Determination of the Sequence Specificity of XIAP BIR Domains by Screening a Combinatorial Peptide Library†

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ABSTRACT: Inhibitor of apoptosis (IAP) proteins regulate programmed cell death by inhibiting members of the caspase family of proteases. The X-chromosome-linked IAP (XIAP) contains three baculovirus IAP repeat (BIR) domains, which bind directly to the N-termini of target proteins including those of caspases-3, -7, and -9. In the present study, we defined the consensus sequences of the motifs that interact with the three BIR domains in an unbiased manner. A combinatorial peptide library containing four random residues at the N-terminus was constructed and screened using BIR domains as probes. We found that the BIR3 domain binds a highly specific motif containing an alanine or valine at the N-terminus (P1 position), an arginine or proline at the P3 position, and a hydrophobic residue (Phe, Ile, and Tyr) at the P4 position. The BIR2-binding motif is less stringent. Although it still requires an N-terminal alanine, it tolerates a wide variety of amino acids at P2−P4 positions. The BIR1 failed to bind to any peptides in the library. SPR analysis of individually synthesized peptides confirmed the library screening results. Database searches with the BIR2- and BIR3-binding consensus sequences revealed a large number of potential target proteins. The combinatorial library method should be readily applicable to other BIR domains or other types of protein modular domains.

Apoptosis, or programmed cell death, fulfills an essential requirement in normal physiology by eliminating undesirable cells during embryogenesis, immune cell development, viral infection, and after environmental insult. As might be expected from its irreversible nature and severe consequence, apoptosis must be tightly regulated. Both insufficient and excessive apoptotic activity can lead to human diseases such as developmental abnormalities and cancers (*1*). The caspases, which are a family of cysteine proteases responsible for the initiation and execution phases of cellular dismemberment, must be held in check until the arrival of appropriate signals. Their regulation is achieved at several levels beginning with their synthesis as inactive zymogens. Under the control of accessory proteins, proteolytic processing yields active caspases consisting of an $\alpha 2\beta 2$ subunit stoichiometry (*2*). The activated proteases are further regulated by a family of inhibitor of apoptosis (IAP)[1] proteins (*3*, *4*). A common feature of IAPs is that they contain one to three baculovirus IAP repeat (BIR) domains (*5*). The BIR domain, which is composed of ∼70 amino acids, mediates protein−protein interactions by interacting with specific N-terminal sequences of its partner proteins (*6*, *7*). The best characterized member of the IAP family is the human X-linked IAP (XIAP), which contains three N-terminal BIR domains (BIR1, BIR2, and BIR3) and a canonical C-terminal RING finger domain (*5*). The BIR3 domain of XIAP binds directly to the small subunit of processed caspase-9, the initiator caspase of the mitochondrial pathway of apoptosis (*8*, *9*). Structural studies show that the BIR domain interacts with the N-terminal tetrapeptide of caspase-9, ATPF, and sequesters caspase-9 in a catalytically inactive monomeric state (*8*, *10*). The BIR2 domain of XIAP, together with the preceding linker sequence, binds to and inhibits the executioner caspases-3 and -7 (*11*−*13*). Caspase inhibition by XIAP is in turn regulated by a small protein, second mitochondrial activator of caspases (SMAC), which binds to the BIR2 and BIR3 domains of XIAP, thereby sequestering the inhibitor and releasing the caspase activity (*6*, *7*, *14*, *15*). Interestingly, the BIR1 domain of XIAP, despite sharing 41% sequence identity with BIR2, has never been observed in any protein−protein interactions.

The physiological functions of other IAPs are much less understood. In addition to their putative function in inhibition of apoptosis, IAPs have been suggested to play additional roles. For example, survivin, which consists largely of a single BIR domain, has been shown to be associated with cytokinesis (*16*, *17*). It is therefore of great interest to determine what other proteins may be recognized by the BIR

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[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BIR domain, baculovirus IAP repeat domain; IAP, inhibitor of apoptosis; MBP, maltose-binding protein; Nic-OSU, *N*-hydroxysuccinimidyl nicotinate; PED/MS, partial Edman degradation/mass spectrometry; PITC, phenyl isothiocyanate; SA-AP, streptavidin−alkaline phosphatase; SMAC, second mitochondrial activator of caspases; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; XIAP, X-linked inhibitor of apoptosis.

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domains of these IAPs. Since BIR domains appear to recognize only the N-terminal few residues of a protein, in principle, one should be able to identify their partner proteins by determining their sequence specificities and then using the resulting binding motifs to search the protein and/or genomic databases. We have recently developed a combinatorial peptide library method to systematically determine the sequence specificity of modular domains such as the SH2 domain (*18*). In the present study, we have applied the library method to identify the peptide motifs and potential protein targets that may interact with the three BIR domains of XIAP.

EXPERIMENTAL PROCEDURES

Materials. The pMAL-c2 vector, all DNA modifying enzymes, and amylose resin were purchased from New England Biolabs. The pET-28a vector and *Escherichia coli* BL21(DE3) Rosetta CodonPlus strain were purchased from Novagen. All oligonucleotides were purchased from Integrated DNA Technologies. Streptavidin−alkaline phosphatase (SA-AP) was purchased from Prozyme. 5-Bromo-4-chloro-3-indolylphosphate (BCIP), antibiotics, *N*-hydroxysuccinimido-biotin, Sephadex G-25 resin, 4-hydroxy-α-cyanocinnamic acid, and organic solvents were obtained from Sigma-Aldrich. Talon resin for metal affinity purification was purchased from Clontech. Reagents for peptide synthesis were from Advanced ChemTech, Peptides International, and NovaBiochem. *N*-Hydroxysuccinimidyl nicotinate (Nic-OSU) was from Advanced ChemTech and was recrystallized from ethyl acetate prior to use. Phenyl isothiocyanate (PITC) was purchased in 1 mL sealed ampules from Sigma-Aldrich and used without purification. Protein concentration was determined by the Bradford method using bovine serum albumin (Sigma-Aldrich) as a standard.

