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Structural Analysis of a Functional DIAP1 Fragment Bound to Grim and Hid Peptides

Jia-Wei Wu,[1] Amy E. Cocina,[1] Jijie Chai,[1] Bruce A. Hay,[2] and Yigong Shi[1,3]
[1]Department of Molecular Biology
Lewis Thomas Laboratory
Princeton University
Princeton, New Jersey 08544
[2]Division of Biology
California Institute of Technology
Pasadena, California 91125

Summary

The inhibitor of apoptosis protein DIAP1 suppresses apoptosis in *Drosophila*, with the second BIR domain (BIR2) playing an important role. Three proteins, Hid, Grim, and Reaper, promote apoptosis, in part by binding to DIAP1 through their conserved N-terminal sequences. The crystal structures of DIAP1-BIR2 by itself and in complex with the N-terminal peptides from Hid and Grim reveal that these peptides bind a surface groove on DIAP1, with the first four amino acids mimicking the binding of the Smac tetrapeptide to XIAP. The next 3 residues also contribute to binding through hydrophobic interactions. Interestingly, peptide binding induces the formation of an additional α helix in DIAP1. Our study reveals the structural conservation and diversity necessary for the binding of IAPs by the *Drosophila* Hid/Grim/Reaper and the mammalian Smac proteins.

Introduction

Apoptosis plays a central role in the development and homeostasis of all multicellular organisms (Abrams, 1999; Bangs and White, 2000; Horvitz, 1999; Jacobson et al., 1997; Steller, 1995). Alterations in apoptotic pathways have been implicated in many types of human pathologies, including developmental disorders, cancer, autoimmune diseases, as well as neurodegenerative disorders (Green and Martin, 1995; Thompson, 1995; Yuan and Yankner, 2000). Central to the induction of apoptosis in all metazoans examined to date is the activation of initiator and effector members of the caspase family of proteases (Budihardjo et al., 1999; Chinnaiyan and Dixit, 1996; Thornberry and Lazebnik, 1998).

Many members of the inhibitor of apoptosis (IAP) family of proteins suppress programmed cell death, at least in part, by physically interacting with and inhibiting the catalytic activity of caspases (Deveraux and Reed, 1999; Miller, 1999). An important functional unit in all death-inhibiting IAP proteins is the so-called baculoviral IAP repeat (BIR), which contains approximately 80 amino acids folded around a zinc atom (Fesik, 2000). The *Drosophila* genome contains four genes that encode proteins with BIR domains (Hay, 2000; Vernooy et al., 2000). The overexpression of two of these, DIAP1 and DIAP2, inhibit both normal developmental cell death and apoptosis induced by expression of proapoptotic genes (Hay et al., 1995). In addition, DIAP1 is required for cell survival in the embryo and in a number of adult tissues (Goyal et al., 2000; Hay et al., 1995; Lisi et al., 2000; Wang et al., 1999). These observations, in conjunction with others showing that DIAP1 binds and inactivates several *Drosophila* caspases (Hawkins et al., 1999; Kaiser et al., 1998; Wang et al., 1999) and that loss of DIAP1 results in an increase in caspase activity in vivo (Wang et al., 1999), argue that DIAP1's function as a caspase inhibitor is required for cell survival. DIAP1 contains two N-terminal BIR repeats and a C-terminal RING domain. DIAP1 fragments containing the BIR2 domain are sufficient to prevent cell death in a number of contexts (Hay et al., 1995; Vucic et al., 1998b). Interestingly, fragments consisting of the BIR2 and surrounding linker sequences also bind multiple proapoptotic proteins, including the apical caspase DRONC (Meier et al., 2000), and Hid, Grim, and Reaper (Vucic et al., 1997, 1998b).

One mechanism by which Hid, Grim, and Reaper promote cell death is by binding to DIAP1, thereby inhibiting its function as a caspase inhibitor (Goyal et al., 2000; Wang et al., 1999). Although Hid, Grim, and Reaper perform a similar function in promoting cell death, they only share homology in the N-terminal 14 residues of their primary sequences (Chen et al., 1996). These N-terminal sequences are sufficient to mediate interactions with DIAP1 (Vucic et al., 1998a) and with several mammalian IAPs (McCarthy and Dixit, 1998). In the case of Hid in insects (Vucic et al., 1998a), and Hid and Reaper in mammalian cells (Haining et al., 1999; McCarthy and Dixit, 1998), these N-terminal sequences are essential for proapoptotic function.

At least seven mammalian IAPs have been identified, including X-linked IAP (XIAP), cIAP1, cIAP2, and survivin (Deveraux and Reed, 1999). Most mammalian IAP proteins contain more than one BIR domain, with the different BIR domains exhibiting distinct functions (Deveraux et al., 1999). For example, in XIAP, the third BIR domain (BIR3) potently inhibits the activity of the processed caspase-9, whereas the linker region between BIR1 and BIR2 selectively targets the active caspase-3 or -7 (Chai et al., 2000, 2001; Srinivasula et al., 2000; Sun et al., 1999, 2000; Takahashi et al., 1998).

In mammalian cells, caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol upon apoptotic stimuli (Du et al., 2000; Verhagen et al., 2000). Smac/DIABLO physically interacts with multiple IAPs and relieves their inhibitory effect on both initiator and effector caspases (Chai et al., 2000; Srinivasula et al., 2000). Thus, Smac/DIABLO represents the mammalian functional homolog of the *Drosophila* Hid, Grim, and Reaper proteins. Recent structural studies reveal that the N-terminal tetrapeptide of Smac/DIABLO binds a surface groove on XIAP-BIR3, thus competitively removing the inhibition of caspase-9 by XIAP (Liu et al., 2000; Srinivasula et al., 2001; Wu et al., 2000). Smac/DIABLO shares sequence homology

