

# The mechanism of peptide-binding specificity of IAP BIR domains

BP Eckelman<sup>1,2</sup>, M Drag<sup>1,3</sup>, SJ Snipas<sup>1</sup> and GS Salvesen<sup>\*1,2</sup>

**We describe the peptide-binding specificity of the baculoviral IAP repeat (BIR) domains of the human inhibitor of apoptosis (IAP) proteins, X-linked IAP, cellular IAP1 and neuronal apoptosis inhibitory protein (NAIP). Synthetic peptide libraries were used to profile each domain, and we distinguish two types of binding specificity, which we refer to as type II and type III BIR domains. Both types have a dominant selectivity for Ala in the first position of the four N-terminal residues of the peptide ligands, which constitute a core recognition motif. Our analysis allows us to define the signature of type III BIRs that demonstrate a preference for Pro in the third residue of the ligand, resembling the classic IAP-binding motif (IBM). The signature of the type II BIRs was similar to type III, but with a striking absence of specificity for Pro in the third position, suggesting that the definition of an IBM must be modified depending on the type of BIR in question. These findings explain how subtle changes in the peptide-binding groove of IAP BIR domains can significantly alter the target protein selectivity. Our analysis allows for prediction of BIR domain protein-binding preferences, provides a context for understanding the mechanism of peptide selection and heightens our knowledge of the specificity of IAP antagonists that are being developed as cancer therapeutics.**

*Cell Death and Differentiation* (2008) 15, 920–928; doi:10.1038/cdd.2008.6; published online 1 February 2008

Apoptosis is a highly regulated cellular signaling pathway that results in the elimination of unwanted cells from multicellular animals. The apoptotic signaling cascades can be initiated by various extracellular (extrinsic pathway) or intracellular (intrinsic pathway) stimuli and ultimately converge on the activation of a family of proteases known as the caspases – reviewed in Fuentes-Prior and Salvesen.<sup>1</sup> Once active, caspases cleave a limited number of substrates that results in the destruction of the cell and its eventual disposal by phagocytic cells.

Members of the inhibitor of apoptosis (IAP) protein family have the unique ability to attenuate apoptosis induced through both intrinsic and extrinsic stimuli. It is well established that the X-linked IAP (XIAP) is capable of blocking apoptosis by direct inhibition of caspase-3, -7, and -9 – reviewed in Salvesen and Duckett<sup>2</sup> and Eckelman *et al.*<sup>3</sup> The means by which the other members of the IAP family inhibit apoptosis is less understood. Many IAPs have the capacity to bind caspases, yet lack the ability to directly inhibit the proteolytic activity of these enzymes – reviewed in Eckelman *et al.*<sup>3</sup> The defining feature of the IAP family is the presence of 1–3 baculoviral IAP repeat (BIR) domains. BIRs are small, ~70-residue zinc-coordinated domains that have been identified as the regions essential to the IAP–caspase interaction. The BIR domain(s) are necessary for the antiapoptotic activity of most IAPs. Often a surface groove on the BIR domain endows it with an affinity toward extreme N-terminal epitopes of defined sequence.<sup>2,4</sup> These

epitopes are denoted as IAP-binding motifs (IBMs) and have classically been defined to match the consensus (NH<sub>2</sub>)AΦPΦ, where Φ represents a hydrophobic amino acid and the Ala is N-terminally exposed and unblocked. This conserved surface cleft is therefore termed the IBM groove.

The exact sequence requirements of the IBM are based primarily on several IAP-interacting proteins and on profiling studies of a few BIR domains.<sup>5–7</sup> The third BIR domain of XIAP has been the most well-studied BIR in terms of defining the consensus IBM sequence.<sup>8–11</sup> Examples of proteins that bind to BIRs in an IBM-dependent manner include the caspases,<sup>11,12</sup> the second mitochondrial activator of caspase (SMAC – also known as DIABLO)<sup>13,14</sup> and HtrA2 (also known as Omi),<sup>15</sup> and the *Drosophila* proteins Hid, Grim and Reaper – reviewed in Bangs *et al.*<sup>16</sup> The most documented function of IAP-binding proteins is to compete with caspases, and thereby abrogate caspase regulation.

Because of the overlapping binding capacities of various BIR domains to IBM-containing proteins it is often reasoned that the binding preference is conserved across BIR domains. Recently, the contrary has been demonstrated in which BIR2 and BIR3 domains of XIAP had different sequence specificities.<sup>5,7</sup> In this present study, we analyzed the peptide-binding selectivity of the second and third BIR domains of XIAP, cellular IAP1 (cIAP1) and neuronal apoptosis inhibitory protein (NAIP) using a degenerative peptide library and N-terminal sequencing. These BIR

<sup>1</sup>Program in Apoptosis and Cell Death Research, Burnham Institute for Medical Research, La Jolla, CA, USA; <sup>2</sup>Graduate Program in Molecular Pathology, University of California, San Diego, La Jolla, CA, USA and <sup>3</sup>Division of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland

\*Corresponding author: GS Salvesen, Program in Apoptosis and Cell Death Research, Burnham Institute for Medical Research, 10901 N Torrey Pines Road, La Jolla, CA 92037, USA. Tel: 858 646 3114; Fax: 858 713 6274; E-mail: gsalvesen@burnham.org

