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I. Tamm, Martin Trepel, M. Cardo-Vila, Y. Sun, K. Welsh, E. Cabezas, A. Swatterthwait, W. Arap, J. C. Reed, R. Pasqualini

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Peptides Targeting Caspase Inhibitors*

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Ingo Tamm‡§¶, Martin Trepel§‖, Marina Cardó-Vila‖, Yan Sun‖, Kate Welsh‡‡, Edelmira Cabezas‡‡, Arnold Swatterthwait‡‡, Wadih Arap‖, John C. Reed‡‡, and Renata Pasqualini‖§§**

From the ‡‡The Burnham Institute, La Jolla, California 97037, ‡Department of Hematology and Oncology, Charité, Virchow-Clinic, Berlin, Germany, and ‖The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Here we report on the identification of peptides targeting the X-inhibitor of apoptosis protein (XIAP). XIAP functions as a caspase inhibitor and is a member of the inhibitors of apoptosis (IAP) family of proteins. IAPs are often overexpressed in cancers and leukemias and are associated with an unfavorable clinical prognosis. We have selected peptides from a phage library by using recombinant full-length human XIAP or a fragment containing only the baculovirus IAP repeat 2 (BIR2) domain. A consensus motif, C(D/E/P)(W/F/Y)-acid/basic-*X*C, was recovered from two independent screenings by using different libraries. Phage-displaying variations of the consensus sequence bound specifically to the BIR2 domain of XIAP but not to other IAPs. The interaction was specific as it could be blocked by the cognate synthetic peptides in a dose-dependent manner. Phage displaying the XIAP-binding motif CEFESC bound to the BIR2 domain of XIAP with an estimated dissociation constant of 1.8 nM as determined by surface plasmon resonance. Protein-protein interaction assays revealed that caspase-3 and caspase-7 (but not caspase-8) blocked the binding of the CEFESC phage to XIAP, indicating that this peptide targets a domain within XIAP that is related to the caspase-binding site. In fact, the sequence EFES is homologous to a loop unique to the executioner caspase-3 and caspase-7 that are targeted by XIAP. Finally, we demonstrated that an internalizing version of the XIAP-binding peptide identified in our screenings (PFKQ) can induce programmed cell death in leukemia cells. Peptides interacting with XIAP could serve as prototypes for the design of low molecular weight modulators of apoptosis.

The inhibitor of apoptosis proteins (IAPs)[1] represent a family of anti-apoptotic proteins found in both vertebrates and invertebrates (reviewed in Ref. 1). All of the human IAP homologs have been shown to inhibit programmed cell death (1, 2). The human IAP family members, XIAP, c-IAP1, and c-IAP2, bind to caspase-3 and caspase-7 with inhibitory constant values (K_i) of 0.2–10 nM (3–5). XIAP also binds to and suppresses specifically caspase-9, an initiator caspase, that is at the apical protease in the cytochrome *c*/mitochondrial pathway for apoptosis (6–9).

All of the IAP family members have at least one and up to three copies of an ≈70-amino acid zinc-binding domain termed the baculovirus IAP repeat (BIR) (1). We have performed mutagenesis-based studies to show that in the context of XIAP, the second of the three BIR domains (BIR2) is necessary and sufficient to inhibit the effector proteases caspase-3 and caspase-7 (10). In contrast, for XIAP to suppress caspase-9, the third BIR domain is required (11). These data suggest that a single BIR domain can mediate anti-apoptotic activity. To map functionally relevant interacting sites within molecules associating with XIAP, we screened peptide libraries using the full-length protein and on the isolated BIR2 domain.

We identified XIAP ligands containing the motif CEFESC. This motif appears to mimic the XIAP-binding site within specific caspases because the binding of this peptide to XIAP is inhibited by preincubation with caspase-3 and caspase-7 but not by caspase 9. Binding assays using individual phage displaying this motif and a panel of purified targets confirmed that the phage interacts specifically with the BIR2 domain in a pattern consistent with caspase-type ligands. No binding was observed when BIR1, BIR3, or the RING finger domain of XIAP were tested nor when other IAP family members such as cIAP1, cIAP2, NAIP, and Survivin were evaluated under the same experimental conditions. We also show that XIAP-binding peptides can affect cell viability. Taken together, our results contribute to the understanding of the structural requirements and functional domains that are important in the regulation of programmed cell death.