Recombinant DNA Constructs. A pET-MAL vector was created by subcloning the *malE* gene from pMAL-c2 into the *Nde*I-*Xho*I site of pET-28a with the retention all pMAL-c2 multiple cloning sites. The DNA sequences coding for the BIR1 domain (amino acids 1−123), BIR2 domain (amino acids 124−240), and BIR3 domain (amino acids 241−356) were isolated by polymerase chain reaction (PCR) from a pGEX plasmid containing the BIR1−3 domains of XIAP (*19*) (kindly provided by Dr. Xiaodong Wang, University of Texas Southwestern Medical Center). The DNA primers used were as follows: BIR1, 5′-GGAATTCATGACTTTTAACAGTTTTGAAGG-3′ and 5′-CTTGAAGCTTGTCCTCAGGATCCCAGATAGTTTTCAAG-3′; BIR2, 5′-GAGAATTCAGAGATCATTTTGCCTTAGACAGG-3′ and 5′-CTGGAAAGCTTATTCACTTCGAATATTAAGATTCC-3′; BIR3, 5′-CGAATTCTCTGATGCTGTGAGTTCTGATAG-3′ and 5′-GTACGAAGCTTAAGTAGTTCTTACCAGACACTCC-3′. The PCR products were digested at the underlined sites with restriction endonucleases *Eco*RI and *Hin*dIII and ligated into their corresponding sites in pET-MAL. This procedure resulted in the fusion of the BIR domains to the C-terminus of maltose-binding protein (MBP), facilitating both purification and biotinylation. The authenticity of all DNA constructs was confirmed by dideoxy sequencing.

Purification and Biotinylation of MBP-BIR Proteins. E. coli BL21(DE3) cells harboring the proper pET-MAL-BIR plasmid were grown in LB medium to the mid-log phase and induced by the addition of 300 μM isopropyl β-D-thiogalactoside (IPTG) for 2.5 h at 30 °C. The cells were harvested by centrifugation and lysed in the presence of protease inhibitors by passing through a French pressure cell. The MBP-BIR protein was purified from the crude lysate on an amylose column according to manufacturer's recommended procedures, except that the elution buffer contained 20 mM HEPES, pH 8.2, 150 mM NaCl, 2 mM 2-mercaptoethanol, and 10 mM maltose. The protein was concentrated in an Amicon concentrator to approximately 4 mg/mL and treated with 2 equiv of *N*-hydroxysuccinimido-biotin at room temperature for 45 min. Excess biotin was removed by passing the protein solution through a Sephadex G-25 column. After concentration and addition of glycerol to a final concentration of ∼40%, the protein was quickly frozen in a dry ice/2-propanol bath and stored as −80 °C. MBP alone was prepared and labeled in the same manner as a control. For binding experiments by surface plasmon resonance (BIAcore), the MBP-BIR fusion proteins were purified as above, but without biotinylation. Instead, following elution from the amylose column, the protein was passed through an XK-16 Superdex-75 column connected to an FPLC system (Pharmacia). The elution buffer contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 10 μM tris-(carboxyethyl)phosphine (TCEP). In this instance, all proteins were flash frozen without the addition of glycerol.

Synthesis of the Peptide Library. The peptide library (NH_2-XXXXLNBBRM-resin, B = β-alanine, X represents randomized amino acids) was synthesized on 5 g of TentaGel S NH_2 resin (130-μm, 0.2−0.5 mmol/g) using standard Fmoc/HBTU/HOBt chemistry. The invariant linker (LNBBRM) was synthesized with 4 equiv of Fmoc-amino acids, and the coupling reaction was terminated after ninhydrin tests were negative. The random positions (indicated by X) were synthesized by the split-synthesis method (*20*−*22*). Five equivalents of Fmoc-amino acids were employed, and reactions were allowed to proceed for 45 min. Each coupling reaction was repeated once to ensure reaction completion. To facilitate unambiguous sequence determination by mass spectrometry, 5% Ac-Gly was added to the coupling reactions of Leu and Lys, whereas 5% Ac-Ala was added to the coupling reactions of Nle (*18*, *23*). After removal of the N-terminal Fmoc group, the resin-bound library was washed exhaustively with dichloromethane and deprotected using a modified reagent K [7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in trifluoroacetic acid (TFA)] at room temperature for 60 min. The library was washed with TFA, dichloromethane, and methanol before being dried and stored at −20 °C.

Library Screening and Hit Identification. In a micro BioSpin column (0.8 mL, Bio-Rad), 50 mg of library was swollen in dichloromethane, washed extensively with methanol, doubly distilled H_2O, and HBST buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), and blocked for 1 h with 800 μL of HBST buffer containing 0.1% gelatin. The resin was drained and resuspended in 800 μL of a biotinylated MBP-BIR domain of interest (1−2000 nM final concentration) in HBST buffer plus 0.1% gelatin. After overnight incubation at 4 °C with gentle mixing, the resin was drained and resuspended in 800 μL of SA-AP buffer (30 mM Tris, pH 7.6, 1 M NaCl, 10 mM $MgCl_2$, 70 μM

$ZnCl_2$) containing 1 μL of SA-AP (~1 mg/mL stock solution). After 10 min of gentle mixing at 4 °C, the resin was rapidly drained and washed with 400 μL of SA-AP buffer, 400 μL of staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$), 400 μL of HBST buffer, and 400 μL of staining buffer again. The resin was then transferred to a 35 mm Petri dish by rinsing with 5 × 300 μL of the staining buffer. Upon the addition of 80 μL of 5 mg/mL BCIP in the staining buffer, intense turquoise color developed on positive beads in ~45 min, at which point the staining reaction was quenched by the addition of 3 mL of 8 M guanidine hydrochloride. The resin was transferred back into the Bio-Spin column, extensively washed with water, and replated in a Petri dish. Colored beads were picked manually using a micropipet under a dissecting microscope, separated into two categories according to their color intensities ("intensely" vs "lightly" colored), and individually sequenced by a partial Edman degradation/mass spectrometry (PED/MS) method (*18*, *23*). Flawed beads containing cracks or nonuniformed coloring were discarded. Control experiments with biotinylated MBP produced no colored beads under identical conditions.

