In Vitro Selection and Characterization of Bcl-X₇-binding Proteins from a Mix of Tissue-specific mRNA Display Libraries*

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The covalent coupling of an mRNA to the protein that it encodes (mRNA display) provides a powerful tool for analysis of protein function in the post-genomic era. This coupling allows the selective enrichment of individual members from libraries of displayed proteins and the subsequent regeneration of an enriched library using the RNA moiety. Tissue-specific libraries from poly(A)⁺ mRNA were prepared by priming first and second strand cDNA synthesis with oligonucleotides containing nine random 3′ nucleotides, the fixed regions of which encoded the requisite sequences for formation of mRNA display constructs and a library-specific sequence tag. Starting with a pool of uniquely tagged libraries from different tissues, an iterative selection was performed for binding partners of the anti-apoptotic protein Bcl-X₇. After four rounds of selection, the pool was deconvoluted by polymerase chain reaction amplification with library-specific primers. Subsequent clonal sequence analysis revealed the selection of three members of the Bcl-2 family known to bind to Bcl-X₇. In addition, several proteins not previously demonstrated to interact with Bcl-X₇ were identified. The relative binding affinities of individual selected peptides were determined, as was their susceptibility to competition with a BH3 domain peptide. Based on these data, a putative BH3 domain was identified in most peptides.

The Bcl-2 family of proteins plays an important role in modulating the cellular program of apoptosis (for reviews see Refs. 1 and 2). Normal cellular homeostasis appears dependent on a balance between the actions of anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-X₇, etc.) and those of pro-apoptotic members (Bax, Bak, Bad, etc.). These proteins all share significant homology to Bcl-2 in a number of regions designated BH1–3 with the antiapoptotic members also having a BH4 domain. An additional sub-family is made up of proteins whose only homology to Bcl-2 is that they have BH3 domains (Bad, Bik, Bid, Bim, etc.; reviewed in Ref. 3). These appear to fall exclusively into the pro-apoptotic class. At least part of this Bcl-2 family balancing act appears to be controlled by direct binding interactions between the family members. Both homom- and heterodimeric interactions between family members are mediated by the binding of the BH3 domain in a hydrophobic cleft formed by three helices in its corresponding partner (4, 5).

The Bcl-2 family proteins containing multiple BH domains may function in part through the regulation of mitochondrial membrane potential and a corresponding release of cytochrome c into the cytoplasm. Due to their structural homology with pore-forming bacterial toxins, it was postulated that these proteins could act directly by forming ion channels in mitochondria (6). Indeed, this was demonstrated in an in vitro lipid membrane system (7). More recently, it was demonstrated that Bcl-2 family proteins can act indirectly to affect membrane potential through interactions with the mitochondrial channel VDAC (8). Unlike the channels formed by Bcl-2 family members themselves, the VDAC is capable of directly passing cytochrome c out of the mitochondria.

However, this model still leaves open the issue of how protein interactions within the Bcl-2 family could regulate apoptosis. One possibility is that BH3 domain binding of a family member causes the competitive release of other interacting proteins. This is a particularly attractive model for the BH3-only class of molecules, as each may serve to monitor a specific cellular activity and respond to insult by stimulating apoptosis (3). Indeed, in addition to forming dimers with other members of the Bcl-2 family, both Bcl-2 and Bcl-X₇ have been shown to interact with a number of heterologous proteins. By discovering what proteins interact with Bcl-2 family members, insight may be gained into the cellular processes that are monitored by the apoptotic machinery. In addition, the identity of proteins released in response to these signals may be discovered.

The major tool used in the identification and characterization of dimerizing pairs within the Bcl-2 family has been the yeast two-hybrid assay (9, 10). Whereas two-hybrid has emerged as the leading technology for mapping protein-protein interactions, it is not without significant limitations that arise because the interaction takes place in the yeast nucleus (11). Display technologies provide a powerful alternative because the interaction between library and target occurs in vitro, allowing optimal binding conditions to be used for different targets (12). Additionally, large libraries are screened iteratively, thus allowing even very low copy number proteins to be identified. However, the use of phage display, the most widely practiced display technology, has been similarly hampered by the limitations of producing libraries in a living system.

