concept

Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family

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Several of the inhibitor of apoptosis protein (IAP) family members regulate apoptosis in response to various cellular assaults. Some members are also involved in cell signalling, mitosis and targeting proteins to the ubiquitin-proteasome degradation machinery. The most intensively studied family member, X-linked IAP (XIAP), is a potent inhibitor of caspase activity; hence, it is generally assumed that direct caspase inhibition is an important conserved function of most members of the family. Biochemical and structural studies have precisely mapped the elements of XIAP required for caspase inhibition. Intriguingly, these elements are not conserved among IAPs. Here, we review current knowledge of the caspase-inhibitory potential of the human IAPs and show that XIAP is probably the only bona fide caspase inhibitor, suggesting that the other family members never gained the ability to directly inhibit caspase activity.

Introduction

The importance of proteolytic signalling pathways in human physiology was first appreciated after the characterization of the coagulation pathway. Proteolytic enzymes transmit signals by hydrolysing the peptide bond between two amino acids. This proteolysis can either initiate signalling by activating a protein, or halt it by inactivating a crucial participant in a given pathway. Unlike kinase and phosphatase pathways, protease signalling is irreversible. For this reason, many points of regulation have evolved to keep proteases silent until their activity is required. For example, in the coagulation pathway, the latent inactive protease zymogens of blood-clotting factors are proteolytically activated in a cascade-like manner. This pathway can also be suppressed at various points by specific protease inhibitors.

It took more than 50 years to identify and characterize the coagulation pathway. Due to massive technological advances in the post-genomic era, it has taken just over 10 years to gain an equivalent understanding of the proteolytic cascade that dismantles cells during apoptosis, through which damaged or unwanted cells are neatly packaged into small membrane-bound fragments for engulfment and disposal by neighbouring phagocytes.

At the heart of the apoptosis pathway are the caspases (formally designated as members of the C14 family of clan CD cysteine proteases), which are so named because of their stringent specificity for cleaving substrates after aspartic-acid residues (Alnemri *et al*, 1996). There are 11 human caspases, and those that have a clear role in apoptosis can be divided into those involved in the initiation (caspase-2, caspase-8, caspase-9 and caspase-10) or the execution (caspase-3, caspase-6 and caspase-7) phase of apoptosis (Fuentes-Prior & Salvesen, 2004).

The initiator caspases are the first to be activated, translating a death-inducing signal into proteolytic activity. Similar to the coagulation cascade, this initial activation event is not proteolysis itself, but rather the recruitment of a caspase to an activating protein, or platform of proteins. On these activation platforms, the initiator caspase zymogens are activated through dimerization, and subsequent autocatalytic processing is required for stabilization of the catalytically competent initiator caspase dimer (Boatright *et al*, 2003).

In humans, there are two main pathways to initiator caspase activation. The 'intrinsic pathway' senses intracellular damage and growth factor or cytokine availability, and activates caspase-9 at the apoptosome (Jiang & Wang, 2004). The 'extrinsic pathway' is a sensor for extracellular signals, and activates caspase-8 through the ligation of tumour necrosis factor transmembrane-receptor family members to form the death-inducing signalling complex (Debatin & Krammer, 2004). Although activated by different cues, caspase-8 and caspase-9 converge on the executioner caspases, caspase-3 and caspase-7, which exist as inactive dimeric zymogens in resting cells (Fuentes-Prior & Salvesen, 2004). Using a mechanism reminiscent of classical protease activation, the executioner caspase zymogens are cleaved in their interchain linker and, once active, are responsible for most of the proteolytic events that destroy the cell (Fischer *et al*, 2003).