Peptide Synthesis. All peptides were synthesized to contain a C-terminal BBK-NH_2 sequence. Each peptide was synthesized on ~65 mg of CLEAR-amide resin using standard Fmoc/HBTU/HOBt chemistry. Deprotection and cleavage from the resin were effected by the treatment with the modified reagent K without removal of the N-terminal Fmoc group. Approximately 3 mg of the crude peptide was dissolved in a minimal volume of DMSO (200−400 μL, with sonication) containing a small amount of diisopropylethylamine and mixed with 1 molar equiv of biotin-(ethylene glycol)$_4$-*N*-hydroxysuccinimide (Quanta Biochem) dissolved in 25 μL of DMSO. After 45 min at room temperature, piperidine was added to 30% final concentration and allowed to react for 20 min. The mixture was acidified with TFA and triturated twice with 20 volumes of Et_2O. The precipitate was collected and dried under vacuum. The biotinylated peptides were purified by reversed-phase HPLC on a C_{18} column (Vydac 300 Å, 4.6 × 250 mm). The identity of each peptide was confirmed by MALDI-TOF mass spectrometric analysis. This procedure resulted in the addition of a 15-atom hydrophilic linker between the side chain of the C-terminal lysine and the carboxyl group of biotin. In the case of peptide VKTFLEABE(PEG-biotin)-NH_2, which contained an internal Lys residue, the above procedure was modified by the replacement of the linker Lys with Glu-(PEG-biotin) during synthesis. This substitution was accomplished using the reagent Fmoc-Glu(PEG-biotin)-OH from NovaBiochem. The terminal Fmoc was removed prior to cleavage/deprotection, and HPLC purification was performed as above.

Determination of Dissociation Constants by Surface Plasmon Resonance (SPR). All measurements were made at room temperature on a BIAcore 3000 instrument. The biotinylated peptides were immobilized onto a streptavidin-coated sensor chip by passing 6 μL of ~8 μM peptide solution in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20). Data for the secondary plot analysis were acquired by passing increasing concentrations (0−10 μM) of MBP-BIR domain protein in HBS-EP buffer over the sensor chip for 6 min at a flow rate of 15 μL/min. A blank flow cell (no immobilized peptide) was used as a control to correct for any signal due to the solvent bulk and/or nonspecific binding interactions. In fact, neither significant bulk effect nor nonspecific binding was observed. In between two runs, the sensor chip surface was regenerated by flowing a strip solution (10 mM NaCl, 2 mM NaOH, and 0.025% SDS in H_2O) for 5−10 s at a flow rate of 100 μL/min. The equilibrium response unit (RU_{eq}) at a given MBP-BIR protein concentration was obtained by subtracting the response of the blank flow cell from that of the sample flow cell. The dissociation constant (K_D) was obtained by nonlinear regression fitting the data to the equation:

$$RU_{eq} = RU_{max}[SH2]/(K_D + [SH2])$$

where RU_{eq} is the measured response unit at a certain MBP-BIR protein concentration and RU_{max} is the maximum response unit. Control runs with MBP showed no significant binding to any of the immobilized peptides.

RESULTS

Library Design, Screening, and Sequencing. Previous biochemical and structural studies suggest that BIR domains bind to their partner proteins through interaction with the four amino acids at their N-terminus (*6*−*13*). We therefore designed a peptide library containing four randomized residues at the N-terminus, XXXXLNBBRM-resin [where X represents norleucine (Nle) or any of the 18 proteinogenic amino acids excluding Met and Cys]. The C-terminal Met allowed for the specific release of a peptide from the resin upon treatment with CNBr, and the neighboring Arg provided a locus for ionization of the released peptide during MS analysis. Methionine was excluded from the random positions to avoid internal cleavage by CNBr and was replaced by the isosteric Nle residue. The two β-alanyl residues were added to increase the flexibility of the linker sequence, thereby making the random region more accessible to a protein receptor. Finally, neutral residues Asn and Leu were incorporated to increase the mass of the linker to >600 Da, avoiding signal overlap between peptides and matrix materials in the MALDI mass spectrum. The split-pool method (*20*−*22*) was employed to produce a "one-bead-one-compound" library on TentaGel S NH_2 resin (130 μm), which has excellent swelling properties in both aqueous and organic solvents. In this library, each resin bead carried ~300 pmol of a unique peptide, ensuring equal representation of all library members. The theoretical diversity of the library is 19^4 or 1.3×10^5.

A typical library screening experiment involved incubating 50 mg of the library (~4.4×10^4 beads) with a biotinylated MBP-BIR domain of interest (1 nM−2 μM) overnight at 4 °C with gentle mixing. Binding of the BIR domain to a resin-bound peptide recruits streptavidin−alkaline phosphatase to the surface of that bead. Upon the addition of phosphatase substrate BCIP, the bound alkaline phosphatase converts BCIP into an indole, which rapidly dimerizes in air to form a turquoise-colored precipitate deposited on the bead surface. Thus, beads that carry high-affinity BIR domain ligands become colored. The stringency of the screening was controlled by adjusting the BIR domain concentration as well as the staining reaction (BCIP) time and was reflected in the number of positive beads obtained. The positive beads

Table 1: Peptides Selected against the BIR3 Domain[a]

500 nM		1000 nM		2000 nM	
AIAF	AAPY	AIAF	ASPI*	AIRA	AVII
AKAF	AHPY	AKAF	ATPI*	AVPE	AKII
AMAF	AVPY*	AKAF	ATPI	ARRE	AQII
AIGF	AYPY	AMGF	AVPI*	AAAF	ARVI
AKGF	ARRY	ARIF	AYKI	AKSF	AVRI
AKSF	ARRY	AKMF	AVRI	AMTF	AIRI
AFPF*	AIPI*	ATPF*	AVRI	ATPF	ARRI
AGPF*	ARPI*	AAPF	AIRI	AVPF*	AKRI
AHPF	ARPI	AHPF	AARI	AAPF	AAPV
AMPF*	AYPI*	AKPF*	AKRI	AVIF	AKIV
ASPF	AIRI	AFKF	ARPG	AIMF	ARAT
ARPF*	AKRI	AKKF	ATRS	AKMF	ALPT
AKIF	ARRI	ARKF	ARPQ	AAVF	AFPT
AKIF	ARPV	AARF	VTGF	AVKF	AAPT
AAMF	ARPA	AGRF	VARF	AFRF	ARPT
AIMF	ARPS	ARRF	VQRF	AFRF	ATRT
AKVF	ARPS	AVRF	VKTF	AGRF	AFPG
AIRF	ARRS	AYRF	SRPF	AYRF	AIPS
AMRF	VKPF	ATPY	ESPW	AKAY	ARPN
AMRF	VRPF	AYPY		ATAY	VAIF
AMRF	VRPI	AFRY		ALRY	VRAF
ASRF	VKTF*	AKRY		AQRY	VRRF*
ATRF*	TKRF	ARRY		ANAI	VQRF
AYRF	FDHI	ASRY		ARTI	TKRI
AIKF	MQII	ATRY*		AAPI*	IRTF
	QGRW			AFPI*	IRRF
	RQTA			ARPI	NHGW
				AVII	

[a] Peptides labeled with asterisks were confirmed for their ability to bind XIAP BIR3 domain.

were manually removed from the library by a pipet under a low-power dissecting microscope and individually sequenced by the PED/MS method (*18*, *23*).

