Hydrophile scanning as a complement to alanine scanning for exploring and manipulating protein–protein recognition: Application to the Bim BH3 domain

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Abstract

Alanine scanning has been widely employed as a method of identifying side chains that play important roles in protein–protein and protein–peptide interactions. Here we show how an analogous and complementary technique, hydrophile scanning, can provide additional insight on such interactions. Mutation of a wild-type residue to alanine removes most of the side-chain atoms, and the effect of this removal is typically interpreted to indicate contribution of the deleted side chain to the stability of the complex. Hydrophile scanning involves systematic mutation of wild-type residues to a cationic or anionic residue (lysine or glutamic acid, in this case). We find that the results of these mutations provide insights on interactions between polypeptide surfaces that are complementary to the information obtained via alanine scanning. We have applied this technique to a peptide that corresponds to the BH3 domain of the pro-apoptotic protein Bim. The wild-type Bim BH3 domain binds strongly to the anti-apoptotic proteins Bcl-x_L and Mcl-1. Combining information from the alanine, lysine, and glutamic acid scans has enabled us to identify Bim BH3 domain mutants containing only two or three sequence changes that bind very selectively either to Bcl-x_L or Mcl-1. Our findings suggest that hydrophile scanning may prove to be a broadly useful tool for revealing sources of protein–protein recognition and for engineering selectivity into natural sequences

Keywords: peptide; apoptosis; Bcl-x_L; Mcl-1; Bim; hydrophile scan

Supplemental material: see www.proteinscience.org

Understanding the factors that control protein–protein binding affinities and selectivities is a central challenge in molecular biology. This challenge becomes especially important in the context of protein interaction networks that contain many homologous members, systems in which the biological outcome depends upon partner selectivity. The B-cell lymphoma 2 (Bcl-2) protein family

constitutes a network that is involved in early steps of the programmed cell death pathway (Coultas and Strasser 2003). Some family members promote apoptosis, while others inhibit apoptosis. The mechanisms by which individual proteins play their characteristic roles remain subjects of debate (Galonek and Hardwick 2006; Willis et al. 2007), but it is well established that dimeric interactions between specific Bcl-2 family proteins are critical for regulation of apoptosis (Letai et al. 2002; Adams and Cory 2007). Inhibition of binding between pro- and anti-apoptotic family members has emerged as an attractive target for anticancer drug development (Garber 2005).

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Interactions between Bcl-2 family members offers an excellent opportunity for fundamental studies of the relationships among sequence, stability, and selectivity in protein–protein complexation (Walensky et al. 2004; Wang et al. 2005; Fu et al. 2007), especially since one of the protein partners can be reduced to a peptide. Associations within this family are relatively simple from a structural standpoint: A single α -helical segment (the Bcl-2 Homology 3 [BH3] domain) from a pro-apoptotic family member binds into a complementary cleft on the surface of an anti-apoptotic family member (Sattler et al. 1997). BH3 domain peptides display considerable variation in their selectivity for binding to anti-apoptotic Bcl-2 family proteins (Chen et al. 2005; Certo et al. 2006), but it is not yet clear how these selectivities are encoded in BH3 domain sequences. Information provided by mutagenesis studies such as the one reported here may provide insight on the factors that control affinity and binding preference within this protein–protein interaction network, information that might ultimately prove useful for elucidating the signaling pathways in the network (Galonek and Hardwick 2006; Willis et al. 2007), and perhaps for medicinally oriented efforts.

Essential features of the BH3 domain motif have been revealed by structural analysis of complexes between BH3 domain peptides and anti-apoptotic family members (Petros et al. 2004), by comparisons among BH3 domain sequences (Huang and Strasser 2000), and by evaluation of BH3 mutation effects on binding affinity (Sattler et al. 1997; Petros et al. 2000; Lee et al. 2007). BH3 domains feature four hydrophobic residues in an $i, i + 4, i + 7, i + 11$ pattern, which results in alignment of the nonpolar side chains along one face of the BH3 α -helix. These four side chains occupy pockets within the BH3-recognition cleft of anti-apoptotic Bcl-2 family proteins. Among the four aligned nonpolar residues, only Leu at the $i + 4$ position is absolutely conserved among BH3 domains. Ile, Leu, Val, and Met are common at the other three nonpolar positions, and aromatic residues are found occasionally as well. Asp is absolutely conserved at the $i + 9$ position. All other sequence positions in a BH3 domain are variable, although trends can be discerned in some cases (e.g., positions $i + 1$ and $i + 8$ are nearly always glycine or alanine).