[3]Correspondence: yshi@molbio.princeton.edu

Table 1. Data Collection and Statistics from Crystallographic Analysis

Data set (space group)	DIAP1-BIR2 (I4)	BIR2-Grim ($P6_522$)	BIR2-Hid ($P6_522$)
Resolution (Å)	99.0–2.7	99.0–1.9	99.0–2.7
Total observations	36,725	244,305	87,781
Unique observations	7,220	12,525	4,535
Data coverage (outer shell)	95.1% (95.5%)	98.8% (97.3%)	98.3% (98.4%)
R_{sym} (outer shell)	0.113 (0.437)	0.052 (0.160)	0.133 (0.381)
Refinement			
Resolution range (Å)	20.0–2.7	20–1.9	20.0–2.7
Number of reflections	7,176 (all)	11,697 ($I>\sigma$)	4,519 (all)
$R_{working}/R_{free}$	24.6%/28.9%	20.2%/24.3%	21.7% (28.7%)
Number of atoms	1,588	1,062	968
Number of waters	18	58	36
R.m.s.d. bond length (Å)	0.008	0.007	0.010
R.m.s.d. bond angles (degree)	1.334	1.253	1.434

$R_{sym} = \Sigma_h\Sigma_i|I_{h,i} - I_h|/\ \Sigma_h\Sigma_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry-related reflections of h. $R = \Sigma|F_{obs} - F_{calc}|/\ \Sigma F_{obs}$, where $F_{obs} = F_P$, and F_{calc} is the calculated protein structure factor from the atomic model (R_{free} was calculated with 5% of the reflections). R.m.s.d. in bond lengths and angles are the deviations from ideal values, and the r.m.s.d. deviation in B factors is calculated between bonded atoms.

with Hid, Grim, and Reaper only in the N-terminal 4 residues, prompting the hypothesis that Hid, Grim, and Reaper interact with DIAP1 using similar tetrapeptides and binding to a similar surface groove on DIAP1 (Wu et al., 2000).

There is currently no structural information on DIAP1 or Hid, Grim, or Reaper. To investigate the structural mechanisms of DIAP1 recognition by the *Drosophila* Hid, Grim, and Reaper proteins, we have crystallized the DIAP1-BIR2 domain by itself and in complex with the N-terminal peptides from both Hid and Grim and determined these structures at 2.7, 2.7, and 1.9 Å resolution, respectively. In analogy to the Smac-XIAP interactions, the first four amino acids of Hid and Grim bind an evolutionarily conserved surface groove on DIAP1-BIR2. The next 3 conserved residues of Hid and Grim also contribute to the interactions with DIAP1 through extensive van der Waals contacts. Interestingly, peptide binding to DIAP1-BIR2 appears to induce the formation of an additional α helix, which appears to stabilize peptide binding. In conjunction with biochemical analysis, our structural study reveals a molecular basis for the conservation and diversity necessary for the recognition of IAPs by the *Drosophila* Hid/Grim/Reaper and the mammalian Smac proteins. These results have important ramifications for the design of IAP inhibitors toward therapeutic applications.

Results

Rationale and Structure Determination

Previous studies suggest that the BIR2 domain of DIAP1 is sufficient for interactions with the N-terminal sequences of the *Drosophila* proteins Hid, Grim, and Reaper. Therefore, we purified the individual BIR1 (residues 1–145) and BIR2 (residues 201–325) domains to homogeneity and incubated these BIR domains with the N-terminal peptides of Hid and Grim. Gel filtration and mass spectroscopic analyses demonstrate that only the BIR2 domain forms a stable complex with the Hid or Grim peptide (data not shown). In contrast, no stable association was detected between the BIR1 domain and either peptide. Although the N-terminal 14 residues of Grim and Reaper share sequence homology, the conserved sequences among all three *Drosophila* proteins are restricted to the first 8 residues, suggesting a consensus binding element to DIAP1. Indeed, longer peptides from Grim exhibit the same binding affinity to DIAP1, as does a minimal 8-residue peptide (data not shown). We chose to use 10-residue peptides as derived from the N-terminal sequences of Grim and Hid. The N-terminal sequence of Reaper closely resembles that of Grim.

We purified binary complexes between Hid or Grim and DIAP1-BIR2 by gel filtration and crystallized both complexes. To assess possible conformational changes associated with peptide binding, we also crystallized the DIAP1-BIR2 domain by itself. The structure of the BIR2 domain by itself was determined by molecular replacement using atomic coordinates of XIAP-BIR3 as the initial search model (Protein Data Bank code 1G73). The structures of peptide-bound BIR2 domains were solved by molecular replacement using the atomic coordinates of the partially refined BIR2 domain. The final atomic models were refined at 2.7, 2.7, and 1.9 Å, respectively, for the BIR2 by itself and in complex with the Hid and Grim peptides. All three structures exhibit excellent stereochemical parameters (Table 1).

Structure of DIAP1-BIR2

The final atomic model of DIAP1-BIR2 by itself contains residues 215–309 comprising five α helices, a three-stranded β sheet, and a zinc atom chelated by three Cys and one His residues (Cys263, Cys266, His283, and Cys290; Figures 1 and 2). The structure is similar to those of other BIR domains in XIAP (Liu et al., 2000; Sun et al., 1999, 2000; Wu et al., 2000) and survivin (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000), with root-mean-square-deviation (r.m.s.d.) of less than 2 Å for all aligned Cα atoms.

An 8-residue peptide from Hid or Grim binds a surface groove in an extended conformation, forming a fourth strand to the three-stranded β sheet on DIAP1 (Figures 1B and 1C). Peptide binding appears to induce a major conformational switch in DIAP1, as shown by the formation of an additional α helix, α6 (Figures 1B and 1C).