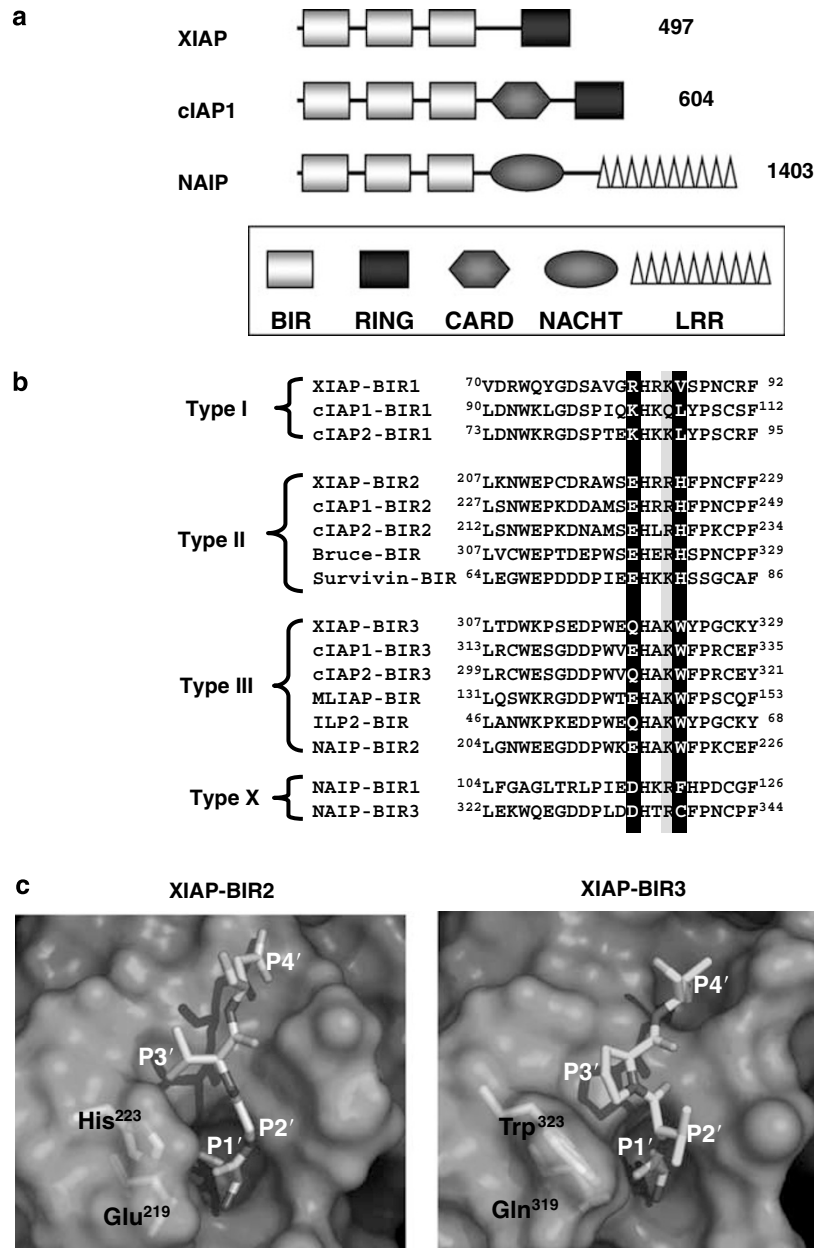
**Keywords:** apoptosis; BIR; IAP antagonists; inhibitor of apoptosis protein (IAP); SMAC

**Abbreviations:** BIR, baculoviral IAP repeat; cIAP, cellular IAP; Dde, 1-4-4-dimethyl-2-6-dioxocyclohex-1-ylidene)ethyl; DMF, dimethyl formamide; HBTU, hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; IAP, inhibitor of apoptosis; NAIP, neuronal apoptosis inhibitory protein; SMAC, second mitochondrial activation of caspase; XIAP, X-linked IAP

Received 20.8.07; revised 10.12.07; accepted 04.1.08; Edited by DR Green; published online 01.2.08

domains, with the exception of NAIP-BIR3, possess critical residues that define the IBM groove, represented by E219/H223 in BIR2 and Q319/W323 in BIR3 of XIAP (Figure 1). In examining the protein sequence and available structures of BIRs, one can recognize subgroups within this domain family. Specifically, some possess a clearly defined peptide-binding groove that is characterized by a His (His<sup>223</sup> within BIR2 of XIAP) or a Trp (Trp<sup>323</sup> within BIR3 of XIAP) required for peptide binding. To simplify discussion of the peptide-binding properties of BIR domains, we here define four types based on

conservation, or lack thereof, of residues that line the IBM groove (Figure 1b). In this study, we focus on the specificity of type II and type III BIR domains, those with a suspected IBM groove that are capable of interacting with N-terminal peptides. Type I BIR domains were excluded from our study, as they lack a His or Trp at the crucial position and do not possess a similar peptide-binding groove. These BIRs are incapable of binding SMACs, caspases or analogous peptides and use distinct sites to interact with their target proteins.<sup>7,17,18</sup>



**Figure 1** (a) Domain composition of the IAP proteins used in this study. (b) Sequence alignment of several human IAP BIRs highlighting the residues that characterize the IBM groove (black-boxed residues). The residue that was mutated along with the primary residues is also highlighted (gray-boxed residues). The IBM groove mutants used in this study have the black-boxed residues mutated to the corresponding residues of XIAP-BIR1. (c) Left, a type II BIR represented by the IBM groove of XIAP-BIR2 with the neo-terminus of the small subunit of caspase-3 bound (PDB 1130); right, type III BIR represented by the IBM groove of XIAP-BIR3 bound to a SMAC peptide (PDB 1G73). The bound peptides are numbered P1'–P4' from the N terminus