We have employed a mutation-based approach in an effort to understand how changes to the BH3 domain sequence influence binding affinity for two anti-apoptotic Bcl-2 family members, Bcl-x_L and Mcl-1. High-resolution crystal structures are available for both of these proteins bound to BH3 domain peptides (Liu et al. 2003; Czabotar et al. 2007; Oberstein et al. 2007), which provide a structural basis for interpreting our results. It should be noted, however, that the Bim/Bcl-x_L crystal structure involves mouse proteins (Liu et al. 2003),

whereas our mutagenesis studies utilized the human sequences. Mcl-1 structural data were obtained for a chimera of murine and human sequences (Czabotar et al. 2007), but the human Mcl-1 sequence was used in our studies. The sequences of the proteins and peptides in the crystal structures, aligned with the sequences utilized in this study, are shown in the Supplemental material. These sequences are highly homologous. We started with a BH3-domain peptide derived from the pro-apoptotic protein Bim, which binds tightly to both Mcl-1 and Bcl-x_L (Chen et al. 2005; Certo et al. 2006). We compared a conventional mutation strategy (alanine scanning) to a nonconventional strategy (hydrophile scanning), which consists of systematic replacement of each natural Bim side chain with either a basic side chain (lysine) or an acidic side chain (glutamic acid).

Hydrophile scanning is intended to provide information that is complementary to the information obtained via alanine scanning, which has been widely pursued for exploration of protein–protein binding interfaces (Cunningham and Wells 1989; Clackson and Wells 1995; Morrison and Weiss 2001). Mutation of nonglycine residues to alanine can be viewed as side-chain deletion, and the results of alanine-scanning studies have usually been interpreted as indicating whether or not a particular side chain contributes to affinity. Hydrophile scanning should provide a useful supplement to the results of alanine scanning for identifying fully buried side chains. If burial of a nonpolar side chain occurs during a complexation event but the packing of the side chain against the partner protein causes strain, then an alanine mutation at this position might not have a large impact on binding (Cunningham and Wells 1989; Clackson and Wells 1995; Petros et al. 2004). On the other hand, replacing the natural nonpolar side chain with a charged side chain (as in mutation to lysine or glutamic acid) should be deleterious to complex stability due to unfavorable desolvation of a charged residue (Bajaj et al. 2005). (However, the pK_a, and therefore the charge of the side chain, may be altered upon complexation [Parsons and Raftery 1972].) Hydrophile scanning should identify positions at which new or altered electrostatic interactions can stabilize or destabilize the complex. In this regard, it should be informative to compare the results of cationic and anionic versions of hydrophile scanning. Of course, changes in binding affinity may also be due to the steric differences between lysine and glutamic acid relative to the natural residue. To evaluate the impact of changing the shape of the residue, isosteric mutations can be made. We choose glutamine as an isostere for glutamic acid and norleucine (Nle) as an isostere for lysine; however, other amino acids, both natural and nonnatural, may be used as isosteric controls. As is the case with alanine scanning, interpretation of hydrophile-scanning results will not

always be straightforward, especially if structural information is not available (Ward et al. 1990), but increases in affinity and/or changes in partner selectivity discovered in this way may nevertheless be valuable for the development of useful protein-binding molecules. Additionally, the increased water solubility to be expected for mutation of a native hydrophobic residue to lysine or glutamic acid may be useful for biological applications of the resulting peptides. We recently introduced the hydrophile-scanning strategy in an effort to determine the role of specific subunits in the binding of an unnatural peptidic oligomer to Bcl-x_L (Sadowsky et al. 2007); here we apply this technique to a conventional peptide derived from the Bim BH3 domain.

Collectively, the alanine-scanning and hydrophile-scanning results enable us to identify Bim BH3 mutants containing two or three sequence changes that bind tightly to and are highly selective for Mcl-1 over Bcl-x_L or vice versa. This study suggests that hydrophile scanning should be considered by other researchers who seek to identify sequence features that influence binding affinity and selectivity in protein–protein or protein–peptide interactions, and to optimize affinity and selectivity.