We have chosen to investigate the binding interactions of the anti-apoptotic protein Bcl-X₇ by direct mRNA display (13), a technology that circumvents many of the difficulties associated with yeast two-hybrid and phage display. In mRNA display, a newly translated mRNA molecule is covalently coupled to its corresponding nascent protein chain through a puromycin moiety ligated to the 3′ end of the RNA. The subsequent synthesis of a cDNA copy stabilizes the RNA and provides a template for PCR amplification. Because mRNA display is a completely in vitro technique, many of the problems inherent in cloning and

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1 The abbreviations used are: VDAC, voltage-dependent anion channel; PCR, polymerase chain reaction; UTR, untranslated region; GST, glutathione S-transferase;
In Vitro Selection of Bcl-X<sub>L</sub>-binding Proteins

In order to increase the diversity of the pool of mRNA displayed proteins used in the selection, it was desirable to mix together libraries prepared from different tissue sources. In order not to lose the potentially valuable information of which starting library a particular isolated clone came from, a tag sequence was incorporated into the 5′-UTR during construction. Unique UTR sequences that are compatible with translation in rabbit reticulocyte lysates were identified by selection from a library of c-myc mRNAs after a partially randomized 5′-UTR. The c-myc cDNA was transcribed by RiboMAX (Promega) and resuspended in 1/10th volume 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 0.05% Triton X-100, 1.0% glycerol, and 8 mM DTT. The library was then adsorbed onto a microcentrifuge tube and washed 3 times with 1 ml of binding buffer containing no EDTA, and then eluted with 100 μl aliquots of 2 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.05% Triton X-100 and incubated with 200 μl of anti-FLAG antibody immobilized on agarose beads (Sigma) for 1 h at 4 °C. The binding reaction was then transferred to a column (Bio-Rad), washed 3 times with 1 ml of binding buffer containing no EDTA, and then eluted with 100-μl aliquots of 2 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.05% Triton X-100 and incubated with 200 μl of anti-FLAG antibody immobilized on agarose beads (Sigma) for 1 h at 4 °C. The binding reaction was then transferred to a column, and the beads were washed 3 times with 1 ml of TBK buffer, mRNA-display constructs were then eluted with 100-μl aliquots of TBK buffer containing 100 μg FLAG-M2 peptide, 0.5 mg/ml bovine serum albumin, and 0.1 mg/ml salmon sperm DNA. The yield of mRNA-protein fusion product was based on an estimated specific activity of methionine in the lysate (5 μmol total concentration) and determined by scintillation counting both the purified product and the eluate from the transcription reactions.

**Choice of UTR Sequence Tag**—In order to increase the diversity of the pool of mRNA displayed proteins used in the selection, it was desirable to mix together libraries prepared from different tissue sources. In order not to lose the potentially valuable information of which starting library a particular isolated clone came from, a tag sequence was incorporated into the 5′-UTR during construction. Unique UTR sequences that are compatible with translation in rabbit reticulocyte lysates were identified by selection from a library of c-myc mRNAs after a partially randomized 5′-UTR. The c-myc cDNA was transcribed by RiboMAX (Promega) and resuspended in 1/10th volume 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 0.05% Triton X-100, 1.0% glycerol, and 8 mM DTT. The library was then adsorbed onto a microcentrifuge tube and washed 3 times with 1 ml of binding buffer containing no EDTA, and then eluted with 100-μl aliquots of 2 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.05% Triton X-100 and incubated with 200 μl of anti-FLAG antibody immobilized on agarose beads (Sigma) for 1 h at 4 °C. The binding reaction was then transferred to a column, and the beads were washed 3 times with 1 ml of TBK buffer, mRNA-display constructs were then eluted with 100-μl aliquots of TBK buffer containing 100 μg FLAG-M2 peptide, 0.5 mg/ml bovine serum albumin, and 0.1 mg/ml salmon sperm DNA. The yield of mRNA-protein fusion product was based on an estimated specific activity of methionine in the lysate (5 μmol total concentration) and determined by scintillation counting both the purified product and the eluate from the transcription reactions.