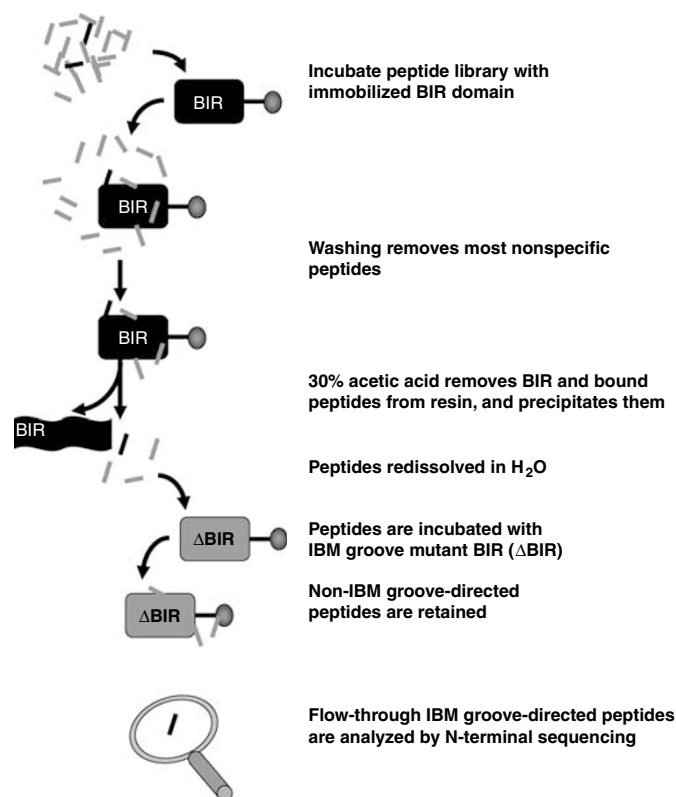
## Results

**BIR domain peptide library screening.** To determine the peptide-binding specificity of IAP BIR domains, we designed a strategy similar to that developed to elucidate protease cleavage motifs.<sup>19</sup> In short, an immobilized BIR domain is incubated with a peptide library, randomized in the four N-terminal positions. Bound peptides are eluted by acid to constitute a primary peptide pool. The primary pool is redissolved and nonspecific interacting peptides are removed on an immobilized mutant BIR domain in which the residues that define the IBM groove were mutated to those of XIAP-BIR1 (Figure 2). We have demonstrated previously that mutation of these crucial residues within IBM groove to those of XIAP-BIR1 ablates protein binding.<sup>12,20</sup> Furthermore, a recent crystal structure of BIR1 confirmed the lack of an IBM groove.<sup>18</sup> Hence, in this study we take advantage of this and utilize these IBM groove mutant BIR domains as a negative selection step in our screen, enabling us to isolate solely IBM groove-directed peptides.

The sequence of the pool of peptides binding to each BIR domain was determined by N-terminal sequencing (Edman degradation). To control for endogenous peptides associated with the *Escherichia coli*-expressed BIR domains, we corrected for the quantity of residue per cycle by using a control

sample in which the respective BIR domain was incubated with H<sub>2</sub>O instead of the peptide library. The total amount of peptides recovered following the binding and selection steps was determined by quantization of the fluorescence of the FITC tag that was included on all peptides in the library. Thus corrected, the enrichment above background in each sequence cycle represents the binding selectivity. Values greater than 1.0 define positively selected binding events (see Materials and Methods section).

Importantly, all the BIR domains tested demonstrated a strong preference for Ala in the first position. This preference was substantially more robust than at any of the subsequent positions, suggesting that Ala in the first position is a limiting factor for binding capacity. We utilized a second peptide library in which the first position was fixed as Ala to determine the specificity in the subsequent three positions. This allowed us to analyze more stringent binding events and make a more sensitive determination of the consensus sequence for each BIR domain. Furthermore, we found that the amount of peptide recovered following our binding studies was on average less than 0.2%, suggesting that we are only analyzing stringently specific binding events. Because all natural proteins that bind BIRs via their N-terminal epitope must have resulted from proteolytic cleavage (methionine aminopeptidase or endoproteolytic cleavage), we used the



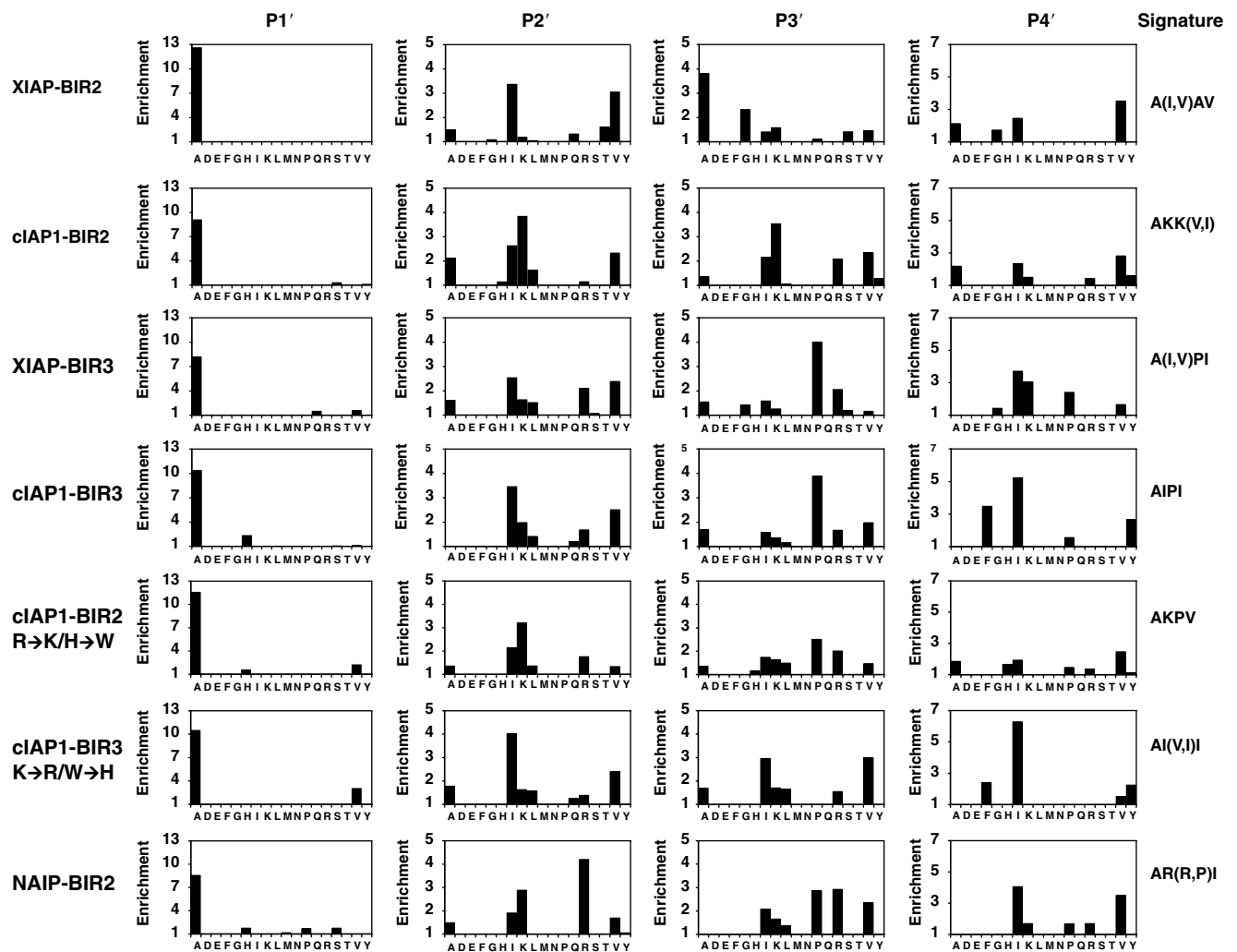
**Figure 2** Flowchart depicting the method for the isolation of IBM groove-directed peptides. The peptide library is incubated with the BIR domain that is immobilized on resin. Washing steps remove nonspecific peptides. A 30% acetic acid treatment removes the BIR and its bound peptides from the resin, which are then dried. The peptides are then redissolved in H<sub>2</sub>O, while the BIR domain remains precipitated. The redissolved peptides are subjected to a negative selection step that entails incubating them with the respective IBM groove mutant BIR domain. This will remove any peptides that are not directed toward the region of interest, that being the IBM groove. Remaining peptides (specific IBM groove binders) are collected and analyzed by Edman degradation N-terminal sequencing