EXPERIMENTAL PROCEDURES

Plasmids, Protein Expression, and Purification—Cloning and synthesis of full-length XIAP and the XIAP fragments BIR1, BIR2, BIR3, BIR1–2, BIR2–3, and RING as well as full-length cIAP1, cIAP2, NAIP, and Survivin have been described previously (4, 10, 12). Recombinant caspase proteins containing His_6 tags were prepared as described previously (8) and were a gift from Dr. G. Salvesen (The Burnham Institute, La Jolla, CA).

Identification of XIAP-binding Peptides by Phage Display—fUSE5-based phage peptide libraries displaying either cyclic or linear random peptides (CX_4C or X_6; C = cysteine and *X* = random amino acid) were made and screened as described previously (13, 14). Polystyrene 96-well plates were coated with 50 μl/well of 1 mg/ml GST-XIAP fusion protein

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§ Both authors contributed equally to this work.

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** Supported by fellowships from the Deutsche Forschungsgemeinschaft and the Susan G. Komen Breast Cancer Foundation. Present address: Dept. of Hematology and Oncology and Institute for Molecular Medicine and Cell Research, University of Freiburg Medical Center, 79106 Freiburg, Germany.

§§ To whom correspondence should be addressed: rpasqual@notes.mdacc.tmc.edu, jreed@burnham-inst.org.

[1] The abbreviations used are: IAP, inhibitor of apoptosis; XIAP, X-inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; GST, glutathione *S*-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TU, transducing units; Fmoc, *N*-(9-fluorenyl) methoxycarbonyl; Pen, penetratin.

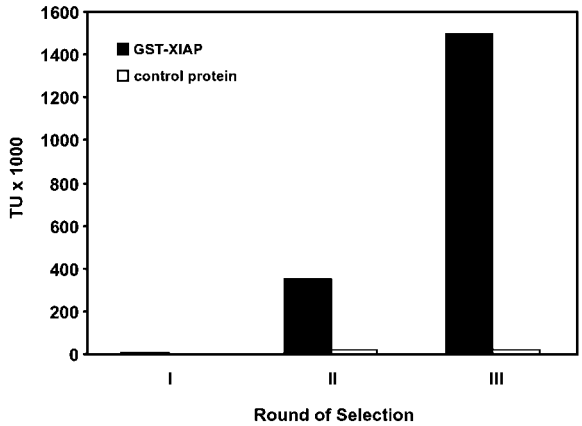

FIG. 1. **Enrichment of XIAP-binding phage on recombinant XIAP.** A CX_4C random phage-display peptide library was selected on immobilized GST-XIAP as described under "Experimental Procedures." Aliquots of the recovered phage-bacteria mixture were plated on LB plates containing tetracycline to determine the number of TU recovered. Data represent the number of TU recovered from wells coated either with GST-XIAP (*black bars*) or a control protein (*white bars*; casein for screening round II, bovine γ-globulins for screening round III).

or GST fusion proteins of various fragments of XIAP, cIAP1, cIAP2, NAIP, Survivin, BSA, or GST in PBS overnight at 4 °C. The wells were washed with PBS, blocked with 3% BSA (first round of panning), casein (second round), or γ-globulins (third round) for 1 h, and incubated with 10^{10} transducing units (TU) of the primary library for 1 h at room temperature. The wells were washed nine times in PBS, 0.01% Tween 20 to remove unbound phage and once with PBS. Bound phages were eluted by adding 200 μl of a log-phase K91Kan terrific broth culture and amplified overnight in 10 ml of LB medium containing 40 μg/ml tetracycline. Randomly selected phage clones from the third round of panning were sequenced as described previously (15). Individual phage clones displaying similar peptide motifs were tested for specific binding to GST fusion proteins of XIAP, fragments of XIAP, cIAP1, cIAP2, NAIP, Survivin, BSA, or GST alone. These binding assays were performed following the protocol of the initial screening. The number of bound phage was determined by plating serial dilutions of the recovered phage-bacteria mixture on LB agar plates containing 40 μg/ml tetracycline.

Peptide Synthesis—Peptides were synthesized with an ACT-350 multiple peptide synthesizer by using Fmoc synthesis on Rink amide MBHA resin or Fmoc-Lys(-LC-D-Biotin)-Rink amide-MBHA resin for C-terminally biotinylated peptides. The peptides were cleaved with 92.5% trifluoroacetic acid, 2.5% water, 2.5% EDTA, and 2.5% TIS and precipitated with cold ethyl ether. Quantitative cyclization was achieved in 20% Me_2SO, H_2O, pH 4.0–7.0 in 1 day as described previously (16). Crude peptides were purified by high pressure liquid chromatography on a reverse phase C-18 column with a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid. The purified peptides showed one peak by analytical high pressure liquid chromatography, and the molecular weight was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