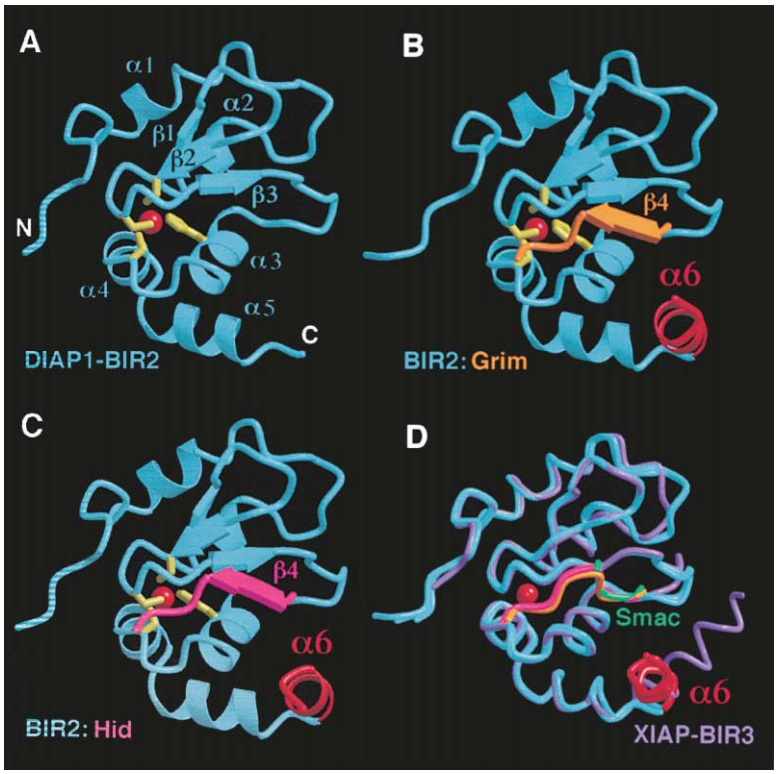


Figure 1. Overall Structure of the DIAP1-BIR2 Domain by Itself (A) and in Complex with the Grim (B) or Hid (C) Peptide

The DIAP1-BIR2 domain is shown in cyan and the bound Grim and Hid peptides are highlighted in orange and pink, respectively. The zinc atom in the BIR domain is colored red, while its coordinating residues are shown in yellow. Some of the secondary structural elements are labeled. In (D), the structures of DIAP1-BIR2 by itself and in complex with the Hid/Grim peptides are superimposed with that of XIAP-BIR3 in complex with the Smac tetrapeptide (Protein Data Bank code 1G73). The DIAP1-BIR2 and XIAP-BIR3 are shown in cyan and purple, respectively. The bound Grim and Hid peptides are highlighted in orange and pink, respectively, while the Smac tetrapeptide is represented in green. Helix α6, highlighted in red, is only present in the peptide-bound BIR2.

This helix (residues 310–316) is disordered in the apo form of BIR2, likely existing as a flexible loop in solution. Upon binding by the Hid or Grim peptide, this region adopts a helical conformation (Figure 1) and stabilizes peptide binding through hydrogen bonds. Residues on this helix (α6) closely pack against surrounding residues on helix α3 through networks of hydrophobic interactions.

Except for the newly formed helix α6, both the backbone and the side chains of the residues that comprise the peptide binding groove remain identical before and after recognition by the Hid or Grim peptide. In this respect, the binding groove is fairly rigid, and the recognition can be described as a precise lock-and-key docking, although helix α6 also contributes significantly to peptide binding. The overall structure of DIAP1-BIR2 by itself can be superimposed to that bound by the Hid or Grim peptides with 0.54 and 0.53 Å r.m.s.d., respectively, for 96 aligned Cα atoms (Figure 1D). On the other hand, DIAP1-BIR2 domains bound by either Hid or Grim peptide are essentially identical, with 0.17 Å r.m.s.d. for all aligned Cα atoms. In addition, the peptide-bound DIAP1-BIR2 structure also closely resembles that of the mammalian XIAP-BIR3 domain bound by the Smac peptide, with approximately 0.8 Å r.m.s.d. for 95 aligned Cα atoms (Figure 1D).

Peptide Binding Interface

Binding to the DIAP1-BIR2 domain results in the burial of 994 and 990 Å^2 surface area for the Hid and Grim peptides, respectively (Figure 2). The recognition involves both hydrogen bond networks and extensive van der Waals contacts between 7 hydrophobic residues on the peptides and surrounding DIAP1 residues. The eighth conserved residue on the peptides, Glu in Hid and Asp in Grim, exhibits well-defined electron density and points into the solvent phase, making no specific contact to DIAP1 (Figure 3).

The peptide binding surface on DIAP1 is enriched in hydrophobic residues (Figure 3). These residues interdigitate with the corresponding hydrophobic residues in the peptides through numerous contacts. The N termini of these peptides are positioned in a highly negatively charged environment (Figure 3), in which 2 acidic residues (Asp277 and Glu314) in DIAP1 play an essential role in binding the Hid/Grim peptides.

For both Hid and Grim, a network of hydrogen bonds centered on Ala1 plays a central role in recognition specificity. In both cases, the amino group of Ala1 donates two hydrogen bonds to the surrounding residues Asp277 and Gln282, while the carbonyl group accepts two hydrogen bonds from the side chains of Trp286 and Glu314 (Figures 4A and 4C). These central interactions are buttressed by two intramolecular contacts between the side chains of Glu314 and Gln282 and between the backbone groups of Asn274 and Asp277, respectively (Figures 4A and 4C). In addition, four intermolecular hydrogen bonds among the backbone groups place the Hid and Grim peptides in an extended β strand conformation (Figures 4A and 4C).

Although hydrogen bonds appear to provide recognition specificity, van der Waals interactions play a dominant role in stabilizing the interactions between the Hid/Grim peptides and the DIAP1-BIR2 surface groove (Figure 4). The methyl group of Ala1 fits tightly in a hydrophobic pocket formed by the side chains of Leu270 and Trp273 and the aliphatic portion of Gln282 (Figure 4). The