**Target Protein Preparation**—Bcl-X<sub>L</sub> was PCR-amplified using the primers AGTAGGATTCTGTCGTTGACACGGCC and TAGCGCATGCTTATC-ACTTTATACTTTATCAATT, in the production of libraries from human kidney, liver, brain and marrow, and brain mRNAs, the tags CTCCTAAC, CTTTCTCT, CT- TACTTC, and ATTTCAAT were used, respectively. Unextended primer 9 (indicates PEG spacer 9; Pu indicates 3′-UTR, TAATACGACTCACTAT-GTGAC, encoding the T7 promoter (underlined) and 5′-UTR upstream of the ATG start codon, to give a library of 38 (6561) different mRNA molecules after in vitro transcription with T7 RNA polymerase. Fusion formation, reverse transcription, and immunoprecipitation with an anti-c-myc antibody were carried out as described (13) to separate mRNAs that had undergone translation from those that had not. The successfully translated and fused sequences were amplified by PCR using the 5′ primer TAA TAC GAC TCA TTA TAG GGA CAA TTA CTA TTT ACA ATT HHI HHH HHI CAAT GGT CGTCTTTGTAATC, encoding the FLAG-M2 epitope (underlined) and a region complementary to the photoligation linker (italics). In the production of libraries from human kidney, liver, brain and marrow, and brain mRNAs, the tags CTCCTAAC, CTTTCTCT, CTTCTATTATAGCGAATTCGACTCACTAT-GTGAC, encoding the T7 promoter (underlined) and 5′-UTR upstream of the ATG start codon, to give a library of 38 (6561) different mRNA molecules after in vitro transcription with T7 RNA polymerase. Fusion formation, reverse transcription, and immunoprecipitation with an anti-c-myc antibody were carried out as described (13) to separate mRNAs that had undergone translation from those that had not. The successfully translated and fused sequences were amplified by PCR using the 5′ primer TAA TAC GAC TCA TTA TAG GGA CAA TTA CTA TTT ACA ATT HHI HHH HHI CAAT GGT CGTCTTTGTAATC, encoding the FLAG-M2 epitope (underlined) and a region complementary to the photoligation linker (italics).
library was then regenerated by PCR using the remainder of the eluate.

Cloning and Sequencing—After selection, PCR products were cloned into the TOPO-TA vector (Invitrogen), and after isolation of individual colonies, the plasmids were purified (Qiagen) and sequenced.

In Vitro Peptide Synthesis—RNAs were prepared from PCR templates and purified as described above. After translation in rabbit reticulocyte lysate (Ambion) peptides were directly from the lysate by immunoprecipitation and peptide elution based on a C-terminal FLAG-M2 epitope (Sigma).

RESULTS

Model Binding Study—In order to demonstrate the feasibility of using mRNA display technology to identify proteins that bind to Bcl-X<sub>L</sub>, a model study was performed using known BH3 domains. The target protein used in this study was the human Bcl-X<sub>L</sub> protein produced as a GST fusion and immobilized on glutathione-Sepharose beads. The BH3 domains of three different Bcl-2 family proteins (Fig. 2) were prepared as mRNA display constructs along with control peptides derived from unrelated proteins Stat-1 and Raf-1. The constructs were radiolabeled by incorporation of [35S]Met during in vitro translation. These mRNA displayed peptides were then incubated with immobilized Bcl-X<sub>L</sub>, and the unbound material was removed by washing. The amount of peptide bound to the beads was determined by scintillation counting and graphed in Fig. 2 as the percent of input counts bound.

Binding was specific to the BH3 helices, with Bak bound most efficiently (40%) followed by Bax (6%); no binding was observed for the BH3 helix from Bcl-2 or either control. The ordering of Bax and Bak is in good agreement with published IC<sub>50</sub> values, which indicate that Bcl-X<sub>L</sub> has an affinity for the Bak BH3 domain that is ~5-fold higher than that for Bax (4). There are several possible explanations for the lack of binding observed for the BH3 domain of Bcl-2. Published interactions between Bcl-2 and Bcl-X<sub>L</sub> (15, 16) utilized full-length proteins, so perhaps the chosen BH3 domain peptide fails to form a helix by itself. Alternatively, previously observed interactions may be due to the recognition of the BH3 domain of Bcl-X<sub>L</sub> by Bcl-2 rather than the reverse. Finally, the affinity may be below that required to generate a signal in this assay.