Caspase Competition Assays—Inhibition of phage binding to XIAP by recombinant caspases was assayed by coating polystyrene 96-well plates with 1 μg/well of recombinant XIAP protein (50 μl of a 1 mg/ml solution), various fragments of XIAP, or control proteins (as indicated) in PBS overnight at 4 °C. The wells were washed once with PBS, blocked with 3% BSA for 1 h, washed again, and incubated with various amounts of recombinant active caspases-3, -7, or -8 for 30 min at room temperature. The wells were then washed once with PBS, and XIAP-binding or control phage was added (10^8 TU/well) and incubated for 30 min. Wells were then washed nine times with PBS, 0.01% Tween 20, and washed once more in PBS. Bound phage were eluted by adding 200 μl of a log-phase K91Kan terrific broth culture. The number of bound phage was determined as described above.

Surface Plasmon Resonance—The interaction of XIAP-binding phage and control phage with the BIR2 domain of XIAP was investigated using the BIAcore 3000 system (BIAcore, Uppsala, Sweden). GST-BIR2 was covalently attached by its primary amine residues to CM5 sensor chips (17). Binding was detected in resonance units after injecting phage in a range of concentrations in HBS buffer (10 mM HEPES, pH 7.4, with 0.15 M NaCl, 3.0 mM EDTA, and 0.05% surfactant P20). After each run, the chip surfaces were regenerated with 10 mM glycine, pH 4.5. Non-linear regression analysis was used to determine equilibrium-binding constants that fit to a single site-binding model (18–20).

Peptide Internalization and Cell Viability Assays—Uptake of penetratin-linked peptides into OCI/AML-4 cells (an acute myeloblastic leukemia cell line, for review see Ref. 21) was monitored as described previously (22). Cells growing in 24-well plates were incubated with increasing doses (1–20 μM) of penetratin alone, synthetic peptides alone (CEFESC, CPFKQC, or ARGKER), or penetratin-linked versions of each peptide. Cell viability was assessed at different time points (12, 24, and 72 h).

TABLE I
Sequences of XIAP-binding peptides

Randomly selected phage clones displaying the XIAP-binding motif were grouped into three subsets based on the amino acid residue at position 2.

	C	D	W	T	H	C
	C	D	W	R	V	C
	C	D	W	L	T	C
	C	D	W	L	T	C
	C	D	W	L	T	C
	C	D	W	N	Q	C
	C	D	F	I	M	C
	C	D	W	V	F	C
	C	D	F	K	A	C
	C	D	F	V	G	C
	C	E	Y	K	E	C
	C	E	E	N	L	C
	C	E	F	L	F	C
	C	E	F	F	F	C
	C	E	K	F	P	C
	C	E	F	A	Q	C
	C	E	F	A	Q	C
	C	E	H	Y	P	C
	C	E	F	E	S	C
	C	P	F	I	R	C
	C	P	F	T	E	C
	C	P	F	D	R	C
	C	P	Y	R	H	C
	C	P	F	M	A	C
	C	P	F	K	E	C
	C	P	F	H	T	C
	C	P	Y	K	Q	C
	C	P	F	K	Q	C
	C	P	F	A	A	C
	C	P	Y	H	E	C
	C	P	Y	R	R	C
	C	P	Y	R	F	C
Motif:	**C**	**D/E/P**	**Aromatic**	**+/−**	**X**	**C**

RESULTS

Identification of XIAP-binding Peptides—XIAP-binding peptides were identified by selecting phage display peptide libraries on immobilized human GST-XIAP. The libraries contained six amino acid inserts in which either all six residues were randomized or the first and last positions were fixed as cysteines to promote cyclization by disulfide bonding (X_6 or CX_4C libraries, respectively). The enrichment of phage on XIAP was monitored by counting the number of TU recovered from the XIAP-coated wells *versus* the number recovered from wells coated with the control protein. We observed a pronounced enrichment for phage binding to GST-XIAP (Fig. 1). The DNA inserts of 36 randomly chosen phage clones (18 from the X_6 library and 18 from the CX_4C library) recovered from the third round of biopanning on XIAP were sequenced. A total of 32 phage clones derived from both libraries displayed the consensus motif C-(E/D/P)-(aromatic amino acid = W/Y/F)-charged amino acid-(X = random)-C (Table I). In addition, marked enrichment was observed for phage displaying the motif (8 of 18 in the second round and 16 of 18 in the third round by using the X_6 library). In contrast, biopanning experiments using the same libraries on other IAPs such as Survivin or on GST protein alone failed to select for phage with the above consen-