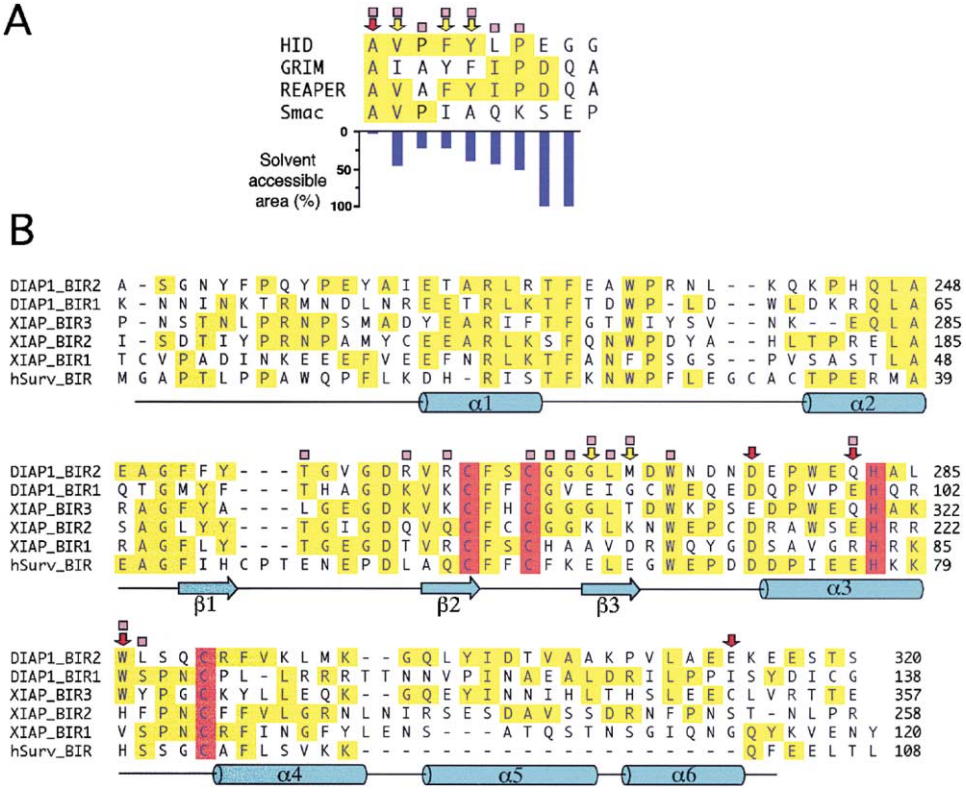


Figure 2. Sequence Alignment of the N-Terminal Peptides from Hid/Grim/Reaper and Smac (A) and of the BIR Domains from DIAP1, XIAP, and Survivin (B)

The zinc-chelating residues are shown in red, whereas the conserved amino acids are highlighted in yellow. Red and yellow arrows identify those residues that make intermolecular hydrogen bonds using their side chain and main chain atoms, respectively. The solvent accessibility for the peptides (A) and the secondary structural elements for the DIAP1-BIR2 domain (B) are indicated below the sequence alignment.

next 6 residues closely follow the hydrophobic surface groove on DIAP1 and make numerous van der Waals contacts (Figure 4). Aside from the invariant Ala1 and Pro7, the intervening 5 residues are also conserved in Hid and Grim. These 5 residues mediate identical sets of interactions with the surrounding residues in DIAP1-BIR2 (Figure 4).

Unique Structural Features

Recent studies on the recognition of XIAP-BIR3 by the Smac tetrapeptide suggested similar modes of interactions between the *Drosophila* proteins DIAP1 and Hid/Grim/Reaper (Liu et al., 2000; Wu et al., 2000). This prediction was supported by reasonable sequence homology among the N-terminal 4 residues of the mammalian protein Smac and the *Drosophila* proteins Hid, Grim, and Reaper (Liu et al., 2000; Wu et al., 2000). Indeed, many of the critical interactions between the Hid/Grim peptides and DIAP1-BIR2 have been observed in the mammalian Smac-XIAP complex, including the anchoring role by Ala1 (Figure 4). Despite this conserved theme, the *Drosophila* complexes exhibit two unique features.

In the mammalian complex, recognition is restricted to the N-terminal tetrapeptide of Smac; the fifth residue is not involved in binding the conserved surface groove on XIAP-BIR3. In the *Drosophila* complexes, however, 3 additional conserved residues make important contributions to DIAP1 binding through van der Waals contacts (Figures 2 and 4). These 3 residues stack against a hydrophobic surface groove comprising Cys266, Gly267, Gly268, Gly269, Trp286, and Leu287 on DIAP1, forming an extensive interface (Figure 4B). The absence of side chains in the 3 consecutive Gly residues is likely a key determinant for the recognition of BIR2 by the Hid/Grim/Reaper peptides, as this feature is not conserved in BIR1. There are also considerable intramolecular packing interactions in the Hid/Grim peptides. For example, the fifth residue, Phe5 in Grim or Tyr5 in Hid, stacks against Pro7 while Ile6/Leu6 reaches out to contact Ala3/Pro3 (Figure 4). The eighth residue, a conserved Glu/Asp in Hid/Grim/Reaper, does not interact directly with DIAP1 and points into the solvent phase. This unique mode of interaction agrees well with the observation that the N-terminal 8 residues are conserved in Hid, Grim, and Reaper.

In the peptide-bound structures of DIAP1-BIR2, there is an additional α helix (α6, residues 310–316). The constituent residues for this helix are completely disordered in the absence of peptide binding and become structured upon binding by the Hid/Grim peptides (Figure 1). Supporting the large conformational switch, residues from this helix pack closely against helix α3 and contribute significantly to peptide binding (Figure 5). At the periphery of the interface between helices α3 and α6,

