Novel Bcl-2 Homology-3 Domain-like Sequences Identified from Screening Randomized Peptide Libraries for Inhibitors of the Pro-survival Bcl-2 Proteins

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Interactions between Bcl-2 homology-3 (BH3)-only proteins and their pro-survival Bcl-2 family binding partners initiate the intrinsic apoptosis pathway. These interactions are mediated by a short helical motif, the BH3 domain, on the BH3-only protein, which inserts into a hydrophobic groove on the pro-survival molecule. To identify novel peptidic ligands that bind Mcl-1, a pro-survival protein relative of Bcl-2, both human and mouse Mcl-1 were screened against large randomized phage-displayed peptide libraries. We identified a number of 16-mer peptides with sub-micromolar affinity that were highly selective for Mcl-1, as well as being somewhat selective for the species of Mcl-1 (human or mouse) against which the library was panned. Interestingly, these sequences all strongly resembled natural BH3 domain sequences. By switching residues within the best of the human Mcl-1-binding sequences, or extending beyond the core sequence identified, we were able to alter the pro-survival protein interaction profile of this peptide such that it now bound all members tightly and was a potent killer when introduced into cells. Introduction of an amide lock constraint within this sequence also increased its helicity and binding to pro-survival proteins. These data provide new insights into the determinants of BH3 domain:pro-survival protein affinity and selectivity.

Cellular response to insult is critically dependent on the Bcl-2 homology-3 (BH3)**-only members of the Bcl-2 family of proteins (1). This sub-family harbors the BH3 domain, a short region of sequence necessary for cell killing. Although many BH3-only proteins are intrinsically unstructured (2), their BH3 domains form amphipathic α-helices that bind tightly to hydrophobic grooves present on pro-survival molecules such as Bcl-2 (3–7). Binding to the pro-survival proteins by the BH3-only proteins is a key step in triggering apoptosis, because this inactivates the pro-survival proteins, allowing the essential cell death mediators, Bax and Bak, to cause mitochondrial damage and thereby initiate cell death.

Although the BH3-only proteins share significant sequence similarities within their BH3 domains, their overall sequences are largely divergent. Moreover, they are not comparable in their ability to bind the pro-survival Bcl-2 proteins (8–10). For example, Bim binds Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1 avidly, whereas Bad preferentially binds Bcl-2, Bcl-xL, and Bcl-w, and Noxa prefers Mcl-1 and Bfl-1 (8). In addition, certain BH3-only proteins, such as Bim, Bid, and perhaps Puma, can also activate the pro-apoptotic proteins Bax and Bak by direct binding, an interaction also mediated by their BH3 domains (10–12).

Small molecules that mimic the BH3-only proteins (BH3 mimetics) have shown enormous potential as anti-cancer therapeutics (13). One such molecule, ABT-737, mimics the pro-survival protein-binding profile of Bad. Because targeting a wider range of pro-survival proteins is required to activate apoptosis in some cell types (14), small molecules that mimic the binding profile of Bim or that can target Mcl-1, which is the key resistance factor for ABT-737 (15), are desirable.

It is envisaged that the identification of pro-survival protein-binding peptides will provide useful insights into the requirements for binding. To further understand the molecular interactions between peptidic ligands and pro-survival protein targets, we screened diverse phage-displayed peptide libraries for novel sequences that bind to pro-survival proteins. We wondered whether peptides that do not bear a canonical BH3 domain could also bind and function like the BH3-only proteins, and whether novel means for antagonizing pro-survival protein function could be identified. In addition to providing useful insights into the requirements for binding, we suspected our studies could provide hints about the evolutionary origin of the BH3-only proteins and also generate ligands that will enable dissection of pro-survival protein biology and proof-of-principle studies for the use of BH3 mimetics.
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Herein, we report on the outcomes of our screen to identify novel peptides that bind Mcl-1. A significant observation was that the best binders that emerged all bore a striking resemblance to the canonical BH3 motif. Moreover, further investigation of these peptides provided insights into how differences in the core BH3 sequence combined with changes in flanking sequences control selectivity and bioactivity.

EXPERIMENTAL PROCEDURES

Cell Lines—The origin of all MEF cell lines has been described previously (14, 16).

Recombinant Proteins—N- and/or C-terminal truncation mutants of Bcl-xL, mMcl-1, hMcl-1, Bcl-2, and Bcl-w, were expressed in Escherichia coli and purified as previously described (3, 8, 16). hMcl-1 is identical to the human sequence with the exception of nine N-terminal amino acids. Importantly, the BH3-binding groove remains of human origin. This construct improves expression and solubility and is hereafter referred to as hMcl-1 (3).

Phage Panning and Analysis—N-terminal gene 8 major coat protein-displayed peptide libraries (>10^10 unique members) were constructed by previously described methods (17). “Hard randomization” of desired positions (denoted by X, supplemental Fig. 1a) was achieved by incorporation of NNK degenerate codons (where N = A/G/C/T and K = T/G), which encode all 20 natural amino acids. The target protein (mMcl-1 or hMcl-1) was immobilized on a Maxisorp immunoplate (Nunc) at 5 μg/ml at 4 °C overnight. Nonspecific binding to the microtiter wells was blocked by either 0.5% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20 in phosphate-buffered saline, or 0.5% (w/v) casein in phosphate-buffered saline. A solution of the phage library was then added, and the mixture was incubated for 2 h at room temperature with gentle shaking. The plates were washed eight times with phosphate-buffered saline, 0.05% (v/v) Tween 20, and the bound phage were eluted with 0.1 M HCl for 10 min and neutralized with 1 M Tris base. Eluted phage was propagated in E. coli XL-1 blue with M13K07 helper phage for further rounds of selection. Phage were analyzed after the third and sixth rounds: individual clones were grown in a 96-well format in 400 μl of 2YT broth (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, and 0.5% (w/v) NaCl) supplemented with carbenicillin and M13K07, and the culture supernatants were used directly in phage enzyme-linked immunosorbent assays to detect peptides that bound specifically to mMcl-1 or hMcl-1. The peptide sequences were determined from the sequences of the encoding DNA.