Results and Discussion

Experimental design

The sequences of pro-apoptotic BH3 domains contain four hydrophobic residues (labeled H1 to H4 in Table 1) that are broadly conserved across the Bcl-2 family. The binding of several BH3 domain peptides from pro-apoptotic proteins (including most of those listed in Table 1) to the five anti-apoptotic proteins Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1 (Chen et al. 2005; Certo et al. 2006) has been characterized. The BH3 domain peptide derived from Bim was observed to bind tightly to all five anti-

apoptotic proteins (Chen et al. 2005; Certo et al. 2006). However, most of the other BH3 domain peptides examined displayed significant selectivity among the anti-apoptotic Bcl-2 family members. We were interested to see whether alanine or hydrophile scanning would identify mutations in the Bim BH3 domain that confer selectivity between Bcl-x_L and Mcl-1, anti-apoptotic proteins that display different patterns of binding preference among most pro-apoptotic family members (Chen et al. 2005; Certo et al. 2006).

We used competition fluorescence polarization (FP) assays to evaluate the binding of Bim 81-106 to Bcl-x_L and to Mcl-1. The data suggested *K_i* values of 7.4 ± 2.8 and 5.5 ± 2.7 nM, respectively, which appears to be consistent with the observations of others (Chen et al. 2005; Certo et al. 2006). Before conducting our alanine- and hydrophile-scanning experiments, we examined the effect of Bim peptide truncation in order to minimize the length of the sequence to be scanned. The 18-mer, Bim 86-103, proved to be comparable to the original 26-mer in terms of affinity for Mcl-1 (*K_i* = 4.0 ± 1.3 nM) but associated somewhat less strongly with Bcl-x_L (*K_i* = 110 ± 40 nM). This result suggests that comparisons of peptide mutation effects among different studies must be carefully made, as the length of the peptide can significantly influence binding affinity and selectivity (Sattler et al. 1997; Petros et al. 2000). We used Bim 86-103, referred to hereafter as Bim^{BH3}, as the starting point for mutational studies. The comparison of BH3 domain sequences in Table 1 is based on alignment with a 23-residue fragment of Bim.

Figure 1 summarizes the results of scanning with alanine (part A), lysine (part B), or glutamic acid (part C). Figure 2 compiles the results without normalization, separated for Mcl-1 (part A) and Bcl-x_L (part B). In each series, all residues in the Bim^{BH3} sequence were individually replaced by the indicated residue, except for closely

Table 1. Homology alignment of various BH3 domains

		H1							H2							H3							H4						
Sequence																													
Bim _L	84-106	P	E	I	W	I	A	Q	E	L	R	R	I	G	D	E	F	N	A	Y	Y	A	R	R					
Puma	133-155	W	A	R	E	I	G	A	Q	L	R	R	M	A	D	D	L	N	A	Q	Y	E	R	R					
Noxa	21-43	L	E	V	E	C	A	T	Q	L	R	R	F	G	D	K	L	N	F	R	Q	K	L	L					
Bmf	129-151	A	E	V	Q	I	A	R	K	L	Q	C	I	A	D	Q	F	H	R	L	H	V	Q	Q					
Bik	53-75	G	S	D	A	L	A	L	R	L	A	C	I	G	D	E	M	D	V	S	L	R	A	P					
Bid	82-104	I	I	R	N	I	A	R	H	L	A	Q	V	G	D	S	M	D	R	S	I	P	P	G					
Bad	103-128	A	A	Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	V	D	S	F	K	K	G					
Bak	70-92	T	M	G	Q	V	G	R	Q	L	A	I	I	G	D	D	I	N	R	R	Y	D	S	E					
Bax	55-77	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	N	M	E	L	Q					
Bok	62-84	R	L	A	E	V	C	A	V	L	L	R	L	G	D	E	L	E	M	I	R	P	S	V					

The amino acids in shaded boxes are conserved across the different BH3 domains. H1, H2, H3, and H4 denote the hydrophobic residues at the *i*, *i* + 4, *i* + 7, and *i* + 11 positions.

Figure 1. (A–C) Normalized binding of the Bim (86-103) and mutant peptides to Mcl-1 and Bcl-x_L plotted on an inverse and logarithmic scale. Normalization consists of division of the K_i of the mutant by the K_i of the wild-type Bim (86-103) sequence. Dashed line signifies the K_i of the wild-type sequence. Broken bar indicates binding below the detectable limit. “*” Denotes aggregation of peptide as evidenced in assay; “X” indicates mutation not made at this residue due to similarity to the wild-type residue; “+” equals $K_i(\text{mutant})/K_i(\text{Bim } 86-103) > 100$.

related residues (neither Arg93 nor Arg94 was replaced by lysine, nor was Asp97 replaced by glutamic acid). In all, 53 new peptides were synthesized and evaluated in the two competition FP assays. K_i values were calculated by a complete competitive binding model (Roehrl et al. 2004). Figure 1 presents the relative K_i , calculated by dividing K_i of the mutant peptide by K_i of wild-type Bim^{BH3} for the appropriate binding partner (Bcl-x_L or Mcl-1). The K_i values themselves are presented in the Supplemental material.