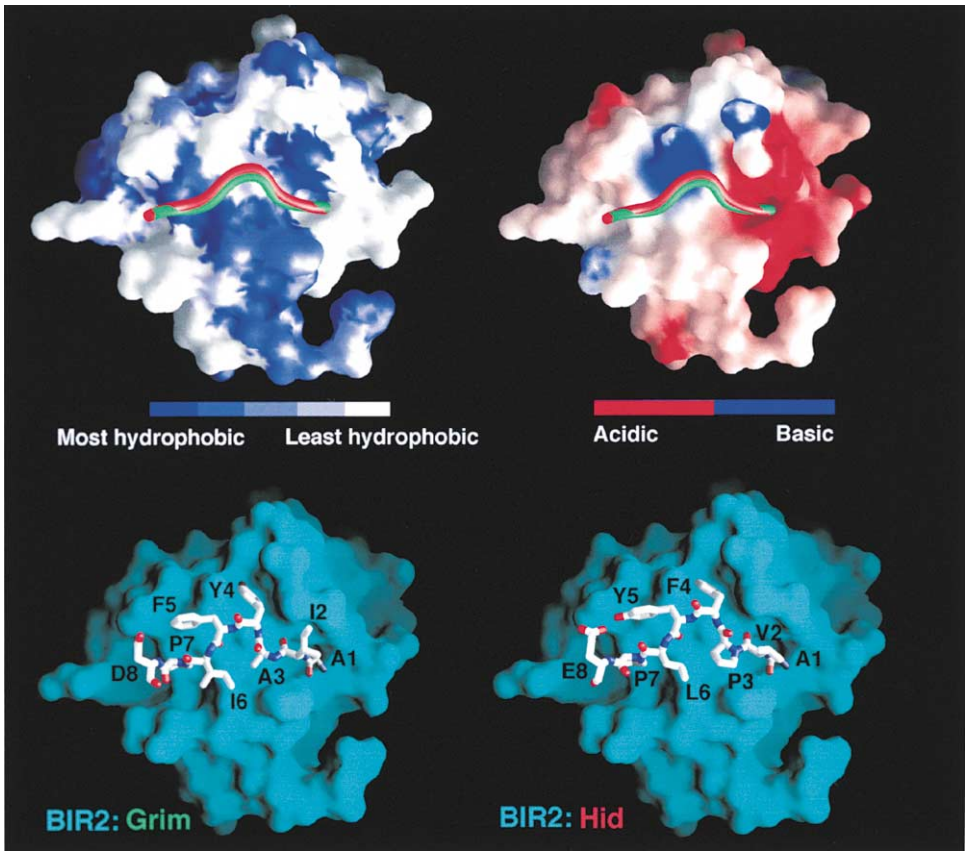


Figure 3. Surface Representation of the DIAP1-BIR2 Domain Bound to the Hid (Red) and Grim (Green) Peptides

The BIR2 surface is colored according to either the scale of hydrophobicity (top left panel) or electrostatic potential (top right panel).

the carboxylate side chain of Glu314 on α6 donates one hydrogen bond to the carbonyl oxygen of Ala1 on the Hid/Grim peptides (Figure 5). Buttressing this interaction, Glu314 makes two intramolecular contacts to Trp286 and Gln282, respectively (Figure 5). In the center, the packing interactions between this additional helix (α6) and the rest of the structure are predominantly hydrophobic in nature, involving 4 residues on α6 (Val310, Leu311, Glu314, and Lys315) and 3 residues on α3 (Gln282, Leu285, and Trp286; Figure 5).

Ligand Diversity and DIAP1 Recognition by Hid/Grim

Since the discovery of an important role by the N-terminal tetrapeptide of Smac (Liu et al., 2000; Wu et al., 2000), another highly conserved tetrapeptide motif has been identified in the mammalian protein procaspase-9, which is generated through proteolytic processing and subsequently involved in binding XIAP (Srinivasula et al., 2001). Together with the N-terminal sequences of Hid/Grim/Reaper, these conserved tetrapeptides define a family of IAP-interacting motifs in both *Drosophila* and mammalian cells (Shi, 2001).

Although these N-terminal sequences are generally conserved, there are considerable variations in the identities of the constituent amino acids in the *Drosophila* proteins Hid/Grim/Reaper and the mammalian proteins Smac and caspase-9. For example, the third residue can be either Ala (Grim/Reaper) or Pro (Hid, Smac, and caspase-9). Although Phe is preferred as the fourth residue (Hid/Reaper and caspase-9), Tyr (Grim) or Ile (Smac) can also be tolerated. The *Drosophila* proteins Hid/Grim/Reaper can induce apoptosis in mammalian cells, suggesting preservation of IAP binding ability. Indeed, the N-terminal peptides of Hid and Grim interact with the mammalian protein XIAP (Figure 6B). This observation raises the obvious question of what sequence elements in these peptides determine recognition specificity to the mammalian protein XIAP and the *Drosophila* protein DIAP1.

To address this issue, we examined the binding affinities between the Hid/Grim peptides and the corresponding BIR domains in DIAP1 and XIAP, using isothermal titration calorimetry (Figure 6). Interestingly, the Hid peptide exhibits a 6-fold higher binding affinity to DIAP1-BIR2 than does Grim. This difference is likely due to the presence of Ala in the third residue position of Grim (Pro3 in Hid), as this is the only significant structural variation in binding (Figure 4). A Pro residue in this position is likely to mediate more van der Waals interactions with Trp286 and Leu270 in DIAP1 (Figure 4). Although both Grim and Hid peptides can bind to the mammalian XIAP-BIR3 domain, the binding affinity is different by nearly 100-fold (Figure 6B). Again, this large discrepancy is most likely due to variation in the third position, from Pro3 in Hid to Ala3 in Grim, because previous studies indicate that the Pro3Ala mutation in Smac peptide re-