Peptide Synthesis—All regular peptides were synthesized on The Symphony® (Protein Technologies, Inc). Standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry was used for the syntheses, and each amino acid was double coupled using O-benzotriazol-N,N,N',N'-tetramethyluronium hexafluorophosphate, as the peptide-coupling reagent. The peptides were cleaved manually with 95% (v/v) trifluoroacetic acid, 2.5% trisopropylsilane (v/v), and 2.5% water for 2 h and purified by preparative high-performance liquid chromatography. A detailed protocol for the synthesis of the “locked” peptide is provided in the supplemental “Experimental Procedures.”

Shotgun Scanning Library Construction and Panning—The shotgun alanine scanning libraries were constructed by first modifying the previously described FLAG-tagged BimBH3 gene 3 construct (16) such that TGA stop codons were introduced in place of each codon in the sequence that was to be mutated. These “stop templates” were then used as templates for Kunkel mutagenesis reactions (18) with mutagenic oligonucleotides designed to replace the stop codons and introduce the appropriate degenerate codon at that site. The choice of degenerate codons for each residue was based on the shotgun alanine scanning series devised by Weiss et al. (19) (see supplemental Table 1 for details). Each library was subjected to three rounds of panning versus purified Bcl-xL or M2 anti-FLAG antibody (Sigma), then approximately 50 individual positive-binding clones were sequenced as described (20, 21). To calculate the functional ratio, the ratio of wild-type clones:mutant clones for each possible codon in the Bcl-xL selection was divided by the corresponding ratio from the anti-FLAG selection (19, 22). The relative affinity (IC_{50}) of selected clones was determined by phage competition enzyme-linked immunosorbent assay as described previously (16).

Biacore and Fluorescent Polarization Assays—Solution competition assays were performed using a Biacore 3000 instrument as described previously (8). Briefly, pro-survival proteins (5–10 nM) were incubated with varying concentrations of synthetic peptide for 2 h in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4) prior to injection onto a CM5 sensor chip on which either a wild-type BimBH3 peptide or an inert BimBH3 mutant peptide was immobilized. Specific binding of the pro-survival protein to the surface in the presence and absence of peptides was quantified by subtracting the signal from the Bim mutant channel from that obtained on the wild-type Bim channel. The ability of the peptides to prevent protein binding to immobilized BimBH3 was expressed as the IC_{50}, calculated by nonlinear curve-fitting of the data with KaleidaGraph (Synergy Software).

Direct binding assays were performed using a Biacore S1 biosensor as described previously (23) with 10 mM NaH_{2}PO_{4}, 40 mM Na_{2}HPO_{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 sensor chip using amine-coupling chemistry. Recombinant GST-tagged Bcl-xL or Mcl-1 (100 μg/ml) was then captured by their GST tag. Several concentrations of peptide around that peptide’s KD were then injected at a flow rate of 90 μl/min. 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 blank injections over the immobilized spot. For KD calculations, corrected response data were fitted using a 1:1 binding site model, including mass transport limitations.

Fluorescence polarization (FP) experiments were performed as previously described (24), using carboxyfluorescein-conjugated murine Bim BH3 (DLRPEIRIAQK(FAM)LRRIGDEFNE) as a probe.

Cytochrome c Release—Mouse embryonic fibroblasts (mcl-1^{-} and bcl-x^{-}) (~2 × 10^6) were permeabilized in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM MgCl_{2}, 1 mM EDTA, 1 mM
EGTA, 250 mM sucrose, 0.05% (w/v) digitonin (Calbiochem) supplemented with protease inhibitors (Roche Applied Science), for 10 min on ice. The mitochondria-containing crude lysates were incubated with 10 μM peptide at 30 °C for 1 h before pelleting. The supernatant was retained as the soluble fraction, while the pellet, which contains intact mitochondria, was solubilized in 1% (v/v) Triton X-100-containing lysis buffer (20 mM Tris, pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol) supplemented with protease inhibitors (Roche Applied Science). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The cytochrome c and Bax proteins were detected with the anti-cytochrome c (7H8.2C12, BD Pharmingen) and rabbit anti-Bak (Sigma) antibodies, respectively.

**CD Spectroscopy**—CD spectra were recorded at 20 °C using an AVIV 410-SF CD spectrometer. Wavelength scans were performed between 190 and 250 nm in aqueous buffer (5 mM HEPES, pH 7.2) ± 30% (v/v) trifluoroethanol with 0.15 mg/ml peptide in a 1-mm quartz cuvette. The α-helix content (25) was calculated from the molar ellipticity at 222 nm ([θ]$_{222}$ nm) according to Equation 1 as follows.

$$\% \alpha\text{-Helix} = -\frac{([\theta]_{222} + 2340)}{303} \quad (\text{Eq. 1})$$

**Cell Killing**—Retroviral expression constructs were made using the pMIG vector (MSCV-IRES-GFP; GFP sequence is that of enhanced GFP) as described previously (8, 26). These plasmids were transiently transfected, using Lipofectamine™ (Invitrogen), into Phoenix ecotropic packaging cells (27). Filtered virus-containing supernatants were used to infect SV40 large T-antigen-transformed mouse embryo fibroblasts by spin inoculation as described previously (14). For short term killing assays, cell viability was determined by flow cytometric analysis in propidium iodide staining profile across Bcl-2 family members, we measured its binding profile with recombinant proteins and Bax were detected with the anti-HA antibody (3F10, Roche Applied Science). In co-immunoprecipitation experiments where $^{35}$S-labeling was used for detection, FLAG-tagged pro-survival proteins were co-transfected with HA-tagged Bim and Bim variants into HEK293T cells. Cells were metabolically labeled with $^{35}$S-methionine/cysteine as described previously (28, 29), then lysed as above. Lysates were incubated with either anti-FLAG or anti-HA antibodies and precipitated with protein G-Sepharose. Bound proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, and then transferred to nitrocellulose. The $^{35}$S-labeled proteins were detected by fluorography using Amplify (Amersham Biosciences).