Sequence Specificity of the BIR3 Domain. In light of the reported K_D values in the high nanomolar to low micromolar range for the BIR3 domain binding to peptides derived from its protein targets (*7*), our initial screening was conducted at 500 nM BIR3 domain. This resulted in 25 positive beads of similar color intensity (~0.057% hit rate). To test whether the screening method was reproducible and to obtain a larger number of binding sequences for consensus analysis, the screening experiment was repeated four times at BIR3 domain concentrations of 0.5 (twice), 1.0, and 2.0 μM, respectively. All together, ~160 colored beads were selected from 250 mg of the library (2.2×10^5 beads; ~0.073% average hit rate); PED/MS sequencing of these beads produced 151 complete sequences (Table 1). The results indicate that the library method was highly reproducible and essentially the same set of sequences was selected from the five independent experiments. In fact, many of the sequences were selected twice (e.g., AIAF, AKAF, AKSF, ATPF, AHPF, AKIF, AIMF, AYPY, ATPI, ARRI, ARPS) or three times from the library (e.g., AVRI, AIRI, AKRI, ARPI, AMRF, AYRF, AKAF, and ARRY). Note that ATPF is the N-terminal sequence of processed caspase-9 (*8*). Peptide AVPI, which corresponds to the N-terminus of SMAC, was also selected from the library (Table 1, 1000 nM). Both peptides have previously been shown to bind to the BIR3 domain with high affinity (*7*).

Overall, the BIR3 domain is highly sequence specific. It has the most stringent specificity at the N-terminal position (P1 position), strongly preferring an alanine residue; out of

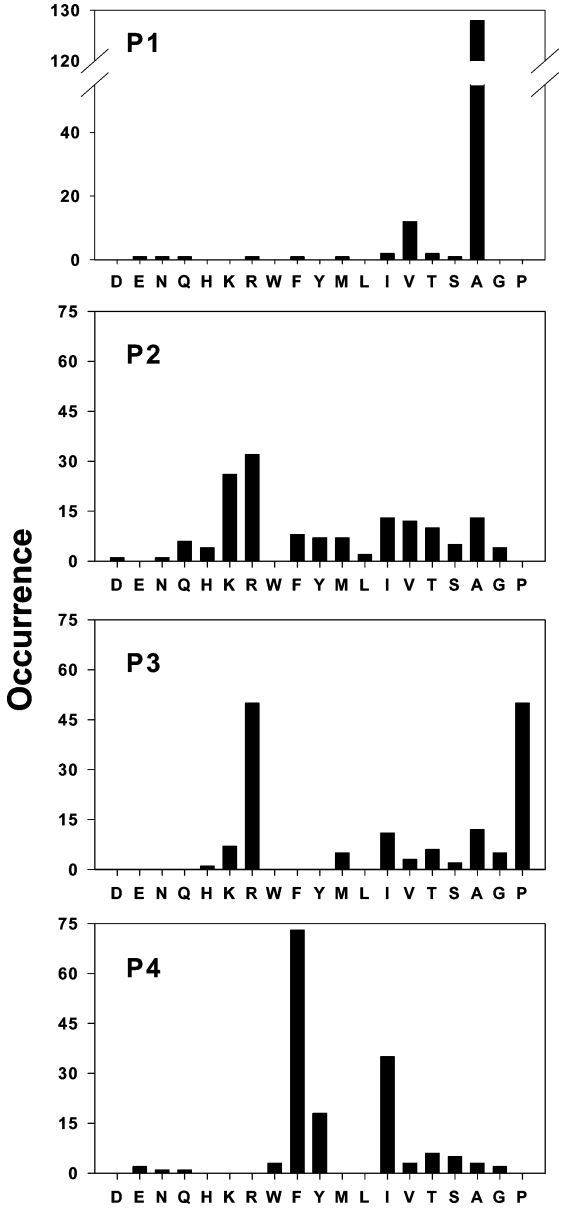

FIGURE 1: Specificity of the BIR3 domain. The histograms display the amino acids identified at each position (P1 to P4). Occurrence on the *y*-axis represents the number of selected sequences that contained a particular amino acid at a certain position. Key: M, norleucine.

the 151 selected sequences, 128 had an alanine at the P1 position (Figure 1). The only other amino acid that appeared in a significant number of sequences (12 peptides) was valine. Five peptides contained threonine, isoleucine, or serine at this position. The second most selective position is the fourth position from the N-terminus (P4). The BIR3 domain favors a large hydrophobic residue at this position, with phenylalanine being the most preferred, followed by isoleucine and tyrosine. Strong selectivity was also evident at the P3 position, where the most frequently selected residues were arginine and proline, which were occasionally replaced by other structurally similar residues (e.g., Arg by Lys and Pro by Ala or Gly). The weakest selectivity is at the P2 position. Although there was some preference for basic residues (e.g., Arg and Lys), most other amino acids were tolerated at this position. Thus, the BIR3 domain of XIAP recognizes peptides of the consensus (A/v)X(R/P)(F/I/y), where lower case letters represent less frequently

Table 2: Peptides Selected against the BIR2 Domain[a]