Selection—By having established the binding to Bcl-X<sub>L</sub> of control peptides in the form of mRNA display constructs, a selection to identify binders from within the complex mixture of an mRNA display library was initiated (Fig. 1). Four libraries, individually prepared from the tissue-specific mRNAs of human kidney, liver, marrow, and brain, were pooled prior to the start of selection. Each library contained a unique 8-nucleotide tag within the 5'-UTR to allow specific amplification of an individual library, as well as to provide for the identification of the tissue of origin during sequence analysis of individual selected clones.

As a target for the selection, a GST fusion protein of Bcl-X<sub>L</sub> was immobilized on glutathione-Sepharose beads. The selection was initiated with a combined library of ~1.5 × 10<sup>11</sup> molecules. After incubation of the library with the target, unbound members of the library were washed away, and the bound material was eluted. An enriched library was then regenerated by PCR, transcription, ligation, translation, fusion, reverse transcription, and purification. This enriched library was then used for the subsequent round of selection.

After four rounds of selection, the enriched pool from the combined libraries bound the Bcl-X<sub>L</sub> target at about 40%, an extent similar to the Bak control construct (Fig. 2). In order to determine if the winning molecules came from one or multiple libraries, the enriched pool was deconvoluted by PCR with library-specific amplification primers. The pool derived from the brain library was omitted due to cross-reaction of the PCR amplification primers. A test of binding revealed that each tissue-specific pool bound to the target to an extent similar to the mixed pool. The bound material from each of the individual pools was then recovered by elution, PCR-amplified, and analyzed by cloning and sequencing.

Sequence Analysis—A total of 378 sequences were obtained as follows: 181 from the kidney, 85 from the liver, and 112 from the marrow library. Initial analysis of the sequences revealed a total of 71 distinct sequence clusters. Six of the clusters originated from all three libraries, 14 from two of the three libraries and the remaining 51 from only one library. The sequences were then subjected to both nBLAST and pBLAST searches to identify the proteins represented by each cluster. Thirty-seven of the clones were from known proteins, 22 from hypothetical or unknown proteins whose nucleic acid sequences were found in the data base, and 12 were unique sequences not yet present in the public data base at the time of the search. Twenty of the most frequently found proteins are given in Table I.

Included among the top 20 were four members of the Bcl-2 family. The most abundant sequence (~25% of the total) was that of Bim, which was originally identified as a partner of...
Bcl-2 in a protein interaction screen and subsequently shown to bind to Bcl-XL (17). Two other proteins, Bak and Bax, contain BH3 domains known to interact with Bcl-XL (4). A fourth member of the Bcl-2 family, BCL2L12, was also found in this screen. However, no data on the interaction of BCL2L12 with Bcl-XL have been reported.

Bcl-XL has recently been reported to bind directly to the mitochondrial voltage-dependent anion channel (VDAC1) (8). Two isoforms of this protein, VDAC2 and VDAC3, have also been reported (18, 19). Among the less frequently observed proteins was a single clone from the latter, VDAC3, which this screen identifies as a binder of Bcl-XL. None of the other proteins found has previously been reported to bind to Bcl-XL.

Further analysis of the known proteins was done to determine whether the selected sequence was from the coding region or UTR and if the reading frame matched that of the native protein. This analysis was used as a filter to eliminate false positives; proteins that failed at this step were not further characterized. Twenty-seven of the 35 clusters from known proteins were in frame and within the native open reading frames. Only 1 of 35, proline/glutamine-rich splicing factor, was from the incorrect reading frame. Two clusters had inserts in the reversed orientation relative to the parent mRNA and probably arose due either to incomplete removal of the first strand primer after cDNA synthesis or re-priming on the cDNA strand after first strand synthesis. An additional six clusters were derived from reportedly noncoding regions of the parent mRNA, either the 3'-UTR or introns.