**RESULTS**

**Identification of a Novel Peptide Selective for Mouse Mcl-1**—To identify novel Mcl-1-binding peptides, we screened a panel of five phage-displayed linear libraries containing randomized peptides ranging from 8 to 16 residues in length, as well as a panel of 8 cyclic libraries in which pairs of cysteine residues were fixed at different positions within 14 residue sequences to give constrained peptide segments of between 3 and 10 residues (supplemental Fig. 1a). All peptides were expressed as polycysteine gene 8 fusions, and recombinant N- and C-terminally truncated mouse Mcl-1 (mMcl-1, 1mMcl-1, see “Experimental Procedures”) was used for these initial screens, because it is generally easier to express and purify than the recombinant human protein.

Our initial strategy involved panning two combined library pools of either linear or cyclic sequences. In this screen, selection was biased toward clones that bound multiple targets nonspecifically, because they contained a large proportion of hydrophobic residues. However, after six rounds of sorting with the linear X$_{14}$ library only, a number of clones that bound specifically emerged, of which $>60\%$ had the identical sequence (A1: SDEDILEAVDLLMSFS).

The A1 sequence was found to bind mMcl-1 with high enough affinity, both in a phage enzyme-linked immunosorbent assay and as a synthetic peptide in a fluorescence polarization assay ($K_i = 16 \mu M$, to warrant further investigation. In the same FP assay, this peptide appeared to be highly selective over Bcl-2 ($K_i \approx 10 \mu M$) and Bcl-xL ($K_i \approx 10 \mu M$).

To confirm the binding of A1 to mMcl-1, and to extend its binding profile across Bcl-2 family members, we measured its IC$_{50}$ in competition binding assays using surface plasmon resonance on a Biacore biosensor, as described previously (8). Here the IC$_{50}$ for mouse Mcl-1 was $\sim 110 \mu M$, but no binding to Bcl-xL, Bcl-2, or Bcl-w was detected at 10 μM, further suggesting that A1 was highly selective for mouse Mcl-1 (supplemental Fig. 1b). Interestingly, when we tested peptide A1 for binding to human Mcl-1, its affinity was $\sim 6$-fold weaker (supplemental Fig. 1b) suggesting that this sequence is relatively selective for mouse Mcl-1. Because selective inhibitors of human Mcl-1 are preferred for functional studies, we performed additional screening studies using the human Mcl-1 as the target protein instead.

**Identification of Novel Human Mcl-1-binding Peptides**—Two strategies were employed to identify sequences that bind human Mcl-1. In the first, a “soft randomization” of A1 was
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TABLE 1
Sequence alignment of peptides identified from the screen against hMcl-1 after six rounds of panning

| h1  | h2  | h3  | h4  | No. of clones |
|-----|-----|-----|-----|--------------|
| SYDDALLRRSDDL | TTTMVMGYSEFLAGDYLDQPFY | DLPSVFILLRNGDSSLG | MGAELVALREGLDMV | 28 (B1) |
| TCMYVYNSFLAVGDYVDFHY | DDLVWYDLVLYDVLYVQV | DLPSVFILLRNGDSSLG | LDYMFNLDDLPTQPF | 13 (B2) |
| DWKWEFEQSLRLVLL | WYSEFLAGDYVDFHY | RYDDALLRRSDDL | YDHWAIELRDVDDAL | 9 (B3) |
| DLRPIPQDNLQFLQMT | DDLVWYDLVLYDVLYVQV | RYDDALLRRSDDL | LALYLMDYFLYDDLPTQPF | 8 (B4) |
| RYDDALLRRSDDL | DDLVWYDLVLYDVLYVQV | YDHWAIELRDVDDAL | FHDHDSFL RVFDWFDNFL | 3 |
| MGAELVALREGLDMV | LDYMFNLDDLPTQPF | IYDDALLRRSDDL | MGADVLEAEGGQFG | 2 |
| WSDVSYEARNRUL | LDYMFNLDDLPTQPF | IYDDALLRRSDDL | WDYDDALMLSIDSDSL | 1 |
| TTYVMNRMIRIDDAML | LLIELRTIGDNDMGLG | PDDMIKERYIDYDYLE | TDYDMNRMIRIDDAML | 1 |
| WYDDALLRRSDDL | LDYMFNLDDLPTQPF | MYDDALLRRSDDL | YWDDALLMLQSIDDSL | 1 |
| MGAELVALREGLDMV | LDYMFNLDDLPTQPF | MGADVLEAEGGQFG | MGADVLEAEGGQFG | 1 |
| FHDHDSFL RVFDWFDNFL | MGADVLEAEGGQFG | FHDHDSFL RVFDWFDNFL | FHDHDSFL RVFDWFDNFL | 1 |

In a 16-amino acid library a given hexapeptide motif (in our case LRXGID) could be positioned in 11 ways to occupy positions 1–6, 2–7, 3–8, …, 11–16 along the sequence. Because any of the 20 amino acids could occur in the remaining 11 positions within the sequence, the chance of observing an LRXGID clone (P) is 11 × 20¹¹/20¹⁶ or ~1/300,000 (0.0003%). Because our library has been constructed by using degenerate oligonucleotides with NNK triplets (N = G, A, T, or C; K = G or T), the genetic code is reduced to 32 codons for all 20 amino acids. If codon degeneracy is taken into account (Leu-3, Arg-3, Ile-1, Gly-2, and Asp-1) P = 11 × (3 × 3 × 3 × 2 × 1) × 3²¹¹/3²¹⁶ or ~1/169,500 (0.0006%).

performed (30, 31). Here, each of 16 positions within the A1 sequence was randomized, with 50% to the native residue and the other 50% to any of the other amino acids. Sorting with this library isolated numerous binding sequences, but there were no preferred sequences, and no significant increase in affinity for human Mcl-1 was achieved.