Mutations at canonical hydrophobic residues in Bim^{BH3} yield surprising results

The alanine-scanning data (Fig. 1A) contain unexpected features. Previous alanine-scanning results for binding of the Bak BH3 domain to Bcl-x_L highlighted the importance of the $i, i + 4, i + 7, i + 11$ hydrophobic side-chain array (i.e., H1 to H4 in Table 1) for the stability of the complex (Sattler et al. 1997). The high degree of conservation of hydrophobic residues at analogous positions in other BH3 domains has strengthened the view that these four positions are essential for proper interactions among Bcl-2 family proteins (Huang and Strasser 2000). In this context, the results of alanine mutations at the $i, i + 7$, and $i + 11$ positions in Bim^{BH3} (I88, I95, and

Figure 2. (A,B) Inhibition constant (K_i) of the Bim (86-103) (white bar) and mutant peptides to Mcl-1 and Bcl-x_L plotted on an inverse and logarithmic scale. Dashed line signifies the K_i of the wild-type sequence. Broken bar indicates binding below the detectable limit. “*” Denotes aggregation of peptide as evidenced in assay; “+” indicates $K_i \geq 10,000$ nM. Absence of symbol or bar indicates that the mutation was not made at this residue due to similarity to the wild-type residue.

F99, respectively) are surprising. Removing the $i + 4$ hydrophobic side chain (Leu92) causes a large drop in affinity for both Bcl-x_L and Mcl-1, as expected. The I88A mutation, however, causes only a very minor decrease (1.5-fold) in affinity for Bcl-x_L or Mcl-1. According to previous results, changing the analogous valine residue to alanine in the Bak BH3 domain resulted in an ~50-fold drop in binding to Bcl-x_L (Sattler et al. 1997). In Bim^{BH3}, I95A causes an ~200-fold decrease in binding to Mcl-1 but a slight improvement in binding to Bcl-x_L, and F99A has the opposite effect, a 90-fold decrease in binding to Bcl-x_L but a slight improvement in binding to Mcl-1. For the Bak BH3 domain, mutation of isoleucine at the $i + 7$ position to alanine caused an ~50-fold drop in affinity for Bcl-x_L, and mutation of isoleucine at the $i + 11$ position to alanine caused an ~300-fold drop (Sattler et al. 1997). The unexpected effect of the F99A mutation on binding to Mcl-1 may be partially rationalized by structural data, which show that the pocket accommodating the Phe side chain is more solvent exposed for Mcl-1 than for Bcl-x_L (Liu et al. 2003; Czabotar et al. 2007). Structures of small molecule ligands bound to Bcl-x_L provide insight on the high binding affinity of the I95A mutant for Bcl-x_L, as the protein can tolerate small hydrophobic groups in this pocket (Bruncko et al. 2007; Lee et al. 2007). The same small molecules do not bind to Mcl-1, so this protein apparently requires a large hydrophobic group to fill this pocket. The moderate affinity of the I88 mutations for both proteins is surprising, as the analogous residue resides in a hydrophobic pocket in the crystal structures of the 33-residue Bim peptide with Bcl-x_L and 26-residue Bim peptide with Mcl-1 (Liu et al. 2003; Czabotar et al. 2007).

The hydrophile-scanning results for canonical hydrophobic residues of Bim^{BH3} offer additional insights on the contributions of these side chains to affinity. Both I88K and I88E mutations in Bim^{BH3} lead to moderate decreases in affinity for Mcl-1 and Bcl-x_L, which is more consistent with expectations than are the I88A mutation effects. The crystal structures show that the I88 side chain is buried in the complex (Liu et al. 2003; Czabotar et al. 2007), but the hydrophilic mutations were required to confirm that this burial is energetically significant for Bim^{BH3} binding. Overall, the scans suggest that the binding interface surrounding I88 is flexible, perhaps more so than around other side chains such as L92. I88K is the only hydrophilic mutation that causes a large decrease in affinity, and only in the case of Bim^{BH3} binding to Mcl-1 (70-fold). This distinctive effect presumably reflects that fact that the Mcl-1 surface (but not the Bcl-x_L surface) is highly cationic in the region of the binding pocket that accommodates the side chain of I88, at least in a chimeric version of Mcl-1 (Czabotar et al. 2007). However, the change in side-chain shape of the I88K mutation may also account for the binding selectivity observed.