Of the clusters with multiple clones, several included more than one unique sequence with distinct N and/or C termini. This variety reflects the random priming used to prepare the library. Alignment of these fragments against parental protein sequences allowed minimal functional regions required for binding to be delineated based on the overlap of individual functional regions.
family members (Fig. 3). For the Bcl-2 family members BimL and Bax, these overlaps clearly define the BH3 domains. In the case of Bax, this overlap domain, Lys58–Gln77, is only 12 amino acids longer than the BH3 core, Leu63–Leu70 (Fig. 3). Greater variety was observed at the C terminus than at the N terminus. This may be due to the different enzymes, reverse transcriptase, and Klenow fragment used for first and second strand synthesis, respectively. The N termini show a distinct pattern of starting immediately after methionine residues in the native protein (Fig. 3, A and C). This is apparently due to annealing of the primer-encoded ATG start codon (immediately adjacent to the nine random nucleotides of the second strand primer) to corresponding sequences in the cDNA.

It should be noted that the information obtained upon cloning and sequencing represents a snapshot of the status of the selection at a specific point. Additional rounds of selection may change the population distribution significantly. A rare sequence from the starting pool that binds tightly might be enriched only to the point of appearing once among the clones, whereas a poorer binding sequence that was abundant in the starting pool might still be found at high copy number. Also, sequencing more clones might lead to the identification of other proteins still present at low copy number.

Affinity and Specificity—Although the most prevalent binder was the known Bcl-XL-binding protein Bim, the number of clones represented in each sequence cluster may not be a good indicator of which clones are biologically relevant. Therefore, post-selection characterization was performed to determine the relevance of selected proteins. One inherent benefit of this in vitro system is the ability to manipulate the binding conditions to obtain additional information. By varying the concentration of the immobilized target protein Bcl-XL, the relative affinities of individual peptides were determined.

Each cluster of sequences was aligned, and the shortest sequence was generally chosen as representing the minimal binding domain for that particular cluster. It should be noted that this shortest fragment may represent only a partial binding sequence, and longer fragments might be tighter binders. The chosen clones were prepared as free peptides and used in the binding assay described below.

Purified protein from these individual clones was incubated with immobilized Bcl-XL for 1 h, and after washing, the amount bound was determined by scintillation counting (Fig. 4). To determine the affinity, the data were fitted to a binding curve using non-linear regression. In this assay, all of the clones except one showed binding that was clearly dependent on target concentration. However, only binding curves that gave a high correlation coefficient ($R$ value) were used to determine an affinity. Binding affinities ranged from $2 \text{ nM}$ to $10 \text{ mM}$, demonstrating the great range of affinities accessible by in vitro selection. The 20 clones with the highest affinity are given in Table II. A comparison of $K_d$ values to the frequency in the pool (Table I) showed a 65% overlap; of the 20 lowest $K_d$ values, 13 were found within the top 20 most abundant winners, indicating a correlation between $K_d$ and frequency. However, 5 of the tightest binders were observed only a single time, and conversely, 4 of the most abundant proteins had affinities lower than $2 \text{ mM}$. Among those was translocated promoter region which, despite appearing 23 times among the sequences, bound very poorly. This disparity between rankings by frequency and affinity emphasizes the importance of post-selection characterization. The final representation of any given protein within the selected pool may be determined by a number of factors as follows: its abundance within the initial mRNA population used to prepare the library; the sum of efficiencies at each step in the mRNA display process (PCR, transcription, translation, fusion, etc.); and its affinity to the target.

Because the target used in this selection was a GST fusion protein of Bcl-XL, the specificity of each selected protein was
tested by binding to immobilized GST. The vast majority of proteins gave background levels of binding (less than 2%) to GST (data not shown). Of the 8 proteins that bound to GST, 5 bound 8–10-fold higher to the Bcl-XL fusion protein and so were deemed specific. The 3 remaining proteins bound poorly to the Bcl-XL fusion protein relative to GST alone and so were deemed nonspecific. Whether these were binding to GST or to the glutathione-Sepharose beads was not determined.

Competition—The Bcl-2 family of proteins has been shown to form homo- and heterodimers through the binding of the BH3 domain of one protein in the corresponding binding pocket on its partner. Only three of the selected proteins (Bim, Bak, and Bax) were previously known to contain a BH3 domain. In order to determine if the other proteins bound to the BH3 domain of the Bcl-XL fusion protein, a competition assay was performed. The Bak BH3 domain peptide used as a positive control was prepared by chemical synthesis and used to compete with individual Bcl-XL binders. This peptide competed effectively with the representative selected peptide Talin at 20 μM or above (Fig. 5).