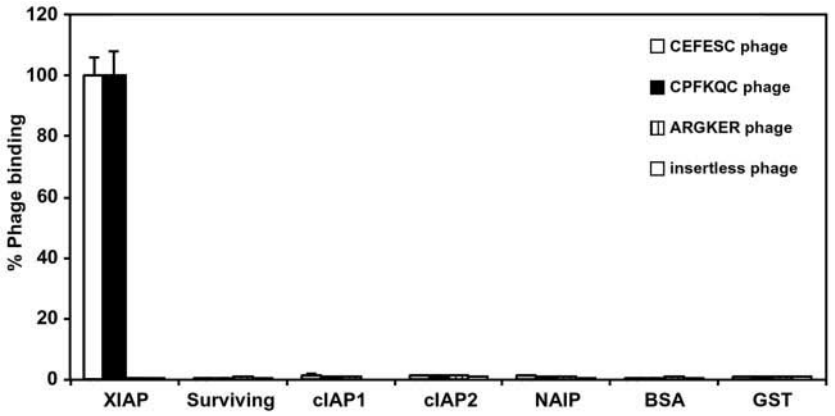

FIG. 2. **CEFESC and CPFKQC selectively bind to XIAP but not to other IAP family members.** Phage displaying no peptide insert (fd-tet), the XIAP-binding sequences CEFESC or CPFKQC, or a control sequence ARGKER were incubated in microtiter plate wells coated with 2 μg of various GST fusion proteins (XIAP, Survivin, cIAP1, cIAP2, and NAIP), GST alone, or BSA. Bound phage were recovered by bacterial infection. Transduced bacteria were grown overnight on LB plates containing tetracycline to determine the number of TU recovered. Data are expressed as the percentage relative to the number of TU recovered from wells coated with GST-XIAP (set to 100%). Data represent the means from triplicate platings ± S.D.

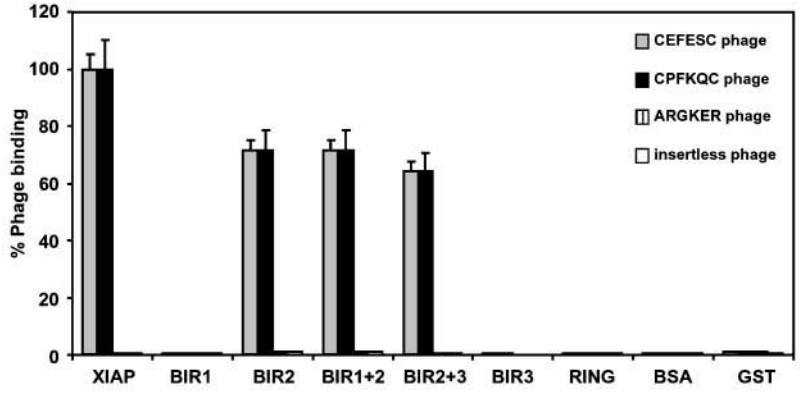

FIG. 3. **XIAP-binding phage interact with the BIR2 domain of XIAP.** Phage were incubated in microtiter plate wells coated with 2 μg of immobilized GST fusion proteins representing full-length XIAP, various fragments of XIAP, or BSA (control). Bound phage were rescued and quantified by colony counting as in Fig. 2.

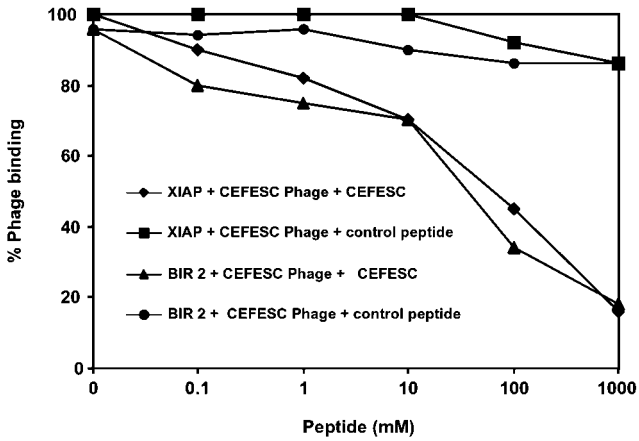

FIG. 4. **Synthetic peptides displaying phage-derived sequences bind XIAP.** Binding of CEFESC phage to XIAP is inhibited by the cognate peptide. Binding of phage displaying the XIAP-binding peptide-CEFESC or a control peptide (ARKGER) to GST-XIAP or GST-BIR2 was measured as in Fig. 2 in the presence or absence of cyclic CEFESC-synthetic peptide. Data represent the means from triplicate platings ± S.D. and are expressed as the percentage relative to the number of TU obtained in the absence of synthetic peptides.