naming convention where the first amino acid is called P1', the second P2' and so on. A minor limitation to our profiling method is the omission of Trp, since this residue is destroyed during Edman degradation, and thus cannot be quantitated.

**Peptide-binding preference of BIR2 and BIR3 of XIAP.** We used the BIR2 (a type II BIR) and BIR3 (a type III BIR) domains of XIAP, the most thoroughly characterized IAP to date, to test the accuracy of our binding assay. Our peptide library screen revealed differential binding preferences between these domains (Figure 3). We found that both the BIR2 and BIR3 domains from XIAP have a substantial preference for Ala in the first position (P1'). Utilizing the Ala-fixed library we found that these BIR domains displayed similar preferences in the second position (P2'), dominated by the  $\beta$ -branched amino acids Val and Ile, and also Arg. This finding is consistent with specificities obtained with peptides based on the natural IAP-binding protein SMAC, and with a SMAC peptide in which the

Val in P2' was replaced by Arg.<sup>21</sup> The preference in the third position (P3') was notably different between BIR2 and BIR3, with the BIR3 domain selecting for Pro, while the BIR2 domain preferred Ala. Arg was the second most preferred residue in P3' by BIR3, consistent with Sweeney *et al.*,<sup>7</sup> while Gly was the second most selected for residue in P3' by BIR2, consistent with Franklin *et al.*<sup>5</sup> We observed some distinctions in selectivities between the BIR2 and BIR3 domains of XIAP in the fourth position (P4') in which the former preferred Val and the latter Ile. In summary, the peptide-binding specificity of XIAP-BIR3 (A-I/V-P-I) very closely resembles the mature N-terminal epitope of SMAC (A-V-P-I), while that of XIAP-BIR2 (A-I-A-V) was distinct from this classic consensus (Figure 3). The differential specificity in the P3' position demonstrates that IAPs have distinct intrinsic binding selectivities.

Our results are in agreement with a recent study that described several novel IBM-containing XIAP-BIR2 interacting proteins, many of which lacked Pro in P3',<sup>22</sup> and also



**Figure 3** Specificity profile of several IAP BIR domains. The binding enrichment of each amino acid at P1'–P4' is representative of the molar fraction of a given residue above background. Values above 1.0 demonstrate a positively selected binding event. The molar fraction is the ratio of a given amino acid to the total quantity of amino acids at the given position

the XIAP-BIR domain-profiling studies mentioned above.<sup>5,7</sup> Although the BIR2 did not select for Pro in P3', it is known that the presence of this residue does not preclude BIR2 binding, since this BIR domain has been shown to bind several proteins with Pro in this position.<sup>11,12,23</sup> Thus, we can conclude that although the Pro does not contribute to the BIR2-IBM interaction, as it does in the case of BIR3, the presence of this residue does not preclude the interaction, it is simply overruled by other residues. This demonstrates that we are determining the optimal binding preference of these BIR domains and not simply allowable binding events. These studies confirm that the basis for the varying binding affinities for SMAC peptides by XIAP-BIR2 and XIAP-BIR3 ( $K_D$  of 6 and 0.4  $\mu$ M, respectively)<sup>8</sup> is due to differences in the inherent specificities of the IBM groove. Having validated our approach, we proceeded to determine whether the IBM grooves of various BIR domains from other IAPs maintain differences in specificity.

**Peptide-binding preference of BIR2 and BIR3 of cIAP1.** Similar to our finding with the XIAP-BIR domains, we found that the BIR2 and BIR3 domains of cIAP1 possess different binding specificities. Again the most notable difference was the preference for Pro in P3' by BIR3 but not by BIR2. Furthermore, the ideal sequence of the cIAP1-BIR3 (A-I-P-I) was essentially the same as XIAP-BIR3 (Figure 3). The BIR2 domain demonstrated a preference for Lys in P2' and P3', and thus seems to have a binding signature (A-K-K-V) that distinguishes it from XIAP-BIR2 (Figure 3). Because P2' and P3' also accommodate aliphatic residues, it is likely that the aliphatic nature of the Lys (and Arg) side-chain dominated interactions, rather than the  $\epsilon$ -amine. These findings, coupled with those of the XIAP-BIR domains, suggests that BIR3 domains select for Pro in P3', whereas BIR2 domains do not, suggesting that there might be distinctions between type II and type III BIRs. Consequently, we predict that cIAP1-BIR3 would bind to SMAC-like peptides with much greater affinity than would cIAP1-BIR2.