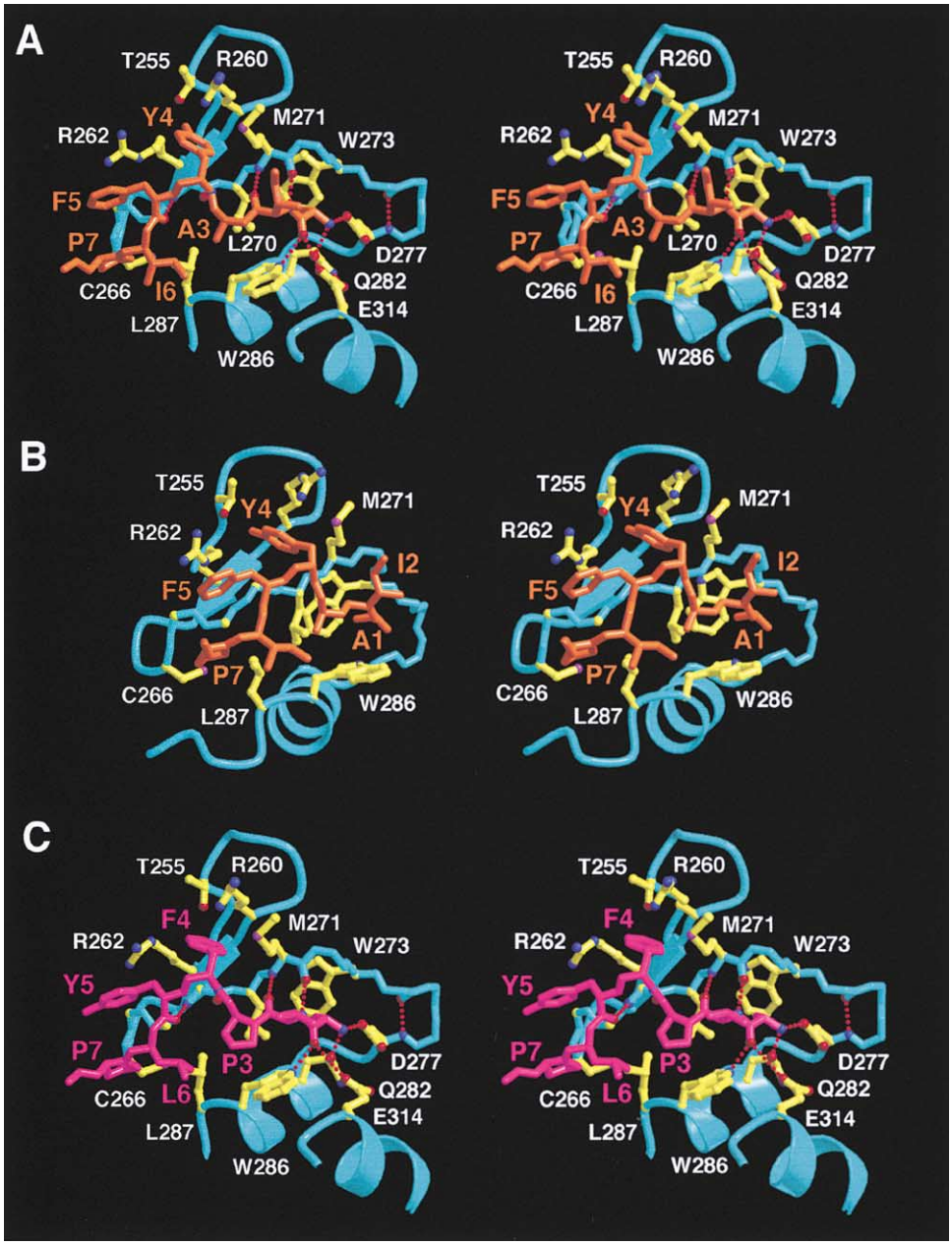


Figure 4. Specific Recognition of DIAP1-BIR2 by the Hid and Grim Peptides

(A) Stereo view of the interface between DIAP1-BIR2, colored cyan, and the bound Grim peptide, in orange. The important residues in DIAP1 are highlighted in yellow. Hydrogen bonds are represented by red dashed lines. The same DIAP1-BIR2 orientation is maintained for (C).

(B) Close up view of the hydrophobic interface between DIAP1-BIR2 and the Grim peptide. The coloring scheme is the same as in (A).

(C) Stereo view of the interface between DIAP1-BIR2 and the bound Hid peptide, shown in pink.

duced the binding affinity by approximately 50-fold (Liu et al., 2000).

Discussion

Although the DIAP1-BIR2 domain is highly homologous to the BIR3 domain of XIAP (Figure 2), the binding affinity difference between Hid and Grim is 6-fold and 100-fold, respectively, for interactions with DIAP1-BIR2 and XIAP-BIR3 (Figure 6B). This is likely due to a minor difference in the surfaces of these two domains. Leu287 in DIAP1 closely packs against Leu/Ile6 and Pro7 in the Hid/Grim peptides (Figure 4). However, Leu287 in DIAP1 is replaced by a bulkier residue, Tyr324 in XIAP, which may restrict binding by the Hid/Grim peptides to their N-terminal four amino acids. In addition, the deleterious effect of Ala3 in Grim-DIAP1 interaction could be lessened by the binding of 3 additional residues after the N-terminal tetrapeptide (Figure 4).

Due to the unique extensive binding interfaces in *Drosophila*, the Grim peptide is able to maintain specific recognition to the DIAP1-BIR2 domain with 0.24 μM

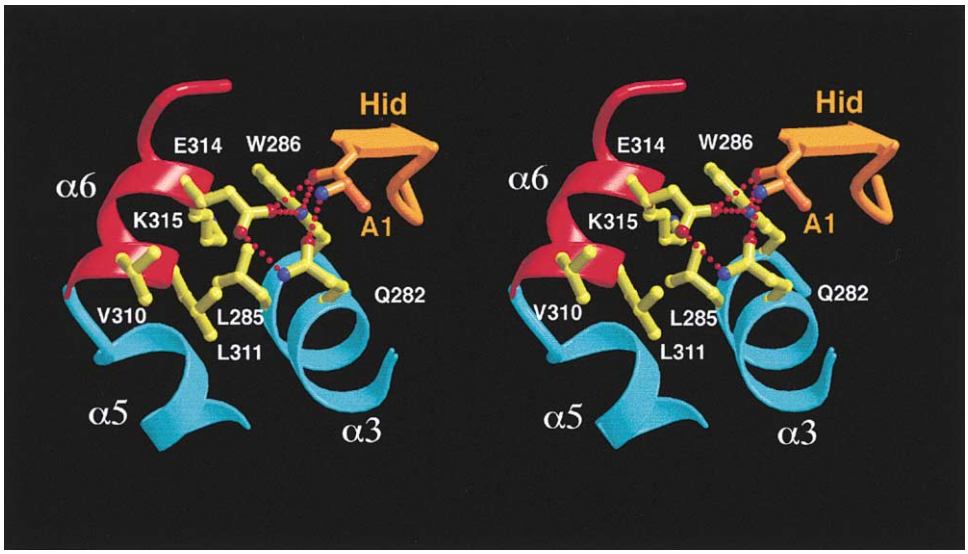


Figure 5. Close Up View on the Packing between Helices α6 and α3, and the Bound Peptide

Helix α6, induced upon peptide binding to DIAP1 and shown in red, makes important contacts to the bound Hid or Grim peptide (shown in orange). Glu314 on α6 hydrogen bonds to A1 of the Grim peptide, and Gln282 and Trp286 of DIAP1. There are numerous van der Waals interactions among the side chains of Val310/Leu311/Lys315 on α6 and Leu285/Trp286/Gln282 of helix α3.

binding affinity (Figure 6B). In contrast, the Grim peptide strongly discriminates against XIAP-BIR3, with a 12.6 μM dissociation constant, presumably because only the N-terminal tetrapeptide is involved in binding. On the other hand, the N-terminal tetrapeptide of Hid already represents the optimal binding element to XIAP, in agreement with the small difference in binding affinities to DIAP1 and XIAP (Figure 6).