The second strategy involved re-screening the linear 16-residue random peptide library from which A1 was identified against human Mcl-1. This approach yielded multiple peptide sequences (Table 1) of which we more closely examined the five preferred sequences, and no significant increase in affinity for the other 50% to any of the other amino acids. Sorting with this library isolated numerous binding sequences, but there were no preferred sequences, and no significant increase in affinity for human Mcl-1 was achieved.

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Because neutralization of Mcl-1 and Bcl-xL is required for activation of Bak and cytochrome c release in MEFs (14), we anticipated that our Mcl-1-selective peptides could release cytochrome c from mitochondria derived from bcl-xL−/− MEFs, but not wild-type MEFs. However, none of the phage-derived peptides caused cytochrome c release, although the BimBH3 control peptide was highly effective (Fig. 1C). A plausible explanation for this is the relatively weak affinity of B1–B5 for mouse Mcl-1 (Fig. 1A). Thus, to improve the biological properties of these peptides, we closely examined their sequences for any obvious changes that could improve binding affinity.

We decided to focus on peptide B1, because it had the highest affinity for human Mcl-1 and, unlike some of the others, was significantly more soluble. The B1 sequence differed from the canonical BH3 motif in that the residue at the h1 position is an alanine (instead of a large hydrophobic) while at position h2 + 1 there is a leucine, although nearly all naturally occurring proapoptotic BH3 domains have a small residue here (generally alanine or glycine) (Fig. 1B). Hence, we swapped the positions of these residues (to give us B6) and found that, in solution competition assays, this modification improved the affinity of the peptide for both mouse and human Mcl-1, while selectivity over Bcl-xL and Bcl-2, though not Bcl-w, was maintained (Fig. 2A). This result was mirrored in kinetic studies using a Biacore S51 biosensor where the B6 peptide showed an increase in affinity for human Mcl-1, resulting from a decreased dissociation rate as compared with the B1 sequence (Fig. 2B).
Unlike B1, B6 was then able to cause cytochrome c release from mitochondria derived from bcl-x<sup>-/-</sup> MEFs, but not from mcl-1<sup>-/-</sup> MEFs (Fig. 2C). In the same assay, Noxa (which selectively targets Mcl-1) promoted cytochrome c release from bcl-x<sup>-/-</sup> mitochondria but not ones from mcl-1<sup>-/-</sup> cells, whereas Bad (selectively targeting Bcl-xL, Bcl-2, and Bcl-w) had the complementary activity. These results confirm the selectivity of the B6 peptide for Mcl-1 over Bcl-xL and provide an insight into the threshold affinity required for biological activity, because B6 (IC<sub>50</sub> for mMcl-1 of 38 nM), but not B1 (IC<sub>50</sub>: 630 nM), was bioactive.

**Cell Killing Activity of Mcl-1 Binding Sequences** — The cytochrome c release assays provided an indication that the phage-derived sequences, in particular, the modified B6 motif, may induce cell killing by neutralizing Mcl-1. To test this hypothesis, we created a chimeric construct in which the BH3 domain of BimS was replaced with the B6 sequence (BimSB6). Similar BimBH3 chimeras have been previously examined with naturally occurring BH3 sequences and shown to adopt the prosurvival protein-binding and cell-killing activities of the BH3 domain's parent protein (8, 16). The placement of the B6 sequence within BimS was determined following alignment of the putative h2 leucine in the phage peptide sequence with the corresponding residue in BimS. This construct was introduced into MEFs by retroviral transduction, and its ability to suppress colony formation examined.
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(A) Table

| B1  | S Y D D A L L M L R S I G D S L | h1 | h2 | h3 | h4 | mMcl-1 | hMcl-1 | hBcl-xL | hBcl-2 | hBcl-w |
|-----|---------------------------------|----|----|----|----|--------|--------|--------|--------|--------|
| Bim | D M R S Y D D A L L M L R S I G D S L N A Y Y A R R | 15 (1) | 18 (5) | 2,000 (200) | 4,000 | 440 (7) |
| Bim/B6 | D M R S Y D D A L L M L R S I G D S L N A Y Y A R R | 11 (3) | 14 (9) | 16 (6) | 90 (45) | 15 (5) |
| Bad/B6 | N L W S Y D D A L L M L R S I G D S L V D S F K K G | ND | 13 (1) | 81 (1) | 1,200 (65) | 28 (1) |
| Noxa/B6 | P A E S Y D D A L L M L R S I G D S L N F R Q K L L | ND | 12 (1) | 160 (1) | 2,300 (460) | 29 (1) |
| Bad | N L W A A R Q R G R L R R M S D E F V D S F K K G | ND | > 1,000 | 14 (1) | 22 (1) | 75 (3) |
| Noxa | P A E L E V E C A T Q L R F G D K L N F R Q K L L | ND | 18 (1) | > 1,000 | > 1,000 | > 1,000 |

(B) Table

| B1  | mMcl-1  | hMcl-1  | hBcl-xL  |
|-----|----------|----------|----------|
|     | $k_a \times 10^7$ (1/s) | $k_d \times 10^7$ (1/Ms) | $K_c$ (nM) kinetic |
|     |          |          |          |
| B1  | 3.6      | 440      | 12       |
| B6  | 4.0      | 53       | 1.3      |
| Bim/B1 | 3.5      | 5.4      | 0.15     |
| Bim/B6 | 3.9      | 8.4      | 0.22     |
| Bim  | 3.2      | 8.1      | 0.25     |

(C) Diagram

WB: cytochrome c
- Pellet
- Soluble

WB: Bak
- Pellet
- Soluble

(D) Graph

No of colonies (% control)

- wild-type MEFs
- mcl-1−/− MEFs
- bcl-x−/− MEFs
Because of the selectivity of this sequence for Mcl-1 over the other pro-survival proteins, it was anticipated that it would only kill cells where Bcl-xL was absent. Indeed, as predicted based on the binding studies and cytochrome c release assays, expression of Bim/B6 caused significant suppression of colony formation in bcl-xL−/− cells (Fig. 2D). Unexpectedly, it also caused potent killing of both mcl-1−/− and wild-type MEFs (Fig. 2D). No killing was observed for bax−/−/bak−/− MEFs (supplemental Fig. 2) suggesting all the cell lines were dying specifically by an apoptotic mechanism. Therefore, in the context of BimS, B6 appeared to neutralize a wider subset of pro-survival proteins than suggested by the peptide binding studies, or alternatively, acts by directly activating Bak or Bax.