A competition assay was performed for many of the selected Bcl-XL-binding proteins using 20 μM competitor (100 μM where indicated) based on the titration shown in Fig. 5. Each peptide was incubated with immobilized Bcl-XL in the presence of competitor and the amount bound normalized to a comparable reaction without competitor (Table III).

Nearly all of the peptides were competed by the BH3 domain, indicating that they probably bind to the same site on Bcl-XL. However, a decrease in binding of the selected protein at one site, due to a change in conformation of the target upon binding the competitor at a different site, cannot be ruled out. Only three of the proteins tested were not competed at all by the BH3 domain, indicating that they may bind to a different site on Bcl-XL.

Alignment of Selected Proteins—Competition for binding with the Bak BH3 domain indicated that most of the peptides selected were binding in the same site. Therefore, each of the selected proteins (Bim, Bak, and Bax) were previously known to contain a BH3 domain. In order to determine if the other proteins bound to the BH3 domain binding site on Bcl-XL, a competition assay was performed. The Bcl-XL domain peptide used as a positive control was prepared by chemical synthesis and used to compete with individual Bcl-XL binders. This peptide competed effectively with the representative selected peptide Talin at 20 μM or above (Fig. 5).

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Alignment of Selected Proteins—Competition for binding with the Bak BH3 domain indicated that most of the peptides selected were binding in the same site. Therefore, each of the peptides was examined for the presence of a BH3 domain sequence. A tentative assignment could be made for most peptides, and those of the tightest binders (Table II) are shown aligned in Table III. The amino acid alignments do not include the initiator methionine or the C-terminal FLAG epitopes which were encoded for by the fixed regions of the library primers. It is possible that for some peptides the region recognized by Bcl-XL included these conserved sequences. Based on this alignment, one such protein might be the Toll-like receptor 3. Most of the peptides have the hallmark periodicity of hydro-
phobic amino acids indicative of an amphipathic α-helix. Additional homologies among the sequences are indicated by shading.

**DISCUSSION**

In four rounds of iterative selection from an mRNA display library, a pool of proteins that bound to the target protein Bcl-XL was isolated. The identity of these selected proteins was readily determined by the sequencing of individual clones and their comparison to the public data base. The more than 70 Bcl-XL-binding proteins identified included both known and novel proteins.

For this selection, four independently prepared libraries derived from human liver, kidney, bone marrow, and brain tissues were mixed prior to selection. The presence of a unique sequence tag in the 5'-UTR of each library allowed simultaneous selection while retaining the information of the tissue of origin within each molecule. Sequencing analysis revealed that ~28% of the selected proteins were represented in two or more tissues, whereas the remaining 72% came from a single tissue. The ability to mix libraries not only increased the size and diversity of the starting pool, but the identification of tissue of origin for each selected protein may provide additional information normally derived from an mRNA expression analysis. Whereas the selection of a protein from a given tissue-specific library clearly demonstrates its presence in that tissue, failure to select it from another library does not prove its absence. Indeed, a comparison of the tissue of origin data for five of the most frequent clones (Table I) to the tissue distribution reported in the Bodymap data base (20) showed no significant correlation (data not shown).

The identities of selected proteins were determined by a comparison to sequences obtained from the public data base. Each fragment of a known protein was aligned to the parental mRNA and/or gene sequence, and its reading frame and location within the message were determined. Fragments found in introns, the UTRs, or in the wrong reading frame were not considered further. This led to the elimination of 8 false positives out of 71 proteins, including the abundant proline/glutamine-rich splicing factor. The sequence data also provided a preliminary means of ranking the clones based on the frequency with which each appeared (Table I). Subsequent characterization of individual clones provided a means of evaluating this initial ranking and revealed both its power and limitations (see below).