In contrast to the slight enhancement of Bim^{BH3} binding to Bcl-x_L seen for I95A mutation, I95K causes a fourfold decline in affinity and I95E causes a 70-fold decline. Without the hydrophile scan and structural data, one might conclude from the I95A results that the side chain of I95 is not buried upon binding to Bcl-x_L. The declines seen for the lysine and glutamic acid mutations at this position of Bim^{BH3} suggest that a hydrophilic side chain is not well tolerated. Significant electrostatic interactions with Mcl-1 are implied by the results of lysine and glutamic acid mutations at F99 of Bim^{BH3}. F99E, like F99A, slightly stabilizes the complex (the relative stabilization cannot be determined because of the detection limits of the FP assay), but F99K moderately destabilizes the complex. Structural data indicate significant positive charge in this region of chimeric Mcl-1 (Czabotar et al. 2007), which is consistent with our hydrophilic mutation effects. In order to probe whether the improvement in binding of F99E is due to sterics or electrostatics, the isosteric mutation to glutamic acid F99Q was tested. The binding affinity of the F99Q mutant is similar to that of the F99A mutant, but less than that of the F99E peptide, indicating that the negative electrostatic potential of the glutamic acid side chain contributes to the binding affinity with Mcl-1.

After our experiments were complete, Lee et al. (2007) reported mutagenesis results for the binding of a Bim^{BH3} peptide to Bcl-x_L and Mcl-1. All three components were somewhat different from the versions we used (e.g., we used human Mcl-1 [171-327] while Lee et al. [2007] used a human-mouse chimeric sequence). These investigators mutated the highly conserved Leu at $i + 4$ to 17 other residues (all proteinogenic possibilities except Cys and Pro), and they mutated the Phe at $i + 11$ to 17 other residues. Their findings can be compared with our observations for mutations at Leu92 and Phe99, which correspond to Leu62 and Phe69 in the numbering of Lee et al. (2007). In most cases that allow direct comparison, the results of these two mutagenesis studies agree. For example, both studies find that mutating Leu at $i + 4$ to lysine or glutamic acid is highly deleterious to binding of a Bim^{BH3} peptide to either Bcl-x_L or Mcl-1. However, Lee et al. (2007) find intriguingly that Leu → Ala at this position has little or no effect on peptide affinity to Mcl-1, in contrast to our observation that L92A causes a drastic drop in binding to Mcl-1 as well as Bcl-x_L. For mutations at $i + 11$, the two studies indicate similar effects on affinity for Mcl-1, but for Phe → Glu at this position, Lee et al. (2007) found significant, albeit reduced, affinity for Bcl-x_L, in contrast to the lack of binding we observed. These differences may result from the variations in Bim^{BH3} peptide length between the two studies and are being explored in ongoing experiments.

Mutations at other sites in Bim^{BH3}: Electrostatic sources of selectivity

Hydrophile scanning can provide new insight, relative to alanine scanning, regarding the electrostatic environment experienced by specific side chains upon complex formation. This feature is illustrated by the effects of mutations at R93 and R94 of Bim^{BH3}. The R93A mutation has no effect on binding to Mcl-1 and only a very small effect on binding to Bcl-x_L. However, R93E causes a 70-fold or more reduction in binding affinity to both proteins. A similar pattern of behavior is observed for the R94A and R94E mutations. The results of the alanine mutations might lead one to conclude that the identity of residues 93 and 94 is unimportant for binding to Mcl-1 or Bcl-x_L, but the results of the glutamic acid mutations suggest that the side chains in these solvent-exposed positions experience a negative electrostatic potential upon binding. When these residues are mutated to the glutamic acid isostere glutamine, the binding affinity is nearly identical to that of the alanine mutation (for Mcl-1 R93Q, $K_i = 7 \pm 2$ nM; R94Q, $K_i = 9 \pm 3$ nM; and for Bcl-x_L R93Q, $K_i = 340 \pm 100$ nM; R94Q, $K_i = 500 \pm 200$ nM). Therefore, the impact of the glutamic acid mutation seems to be due to changes in electrostatic potential, not sterics. The crystal structures of Mcl-1 and Bcl-x_L bound to Bim^{BH3} indicate that R93 and R94 are in close proximity to negatively charged amino acids on the proteins (Liu et al. 2003; Czabotar et al. 2007). Extensive analysis of electrostatic effects on pairing selectivity among leucine zipper sequences has shown that unfavorable electrostatic interactions can destabilize coiled-coil formation even though complementary and presumably favorable electrostatic interactions do not contribute to coiled-coil stability (Lumb and Kim 1995; Krylov et al. 1998; Marti and Bosshard 2004). We propose a similar mechanism for side-chains at positions 93 and 94 of Bim^{BH3}, i.e., mutations to glutamic acid lead to repulsion, but arginines at these positions are not stabilizing relative to a neutral residue (alanine).