**Specificity determinants of cIAP1-BIR domains.** Analysis of BIR domain structures reveals that this BIR type-defining residue (His or Trp) is in juxtaposition with the P3' residue of bound peptides. We sought to examine whether they serve to determine the distinct P3' specificity of BIR domains. Using

the cIAP1-BIR2 and -BIR3 domains as templates, we switched the Trp and His residues by mutagenesis. Sequence alignment of several IAP BIR domains shows that the BIR2 His residue is usually preceded by Arg, while the BIR3 domain's Trp in this position is usually preceded by Lys (Figure 1). On the grounds that the Arg or Lys residues adjacent to the respective His and Trp residues may provide an important role in the integrity of the BIR domain, we made the double mutant instead of the single mutations of His to Trp and vice versa (i.e. cIAP1-BIR2 R242K/H243W and cIAP1-BIR3 K328R/W329H).

We tested the binding specificities of these mutant cIAP1-BIR domains and as predicted, the mutation in the BIR2 domain (cIAP1-BIR2 R $\rightarrow$ K/H $\rightarrow$ W) endowed this protein the capacity to select for Pro in P3', whereas the converse mutation in the BIR3 domain (cIAP1-BIR3 K $\rightarrow$ R/W $\rightarrow$ H) resulted in loss of Pro selection at this position (Figure 3). This alteration in the selection for Pro binding seemed to be the only major change in binding preference of these mutant BIR domains when compared to their wild-type counterparts. The BIR2 domain still maintained the capacity to bind Lys and Arg in the second and third positions, and the mutated BIR3 domain still demonstrated a preference for binding hydrophobic residues, with the preference for Pro in P3' simply ablated by the mutation.

**Determination of binding affinity with optimal peptides.** To validate specificity determinations of BIR domains, we synthesized individual fluorescent peptides based on the predicted optimal sequences from the library screens. We utilized a fluorescence polarization assay to determine the binding affinities of the optimal peptides, using cIAP1-BIR2 and -BIR3 and their specificity-switched mutants as a model. Interestingly, we found that the BIR3 domains demonstrated higher intrinsic binding affinities for all Ala peptides compared to BIR2 (Table 1). These differences in intrinsic binding affinities are consistent with previous affinity measurements comparing the BIR2 and BIR3 domains of XIAP.<sup>11,13,14</sup>

We asked what effect Pro at P3' would have on the binding affinities of BIRs to their optimal peptides. BIR2 preferred not to have Pro in P3', but the BIR2 specificity-switched mutant demonstrated a substantial gain in affinity for Pro at P3' (Table 1). This confirms the importance of the specificity-defining Trp within the IBM groove. In contrast to BIR2, BIR3

**Table 1** Dissociation constants of the cIAP1 BIR2 and BIR3 domains and the specificity-switched mutants toward optimal peptides elucidated by our profiling studies

XXXX=	$K_D$ ( $\mu$ M) -XXXXGGK(FITC)R			
	AKKV	AKPV	AIPI	AIVI
cIAP1-BIR2 wt	4.18 (0.25)	7.22 (0.53)	8.40 (1.3)	5.60 (0.46)
cIAP1-BIR2 R242K/H243W	17.52 (2.23)	4.70 (0.46)	4.99 (0.47)	17.32 (2.17)
cIAP1-BIR3	2.09 (0.16)	0.135 (0.012)	0.13 (0.013)	0.75 (0.055)
cIAP1-BIR3 K328R/W329H	0.476 (0.04)	0.156 (0.013)	0.16 (0.015)	0.22 (0.015)

The peptide backbone was kept the same as used in profiling assays and the four N-terminal positions were selected according to the library profile signature. A fluorescence polarization assay was used to determine millipolarization (mP) units.  $K_D$  ( $\pm$  S.E. in brackets) was determined using a nonlinear fit of the mP dependency on the BIR concentration. Highlighted table cells represent the optimal peptide sequences for the respective BIR domains predicted from the library screens

bound more tightly to peptides containing Pro at P3', but the specificity-switched BIR3 preserved this affinity, while also gaining affinity for non-Pro-containing peptides. Thus, it seems that the presence of the Trp within this groove serves to exclude binding to non-optimal peptides rather than contribute to the overall binding affinity.

**Peptide-binding preference of BIR2 and BIR3 of NAIP.** Armed with our analysis of the requirements for peptide binding in the IBM groove of BIR domains, we set out to predict and test the specificity of the BIR domains of NAIP. These domains are somewhat of an anomaly within the IAP family, with only BIR2 appearing to possess the residues defining classic IBM groove (Figure 1b). Interestingly, NAIP-BIR2 has an IBM groove that more closely resembles a type III BIR domain and based on our profiling, mutagenesis and kinetic experimentation, we predicted it should select for Pro in P3'. We found that NAIP-BIR2 did indeed select for the Pro in P3', thereby confirming our prediction that type III BIR domains have a P3'-Pro selectivity (Figure 3). Like XIAP-BIR3, NAIP-BIR2 also demonstrated a selection for Arg in P3', indeed, this was more enriched than Pro at this position. The BIR3 domain of NAIP is distinct from all other BIR domains in that it has a Cys residue that replaces the Trp or the His and we have classified as a type X BIR. When we analyzed the peptide-binding specificity of NAIP-BIR3 with both peptide libraries, we found that it was unable to bind peptides with any preference (data not shown), suggesting that this BIR domain does not possess the IBM peptide-binding surface groove.