500 nM		50 nM			10 nM	1 nM
AVKV	**ATVG**	AQAV*	**ATAA**	AVVP	**ASAV**	ATPV
ATKV	**AQPG**	AKAV	AYAA	AVNP	AYPV	AVVV
ASRV	**AQKG**	AYPV	AAIA	**AFNP**	**AVVV***	AIKV
ANAV	AEAG	ARSV	ARIA	**AYNP**	AQYV	
AWAV	AEIG	ADVV	ATYA	**AYNP**	AQKV	
ANAI	ADAG	ASMV	AMYA	AVSF	ARAI	
AVHI	ARQG	AYMV	ARYA	AKRF	**ARAT**	
AVKI	ALRG	AVWV	ARVA	AIPY	**ATIA**	
AMKI	AMRG	ANYV	AQNA	ATPY	AINA	
ATKT	ATSS	AVNV	AVQA	**AIAY**	**ATKG**	
ATAT	**ATRP***	AIQV	**ATAS**	AIAY	AVKG	
AVGT	AIRP	AKRV	AYPS	ARAY	AQYG	
AVMT	AYNP	ANRV	AAVS	AEGY	AIKE	
AQAA	AKVP	AWRV	ARVS	AMGY	AVVE	
AQKA	AEPF	**AVRV***	AQYS	**ARGY**	AIAK	
AVHA	ATAN	AYRV	AYNS	AISY		
ARNA	AEAD	AHKV	AVRS	AVSY		
ASVA	ARKE	AKAI	ARIG	AIVD		
AQYA	ATRH	AEMI	AEVG	AIKD		
ASYA	ASAK	ATRI	ASYG	AYAE		
	AQKR	AKAT	ARYG	AQGE		
		AQGT	AYSG	AQPE		
		AQST	ADKG	AQAQ		
		ASRT	AEKG	EFAS		
		ATRT	**AERG**	FVAG		
		ATVT	**ATRG**	**INDH**		
				NQQD		

[a] Sequences in boldfaced letters were derived from intensely colored beads, whereas those labeled with asterisks were confirmed for their ability to bind XIAP BIR2 domain.

selected amino acids whereas X is any amino acid except for proline. Inspection of the selected sequences in Table 1 reveals that 145 of them conform to the above consensus and are most likely the bona fide ligands of the BIR3 domain. The remaining six peptides (FDHI, MQII, QGRW, RQTA, ESPW, and NHGW) differ significantly from the consensus sequence and are likely weak binders or false positives.

Sequence Specificity of the BIR2 Domain. Screening 50 mg of the peptide library at 500 nM BIR2 domain produced 14 "intensely colored" beads and ~220 "lightly colored" beads. All 14 intensely colored beads and 31 of the lightly colored beads (randomly selected) were sequenced by PED/MS to give 41 complete sequences (Table 2, 500 nM). Inspection of these sequences revealed that other than the absolute requirement of an alanine at position P1, there was no clear consensus at any other position. We theorized that more stringent screening conditions would reduce the number of "hits" and a consensus might emerge. Thus, three additional screening experiments were performed at MBP-BIR2 concentrations of 50, 10, and 1 nM. Indeed, the number of binding events decreased substantially as the BIR2 domain concentration was reduced, yielding 79, 15, and 3 binding sequences, respectively (Table 2). As was the case in the 500 nM screening, BIR2 showed exquisite selectivity for an Ala at the P1 position (134 out of 138 sequences) (Figure 2). At the P4 position, Val and other amino acids with small side chains (e.g., Gly, Ala, Thr, Ser, and Pro) were the most frequently selected residues. The P2 position has some preference for β-branched residues (Val, Thr, Ile) or amino acids bearing side chains that have both hydrophobic and hydrophilic moieties (e.g., Gln, Arg, Tyr, Glu, and Lys). This trend is especially evident among the sequences selected under the more stringent conditions (1 and 10 nM) (Table 2). The P3 position favors small residues (e.g., Ala, Val, Pro, and Ser) and amino acids containing amphipathic side chains (e.g., Arg, Lys, and Tyr). Overall, the BIR2 domain has significantly broader sequence specificity as compared to the BIR3 domain, with a consensus sequence of A(r/k/v/t/i/q/y)(A/r/k)(V/g/a/p).

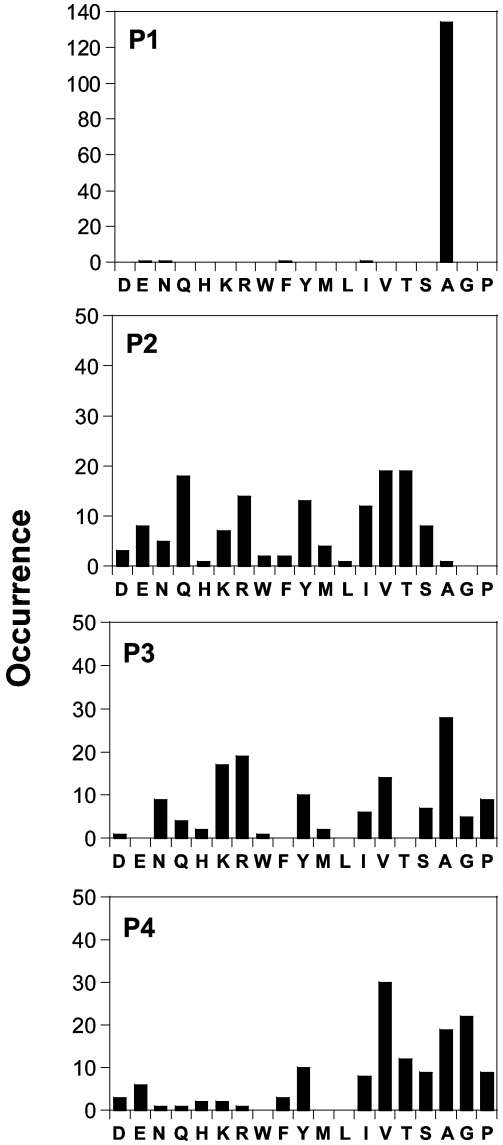

FIGURE 2: Specificity of the BIR2 domain. The histograms display the amino acids identified at each position (P1 to P4). Occurrence on the *y*-axis represents the number of selected sequences that contained a particular amino acid at a certain position. Key: M, norleucine.

BIR1 Domain. Screening of the peptide library with the BIR1 domain did not produce colored beads under any conditions tested (up to 2 μM BIR1 protein). Suspecting that this BIR domain may not recognize peptides with a free N-terminus, we prepared and screened a peptide library containing N-acetylated peptides. Again, no colored bead was found. Since the BIR1 domain has not yet been shown to bind to any ligands, we conclude that it is perhaps not a functional BIR domain. It is possible, however, that binding to the BIR1 domain may require some other posttranslational modification.