Our limited biochemical analysis sheds light on the diversity and conservation of the IAP binding peptides. It is now apparent that the first residue, Ala1, plays an indispensable role in anchoring the peptide recognition of the BIR surface. There is a strong preference for Pro in the third position, particularly for binding to the mammalian protein XIAP. β-Branched side chains appear to be necessary for the second position (Val, Thr, and Ile), while the fourth residue can tolerate Tyr or Ile but prefers Phe. Thus, the optimal tetrapeptide is Ala1-Val2-Pro3-Phe4 for both DIAP1 and XIAP. Longer peptides derived from Hid or Grim do not improve the binding affinity to XIAP because the surface groove on the XIAP-BIR3 domain is optimized for tetrapeptide binding. However, in the *Drosophila* protein DIAP1, additional hydrophobic residues can significantly improve the binding affinity, especially if the third residue in the peptide is Ala. The N-terminal 7-residue peptide of Hid likely represents the optimal sequence for binding DIAP1.

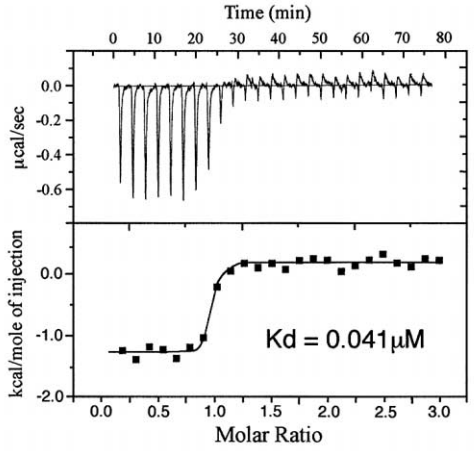
Previous work has shown that mutations in the DIAP1-BIR1 domain partially disrupt the binding of Hid and Reaper to full-length DIAP1 (Goyal et al., 2000). In contrast, our biochemical and structural analysis demonstrates that the N-terminal sequences of Hid, Grim, and Reaper are sufficient to interact with the BIR2 but not the BIR1 domain of DIAP1. These observations strongly suggest that the BIR1 domain plays a regulatory role in the full-length DIAP1, perhaps through interactions with the BIR2 domain. Although the N-terminal sequences of Hid, Grim, and Reaper are sufficient for interactions with DIAP1, this interface may not be exclusive. Indeed, a mutant Grim with its N-terminal 14 amino acids deleted retained significant interactions with DIAP1 (Vucic et al., 1998a), suggesting a second interface. Interestingly, the mammalian protein Smac also uses at least two interfaces to interact with the BIR2 or BIR3 domain of XIAP, and a mutant Smac with the N-terminal tetrapeptide deleted retains interaction with XIAP (Chai et al., 2000).

Crystals of the BIR2 domain by itself and in complex with Hid/Grim peptides grew in the space groups I4 and $P6_522$, respectively. Although helix α6 is involved in some minor crystal packing in the space group $P6_522$, crystal packing by itself does not appear to induce the formation of this α helix, because the BIR2 domain alone was never crystallized in this space group despite numerous attempts. Thus, peptide binding appears to induce the formation of this additional α helix in DIAP1-BIR2. Indeed, residues from this helix make significant contributions to peptide binding through hydrogen bond interactions and this helix stack against α3 through van der Waals contacts (Figure 5).

Our structural analysis also provides a plausible explanation for the observation that the DIAP1-BIR1 domain does not form a stable complex with the conserved peptides. Several important residues in the DIAP1-BIR2 domain, including Gly268, Gly269, and Met271, are replaced by Val85, Glu86, and Gly88, respectively, in the BIR1 domain (Figure 2). These changes would likely destabilize interactions with the second, fourth, fifth, and seventh residues in the Hid/Grim peptides. Similar arguments explain why the XIAP-BIR1 domain or survivin does not interact with the Smac peptides (Figure 2).

The BIR2 domain is just a small part of the 336-amino acid protein DIAP1, which includes the BIR1 domain at the N terminus, the RING finger at the C terminus, and a long linker between BIR1 and BIR2. Although the over-

A



B

Peptide		Dissociation constants (µM) DIAP1-BIR2	XIAP-BIR3
Grim	(AIAYFIPD)	0.24	12.6
Hid	(AVPFYLPE)	0.041	0.12
Smac	(AVPIAQKS)	n/a	0.55
Casp-9	(ATPFQEGL)	n/a	0.25

Figure 6. Quantification of the Interactions between DIAP1-BIR2 and the Hid/Grim Peptides

(A) Isothermal titration calorimetry for the interaction between DIAP1-BIR2 and the Hid peptide. The top part shows the actual titration data, whereas the bottom part shows the curve fitting and the dissociation constant.

(B) Dissociation constants for the binding of four different peptides to the BIR2 domain of DIAP1 and the BIR3 domain of XIAP. The estimated errors are approximately 15% for the tabulated values.

expression of the DIAP1-BIR2 domain by itself is sufficient to confer resistance to some cell death stimuli in lepidopteran cells (Vucic et al., 1998b), other parts of DIAP1 must be required in other contexts (Hay, 2000). In many proteins with RING fingers, the RING domain confers E3 ubiquitin ligase activity (Joazeiro and Weissman, 2000). This list now includes several mammalian IAPs (Huang et al., 2000; Yang et al., 2000) as well as DIAP1 (S. Yoo and B.A.H., unpublished data). The precise function of the BIR1 domain, however, has remained elusive. Both gain-of-function and loss-of-function missense mutations have been identified in the BIR1 domain (Goyal et al., 2000; Lisi et al., 2000). Several of these mutants show decreased binding to Hid and Reaper. Our structural analysis shows that Hid, Grim, and Reaper should be unable to bind to DIAP1 BIR1 via their N termini, and peptides corresponding to these sequences indeed failed to bind BIR1 (data not shown). It is noteworthy, however, that N-terminally truncated Grim and Reaper still have proapoptotic activities in insects (Hay, 2000; Vernooy et al., 2000). As already discussed, this could be due to a second interface between DIAP1 and the Grim and Reaper proteins. It is also possible that the BIR1 domain is involved in mediating the inhibitory effect of DIAP1, perhaps through binding directly to a *Drosophila* caspase. Finally, the BIR1 domain may be important for regulating DIAP1 function as a ubiquitin-protein ligase.