Residues Flanking the Core BH3 Sequence Influence Binding Affinity and Selectivity—To establish whether the addition of flanking residues derived from the native BimS to B6 could alter its binding affinity and/or pro-survival protein selectivity profile, a new B6 peptide was synthesized with additional flanking Bim residues on the N and C termini (Bim/B6) so that it was then 26 residues in length, identical to most of the BH3 peptide sequences we have used in previous studies (8, 16). In Biacore competition assays we then observed a significant increase in binding affinity for all pro-survival proteins with Bim/B6 (Fig. 2A). Similarly, binding of the parent B1 sequence was improved by addition of Bim flanking residues (Bim/B1). The increase in affinity of Bim/B6 for Mcl-1 and Bcl-xL was also observed in direct binding studies using the Biacore S51 biosensor (Fig. 2B) and reflected in co-immunoprecipitation studies using full-length proteins expressed in 293T cells (Fig. 3A). Moreover, the longer Bim/B6 peptide was then able to cause cytochrome c release from both mcl-1−/− and bcl-xL−/− cells (Fig. 2C), consistent with the observed significant increase in affinity for Bcl-xL following addition of BimS N- and C-terminal residues (Fig. 2, A and B). Bim/B1 then caused cytochrome c release from bcl-xL−/− but not mcl-1−/− cells, consistent with the binding data showing relatively weak affinity for Bcl-xL (Fig. 2, A and B). Hence it is likely that the killing activity of BimS/B6 we observed in a range of MEF cell types was due to this increased binding affinity for a larger range of pro-survival proteins compared with that seen with the shorter peptides. In aqueous solution the Bim-flanked sequences (Bim/B1 and Bim/B6) had a higher helical content compared with the shorter sequences (B1 and B6) as measured by CD (supplemental Fig. 3). This could account, at least in part, for their higher affinity/activity, although both the 16-mer and 26-mer peptides had approximately equal propensity to form helices as suggested by the CD spectra measured in the presence of the helix-stabilizing solvent trifluoroethanol (supplemental Fig. 3). Notably, Bim/B6 (or BimS/B1) did not directly interact with Bax in co-immunoprecipitation studies, unlike wild-type BimS (Fig. 3B), therefore the killing activity seen for BimS/B6 is unlikely due to a “direct activation” mechanism.

To examine which of the additional residues on the N- or C-terminal ends of Bim/B6 were most important for the gain in affinity observed for this peptide, several truncations of the 26-mer sequence were examined for binding to Bcl-xL, which was significantly affected by the addition of the flanking residues. Both N- and C-terminal additions affected binding (Fig. 3C), although the addition of C-terminal residues was most influential in determining binding affinity. Indeed, removal of the last four residues ((Bim(DC4)/B6)) from the 26-mer sequence led to a nearly 20-fold decrease in binding affinity while a similar truncation from the N-terminal end ((Bim(DN3)/B6)) decreased binding by 2.5-fold.

Finally, to gain some insight into whether our phage-derived peptides are binding in the hydrophobic groove on the pro-survival protein engaged by natural BH3 sequences, we examined the binding of peptides with point mutations at sites known to affect natural BH3 peptide binding (7, 16). Interestingly, we found that alanine substitution of the canonical hydrophobic residues or the conserved aspartate only weakly affected binding to Mcl-1 in a solution competition assay (Fig. 3C). By contrast, binding of Bcl-xL to the same mutants was significantly reduced in most cases, especially the alanine substitutions in the h1 (L8A), h2 (L12A), h4 (L19A), and conserved aspartate (D17A) positions. Therefore our data are consistent with phage-derived sequences engaging the hydrophobic groove on pro-survival proteins, although as we have seen previously with BimBH3 binding, Mcl-1 is more tolerant of side-chain truncations at the BH3 domain-defining sequence positions (16).

**BH3 Domain-flanking Residues Differentially Influence Binding to Pro-survival Proteins**—The addition of Bim-flanking residues on the B1 core sequence resulted in a significant increase in binding affinity for Bcl-xL and Bcl-2 compared with the B6 core sequence alone (Fig. 2A). Both Bcl-w and hMcl-1 bound the B6 sequence relatively tightly already, and smaller increases in binding were observed for these proteins with the Bim flank. Because Bim binds pro-survival proteins promiscuously, we wondered about the consequences of adding flanking residues to B6 from more selective BH3-only proteins such as Bad (which only binds Bcl-xL, Bcl-2, and Bcl-w) and Noxa (which only binds Mcl-1 and Bcl-2). Interestingly, both of these flanking sequences resulted in an increase in peptide helicity in aqueous solution compared with the parental B6 sequence (supplemental Fig. 3), and an increase in affinity for Bcl-xL, although the Bad flank was ~2-fold more effective (Fig. 2A). However, only a very minor improvement was observed for Bcl-2 binding.
A Covalent Constraint within Peptide B6 Increases Peptide Helicity and Binding Affinity for Mcl-1—Covalent constraints can be incorporated into helix-forming peptides to stabilize or “lock” them into a helical conformation. This can be accompanied by an increase in binding affinity, because the relative concentration of the binding-competent form is increased. A number of different constraints have been examined in the context of BH3 peptides (e.g. lactam bridges and hydrocarbon staples) (34, 35) with varying results. We therefore decided to insert an i to i+4 amide lock constraint (36) into peptide B6 (B6/lock), tethering residues 11 and 15 (see supplemental Fig. 4α), to determine whether this would also lead to increased activity.