Binding Affinities of Selected Sequences. Many of the peptide sequences identified by using the BIR3 domain have

Table 3: Dissociation Constants (K_D, μM) of Selected Peptides toward BIR2 and BIR3 Domains[a]

no.	peptide sequence	BIR2	BIR3
1	AVPFY	4.9 ± 0.8[b]	0.13 ± 0.03[b] 0.06[c]
2	AVPIY	6.0 ± 0.9[b]	0.43 ± 0.06[b] 0.36[c]
3	AFPFY	ND	0.12[c]
4	AGPFY	ND	2.7[c]
5	AMPFY	ND	0.12[c]
6	AKPFY	ND	0.05[c]
7	AVPYY	ND	0.3[c]
8	ARPF	ND	0.02[d]
9	AIPI	ND	0.39[d]
10	ARPI	ND	0.18[d]
11	AYPI	ND	0.59[d]
12	AFPI	ND	0.79[d]
13	AAPI	ND	2.8[d]
14	ASPI	ND	1.4[d]
15	AQAV	4.2[e]	>64[e]
16	ATPFBBK-NH_2	2.3 ± 0.3	0.46 ± 0.03
17	ATPIBBK-NH_2	2.7 ± 0.2	1.0 ± 0.08
18	ATRFBBK-NH_2	4.6 ± 0.3	1.1 ± 0.2
19	ATRYBBK-NH_2	6.9 ± 0.9	1.4 ± 0.2
20	VRRFBBK-NH_2	NB	0.85 ± 0.07
21	VKTFLEABE-NH_2	NB	2.1 ± 0.2
22	ATRPBBK-NH_2	3.8 ± 0.2	NB
23	AVVVBBK-NH_2	0.87 ± 0.3	NB
24	AVRVBBK-NH_2	1.0 ± 0.1	1.5 ± 0.2
25	FNYRBBK$-NH_2$	NB	NB

[a] Peptides 16−25 were biotinylated at their C-terminal lysine or glutamate side chain for immobilization onto streptavidin sensor chips. ND, not determined; NB, no detectable binding under the assay conditions. [b] Data from ref *7*. [c] Data from ref *24*. [d] Data from ref *25*. [e] Data from ref *26*.

previously been shown to bind to the domain with high affinity (K_D values of 0.06−2.8 μM) (*7*, *24*−*26*). These sequences include AVPF, AVPI, AFPF, AGPF, AMPF, AKPF, AVPY, ARPF, AIPI, ARPI, AYPI, AFPI, AAPI, and ASPI (Table 3, peptides 1−14). Likewise, the K_D value (4.2 μM) for one of the BIR2-binding sequences (AQAV; Table 3, peptide 15) was previously reported (*26*). To further verify the screening results, six additional BIR3 domain-binding sequences (Table 3, peptides 16−21) and three BIR2 domain-binding peptides (peptides 22−24) were individually synthesized and tested for binding to both BIR domains using the SPR technique. Figure 3 shows an example of the binding interaction between the BIR3 domain and peptide 16, ATPFBBK-NH_2 (K_D = 0.46 μM). As expected, all of the selected peptides (Table 3, peptides 16−24) bound to their cognate BIR domains, with K_D values of ~1 μM. These K_D values were somewhat higher than those previously reported for peptides 1−14 (Table 3). We speculate that the difference was due, at least in part, to the different assay methods employed. The K_D values for peptides 1−15 were determined by a fluorescence polarization assay in the presence of peptides 1−15 as competitors (*7*, *24*−*26*). Note that all of the BIR3 domain-binding peptides that contain an N-terminal alanine also bound to the BIR2 domain, albeit with lower affinities (2.7−35-fold lower) (Table 3, peptides 1, 2, and 16−19). In contrast, three out of the four peptides selected against the BIR2 domain (AVVV, AQAV, and ATRP) had either no detectable binding or much weaker binding to the BIR3 domain (Table 3 and Figure 4). The fourth peptide (AVRV) is very similar to one of the BIR3-binding peptides, AVRI (Table 1, 1000 nM), and indeed bound to both BIR2

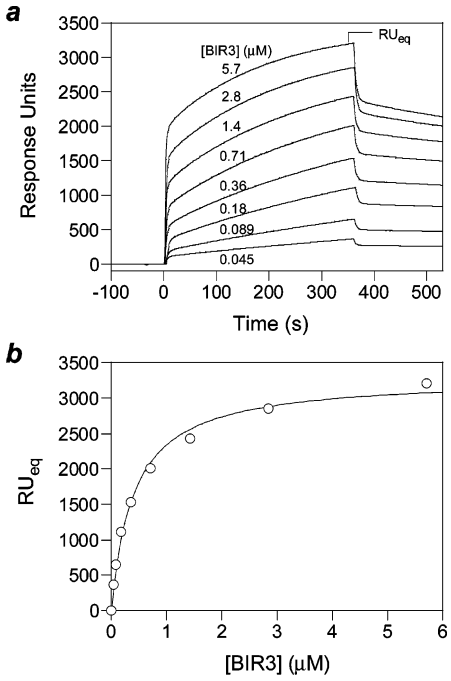

FIGURE 3: SPR analysis of the binding interaction between the BIR3 domain and immobilized peptide ATPFBBK-NH_2. (a) Overlaid sensorgrams at indicated concentrations of BIR3 protein (0.045−5.7 μM). (b) Secondary plot of the resonance signal under equilibrium conditions against BIR protein concentration. The data were fitted to the equation $RU_{eq} = RU_{max}[BIR]/(K_D + [BIR])$.

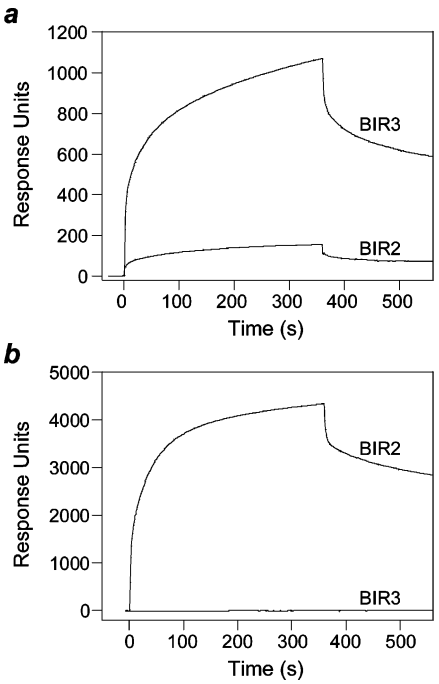

FIGURE 4: SPR sensorgrams showing differential binding of BIR2 and BIR3 domains to immobilized peptides VRRFBBK-NH_2 (a) and AVVVBBK-NH_2 (b). Approximately 6 μM BIR domain was injected in each experiment.

and BIR3 domains with similar affinities. Peptides VRRF and VKTF (Table 3, peptides 20 and 21), which belong to the minor class of BIR3 domain-binding sequences, bound to the BIR3 domain with high affinity (K_D = 0.85 and 2.1 μM, respectively) but not to the BIR2 domain (Table 3 and Figure 4). In light of the absolute requirement for an N-terminal alanine by the BIR2 domain, we believe that all of the N-terminal valine-containing peptides in Table 1 are

specific BIR3 ligands (as opposed to the BIR2 domain). Peptide FNYRBBK-NH_2 (which was selected from one of the pilot screening experiments) showed no detectable binding to either BIR2 or BIR3 domain. In sum, the literature data and the new SPR data confirmed our library screening results. The two BIR domains have overlapping sequence specificities, although the BIR2 domain can bind to a much broader range of sequences than the BIR3 domain. Each BIR domain, however, also recognizes a subset of specific ligands.