## Discussion

We have developed a method to define the binding specificity of the IBM groove possessed by many IAP BIR domains. Previous studies have demonstrated that the minimal IBM sequence is a tetrapeptide.<sup>8–11</sup> The dominant site in the IBM groove binds an N-terminal Ala of this peptide, and the other sites in the groove are important in selecting optimal peptide binding. Our studies have revealed that BIR domains from various IAPs possess distinct binding signatures. Therefore, the concept of a single IAP-binding motif must be modified. We have also formulated a set of rules that will allow for a prediction of IBM groove specificity. Type III BIR domains (which possess a Trp corresponding to residue 323 of XIAP) demonstrate the classic SMAC-like binding signatures, while type II BIR domains (which possess a His corresponding to residue 223 of XIAP) have more varied binding signatures. We show that the characteristic of type III BIRs is the selection for Pro in P3', which is also seen in the single BIR domain of ML-IAP.<sup>5</sup> In contrast, the type II BIR domains tested, XIAP-BIR2 and cIAP1-BIR2, selected against Pro, but otherwise demonstrated no dominant specificity for P3'. Therefore, classifying BIR domains based on the presence of the defining residue within the IBM groove, His for type II and Trp for type III, is consistent with functionality. Using cIAP1 as a model, it is clear that BIR2 and BIR3 domains demonstrate different intrinsic binding affinities: the BIR3 domain bound all Ala peptides substantially tighter. Interestingly, the presence of Trp that forms part of the wall of the IBM groove seems to

largely contribute to specificity, and only minimally to overall affinity, by prohibiting binding to non-optimal peptides.

The basis for the differential intrinsic binding affinities of BIR2 and BIR3 domains is unclear. By examining the structures of several BIR domains in complex with peptides it became apparent that the type III domains possess a pocket that accepts the fourth residue of the peptide (P4' – see Figure 1c). In our hands, the preferred P4' residues of type III domains are  $\beta$ -branched hydrophobes (Ile, Val and Phe). The related hydrophobic residue Trp (not present in our library for technical reasons) has also been found to occupy this site previously.<sup>24</sup> This pocket is substantially shallower in type II BIRs, and this may be the reason for the skewed affinities between these two types of BIR domains. Interestingly, in the type III BIRs, proper positioning of P4' into the pocket necessitates a kink in the peptide chain at the third residue (Figure 1c). Pro in the third position is ideal to obtain the proper peptide conformation to align the fourth residue into this pocket. Two factors seem to contribute to the P3' Pro-specificity of the type III BIRs, the narrowing of the groove by the Trp residue and the requirement of a kinked peptide chain to properly align the P4' residue into its pocket.

The importance of relationship between P3' Pro and the P4' pocket is also highlighted by BIRs of the *Drosophila* IAP1 (DIAP1). Both domains of DIAP1 have a Trp residue lining the IBM groove wall, and are therefore type III domains. Pulldown experiments with the IAP antagonists Hid, Rpr, Grim and Jafrac, although they are not quantitative, suggest qualitative differences in specificity between the DIAP1 BIRs.<sup>25</sup> Crystal structures of DIAP1 BIRs show that the BIR1 pocket is occluded, cannot accommodate a bulky residue and therefore cannot take advantage of a Pro in P3' to orient the peptide chain.<sup>26,27</sup> This fits with its preference for interacting with Rpr and Grim, which do not have Pro in P3'. In contrast, the BIR2 domain has a fully formed P4' pocket, and is a typical type III domain. Unfortunately, quantitative data on peptide binding to DIAP1 is unavailable to allow us to dissect the binding modes in more detail.

Given the distinctions in binding specificity between type II and type III BIRs, it is natural to ask what the biological consequences are. It has been suggested that different types of BIR domains select different target proteins.<sup>22</sup> Several IAPs contain tandem type II and type III BIRs, and this seems to be important in enhancing avidity toward target proteins. For example, SMAC and caspase-9 have an IBM that is optimal for binding type III BIRs, indeed this is part of the mechanism of inhibition of caspase-9 by XIAP-BIR3, as well as derepression of inhibition of caspase-9 by SMAC.<sup>8,9,13,14</sup> The  $K_D$  for SMAC and BIR3 is in the 200–700 nM range, which is above the  $K_i$  for caspase-9 inhibition.<sup>28</sup> Therefore, to be effective the  $K_D$  of the SMAC–IAP interaction must be decreased *in vivo*. This is accomplished by the natural dimeric conformation of SMAC where one IBM interacts with BIR3 and the other with BIR2 of XIAP.<sup>29</sup> This substantially decreases its  $K_D$  to 0.31 nM because of the two-site interaction-driven avidity. A recent study utilizing a dimeric SMAC peptide further confirmed the requirement of a two-site binding mechanism for efficient derepression of XIAP-mediated inhibition of caspase-3 and -7.<sup>30</sup> Interestingly, when screening the dimeric human IAP survivin, we were unable to detect any peptide hits above

background (data not shown), suggesting that survivin may not possess a functional IBM groove, in line with its very weak binding of IBMs.<sup>31</sup>

Although there are many published BIR domain affinity measurements, with  $K_D$ 's in the hundreds of nanomolar to micromolar range, these are almost always carried out on peptides and not full-length natural proteins.<sup>5,7,8</sup> Some full-length proteins may have interactions with IAPs in addition to the N-terminal epitope, indeed the property leading to a decrease in  $K_D$  into the physiological range is driven largely by two-site interactions.<sup>3,29</sup> On the basis of the findings with SMAC, we suggest that the best candidate target proteins that interact with multi-BIR IAPs, would also likely be dimeric, or even polymeric. It follows that single BIR-containing IAPs would require an additional non-IBM groove-directed interaction to bind target proteins in a physiological setting.

It is interesting to compare BIR domains with PDZ domains, both of which interact with terminal peptide extensions found on their target proteins. BIR domains have an affinity for N-terminal epitopes, whereas PDZ bind C-terminal peptide extensions. Binding studies using simple peptides reveal affinities in the micromolar range, similar to BIR domains and peptides.<sup>32</sup> At one stage, PDZ domains were classified into two classes (type I and type II), but a more recent study concluded that binding specificity is more widely distributed across a sample of 157 mouse PDZ domains.<sup>33</sup> Moreover, it has been discussed that PDZ domain/peptide analyses may have oversimplified the understanding of these interactions *in vivo*, because two-site interactions that are likely a critical determinant of physiological PDZ binding are often overlooked.<sup>34</sup> Indeed, multimerization of PDZ-containing proteins and their receptor targets might constitute physiological regulatory mechanisms, and so the enhancement of avidity by multiple interactions of inherently weak ligands may be a common theme in BIRs and PDZs.