**TABLE III**

**Competition with a known BH3 domain and corresponding domain alignment**

| Clone | Protein                  | Compe d binding | Selected sequences aligned to BH3 domains of Bim, Bak and Bad |
|-------|-------------------------|-----------------|-------------------------------------------------------------|
| B4    | Bim.                     | 0.64*           | PEINWILDANLVDHGFRATYRKK                                      |
| 785   | Neutrophil Cyto. factor  | 0.66            | QGRHMFGTQESTSDLKELHDNLQRMN                                 |
| V47   | Hypothalamic protein     | 0.36            | RMMHCGRSMNLMGCCVKSN                                         |
| C21   | Novel protein I          | 0.44*           | IACATKAVDWVCEKLEKHFYLKRSAS                                 |
| V18   | Novel protein A          | 0.27            | VYDYPDDVLWLEAVADL                              |
| X58   | Talin                    | 0.21            | GQSDCTOPEQUGHAKLAKAYSMKALLAYTKRGAQ                                      |
| V73   | Unknown protein          | 0.2             | AAAEMREGETEVCEDMLENMMLVFKWCCYVDTGPFTPHS                    |
| c32   | Bak                      | 0.36            | GQVGKLALIGDDERTY                                            |
| Y37   | Unknown protein          | ND              | AGLKNNMEQRHNLKLLARTERCPORLDMILHCSVMNLCAHP                  |
| Y75   | BCL2L12                   | ND              | YOREAILRLVALRHENEDVEHMRKLADPRLRLVRodaFAR                   |
| V06   | Gag SNAP receptor        | 0.12            | GTQDGRRFTPAKSNHQLARTKTVQEMAYY                              |
| C68   | HSPC305                  | 0.21            | AVOEFDYQRTGLGMDARRETIFSSEKAKYRALY                |
| US8   | Synaptin 4A              | 0.14            | ATRQALNHSRMEIQQLHEFSNKLHDFPLFL                      |
| V50   | Tumor protein            | 0.18            | MFSQTVGFIEAGLCELCEGEKNRPR                  |
| C49   | Bax                      | 0.66            | KELCNFRKGDSDLKMNLAQMAAUVQVZEF                  |
| U15   | Talin                    | 0.6             | TMWREDFCAACUFELAESNKS                          |
| W06   | Unknown protein          | 0.08            | LFSYLLYLANLFLQCS                        |
| V68   | Novel B                  | ND              | MFMOTDKNLTVFETWHELAEKDLGTDFEYPYMNVLHAPH          |
| T25   | Novel D                  | 0.26            | CREQAKFLYRGKACAGLGEPEFPRIVHSSTMRAJTH                  |

**FIG. 5. Competition with a known BH3 domain.** At a fixed concentration of immobilized Bcl-XL, the Bak BH3 domain (underlined) containing peptide MGQVG-RQLAIIGDDINRDYKDDDDKASA was added at the indicated concentration along with a trace amount of a selected Talin fragment. After binding for 1 h, the unbound material was removed and the bound protein quantitated. The bound protein was assayed by scintillation counting, normalized to that bound in the absence of competitor, and plotted versus competitor concentration.
Four members of the Bcl-2 family were among the most frequently observed clones (Table I), three of which have been reported previously to bind to Bcl-X<sub>L</sub> as follows: Bim, Bak, and Bax (10, 17). Each of these was represented by multiple fragments, all containing the BH3 domain responsible for heterodimer formation among Bcl-2 family members (Fig. 3). The fourth newly reported family member, BCL2L12, was not present in the data base during the initial search. It was therefore quite exciting to find that a protein initially categorized as novel is indeed a member of the Bcl-2 family. This result reinforces the possibility that the other proteins that currently fall into the novel category may also be validated as members of the Bcl-2 family.

The heterologous protein VDAC3 was found only a single time; however, it was later demonstrated to be a high affinity binder of Bcl-X<sub>L</sub> (Table II). The isolated fragment of VDAC3 (amino acids 85–123) competed for binding with the BH3 domain of Bak (Table III). An alignment with the three isoforms of VDAC reveals the selected fragment to be in a region spanning exon 4 through exon 6 (data not shown). Although variability is seen in this region, many conserved elements of the BH3-like motif identified in VDAC3 (Table III) can also be found in VDAC1 and VDAC2. The idea that VDAC3 is binding to Bcl-X<sub>L</sub> by donation of a BH3-like domain is consistent with the observation that the BH3-only proteins Bid and Bik do not bind VDAC1 (8).