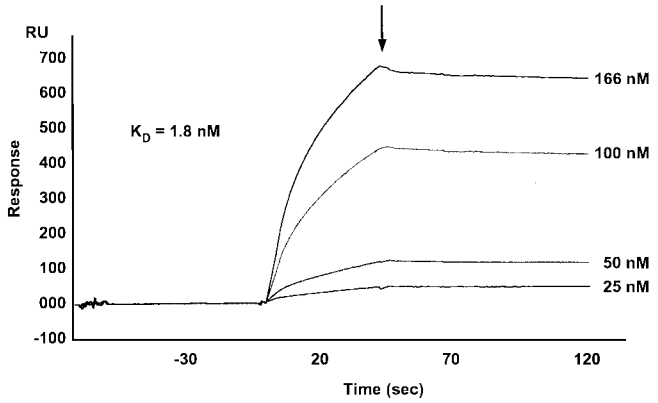

FIG. 5. **Surface plasmon resonance characterizes the binding of the CEFESC-phage to the BIR2 domain of XIAP.** Recombinant BIR2 of XIAP was immobilized on a sensor chip, and a range of concentrations of CEFESC phage was injected over the chip surface. Data represent resonance units plotted as a function of time. The *arrow* indicates the time at which the mobile phase was switched to the buffer lacking phage. The binding constant (K_D) was estimated from association and dissociation rate constants using sensograms analyzed by Bioevaluation 3.0 (BIAcore) software.

sus motif, demonstrating the specificity of the selection process.

Phage Specificity and Mapping of Binding Sites on XIAP—The specificity of XIAP-binding phage was tested in individual phage-binding assays on immobilized GST-XIAP or GST fusion proteins of other members of the IAP family such as Survivin, cIAP1, cIAP2, and NAIP. Representative data are shown for two of the XIAP-binding phage displaying the CEFESC- or the CPFKQC-peptide. Controls were insertless fd-tet phage and phage-displaying ARGKER, an unrelated control insert. In these assays, both the CEFESC and CPFKQC phage bound to XIAP but not to the other IAP family members, BSA, or GST. Control phage did not bind to GST-XIAP, indicating specificity. These data confirm the specificity of phage clones displaying the consensus motif for XIAP binding (Fig. 2).

We next mapped the domain within XIAP that mediates the binding of the isolated phage clones using a panel of XIAP deletion mutants expressed as GST fusion proteins. XIAP-binding phage bound to GST fusion proteins containing the BIR2 domain of XIAP alone or the BIR2 domain in combination with flanking domains. In contrast, fragments of XIAP containing only the BIR1, BIR3, or RING domains failed to adsorb XIAP-binding phage. The insertless fd-tet phage as well as the control ARGKER-phage did not bind to either GST-XIAP or

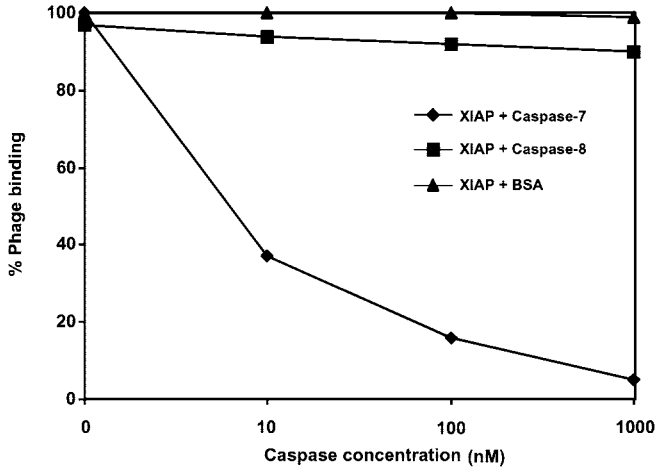

FIG. 6. **Caspase-7 but not caspase-8 inhibits binding of phage to XIAP.** GST-XIAP or GST alone were immobilized on microtiter plate wells (100 ng/well) and incubated with recombinant caspase-7 or caspase-8 (control) in a total volume of 50 μl at the indicated concentrations. XIAP-binding CEFESC-phage were added at 10^7 TU/well and incubated for 1 h, and bound phage were recovered after washing. Recovered phage were quantified as in Fig. 2.