The fact that IBM groove-directed binding necessitates exposed N-terminal motifs places further restrictions on potential binding partners. There are two subsets of proteins that could possess an exposed N-terminal Ala residue. First, those that possess an Ala generated by the removal of the initiator Met by methionine aminopeptidase, which always occurs with cytosolic proteins if an Ala is present in the second position. However, the resulting N-terminal Ala is usually acetylated in the cytosol, thereby making it unavailable as an IBM.<sup>35</sup> The second subset is defined by proteins that have undergone an endoproteolytic cleavage, exemplified by the caspases. Mitochondrial proteins often have their signal peptides removed once that have translocated into the mitochondria. These proteins remain unacetylated and comprise the most likely pool of candidate IAP target proteins, exemplified by SMAC and HtrA2. Figure 4 presents a collective set of previously identified IBM-containing proteins<sup>11–15,20,22,23,36,37</sup> which we have divided based on whether they were shown to interact with type II or type III BIRs. The sequences of these proteins naturally fall into two categories that closely resemble the profiles elucidated for type II and type III BIR domains in our studies. The results of our studies provide a context for understanding the natural binding mechanism of BIR domain and various IBM-containing proteins.

	Binding Specificity
CLPX	ASKD
LRPPR	AIAA
3HB	ASKT
Nsp4	ATGP
GdH	SEAV
Caspase-3	SGVD
Caspase-7	ANPR
GSPT	AKPF
SMAC	AVPI
HtrA2	AVPS
Caspase-9	ATPF

**Figure 4** Mammalian IAP-binding motif-containing proteins. Data collected from the literature where specific proteins have been demonstrated to interact with individual BIR domains.<sup>11–15,20,22,23,36,37</sup> We have divided the interactions according to the type of domain reported to interact. Note the preference for Pro in the third position of the natural proteins that interact with type III BIRs, which is in agreement with our positional scanning data

Our definition of the peptide-binding preference of various BIR domains can be utilized to create tools to dissect the roles of distinct IAP in signaling pathways, elucidate new binding partners and provide insight into the cellular roles of the IAPs. IAP antagonists, namely SMAC mimetics, are currently being explored as cancer therapeutics – reviewed in Vucic and Fairbrother<sup>38</sup> – and our findings here provide a focus point for knowledge-based drug design in this arena. Finally, we point out that, despite the two-site avidity enhancement of natural partners, monomeric small molecule IAP antagonists, targeting the IBM groove or ancillary sites, – reviewed in Schimmer *et al.*<sup>39</sup> – are clearly effective in reactivating apoptosis in cancer cells. Targeting a single site on the BIR should be sufficient to ablate that site, force the IAP to rely on the remaining binding site, and thereby increase the  $K_D$  for the overall target protein interaction above the physiological range.

#### Materials and Methods

**Peptide library synthesis.**  $\text{NH}_2\text{-XXXXGGK(FITC)R-CONH}_2$  (library 1) and  $\text{NH}_2\text{-AXXXGGK(FITC)R-CONH}_2$  (library 2) libraries, where X denotes an isokinetic mixture of all natural amino acids with the exception of Trp and Cys, were synthesized as follows in Burnham Institute's peptide synthesis facility and the laboratory of Dr. Ziwei Huang. Both protected peptide libraries were synthesized using an ABI 433 Peptide Synthesizer by Fmoc-based solid-phase peptide synthesis on NovaSyn TGR Rink-amide resin (0.3 mmol/g, 830 mg, 0.25 mmol) with real-time monitoring of deprotection efficiencies. 1-(4-4-Dimethyl-2-6-dioxocyclohex-1-ylidene)ethyl (Dde) was employed for side-chain protection of Lys in the second position. Fmoc amino acids (1.25 mmol, 5.0 equiv.) were sequentially coupled to a free amino group in dimethyl formamide (DMF) for 2 h, using 2-(1*H*-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.25 mmol, 5.0 equiv.) in the presence of *N*-hydroxybenzotriazole (HOBT) (2.5 mmol, 10.0 equiv.). For residues in the random positions, an isokinetic mixture was created and applied by using a ratio of equivalents of amino acids based on their reported coupling rates.<sup>40</sup> All the natural amino acids were included into isokinetic mixture except of the Trp and Cys, because Trp cannot be quantitated by Edman degradation, while Cys presents oxidation problems. Fmoc deprotection was performed by 20% piperidine in DMF ( $2 \times 15$  min). The last amino acid coupled to the peptide sequence was N-terminally Boc protected. The resulting protected resin was transferred to a glass reactor and all the steps from this point were performed manually. The resin was treated with 2% hydrazine in DMF ( $2 \times 20$  min) to selectively remove the Lys side-chain-protecting group. After washing with DMF (three times), and a positive hydrazine test, the resin was treated with FITC (3 equiv.), in the presence of diisopropylethylamine (6 equiv.) in a minimal amount of DMF (just to form a slurry) for 24 h and protection from light. After

washing with DMF (three times) and ninhydrin test confirming full substitution, the resin was washed with dichloromethane (three times), tetrahydrofuran (three times) and MeOH (three times) and dried overnight in a vacuum. Cleavage from the resin was carried out using TFA (92.5%) in the presence of *m*-cresol (2.5%), triisopropylsilane (2.5%) and H<sub>2</sub>O (2.5%) for 3 h. After removal of the resin by filtration and washing with TFA with H<sub>2</sub>O (two times), the filtrate was poured into ice-cold dry *tert*-butyl methyl ether. The resulting powder was collected by centrifugation and washed with ice-cold dry diethyl ether. The final product was dissolved in water and lyophilized. Library 1 consists of the 104 976 individual peptide sequences and library 2 consists of 5832 peptide sequences.