Viruses have learned that interfering with apoptotic caspases is a good way to keep the host cell alive long enough for viral replication to occur. For example, p35 and p49 from baculovirus are potent inhibitors of most caspases (Bump *et al*, 1995; Jabbour
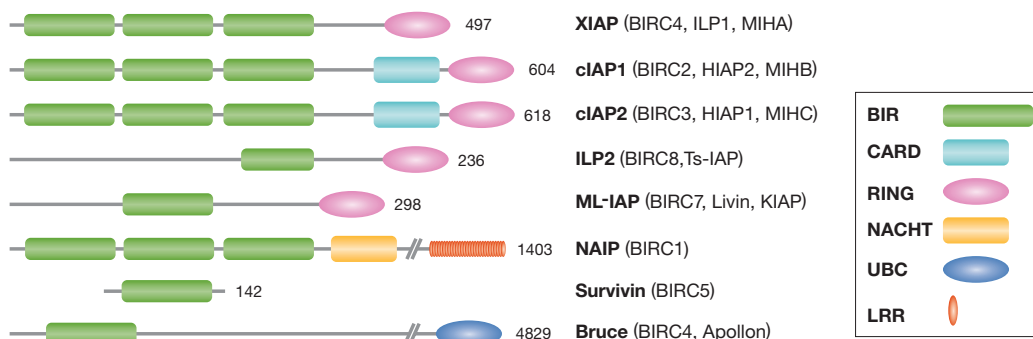
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Fig 1 | Schematic representation of the human inhibitor of apoptosis protein family. In addition to at least one baculoviral IAP repeat (BIR) domain, most IAPs have other distinct functional domains. The really interesting new gene (RING) domain found in many IAPs is an E3 ligase that presumably directs targets to the ubiquitin-proteasome degradation system. Caspase-recruitment domains (CARDs) can mediate homotypic protein–protein interactions, although the binding partners for the cellular IAP (cIAP) CARDs have not yet been elucidated. Bruce has a ubiquitin-conjugation (UBC) domain that is found in many ubiquitin-conjugating enzymes. The NACHT domain of neuronal apoptosis-inhibitory protein (NAIP) resembles a nucleotide-oligomerization domain related to the AAA+ NTPases, whereas the leucine-rich repeats (LRRs) are similar to those of the Toll-like receptors that function as pathogen sensors. BIRC, baculoviral IAP-repeat-containing; HIAP, human IAP; IAP, inhibitor of apoptosis protein; ILP, IAP-like protein; KIAP, kidney IAP; MIH, mammalian IAP homologue; ML-IAP, melanoma IAP; NACHT, domain found in NAIP, CIITA, HET-E and TP-1; Ts-IAP, testicular IAP; XIAP, X-linked IAP.