CD analysis suggested a significant increase in helicity for B6/lock compared with the parent B6 sequence (supplemental Fig. 3), indicating that the covalent constraint functioned as anticipated. In solution competition assays, an increase in binding affinity was observed for Mcl-1 and Bcl-xL compared with the unlocked B6 sequence, whereas essentially no change in Bcl-2 binding was seen (supplemental Fig. 4b). Interestingly, binding to Bcl-w was reduced compared with B6 suggesting that the covalent constraint could be interfering with this interaction.

FIGURE 3. A, binding of BimS,B1 and BimS,B6 chimeras in co-immunoprecipitation experiments using full-length proteins. HEK293T cells were co-transfected with HA-tagged BimS, BimS,B1, or B6 chimeras and FLAG-tagged pro-survival proteins, and then lysates were immunoprecipitated with anti-FLAG affinity resin. Blots were reprobed with anti-FLAG as immunoprecipitation controls and whole cell lysates (WCL) probed with anti-HA and anti-FLAG as expression controls. B, unlike wild-type BimS, the BimS,B1 and B6 chimeras do not interact with Bax in co-immunoprecipitation reactions. C, truncation and alanine-scanning mutagenesis of Bim/B6 peptides. Substitution of most of the BH3-defining residues (shown in red) had a significant effect on Bcl-xL binding in solution competition assays, as did C-terminal (DC) but not N-terminal (DN) truncations. Values represent IC50 (in nanomolar, 1 S.D. in parentheses) from at least three independent assays.

CD analysis suggested a significant increase in helicity for B6/lock compared with the parent B6 sequence (supplemental Fig. 3), indicating that the covalent constraint functioned as anticipated. In solution competition assays, an increase in binding affinity was observed for Mcl-1 and Bcl-xL compared with the unlocked B6 sequence, whereas essentially no change in Bcl-2 binding was seen (supplemental Fig. 4b). Interestingly, binding to Bcl-w was reduced compared with B6 suggesting that the covalent constraint could be interfering with this interaction.

Shotgun Alanine-scanning Mutagenesis of Bim/BH3—Analysis of the peptides that emerged from the random peptide libraries suggested, perhaps not surprisingly, that there is strong selection pressure for sequences that possess hydrophobic residues at the canonical BH3 h1–h4 positions as well as the “GD” doublet present in most BH3 domains (Table 1). The characteristics of the intervening residues varied as occurs in

with both Bad and Noxa flanks (Fig. 2A). No significant effects on Mcl-1 or Bcl-w binding were seen with either addition. Hence, Bcl-xL binding to BH3 sequences appears to be most dramatically influenced by the nature of the residues flanking the core BH3 domain region.

A Covalent Constraint within Peptide B6 Increases Peptide Helicity and Binding Affinity for Mcl-1—Covalent constraints can be incorporated into helix-forming peptides to stabilize or “lock” them into a helical conformation. This can be accompanied by an increase in binding affinity, because the relative concentration of the binding-competent form is increased. A number of different constraints have been examined in the context of BH3 peptides (e.g. lactam bridges and hydrocarbon staples) (34, 35) with varying results. We therefore decided to insert an i to i+4 amide lock constraint (36) into peptide B6 (B6/lock), tethering residues 11 and 15 (see supplemental Fig. 4α), to determine whether this would also lead to increased activity.
The natural BH3 sequences. Hence it might be predicted that sequences that contain the conserved BH3 motif in a helical structure might be capable of engaging pro-survival proteins of the Bcl-2 family regardless of the composition of the intervening residues.

To investigate this hypothesis, we performed a shotgun ala nine scan on the BimBH3 sequence, which, unlike site-specific alanine scanning, allows the importance of a range of residues across a peptide sequence to be probed simultaneously (19, 22, 37). To achieve this we displayed a 26-residue sequence encompassing a FLAG-tagged BimBH3 domain on the surface of M13 phage particles as a fusion to the minor coat protein gene 3.

Two libraries were then created in which either residues 1–13 (Library 1) or 14–26 (Library 2) were all simultaneously mutated using oligonucleotides with degenerate codons possessing limited diversity such that each codon could only encode the wild-type residue at that position, alanine (x1), or depending on the codon employed, up to two other residues (x2 and x3; see supplemental Table 1) (19). These libraries were then panned against Bcl-xL as well as the anti-FLAG antibody to allow a “function ratio” to be calculated as described under “Experimental Procedures.” Function ratios significantly greater than or less than 1 indicate a deleterious or beneficial mutation, respectively (19, 22, 37).

An important outcome of this experiment was that it demonstrated that BH3 domains can be highly tolerant of side-chain truncations at most positions within the sequence, apart from the substitutions described above. Indeed, the sequences of a number of individual clones (e.g. C1–C6) that emerged from sorting contained a majority of non-native residues (i.e. up to 10 of 13 possible substitutions from each library), and retained high affinity binding for Bcl-xL (Fig. 4A).

**Functioning of Highly Mutated BH3 Sequences**—To determine whether any of the highly mutated BH3 sequences retained pro-apoptotic activity, clone C1 from Library 1 (with 10 of 13 substitutions) and clone C6 from Library 2 (with 8 of 13 substitutions) were chosen for further investigation. The corresponding segment of the BH3 domain in full-length BimS was replaced with each of these sequences (BimS,C1 and BimS,C6), Asp-17 (i.e. the absolutely conserved aspartate), and Phe-19 (h4) were most sensitive to mutation to alanine, as we previously reported. Indeed, no clones with mutations to any of these residues were selected (Table 2). Residues Ile-8 (h1) and Ile-15 (h3) were sensitive to substitution to a polar residue (threonine). Substitutions in positions in which the mutating codon could encode a proline (i.e. where there is a wild-type arginine or glutamine, residues 3, 10, 13, 14, 25, and 26) mostly showed strong bias against the proline being selected at that position. A rationale for this is that proline residues can be helix destabilizing; hence this result also provides validation that the clone enrichment/selection process has been effective.