Database Search of Potential XIAP-Binding Proteins. We searched the Protein Information Resource database (web site: http://pir.georgetown.edu/) for potential XIAP BIR domain-binding proteins. Since binding to a BIR domain requires a free N-terminus, we performed the searches in two different ways. The first attempt was the most direct, searching for the N-terminally anchored sequences NH_2-AX-(R/K/P/V/A/Y)(V/T/Y/A/G) for the BIR2 domain and NH_2-(A/V)X(R/K/P)(F/I/Y) for the BIR3 domain. This approach returned 347 and 73 candidate proteins, respectively. However, the majority of these sequences were immunoglobulins or fragments more likely representing incomplete DNA sequencing than proteolytic processing, and therefore, the likelihood of a true free N-terminus was unknown. Several known IAP antagonists are processed by the removal of their N-terminal Met (e.g., *Drosophila* proteins Hid, Reaper, and Grim) (*27*). Thus, database searches were performed by the addition of an N-terminal Met residue to the BIR domain consensus sequences [NH_2-MA(R/K/V/T/I)(R/K/P/A)(VPAG) for BIR2 and NH_2-MAX(R/K/P)(F/I/Y) for BIR3 domains]. On the basis of the substrate specificity of methionine aminopeptidases (*28*, *29*), proteins containing these N-terminal sequences are expected to undergo complete N-terminal methionyl cleavage. These searches returned 387 and 238 "hit" proteins, for BIR2 and BIR3 domains, respectively. After removing redundant and fragment sequences, hypothetical proteins, and "obviously" false positives (e.g., extracellular proteins), we obtained 36 and 83 potential targets for BIR3 (Table 4) and BIR2 domains (Table S1 in Supporting Information), respectively. Among the 36 predicted BIR3 targets, checkpoint kinase 1 (Chk1), which has an N-terminal sequence of MAVPF, has recently been shown to interact with the BIR3 domain of XIAP (*30*). It should be noted that most of the known human XIAP-binding proteins bind to the BIR domains via N-terminal motifs generated by endoproteases (e.g., caspase-9 and SMAC). These proteins were returned by our first search but not by the second search.

One additional database search was performed for the BIR3 domain. In this case, some of the N-terminal valine-containing motifs including VKTF (which was selected twice from the peptide library) and VRRF were searched against the human genome. Among the many candidate proteins returned was caspase-10d, which upon proteolytic processing, contains a large catalytic subunit with VKTFLEA at its N-terminus (*31*).

Probing BIR3−Caspase-10d Interactions. We synthesized a peptide VKTFLEABE (Table 3, peptide 21) and tested its ability to bind the BIR3 domain. SPR analysis showed that the BIR3 domain of XIAP bound to this peptide with a K_D of 2.1 μM, whereas the BIR2 domain had no detectable binding (Table 3). Co-immunoprecipitation of caspase-10d and XIAP from HeLa cells was unsuccessful (data not shown). Next, the BIR domains were tested for their ability to inhibit the catalytic activity of caspases-3 and -10 in vitro. The GST-BIR1−3 fusion protein (which contains BIR1−3 domains but not the RING domain of XIAP) exhibited weak but reproducible inhibition of caspase-10d from transfected HeLa cell lysate (Figure S1 in Supporting Information). In a control experiment, we were able to confirm the ability of GST-BIR1−3 to inhibit recombinant caspase-3 (Figure S2 in Supporting Information). Finally, the BIR3 domain alone (as MBP-BIR3 fusion) showed weak inhibition of recombinant caspase-10d (Figure S3 in Supporting Information).

Table 4: Potential BIR3-Mediated Binding Partners Derived from the Protein Interaction Resource Database Search

protein	motif
checkpoint kinase (Chk1)	MAVPF
creatine kinase, mitochondrial 1	MAGPF
nuclear prelamin A recognition factor-like variant	MASPF
tropomodulin 3	MALPF
HMBA-inducible protein HEXIM1	MAEPF
similar to hephaestin	MAQPF
translocase of outer mitochondrial membrane 34	MAPKF
Sec61 homologue	MAIKF
heart α-kinase	MASKF
mitogen-activated protein kinase 6	MAEKF
four and a half LIM domains 1 (FHL-1, SLIM1)	MAEKF
aldolase B	MAHRF
purigernic receptor P2X1	MARRF
39S ribosomal protein L45, mitochondrial precursor	MAAPI
microcephalin	MAAPI
RAB33A, RAS oncogene family member	MAQPI
GTP-binding protein S10	MAQPI
interferon-responsive finger protein 1, short form	MASKI
ring finger protein 38	MACKI
similar to schlafen 5	MAMKI
similar to 40S ribosomal protein S3	MAARI
F-box protein 47	MASRI
transcription factor EB	MASRI
myeloma overexpressed	MALRI
acyl-CoA synthase 4	MAKRI
sorting nexin 16	MATPY
NPAS1 protein	MAAPY
hexaribonucleotide binding protein 1	MAQPY
phospholipase C beta 4 isoform A	MAKPY
carboxypeptidase Z isoform 3	MAWPY
proteosome (prosome, macropain) subunit, alpha type 8 (PMSA 8)	MASRY
pheromone receptor	MASRY
60S ribosomal protein L36	MALRY
Meis2	MAQRY
myeloid ecotropic viral integration site 1 homologue 2 isoform 1 (Meis1)	MARRY
Meis1-related protein 2 (Meis3)	MARRY