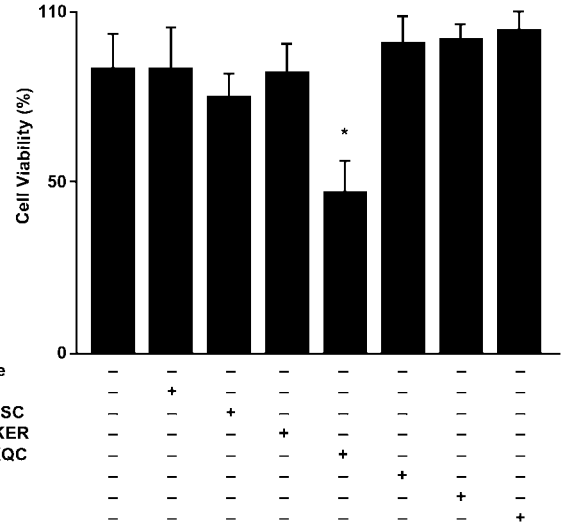

FIG. 7. **An internalizing version of the XIAP-binding peptide triggers cell death.** Internalization of Pen-CPFKQC leads to growth arrest and induces programmed cell death in OCI/AML-4 cells. Cell viability (%) was evaluated at 72 h after no treatment (*No peptide*) or incubation with multiple peptides as indicated. Pen-CPFKQC decreased cell viability. *, $p < 0.01$. Shown are mean ± S.E. obtained from triplicate wells.

GST-BIR2, indicating that the target proteins do not bind recombinant phage nonspecifically (Fig. 3).

Synthetic Peptides Representing Phage-derived Sequences Bind XIAP—To further confirm that the peptides displayed on XIAP-binding phage interact with XIAP independently of other phage components, experiments were performed using synthetic peptides. Cyclic peptides corresponding to the sequences displayed by XIAP-binding phage clones were synthesized and tested for their ability to specifically inhibit phage binding to GST-XIAP and GST-BIR2. The cyclic peptides but not the irrelevant control peptides inhibited phage binding of the corresponding phage clones in a concentration-dependent manner (Fig. 4). Non-cyclic versions of the same peptides failed to prevent phage binding.

Binding Properties of XIAP-binding Phage—To further characterize the interaction of XIAP-binding phage with the BIR2 domain, we used surface plasmon resonance. The BIR2 domain of XIAP was conjugated to the chip surface (solid phase) and phage displaying a XIAP-binding peptide were used as an analyte (mobile phase). A variety of concentrations of phage displaying CEFESC, CPFKQC, or CDFKAC were injected, and the CEFESC-phage clone was the strongest binder of the three phages analyzed (Fig. 5). This phage bound to BIR2 with an estimated k_a of 4.9×10^5 $M^{-1}s^{-1}$. Upon switching to phage-free flow solution, a very slow dissociation of phage from the chip surface was observed with an approximate k_d of 8.6×10^{-4}. A K_D of 1.8 nM was estimated for binding of the CEFESC-phage to BIR2 as compared with a K_D of >10 μM for either insertless fd-tet phage or phage displaying a control peptide-ARGKER.

XIAP-binding Phage and Caspases Compete for Binding to XIAP—Because the BIR2 domain of XIAP has been shown to be necessary and sufficient for binding caspases-3 and -7 (10), we asked whether these caspases compete with XIAP-binding phage for binding to XIAP. GST-BIR2 or various GST control proteins were immobilized on glutathione-Sepharose and incubated with XIAP-binding CEFESC-phage in the presence or absence of recombinant caspase-3 or -7. Recombinant caspase-8 was used as a control because it does not bind XIAP (3). The binding of CEFESC-phage to BIR2 was inhibited in a concentration-dependent manner upon the addition of caspase-7 or caspase-3. In contrast, neither BSA nor caspase-8 blocked the binding of CEFESC-phage to XIAP (Fig. 6). These data suggest that phage displaying the CEFESC-peptide occupy sites on the BIR2 domain that overlap with or are adjacent to sites involved in caspase binding.

To determine whether the XIAP-targeting peptides could affect cell viability, we designed and synthesized internalizing versions of the CEFESC-, CPFKQC-, or ARGKER-peptides (negative control) by using the penetratin system for intracellular delivery. Penetratin (Pen) is a peptide containing 16 amino acids that is part of the third helix of the antennapedia protein homeodomain (22). Because penetratin has translocating properties, it is capable of carrying hydrophilic compounds across the plasma membrane and delivering them directly to the cytoplasm without degradation (22). We fused the XIAP-binding peptides or a control unrelated peptide to penetratin and added a biotin moiety to visualize internalization. We used leukemia cells (OCI/AML-4) as a model system to test the effect of the internalizing peptides because these cells (i) express XIAP and caspases and (ii) respond to regulators of apoptosis (21). Pen-linked peptides were internalized and remained in the cytoplasm (data not shown). Penetratin alone was also internalized and uniformly distributed in the cytoplasm. Peptides lacking penetratin were not internalized (data not shown). We evaluated cell survival in the cells exposed, internalizing versions of the XIAP-binding peptides. After a 72-h incubation, reduced cell viability was observed in cells treated with Pen-CPFKQC (Fig. 7). No cell death was observed when cells were untreated, treated with penetratin alone, or treated with an unrelated control peptide fused to penetratin. Moreover, incubation with non-internalizing versions of each synthetic peptide had no effect (Fig. 7).