**TABLE 2**

Shotgun-scanning mutagenesis

The ratio of wild type/mutant ratios (see supplemental Table 1 for identity of x1, x2, and x3) were determined from the sequences of clones isolated following panning against Bcl-xL or anti-FLAG antibody. These were then used to calculate the functional ratio (\(F_{wt/mut}\)). Fields with an X indicate that no clones were selected with that sequence (i.e. all clones contained the wild-type residue at that position), hence these residues are critical for binding to Bcl-xL.

| Peptide No. | Bcl-xL selection | anti-FLAG selection | \(F_{wt/mut}\) |
|-------------|------------------|---------------------|----------------|
|             | wt / x1         | wt / x2             | wt / x3        |
| Library 1   |                  |                     |                |
| D1          | 1.0              | 3.3                 | 0.3            |
| M2          | 1.8              | 0.9                 | 0.7            |
| R3          | 0.8              | 1.3                 | 1.6            |
| P4          | 0.5              | 0.6                 | 0.9            |
| E5          | 0.6              | 1.7                 | 0.4            |
| I6          | 2.8              | 1.8                 | 1.2            |
| W7          | 0.9              | 0.9                 | 0.7            |
| I8          | 2.2              | X                   | 1.2            |
| A9          | 0.2              | 0.4                 |                |
| Q10         | 0.4              | 0.3                 | X              |
| E11         | 0.9              | 2.3                 |                |
| L12         | X                | X                   | 0.6            |
| R13         | 2.0              | 1.6                 | X              |
| Library 2   |                  |                     |                |
| R14         | 2.1              | 5.8                 | X              |
| I15         | 0.4              | X                   | 5.5            |
| G16         | 1.0              | 1.1                 |                |
| D17         | X                | 1.1                 |                |
| E18         | 1.4              | 1.5                 |                |
| F19         | X                | X                   | X              |
| N20         | 1.3              | 2.8                 | 3.4            |
| A21         | 1.3              | 0.8                 |                |
| Y22         | 1.3              | 0.8                 | 0.8            |
| Y23         | 2.8              | 11.0                | 2.8            |
| A24         | 0.7              | 1.2                 |                |
| R25         | 1.8              | 1.3                 | 8.0            |
| R26         | 1.8              | 0.5                 | 1.3            |

The results (Table 2) were entirely consistent with a previous site-directed alanine scan we have performed against the identical BimBH3 sequence (16). In particular, residues Leu-12 (h2),
and a third Bim₈ construct in which the C₁ and C₆ sequences were combined to replace the entire BH₃ domain region was also made (Bim₈/C₁/C₆) (Fig. 4A).

In co-immunoprecipitation experiments, substitution of the first half of the BH₃ domain of Bim₈ with the C₁ sequence has little or no apparent effect on binding to Bcl-x₁, Bcl-2, or Mcl-1, whereas the construct with the substitution encompassing the second half of the BH₃ domain (Bim₈/C₆) only bound to Bcl-x₁ (Fig. 4B). Bim₈ with its entire BH₃ sequence substituted with the C₁/C₆ combination, also only bound to Bcl-x₁ (Fig. 4B).

To examine whether this binding activity would translate into killing activity, we performed killing assays in which the viability of MEFs was determined following retroviral transduction of the same Bim₈ constructs. For these experiments we used wild-type MEFs, MEFs expressing Bad to neutralize Bcl-x₁, Bcl-2, and Bcl-w, as well as MEFs expressing Noxa or Bim₈₂A to specifically neutralize Mcl-1 (8, 16). As predicted based on their binding profiles, only the Bim₈/C₁ construct was able to kill all cell lines (because it was capable of binding both Bcl-x₁ and Mcl-1), whereas the Bim₈/C₆ and Bim₈/C₁/C₆ constructs could only kill cells in which Mcl-1 was neutralized (Fig. 4C), which is consistent with the co-immunoprecipitation results showing these sequences to be Bcl-x₁-specific (Fig. 4B). Hence, these data demonstrate that sequences that possess only the key interacting side chains retain killing function.

**DISCUSSION**

The binding of the BH₃ domain of a BH₃-only protein into the hydrophobic groove on pro-survival proteins represents a key event in the initiation of the apoptotic cascade. However, the spectrum of pro-survival proteins engaged by the BH₃-only molecules is critical in determining whether cell death will occur (8, 14, 38, 39). BH₃ domain sequence selectivity was

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**FIGURE 4.** A, some peptides (C₁–C₆) identified from the shotgun scanning mutagenesis of Bim contain a high proportion of non-native residues, which retain high affinity for Bcl-x₁ in phage competition assays. Residues shaded gray are wild-type Bim residues.

B, co-immunoprecipitation experiments demonstrate that the highly mutated BH₃ sequence, C₁/C₆ is able to bind Bcl-x₁ but not Bcl-2 or Mcl-1, in the context of full-length Bim₈. C, the binding profiles of the Bim₈/C₁, Bim₈/C₆, and Bim₈/C₁/C₆ is reflected in their killing activities. Bim₈/C₁, which binds Bcl-x₁, Bcl-2, and Mcl-1, is a potent killer of MEFs while Bim₈/C₆ and Bim₈/C₁/C₆ only kill if Mcl-1 is neutralized by Noxa or a Mcl-1-selective ligand Bim₈₂A (16), consistent with their selectivity for Bcl-x₁.
only recently uncovered (8–10), and consequently we still have an incomplete understanding about how each BH3 sequence determines its binding profile. This is even true for those sequences for which we have structures in complex with different pro-survival proteins (3–7). Understanding the basis of this selectivity is important for the design and development of highly selective BH3-mimetic drugs, some of which are currently showing significant promise in both pre-clinical and early clinical studies (13, 40).

Most of the peptides we identified from the hMcl-1 screen possessed the h2 (Leu in natural pro-apoptotic sequences and all phage peptides except B3 where it was a phenylalanine), h3 (variably hydrophobic in natural BH3 domains, predominantly Ile in the phage peptides), h4 (variably hydrophobic in natural BH3 domains, predominantly Leu in the phage peptides), as well as the GD doublet found in most BH3 domains. The peptide identified from the mouse Mcl-1 screen (A1) bore only vague resemblance to canonical BH3 domains, although several features were preserved such as the spacing between a hydrophobic residue at h2 (an Ile in A1) and the conserved aspartate, as well as a hydrophobic at h4.

