CED-4/CED-9 complex was performed by incubation of 100 μg of protein (0.5 mg/ml) with a twofold molar excess of synthetic BH3 domain peptide in a total volume of 200 μl for 1 h at room temperature.[22] The reaction mixture was then centrifuged for 5 min at 13 000 r.p.m. in a bench-top microfuge prior to chromatography on a Superose 6 10/30 column equilibrated and run in TBS at 0.5 ml/min.

Killing assays. Retroviral expression constructs were made using the pMIG vector (MSCV-IRES-GFP; GFP sequence is that of EGFP) as described previously.[23,36] Following transient transfection into Phoenix Ecotropic packaging cells,[37] filtered virus-containing supernatants were used to infect SV40 large T-antigen transformed MEFs by spin inoculation as described previously[23] and cell viability was determined by flow cytometric analyses of infected cells (GFP^+) that excluded 5 μg/mL propidium iodide (Sigma) which was analysed using FACScan® (Becton Dickinson).

For long-term survival (colony) assays, wild-type, *mcl-1*$^{-/-}$, *bcl-x*$^{-/-}$ MEF cell lines or those expressing either, human Noxa or mouse Bad were infected with retroviruses as above, grown in the presence of 50 μM QVD-OPH for 24 h and then 150 GFP^+ cells were sorted into six-well plates in the absence of QVD-OPH. Colonies were allowed to grow for 6 days prior to staining with Coomassie brilliant blue and were counted.

EGL-1 stability experiments. MEFs were infected with retroviruses in which expression of HA-tagged EGL-1, EGL-1-C62G, Bim_SEGL-1BH3 chimeras or Bim_S was linked to GFP in the pMIG vector. GFP^+ cells were selected by FACS sorting and were expanded. Equivalent numbers of cells (200 000) were then treated with 50 μg/ml cycloheximide for 0–9 h after which time they were lysed, and analysed for HA-tagged protein levels by western blotting using the anti-HA antibody (HA.11; Covance Research Products). Blots were re-probed with anti-HSP-70 as a loading control.

***In vitro* cytochrome *c* release assays.** Cytochrome *c* release assays were performed as previously described[38] using mitochondria-containing crude lysates from digitonin-permeabilized MEFs that were stably transfected with either human Noxa or mouse Bad. The lysates were either left untreated or incubated with the synthetic peptide (10 μM) at 30°C for 1 h before pelleting. The supernatant was retained as the soluble (S) fraction whereas the pellet (P) fraction, which contains unpermeabilized mitochondria, was solubilized in RIPA buffer. Both the soluble and pellet fractions were then subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes, which were immunoblotted using a mouse monoclonal anti-cytochrome *c* (7H8.2C12; BD Pharmingen) antibody followed by rabbit polyclonal anti-Bak (B5929; Sigma) antibody.

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