It was suggested that the *Drosophila* proteins Hid/Grim/Reaper interact with DIAP1 in a manner similar to the Smac-XIAP interactions (Liu et al., 2000; Wu et al., 2000). Through structure determination of DIAP1-BIR2 by itself and in complex with the N-terminal peptides from Hid and Grim, we establish a detailed molecular basis for this recognition in *Drosophila*. Our results not only prove the evolutionarily conserved mode of peptide binding from fruit flies to humans, but also reveal an interesting mechanism for the *Drosophila*, which involves DIAP1 binding by three additional conserved residues in Hid/Grim/Reaper and a conformational switch induced by peptide binding that subsequently stabilizes peptide binding.

Experimental Procedures

Protein and Peptide Preparation

All constructs were generated using a standard PCR-based cloning strategy, and the identities of individual clones were verified through double-stranded plasmid sequencing. Recombinant DIAP1-BIR1 (residues 1–145) and BIR2 (residues 201–325) were overexpressed as GST fusion proteins using pGEX-2T (Pharmacia). The soluble fraction of the GST-DIAP1 fusion in the *E. coli* lysate was purified over a glutathione sepharose column, and further purified by anion exchange chromatography (Source-15Q, Pharmacia). The GST moiety was removed by thrombin cleavage, and the resulting BIR domain was further purified by gel filtration (Superdex-200, Pharmacia). The 10-residue Hid and Grim peptides were chemically synthesized, purified by reverse-phase HPLC, and lyophilized. The homogeneous DIAP1-BIR2 domain was mixed with an equimolar amount of synthetic peptide and diluted to a final concentration of 10 mg/ml.

Crystallization and Data Collection

Crystals for DIAP1-BIR2 by itself were grown by the hanging drop vapor diffusion method by mixing the protein (10 mg/ml) with an equal volume of reservoir solution containing 100 mM Tris (pH 8.0), 60% 2,4-methyl-pentane-diol (v/v), and 10 mM DTT. Crystals, with a typical dimension of $0.2 \times 0.2 \times 0.08$ mm^3, are in the space group I4 and contain two molecules in each asymmetric unit. The unit cell dimensions are a = b = 96.1 Å, and c = 59.3 Å. Crystals of DIAP1-BIR2 in complex with the Hid peptide were grown by the hanging drop vapor diffusion method by mixing the complex (10 mg/ml) with an equal volume of reservoir solution containing 100 mM Tris (pH 8.5), 1.4 M $NH_4H_2PO_4$, and 10 mM DTT. Multiple crystals appeared overnight in the midst of heavy precipitation. Macroseeding yielded crystals with a maximum size of $0.2 \times 0.2 \times 0.4$ mm^3 over a period of 6–7 days. The crystals are in the primitive hexagonal space group $P6_522$, with unit cell dimensions a = b = 62.7 Å, c = 130.7 Å. The BIR2-Grim crystals were obtained using tiny BIR2-Hid crystals as microseeds, and grew to a much larger size. The unit cell dimensions are very similar to the BIR2-Hid crystals. Diffraction data were collected using an R-AXIS-IV imaging plate detector mounted on a Rigaku 200HB generator. For all data sets, crystals were equilibrated in a cryoprotectant buffer containing well buffer plus 20% glycerol, and were flash-frozen in a −170°C nitrogen stream.

Structure Determination

The structure of DIAP1-BIR2 by itself was determined by Molecular Replacement, using the software AMORE (Navaza, 1994). The atomic coordinates of the XIAP-BIR3 domain (Protein Data Bank code 1G73) was used for rotational research against the 12–3.0 Å data set. The top 50 solutions from the rotational search were individually used for a subsequent translational search, which yielded

two promising solutions with correlation factors of 23–24 and R factors of 52%–53%. These two solutions turn out to be the two molecules in each asymmetric unit in the crystals; together, they give a combined correlation factor of 36.5 and an R factor of 46%. The peptide-bound BIR2 structures were solved using the partially refined BIR2 structure as the initial search model. All solutions were examined with the program O (Jones et al., 1991). Refinement by the program XPLOR (Brunger, 1991) quickly reduced the R factor and R_{free} to the ranges of 28%–32% and 31%–35%, respectively. The electron density for the bound peptide fragments became clear and unambiguous. A model was built with the program O (Jones et al., 1991) and refined further by simulated annealing using XPLOR. For DIAP1-BIR2 by itself, the final refined atomic model contains residues 215–310 and 18 ordered water molecules at 2.7 Å resolution. For DIAP-BIR2 in complex with the Grim peptide, the final model contains residues 214–318 from DIAP1, the N-terminal 8 residues from Grim, and 58 ordered water molecules at 1.9 Å resolution. For DIAP-BIR2 in complex with the Hid peptide, the final model contains residues 215–316 from DIAP1, the N-terminal 8 residues from Hid, and 36 water molecules at 2.7 Å resolution. The N-terminal 13–14 residues and the C-terminal 7–16 residues in DIAP1 have no electron density in the maps, and we presume that these regions are disordered in the crystals.

In Vitro Interaction Assay

Interaction between the individual BIR domain of DIAP1 and the Hid/Grim peptides was examined by gel filtration chromatography. Approximately 0.5 mg of a recombinant BIR domain was incubated with excess Hid or Grim peptide for 20 min at room temperature. The mixture was fractionated by gel filtration chromatography (Superdex-200, Pharmacia). The protein-containing fractions were analyzed by reverse-phase HPLC and mass spectroscopy for detection of the bound peptide.

Isothermal Microcalorimetry Titration

All proteins and peptides were prepared in 50 mM sodium phosphate buffer (pH 7.5). The Micro Calorimetry System (Microcal) was used to perform the ITC measurements for the interaction between DIAP1-BIR2 and various synthetic peptides. The titration data, collected at 23°C, were analyzed using the ORIGIN data analysis software (Microcal Software).

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Accession Numbers

Protein Data Bank codes for DIAP1-BIR2 by itself and in complex with Grim and Hid peptides are 1JD4, 1JD5, and 1JD6, respectively.