Interestingly the h1 and h1 + 1 residues of the tightest hMcl-1-binding peptide (B1) were reversed relative to their positions in natural BH3 ligands; most natural BH3 domains have a small (usually alanine or glycine) residue at the h1 + 1 position. We have previously shown that Mcl-1 is more tolerant of a large residue at h1 + 1 compared with other pro-survival proteins (16). Hence, one strategy by which Mcl-1 selectivity could be derived would be to increase the bulkiness on groove-binding ligands at a site that contacts the protein at a position similar to the h1 + 1 residue in BH3 ligands. This hypothesis is supported by our finding that reversal of h1 and h1 + 1 in B1 increased its affinity for all other pro-survival proteins tested (Bcl-xL, Bcl-2, and Bcl-w).

A further clue to the determinants of BH3 domain binding affinity/selectivity was uncovered upon variation of peptide length. Both N- and C-terminal extensions of the B6 sequence beyond the canonical BH3 region increased its affinity for all pro-survival proteins, although only Bcl-2 binding was influenced by the nature of these flanking regions. Most surprisingly, the addition of the Bad BH3 flank residues did not improve Bcl-2 binding as observed for the Bim flank, even though Bad BH3 (like Bim BH3) binds Bcl-2 with high affinity (8). Petros et al. (6) have previously demonstrated that longer BH3 peptides bind their targets with higher affinity, because they have higher helical propensity. Indeed all of our extended peptides showed higher (∼2- to 6-fold) helical content in aqueous solution compared with the shorter B6 sequence. Hence, BH3 domain binding to Bcl-2 is dependent on the nature of both the core BH3 sequence together with the flank region. It is unclear why Bcl-2 is somewhat different in this regard, although one implication of our results is that the Bcl-2 binding groove possesses idiosyncrasies that might allow it to be selectively targeted. Interestingly, the short (16-mer) B6 sequence bound both Mcl-1 and Bcl-w relatively tightly, but not Bcl-xL. Previous studies have shown that Bcl-xL and Bcl-w are very similar in the way they engage BH3 domains (8, 16). However, these data demonstrate that ligands selective for Bcl-w over Bcl-xL can be generated.

The introduction of the amide lock into the B6 peptide resulted in significantly increased helical content compared with the unlocked sequence (∼10-fold). Although this did not translate into such a dramatic improvement in affinity for Mcl-1 (∼2-fold), the IC50 we measured for the locked peptide is at the lower limit of the assay, hence greater improvements may have occurred than the results suggest. Interestingly, improved binding to Bcl-xL was also observed while the locked peptide bound Bcl-w more weakly. No affect on Bcl-2 binding was seen. Hence, our results again highlight differences in the way these proteins engage their ligands. Moreover, they provide evidence that covalent constraints are useful for improving the activity of short BH3 peptides.

The shotgun alanine scan of Bim was performed to simultaneously examine the contribution of multiple residues within a known peptide ligand for binding to Bcl-xL. These data highlighted the importance of just three key residues: the conserved leucine at h2, the conserved aspartate, and the phenylalanine at h4. The present study is entirely consistent with our previous Bim mutagenesis studies where individual mutation of these residues had significant effects on binding affinity (16). Moreover, we have now shown that replacement of a relatively long sequence (26 residues) encompassing the BH3 domain of full-length Bim with a sequence that possesses little chemical diversity in the side chains other than at the key positions described above, did not significantly impact on Bcl-xL binding in cells, but they did influence binding to Mcl-1 and Bcl-2. This further demonstrates that residues outside of the core BH3 motif can influence pro-survival protein binding affinity/selectivity (consistent with our phage peptide truncation studies) and, as a consequence, killing activity. Furthermore these data also indicate that the conservation of intervening residues between the canonical BH3 defining residues (for example the double-basic doublet following h2 seen in a number of natural BH3 domains) may be due to some yet-to-be-discovered interaction in which they are involved, but may also suggest that a negative (disruptive) interaction may be important for dictating selectivity and functioning.

Mcl-1 is an excellent therapeutic target, because it is overexpressed in a number of cancers as well as being a key resistance factor for ABT-737. Here we initially aimed to identify novel peptidic ligands for Mcl-1 that could be used as tools to study how Mcl-1-selective drugs might function and potentially serve as templates for the development of new small molecules that selectively target it. Although the majority of peptides that were isolated from our very large randomized peptide libraries resembled those of native BH3 domain ligands, the native BH3 consensus is found in only a vanishingly small fraction (10−4%) of the initial naïve library. This observation suggests the human Mcl-1 evolved to recognize this sequence, possibly from a common ancestor. In addition it appears the hydrophobic groove on Mcl-1 is the single major “hotspot,” at least for peptidic ligands. As described above, however, the peptide obtained from our screen against mouse Mcl-1 did not contain the characteristic BH3 sequence. Moreover, despite 94% sequence identity between the mouse and human proteins, the peptides isolated...
from the screens against either species of Mcl-1 protein showed >5-fold species selectivity, as determined by their binding affinity. This highlights how subtle changes in the “receptor” (i.e., pro-survival protein) can alter ligand affinity (and hence selectivity) and is consistent with our previous observation that very minor changes in BH3 ligands can have dramatic affects on their binding affinity (41). These data are useful to bear in mind for the drug development process, because small molecule ligands are likely to be similarly influenced. In summary, this study identified novel Mcl-1-selective ligands and provides new insights into factors influencing BH3 domain affinity and selectivity.

Acknowledgments—We thank Lisa Bernstein from Genentech for help with the statistical analysis; Mark van Delft, Priscilla Kelly, Philippe Bouillet, and Andreas Strasser for mcl-1−/− cells; and Marco Evangelista for technical assistance.

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