The hydrophile-scanning results at Y103 of Bim^{BH3}, which is not among the four canonical nonpolar positions, suggest a way to encode a binding preference for Bcl-x_L relative to Mcl-1. The Tyr side chain packs into a shallow pocket in complexes formed with both anti-apoptotic proteins (Liu et al. 2003; Czabotar et al. 2007). Y103A mutation has only a small destabilizing effect on binding to Mcl-1 (threefold) and no significant effect on binding to Bcl-x_L. Y103E is more significantly destabilizing (fivefold or more) for both partner proteins than is Y103A, which suggests a significant role for nonpolar surface area burial at this point of contact. The most interesting effects, however, are observed for Y103K. This mutation causes a threefold *decrease* in affinity for Mcl-1

but a sevenfold *increase* in affinity for Bcl-x_L. Nle was chosen as an uncharged isosteric control for the Y103K mutation. The Y103Nle mutant has similar binding affinity to the Y103K mutant for both proteins. Thus, the impact of the Y103K on the binding affinity appears to be due to the shape of the residue, not the electrostatic potential.

Maximizing selectivity for Mcl-1 or Bcl-x_L

The conclusions outlined above regarding the binding of Bim^{BH3} mutant peptides to Mcl-1 or Bcl-x_L are derived from competition FP assays, in which each Bim^{BH3} mutant is asked to displace a fluorescently labeled tracer peptide from the BH3-recognition cleft of the anti-apoptotic protein. We further examined the affinities of Bim^{BH3} and selected mutants via direct-binding FP assays, in which the peptide of interest bears a fluorophore. The dissociation constant measured in this way, K_d , is not necessarily directly equivalent to K_i measured in the competition assay, because contacts between the fluorophore and the protein partner (Mcl-1 or Bcl-x_L) can influence K_d . Nevertheless, we observe comparable trends in the effect of Bim^{BH3} mutation on K_i and K_d for selected peptides (Table 2), which provides strong support for our conclusions.

These comparative studies focused on mutations that conferred selectivity for Mcl-1 or Bcl-x_L. Thus, Bim^{BH3} mutants I86E, Q90E, and F99E were examined because all provide enhanced binding to Mcl-1 relative to wild-type Bim^{BH3}. However, these three mutations varied in their effect on affinity for Bcl-x_L, with Q90E having little effect, I86E causing a modest decrease in binding, and F99E causing a large decrease in binding to Bcl-x_L. We prepared the I86E/Q90E/F99E triple mutant of Bim^{BH3} (peptide 1) to determine whether this peptide would be especially selective for Mcl-1 relative to Bcl-x_L. The I95A mutant and the I95A/Y103K double mutant of Bim^{BH3} (peptide 2) were examined as potentially selective ligands for Bcl-x_L.

The results in Table 2 indicate that making just two or three mutations to the Bim^{BH3} sequence can generate considerable selectivity for Bcl-x_L relative to Mcl-1 or vice versa. Thus, the I86E/Q90E/F99E triple mutant is highly selective for Mcl-1 (as is the F99E mutant), while the I95A/Y103K double mutant is highly selective for Bcl-x_L. These findings raise the intriguing possibility that hydrophile scanning (perhaps in concert with alanine scanning) may provide a generally effective strategy for engineering selectivity into mutant peptides or proteins, starting from sequences that naturally bind to more than one partner.