**Optimal peptide synthesis.** Peptides of the following sequences H<sub>2</sub>N-AKKVGGK(FITC)R-COOH<sub>2</sub>, H<sub>2</sub>N-AKPVGGK(FITC)R-COOH<sub>2</sub>, H<sub>2</sub>N-AIPIGGK(FITC)R-COOH<sub>2</sub> and H<sub>2</sub>N-AIVIGGK(FITC)R-COOH<sub>2</sub> were synthesized manually using a classic mixed Boc and Fmoc strategy, with the help of the Burnham Institute Peptide Synthesis Facility. The PEG Rink Amide resin (EMD, San Diego, CA, USA) was derivatized with Fmoc-Arg(Pbf)-OH using HBTU-HOBt coupling reagents and after all washing steps deprotected with 20% piperidine in DMF. Subsequent amino acids were loaded using the same protocol. The Lys side chain was protected with a Dde-protecting group as described above. The N-terminal amino acid was Boc protected. After synthesis of the full sequence, the Lys side chain was deprotected using 2% hydrazine in DMF and washed. Overnight FITC derivatization (double excess) of the Lys side chain gave the desired peptide, which after washing and drying was deprotected using the mixture TFA/water/TIPS (95:2.5:2.5). The purification using preparative HPLC and subsequent lyophilization gave the desired peptide. Yellow powder. Mass determination: H<sub>2</sub>N-AKKVGGK(FITC)R-COOH<sub>2</sub>: calculated 1231.8, ESI = 1233.8; H<sub>2</sub>N-AKPVGGK(FITC)R-COOH<sub>2</sub>: calculated 1200.4, ESI = 1200.6; H<sub>2</sub>N-AIPIGGK(FITC)R-COOH<sub>2</sub>: calculated 1199.1, ESI = 1199.4; H<sub>2</sub>N-AIVIGGK(FITC)R-COOH<sub>2</sub>: calculated 1200.4, ESI = 1201.6.

**Recombinant protein production and purification.** IAP BIR domains and mutants thereof were cloned into a modified pET15b (Novagen, Madison, WI, USA) that provides a N-terminal His tag. The domain limits and IBM groove mutants for each BIR domain tested are as follows: XIAP-BIR2: 124–237, E219R/H223V; XIAP-BIR3: 252–348, Q319R/W323V; cIAP1-BIR2: 146–260, E239R/H243V; cIAP1-BIR3: 257–354, E325R/W329V; NAIP-BIR2: 128–248, E216V/W220R; NAIP-BIR3: 256–363, D334R/C338V. Recombinant proteins were synthesized in *E. coli* strain BL21. Cells were grown at 37°C and protein expression was induced with the addition of 0.4 mM IPTG when cells reached an OD<sub>600</sub> of 0.6. Cultures were incubated for 4 h at 30°C following IPTG induction. Proteins were purified using chelating Sepharose (APBiotec Inc.) charged with NiSO<sub>4</sub> in a buffer containing 50 mM HEPES and 100 mM NaCl at pH 7.4. For binding assays, proteins were left on beads and were washed three times in each of the following buffers: (1) 50 mM HEPES, 100 mM NaCl, 20 mM imidazole pH 7.4, (2) 50 mM HEPES, 100 mM NaCl, 1% Triton X-100 pH 7.4 and (3) 50 mM HEPES, 100 mM NaCl pH 7.4. For fluorescence polarization, proteins were eluted in a buffer containing 50 mM HEPES, 100 mM NaCl, 200 mM imidazole pH 7.4.

**Determination of the peptide-binding preference of BIR domains.** Recombinant BIR domains immobilized on Ni-charged resin were incubated at a concentration of 20 μM with the peptide library at 2 mg/ml in H<sub>2</sub>O. Following a 30-min incubation at room temperature with rocking, beads were washed three times with 20 mM imidazole, 50 mM HEPES, 100 mM NaCl pH 7.4 to remove nonspecific interactions. Beads were then washed an additional five times in the same buffer without imidazole. Peptides were eluted using 30% acetic acid and dried by speed vac. Dried peptides were redissolved in H<sub>2</sub>O and incubated for 30 min with the IBM groove mutant BIR domain immobilized on Ni-charged resin to remove any non-IBM groove-directed peptides. The supernatant was subsequently dried by speed vac and redissolved in H<sub>2</sub>O. Peptide sequences were determined by Edman degradation N-terminal sequencing in a Procise 492 protein sequencer (Applied Biosystems). The quantity of each amino acid from the selected peptide sample was determined by subtracting the background of each amino acid from a control sample in which the respective BIR domain was incubated with H<sub>2</sub>O instead of the peptide library. The adjusted quantity of each amino acid was normalized to yield a ratio of relative abundance at each position. Binding enrichment in each cycle was determined by multiplying this ratio by 18 (the number of individual amino acids in each position) to display positive binding events as values above 1.0.

**Binding recovery analysis.** Total quantity of peptide recovered after binding was determined using an Fmax spectrophotometer (Molecular Devices) at an excitation of 485 nm and an emission of 538 nm for the FITC tag and compared with the standard curve.

**Fluorescence polarization assays.** Fluorescence polarization studies were carried out on the optimal peptides of cIAP1-BIR2, cIAP1-BIR2 R242K/H243W, cIAP1-BIR3 and cIAP1-BIR3 K328R/W329H. These peptides all had the same backbone as used in the profiling screen (XXXXGGK(FITC)R), and the four N-terminal positions (P1'–P4') were varied to match the optimal signatures. Peptide (5 nM) was used and the concentration of the BIR domain was varied from 1 nM to 27 μM. The ratio of bound ligand to free ligand was determined using a LJI Analyst (Molecular Devices) with excitation at 485 nm and emission at 530 nm. K<sub>D</sub> was determined using a nonlinear fit of the curve generated by plot of millipolarization (mP) units versus BIR concentration. The equation used was  $mP = A_f + [BIR] (A_b - A_f) / (K_D + [BIR])$  where mP is the measured millipolarization units, A<sub>f</sub> is the free fluorescent molecule, A<sub>b</sub> is the bound fluorescent molecule and K<sub>D</sub> represents the dissociation constant of the BIR and peptide.

**Acknowledgements.** We thank Annemarie Price for her help with protein expression, Dr. Ben Turk (Yale University) for his helpful advice during assay design, Dr. Christina Pop for her assistance with data analysis, Dr. Ziwei Huang for advice on peptide synthesis, and Kate Welsh for help with the FPA assays. This study was supported by National Institutes of Health Grants NS37878, AI61139 and 2T32CA77109.

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