DISCUSSION

The sequence specificity of XIAP BIR2 and BIR3 domains has previously been studied using other library methods. Kipp et al. (*25*) and Oost et al. (*24*) applied a "position scanning" method to the BIR3 domain. Beginning with a known binding sequence AVPI or AVPF, these investigators substituted each position with a variety of natural and unnatural amino acids, generating peptide sublibraries of the form XVPI, AXPI, AVXI, and AVPX (X = randomized amino acid). The library members were individually synthesized and tested for binding to the BIR domain, and the most preferred amino acid(s) at each position was (were) identified. A major limitation of this method is its inability to identify truly novel ligands. Furthermore, many modular domains

(e.g., SH2 domains) can recognize two or more distinct consensus sequences, and a combination of the most preferred residues at each position may not necessarily produce a high-affinity ligand for the domain (*18*, *32*). Phage display has also been employed to determine the binding specificity of BIR domains (*26*, *33*). In this method, a random sequence of 4−18 amino acids was inserted between the leader sequence and the N-terminus of mature phage gene-III or -VIII coat protein. Although phage display has been successfully applied to define the specificity of many receptors and enzymes, its application to BIR domains is problematic. Because BIR domains recognize the extreme N-terminus of a protein, the random sequence must be inserted immediately after the leader peptidase cleavage site (usually after AXA motif). Since the leader peptidase is not completely nonspecific at the P′ side, this biases the library against sequences that are poor substrates of the leader peptidase. For example, the *E. coli* leader peptidase does not cleave the leader sequence if the +1 residue is proline and cleaves very poorly when the +1 residue is threonine (*34*). Sequence bias can also arise from the different efficiency in transporting the phage coat protein across the cytoplasmic membrane. It is known that addition of certain sequences (e.g., positively charged amino acids) after the leader peptidase cleavage site can block membrane insertion (*35*). Finally, phage display is generally limited to the 20 proteinogenic amino acids (*36*). Our method does not have any of these limitations. Since the library is chemically synthesized, both natural and unnatural amino acids can be incorporated into the library. The split-pool method ensures that all library members are equally represented (~300 pmol of a unique peptide on each bead in this work). In addition, our method is high throughput, inexpensive, and relatively simple operationally.

A comparison of our specificity data with the literature results reveals a general agreement, but also some notable differences. All of the methods correctly predicted a strong preference for an alanine at the P1 position and a hydrophobic residue at the P4 position for the BIR3 domain and that there are marked differences between the BIR2 and BIR3 domains. One major difference is at the P1 position for the BIR3 domain. Our results show that, in addition to the major N-terminal Ala-containing consensus, the BIR3 domain also recognizes V(R/K)(R/P)F as a minor consensus (Table 1 and Figure 1). This minor consensus was not reported by any of the previous studies (*24*−*26*, *33*). The valine-containing peptides bind the BIR3 domain with K_D values that are largely on a par with the alanine-containing peptides (Table 3), suggesting that they are legitimate potential targets. Moreover, unlike the major consensus peptides, many of which also bind to the BIR2 domain, the valine-containing peptides are specific for the BIR3 domain. Another major difference is our finding that the BIR3 domain favors positively charged residues (Arg and Lys) at P2 and P3 positions (Table 1 and Figure 1). One of the position-scanning studies had correctly predicted the preference for positively charged residues at the P2 position (*25*). However, both phage display studies failed to select any Arg or Lys at the P3 position and greatly suppressed the number of Arg and Lys residues at the P2 position (*26*, *33*). It is likely that the positively charged residues at P2 and P3 positions blocked the membrane insertion of the phage coat proteins and therefore the release of mature phage from the host cell. This finding mandates that proper caution should be taken when interpreting data from phage display libraries.

The sequence specificity data of BIR domains have at least two applications. The optimal binding sequences may serve as the lead for developing specific inhibitors against BIR domain proteins. These inhibitors will be useful as research tools or potential therapeutic agents (e.g., anticancer agents) (*24*). The consensus sequences may also be used to search the databases for potential protein targets. A search with the sequence NH_2-MAX(R/K/P)(F/I/Y) identified 36 potential BIR3 domain-binding proteins. One of the proteins (Chk1) has been shown to be a bona fide XIAP target (*30*). In eukaryotic cells, ~70% of soluble proteins are N-terminally acetylated (*37*). Therefore, only those proteins that escape the N-terminal acetylation pathway, at least transiently, will bind to XIAP. Note that several known BIR3-binding partners including caspases-9 (*8*), SMAC (*14*, *15*), HtrA2 (*38*), and GSPT1 (*39*) were not recovered by the database search, because their IAP-binding motifs are embedded in their sequences and require proteolytic processing to become exposed. Database search with a BIR3 minor consensus motif (VKTF) returned caspase-10 as a candidate. Interestingly, while the N-terminal peptide derived from mature caspase-10d interacts with the BIR3 domain with an affinity similar to those reported for other bona fide target proteins, we have not been able to demonstrate tight binding between caspase-10d and XIAP by co-immunoprecipitation. A possible explanation is that the N-terminus of caspase-10d subunit is not accessible for BIR3 binding in the intact protein. Alternatively, the affinity between the BIR3 domain and the N-terminus of caspase-10d may not be strong enough for detection in our immunoprecipitation assays. However, the inhibition of the biochemical activity of recombinant caspase-10d by BIR3 in vitro, albeit relatively weak, suggests that the interaction between caspase-10d and BIR3 is biochemically significant. Caspase-10 has four isoforms, which all contain a VKTFLEA motif at the N-terminus of their large catalytic subunit (*31*). It has been reported that caspase-10c, which has only the first 21 amino acids of the catalytic domain and is devoid of any caspase activity, is highly efficient in causing apoptosis when transiently overexpressed in MCF7 cells (*31*). A possible explanation for this unusual apoptotic activity is that caspase-10c is processed in vivo, perhaps by another protease, to release the VKTFLEA-containing peptide. The peptide, which should be free of any tertiary structure, may bind to the BIR3 domain of XIAP and trigger the release of caspase-9.

In conclusion, we have applied a powerful combinatorial library method to determine the sequence specificity of the three BIR domains of XIAP. Our results both confirmed the earlier reports and discovered several unique new features. This method is ideally suited for the interactions between protein modules and peptide sequences (e.g., SH2 and BIR domains).

SUPPORTING INFORMATION AVAILABLE

A list of potential BIR2 domain-binding proteins and data showing the inhibition of caspase-10d by XIAP BIR domains. This material is available free of charge via the Internet at http://pubs.acs.org.

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