Helical propensities

Previous studies have suggested that the affinity of a BH3 domain peptide for anti-apoptotic members of the Bcl-2

Table 2. K_i and K_d values for binding of Bim^{BH3} and selected mutants to Mcl-1 and Bcl-x_L

Mcl-1				Bcl-x _L	
K_d (nM)	K_i (nM)			K_d (nM)	K_i (nM)
<2	3.9 ± 1.3	Bim (86-103)	IWIAQELRRIGDEFNAYY-NH ₂ ^a	2.0 ± 0.5	110 ± 40
3 ± 2	<1	I86E	EWIAQELRRIGDEFNAYY-NH ₂ ^a	59 ± 11	250 ± 40
2 ± 1	<1	Q90E	IWIAEELRRIGDEFNAYY-NH ₂ ^a	23 ± 2	71 ± 12
10 ± 4	<1	F99E	IWIAQELRRIGDEENAYY-NH ₂ ^a	>5000	>5000
6 ± 2	2 ± 2	F99Q	IWIAQELRRIGDEQNAYY-NH ₂ ^a	>5000	>5000
<2	1.6 ± 0.5	1	EWIAEELRRIGDEENAYY-NH ₂ ^b	>5000	>5000
76 ± 20	920 ± 30	I95A	IWIAQELRRAGDEFNAYY-NH ₂ ^a	17 ± 2	30 ± 5
<2	18 ± 2	Y103K	IWIAQELRRIGDEFNAYK-NH ₂ ^a	16 ± 4	14 ± 2
<2	23 ± 5.7	Y103Nle	IWIAQELRRIGDEFNAYNle-NH ₂ ^b	2.0 ± 0.2	28 ± 12
340 ± 80	1900 ± 420	2	IWIAQELRRAGDEFNAYK-NH ₂ ^c	3.3 ± 0.6	3.8 ± 1.1

The entries in bold indicate mutant peptides with significant specificity for Bcl-x_L or Mcl-1.

^aPeptide was labeled with 6-carboxyfluorescein on its N terminus.

^bPeptide was labeled with Bodipy TMR on its N terminus.

^cPeptide was labeled with 6-carboxyfluorescein and a short linker on its N terminus.

protein family is strongly influenced by the propensity of the peptide to adopt the α -helical conformation required for binding (Petros et al. 2000). We used circular dichroism (CD) to assess the α -helical propensity of Bim 86-103 (Bim^{BH3}) and mutants **1** and **2**. The CD data in Figure 3 indicate that the Bim^{BH3} peptide is not very α -helical in aqueous buffer, with weak minima near 208 and 222 nm. Nevertheless, Bim^{BH3} displays tight binding to Mcl-1 and moderate binding to Bcl-x_L. The CD data for Bim^{BH3} mutants **1** and **2** show that neither peptide displays significant α -helicity in isolation. Nevertheless, **1** binds tightly to Mcl-1, and **2** binds tightly to Bcl-x_L. These results indicate that a strong α -helical propensity is not required for tight binding to either anti-apoptotic protein (Shangary et al. 2004; Lee et al. 2007).

Conclusions

The results reported here suggest that hydrophile scanning can provide insights regarding affinity and selectivity in protein–protein interactions. For example, our findings indicate that hydrophile scanning can highlight electrostatic repulsions that contribute to natural selectivity or can be used to create artificial selectivity. This technique can be broadly applied since the scan can be accomplished via standard parallel peptide synthesis or mutagenesis (for larger proteins).

Starting from the natural Bim BH3 domain sequence, which binds to both Bcl-x_L and Mcl-1, we have used the combined information from alanine and hydrophile scanning to identify 18-residue peptides that bind tightly and selectively to either Bcl-x_L or Mcl-1. Peptides with selective affinities for particular anti-apoptotic members of the Bcl-2 family could represent useful tools for resolving the ongoing debate regarding the nature of signaling

interactions within this family (Galonek and Hardwick 2006; Willis et al. 2007). In addition, the unexpected discovery that alanine, lysine, and/or glutamic acid can be tolerated at some of the four positions that have been regarded as obligatory hydrophobic sites within a BH3 domain suggests that some natural BH3-domains remain to be discovered, because current sequence-based definitions of BH3 sequences may be too restrictive.