Although previously identified binders of Bcl-X<sub>L</sub> may be considered true positives from this selection, additional filters based on readily assayable characteristics were used to eliminate potential false positives. The generation of a revised ranking of clones based on binding affinity was straightforward. Individual clones were prepared by <i>in vitro</i> translation using much the same methods as used for the mRNA displayed pool. Application of the same basic assay that was used for the selection, while varying the concentration of immobilized target, gave a relative <i>K<sub>d</sub></i> for each clone. Although the choice of a cut-off is somewhat arbitrary, the known BH3 domains all fell within the top 20 with affinities below 1 µM. For a case in which no prior knowledge of binding partners exists, an affinity consistent with the cellular concentrations of the interacting proteins has been proposed as a litmus test for biological significance (21).

The relative affinities of the known Bcl-2 family members may be of particular interest. Apoptosis assays using Bak and Bax have implicated them as significant effectors of Bcl-X<sub>L</sub>. The affinities determined in our binding assay correlate with the extents of binding of the control constructs (Fig. 2) and with published <i>I<sub>50</i></sub> values (4). However, the observation that the BH3 domain-containing fragment of Bim binds to Bcl-X<sub>L</sub> more than 100-fold more tightly than either Bak or Bax may imply that it is a particularly important counterpart. The affinity data provide a biochemical basis for the observation that, uniquely among Bcl-2 family members, stable overexpressing cell lines of Bim could not be made (17).

As discussed previously, the BH3 domain of Bcl-2 family proteins provides the means of heterodimerization. Therefore, new members of the Bcl-2 family identified in this selection could reasonably be assumed to interact with Bcl-X<sub>L</sub> in a similar manner. To determine the mode of binding, the BH3 domain of the known Bcl-X<sub>L</sub>-binding protein Bak was used in a competition assay. The results showed that the majority of selected proteins were interacting at the BH3 domain binding site of Bcl-X<sub>L</sub>. A corresponding region of homology to the known BH3 domains could be found in most proteins (Table III). For selection targets for which there is no known interactor, selected proteins could be tested in competition with each other to establish groups of proteins that compete for the same binding sites.

Applying this series of filters leaves 16 proteins with affinities of less than 1 µM for Bcl-X<sub>L</sub>. Those that were tested in a competition assay all competed for binding with a known BH3 domain. Of these, 25% are known Bcl-2 family members, a very promising success rate. Among the remaining 12 proteins are five for which no function is yet known. Perhaps additional characterization of these newly identified Bcl-X<sub>L</sub>-binding proteins will help to clarify the role of the Bcl-2 family in apoptosis. The use of an <i>in vitro</i> selection method greatly simplified the subsequent performance of <i>in vitro</i> assays, thereby allowing us to quickly identify a subset of proteins for more laborious and time-consuming assays such as those that address <i>in vivo</i> activity.

With the recent publication of the human genome sequence interest is shifting to the emergent field of proteomics, one critical aspect of which is the creation of a comprehensive map of protein-protein interactions. Such interactions are responsible for most signal transductions, making them attractive targets for drug therapy. The creation of such a map presents an enormous task for which the primary methodology currently in use is the yeast two-hybrid assay. Recently, genome-wide efforts to map protein-protein interactions have been reported for <i>Saccharomyces cerevisiae</i> (22, 23), Helicobacter pylori (24), and to a more limited extent for <i>Caenorhabditis elegans</i> (25).

The work described herein has demonstrated the utility of a new methodology, mRNA display, for rapidly mapping these interactions. The great flexibility and precise control over assay conditions, such as target concentration and the presence of additives, is just one of the advantages of this <i>in vitro</i> selection method. All of the procedures used in these experiments were essentially microcentrifuge tube-based. Such systems are readily scalable through the use of microtiter techniques and are amenable to automation. In addition, the relatively laborious step of sequencing can be supplemented or replaced by array-based analysis of the pool.2 These ongoing refinements to mRNA display technology will enable its application to high-throughput, genome-wide identification of protein-protein interactions.

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