DISCUSSION

IAPs are overexpressed in a variety of cancers and leukemias, and antagonistic small molecules could be useful for modifying the antiapoptotic activity of XIAP *in vivo*. In this study, we screened phage-display peptide libraries in search of short peptide motifs capable of binding this caspase-inhibitory protein. An analysis of the peptides displayed by phage binding to XIAP revealed a consensus sequence in which position 1 is invariably a cysteine, position 2 is either an acidic residue (aspartic acid or glutamic acid) or a proline, and position 3 is

usually an aromatic amino acid (tryptophan, phenylalanine, or tyrosine) followed by two variable amino acids and finally a cysteine. Although position 4 was variable among the XIAP-binding phage randomly sequenced, the most highly represented types of amino acids at position 4 were charged (lysine, arginine, histidine, aspartic acid, or glutamic acid in 13 of 32 cases). The binding of these phage to XIAP was highly selective with no evidence of significant interactions with other members of the IAP family of proteins such as cIAP1, cIAP2, NAIP, or Survivin. Thus, the XIAP-binding phage recognize a binding site unique to XIAP, which may be exploitable for applications aimed either at detecting XIAP or selectively inhibiting XIAP function.

We showed that the isolated peptides bind to the BIR2 domain of XIAP. The specificity of the motif for BIR2 was confirmed independently by repeating library screenings using GST-BIR2 as a target, resulting in the selection of phage whose inserts shared the same consensus sequence, which was obtained originally using GST full-length XIAP. Interactions of these peptide motifs with BIR2 are biologically relevant, because our previous studies indicate that the BIR2 domain is necessary and sufficient for inhibiting caspases-3 and -7 (10). Functional studies using the cyclic XIAP-binding peptides show that these probes do not act as repressors of XIAP-mediated caspase inhibition. We also show that an internalizing version of the XIAP-binding peptide identified in our screenings (PFKQ) can induce programmed cell death in leukemia cells in a specific, dose-dependent, and time-dependent manner. These findings suggest that development of peptidomimetics following affinity maturation strategies to increase binding affinity may lead to tools that can be used as modulators of programmed cell death.

During programmed cell death, effector caspase zymogens are cleaved at conserved aspartic acid residues, generating large and small subunits, which together constitute the active protease. The activation of effector caspases such as caspases-3 and -7 is a nearly universal event associated with apoptosis induced by multiple stimuli. Our previous observation that XIAP and other IAP family proteins directly bind active executioner caspases-3 and -7, resulting in their potent suppression *in vitro* and in cultured cells (3, 4), suggests a general mechanism for IAP-mediated apoptosis inhibition. Recently, a naturally occurring IAP inhibitor termed Smac/DIABLO has been identified (23, 24). Smac performs a critical function in apoptosis by eliminating the inhibitory effect of IAPs on caspases. Smac promotes not only the proteolytic activation of procaspase-3 but also the enzymatic activity of mature caspase-3, both of which depend upon the ability of Smac to interact physically with IAPs. A seven-residue peptide derived from the N terminus of Smac promotes procaspase-3 activation *in vitro* (23). Although the amino acid sequence of this peptide is different from our sequences, identification of Smac and Omi provides further evidence that inhibition of IAPs by peptides represents a viable approach to induction of apoptosis in mammalian cells.

Interestingly, the sequence EFES, which is embedded in one of the XIAP-binding phage in a cyclic context (CEFESC), occurs in caspase-3, a protease that binds to BIR2 of XIAP with a K_D $\leq$5 nM (10). This sequence is located in the 381-loop of caspase-3 in a region only found in caspases-3 and -7. Because this loop is important in the binding of BIR2 to caspases-3 and -7 (5, 25, 26) (for review see Ref. 27), one might speculate that the EFES-containing peptide corresponds to an important region within the caspase-3-XIAP interacting site. In fact, the recently reported x-ray crystallographic structure of the XIAP-BIR2 domain/caspases-3 and -7 complex shows that the substrate-binding pocket within these caspases is formed by four surface loops, L1, L2, L3, and L4 (5, 25, 26). The sequence EFES is exposed for binding within the 381-loop, and the second Glu residue in the sequence corresponds to an interaction site between caspase-3 and BIR2 (5). This observation underscores the power of phage display technology for the mapping for biologically relevant protein-interacting sites.