Materials and Methods

Protein expression

Human Mcl-1 (residues 171-327) and Bcl-x_L (residues 1-196) were expressed in *Escherichia coli* with a modified pET vector encoding His tags. After growth and induction, the cells were harvested, lysed, and centrifuged. The supernatant was applied

Figure 3. Circular dichroism of Bim^{BH3} and mutant peptides in 10 mM sodium phosphate buffer (pH 7.0). Bim (86-103) is shown in white squares, peptide **1** is shown in gray triangles, and peptide **2** is shown in crosses.

to Ni-NTA resin, washed with 50 mM Tris pH 8.0, 500 mM NaCl, and the protein was eluted with 50 mM Tris pH 8.0, 200 mM NaCl, and 200 mM imidazole. The proteins were further purified via a FF Q column with a gradient of 25 mM Tris pH 8.0, 1 mM DTT with 25 mM Tris pH 8.0, 1 mM DTT, 1 M NaCl and dialyzed into PBS with 2 mM EDTA and 5 mM DTT.

Peptide synthesis

Standard Fmoc solid phase parallel synthesis was used to generate the peptides on Novasyn TGR resin in a fritted 96-well plate (Arctic White). After global cleavage and deprotection, the excess TFA was removed and the peptides were dissolved and injected onto preparative C18 HPLC for purification. The identity and purity of the peptides was confirmed by MALDI-TOF-MS and analytical C18 HPLC, respectively. After lyophilization, the peptides were dissolved, and the concentration of peptide was determined by UV spectroscopy (Gill and Von Hippel 1989).

Competitive FP

The peptides were diluted serially fivefold in 384-well plates. The peptide stock solution (2 μ L) was added to 48 μ L of the mixture of protein, tracer, and buffer in a separate 384-well plate for assay. The assay buffer consisted of 20 mM phosphate, pH 7.4, 1 mM EDTA, 50 mM NaCl, 0.2 mM NaN_3 , 0.5 mg/mL (0.05%), and Pluronic F-68 (Sigma-Aldrich). The Bcl-x_L assay mixture consisted of 30 nM Bcl-x_L and 33 nM Bcl-x_L tracer peptide (Bodipy-TMR-X-GQVGRQLAIGDDINR-NH₂) in assay buffer. The Mcl-1 assay mixture consisted of 10 nM Mcl-1 and 10 nM Mcl-1 tracer peptide (6-carboxyfluorescein-WIAQLRRIGDEFNA) in assay buffer.

Fluorescent labeling of peptides

The fluorescein labeled peptides (Bim, I86E, Q90E, F99E, F99Q, I95A, Y013K, Y103Nle, and Mcl-1 tracer peptide) for direct binding FP were generated via incubation of the Fmoc deprotected peptide with three equivalents 6-carboxyfluorescein and diisopropylcarbodiimide overnight followed by washing with 20% piperidine in dimethylformamide. The peptides were subsequently deprotected, cleaved, and purified. The double-mutant peptide **2** was labeled on resin with a short linker ({2-[2-(Fmoc-amino)-ethoxy]-ethoxy}-acetic acid; Bachem) followed by 5-carboxyfluorescein. We observed that binding to both Mcl-1 and Bcl-x_L was diminished for a fluorescein-bearing derivative of **2** lacking the linker. Bcl-x_L tracer peptide for competition studies and peptide **1** were labeled with Bodipy TMR-X-SE (Invitrogen) in dimethylformamide and diisopropylethylamine after the peptides had been deprotected, cleaved from the resin, and purified.

Direct binding analyzed via FP

Direct binding experiments were conducted with twofold serial dilutions of protein in assay buffer (40 μ L) with 10 μ L of 10 nM or 100 nM fluorescently labeled peptide. When the K_d of the peptide was expected to be <20 nM based on the K_i data, a final concentration of 2 nM of labeled peptide was chosen. Otherwise a final concentration of 20 nM of peptide was used.

Circular dichroism

CD measurements were performed on an AVIV 202SF CD spectrophotometer. Samples were prepared in 10 mM sodium phosphate buffer pH 7.0 with peptide concentrations near 50 μ M as determined by UV spectroscopy (Gill and Von Hippel 1989). Spectra were recorded in a 0.1-cm path length cell used with a step size of 1nm and averaging time of 5 sec. Signals were truncated to dynode voltages of <450 V.

Electronic supplemental material

The K_i of the Bim^{BH3} peptides and mutants for Mcl-1 and Bcl-x_L is shown in Supplemental Table 1. The complete competitive binding calculation, used to determine K_i , and the normalization and error propagation, are present in the Supplemental material. Supplemental Figure 1 is the homology alignment of the proteins and peptides in the crystal structures with those used in our study.

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