Although XIAP is widely expressed, abnormally high levels of XIAP have been observed in certain types of leukemias and solid tumors (28). Overexpression of XIAP has also been shown to render tumor cells more resistant to apoptosis induction by anti-cancer drugs *in vitro* (2). Thus, agents that interfere with XIAP activity may be useful to treat cancers. The phage-derived peptides described here could provide a starting point for the generation of small molecule compounds that bind to and inhibit XIAP, thereby providing a new approach to the treatment of malignancies. Alternatively, cell membrane-penetrating versions of XIAP-binding peptides or tumor-targeted delivery of genes expressing XIAP-binding peptides fused to ubiquitin ligases could potentially be exploited as mechanism-based strategies for improved cancer treatment.

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REFERENCES

1. Salvesen G. S., and Duckett C. S. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 401–410
2. LaCasse, E. C., Baird, S., Korneluk, R. G., and MacKenzie, A. E. (1998) *Oncogene* **17,** 3247–3259
3. Deveraux, Q., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *Nature* **388,** 300–303
4. Roy, N., Deveraux, Q. L., Takashashi, R., Salvesen, G. S., and Reed, J. C. (1997) *EMBO J.* **16,** 6914–6925
5. Riedl, S. J., Renatus, M., Schwarzenbacher , R., Zhou, Q., Sun, C., Fesik, S. W., Liddington, R. C., and Salvesen, G. S. (2001) *Cell* **104,** 791–800
6. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends Biol. Sci.* **22,** 299–306
7. Salvesen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446
8. Thornberry, N., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
9. Stennicke H. R., and Salvesen G. S. (2000) *Methods Enzymol.* **322,** 91–100
10. Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) *J. Biol. Chem.* **273,** 7787–7790
11. Deveraux, Q. L., Leo, E., Stennicke, H., Welsh, K., Salvesen, G., and Reed, J. C. (1999) *EMBO J.* **18,** 5242–5251
12. Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., and Reed, J. C. (1998) *Cancer Res.* **58,** 5315–5320
13. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–389
14. Koivunen, E., Wang, B., Dickinson, C. D., and Ruoslahti, E. (1994) *Methods Enzymol.* **245,** 346–369
15. Koivunen, E., Wang, B., and Ruoslahti, E. (1995) *Biotechnology* **13,** 265–270
16. Tam, J. P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5409–5413
17. Xie, Z., Schendel, S., Matsuyama, S., and Reed, J. C. (1998) *Biochemistry* **37,** 6410–6418
18. Kalinin, N., Ward, L., and Winzor, D. (1995) *Anal. Biochem.* **228,** 238–244
19. Morelock, M. M., Ingraham, R. H., Betageri, R., and Jakes, S. (1995) *J. Med. Chem.* **38,** 1309–1318
20. Myszka, D., Arulananthm, P., Sana, T., Wu, Z., Morton, T., and Ciardelli, T. (1996) *Protein Sci.* **5,** 2468–2478
21. Koinstinen P., Wang C., Yang G. S., Wang Y. F., Williams D. E., Lyman S. D., Minden M. D., and McCulloch E. A. (1991) *Leukemia* **5,** 704–711
22. Derossi D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J. Biol. Chem.* **269,** 10444–10450
23. Chai, J., Du, C., Wu, J.-W., Kyin, S., Wang, X., and Shi, Y. (2000) *Nature* **406,** 855–862
24. Hegde, R., Srinivasula, S., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A., Fernandes-Alnemri, T., and Alnemri, E. (2002) *J. Biol. Chem.* **277,** 432–435
25. Huang, Y., Park, Y. C., Rich, R. L., Segal, D., Myszka, D. G., and Wu, H. (2001) *Cell* **104,** 781–790
26. Chai , J., Shiozaki, E., Srinivasula, S. M., Wu, Q., Dataa, P., Alnemri, E. S., and Shi, Y. (2001) *Cell* **104,** 769–780
27. Stennicke H. R., Ryan C. A., and Salvesen, G. S. (2002) *Trends Biochem. Sci.* **27,** 94–101
28. Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Scudiero, D. A., Tudor, G., Myers, T., Qui, Y. H., Monks, A., Sausville, E., Andreeff, M., and Reed, J. C. (2000) *Clin. Cancer Res.* **6,** 1796–1803