et al, 2002; Ryan *et al*, 2002; Zhou *et al*, 1998). Orthopox viruses contain a serpin, cytokine response-modifier A (CrmA), which also directly inhibits several caspases (Kamada *et al*, 1997; Zhou *et al*, 1997). Although they have no structural similarity, CrmA and p35 both inactivate their cognate proteases in a mechanism-based manner (Stennicke *et al*, 2002). The reactive-site loop binds to the active site of the protease and is cleaved, thereby inducing a conformational change that irreversibly locks the protease in an inactive conformation.
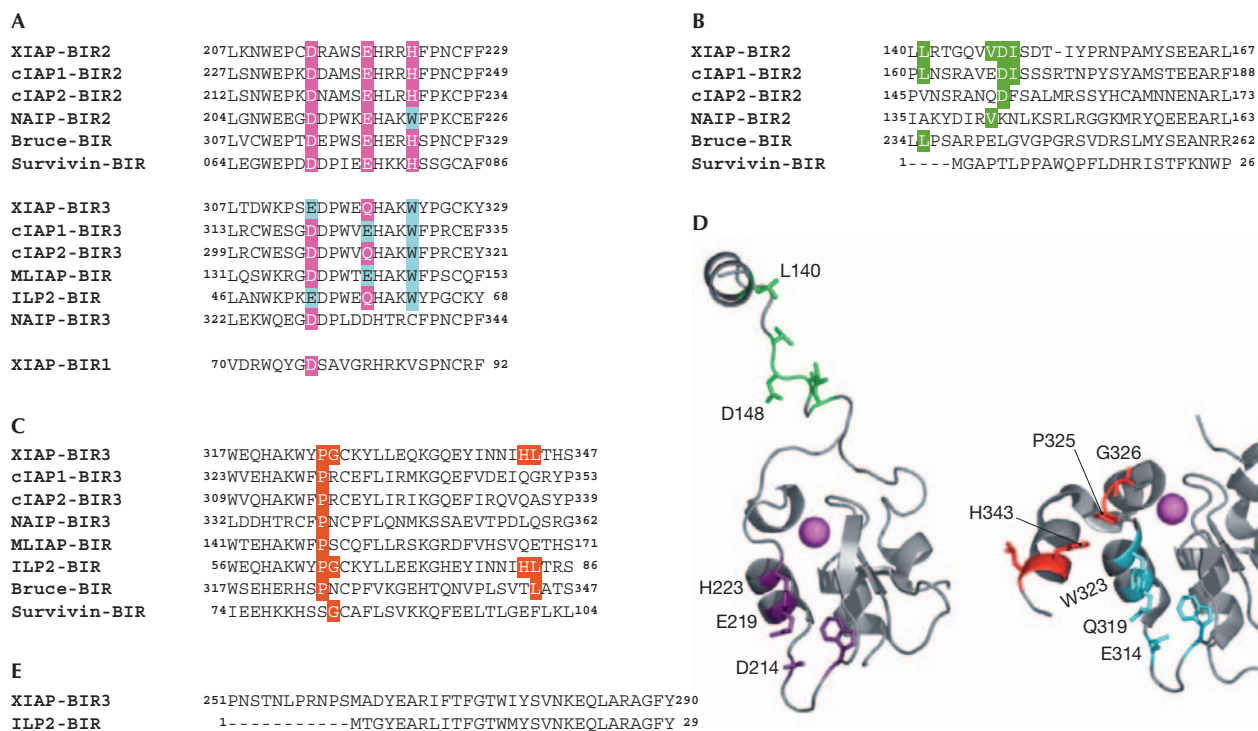
In principle, there are two ways to terminate the activity of a caspase: first, remove it from the cell by using the ubiquitin-targeted proteasome degradation machinery; or second, directly inhibit its enzymatic activity. Intriguingly, there is evidence that members of the inhibitor of apoptosis protein (IAP) family (Fig 1) are capable of both functions (Riedl & Shi, 2004; Salvesen & Duckett, 2002; Vaux & Silke, 2005a). The evolutionarily conserved mechanism seems to be caspase removal. The ubiquitin-targeting function of *Drosophila melanogaster* IAP (DIAP1) is essential for apoptosis inhibition *in vivo*, whereas *in vitro* it only weakly inhibits *D. melanogaster* caspase activity (Goyal *et al*, 2000; Hawkins *et al*, 1999; Lisi *et al*, 2000; Yan *et al*, 2004). By contrast, the most studied of the mammalian IAPs, X-linked IAP (XIAP), has evolved to potently inhibit the enzymatic activity of mammalian caspases at both the initiation phase (caspase-9) and the execution phase (caspase-3 and caspase-7) of apoptosis (Deveraux *et al*, 1997). There is some evidence that XIAP might participate in caspase polyubiquitination and monoubiquitination *in vitro*; however, this function is yet to be confirmed *in vivo* with endogenous proteins. Moreover, XIAP-directed caspase ubiquitination might not result in degradation by the proteasome (Creagh *et al*, 2004; Hao *et al*, 2004; Morizane *et al*, 2005; Suzuki *et al*, 2001). In this review, we discuss recent structural and mechanistic studies indicating that XIAP is the only mammalian IAP that has adopted the ability to directly inhibit the enzymatic activity of caspases.

XIAP: a potent natural caspase inhibitor

Biochemical and structural analyses have precisely mapped the elements in XIAP that are required for the inhibition of caspase activity. Initial biochemical studies determined that a region encompassing the second baculoviral IAP repeat (BIR) domain (BIR2) inhibits caspase-3 and caspase-7, whereas the third BIR domain (BIR3) inhibits caspase-9 (Deveraux *et al*, 1999). Although these BIR domains share close to 40% identity, structural analyses revealed what initially seemed to be strikingly different methods for inhibiting proteases of the same mechanistic family. However, further biochemical dissection has shown that there are, in fact, some shared elements.

Both BIR domains use a two-site binding mechanism for potent caspase inhibition. One of these sites is a conserved surface groove found in BIR2 and BIR3 of XIAP, and in other BIR domains (Fig 2A,D). We have defined this site as the IAP-binding motif (IBM)-interacting groove, because of its specificity for proteins containing an IBM at their immediate amino terminus (Scott *et al*, 2005). These include the pro-apoptotic mammalian IAP antagonists Smac/DIABLO and HtrA2, and the *Drosophila* proteins Hid, Grim and Reaper (Vaux & Silke, 2003). Although no structures are known for the BIR1 domain of XIAP, sequence constraints indicate that the IBM-interacting groove is absent from this domain (Fig 2A).

On activation, caspases are proteolytically processed between their large and small subunits as a result of either auto-catalytic processing following dimerization or the activity of upstream proteases. For caspase-3, caspase-7 and caspase-9, the newly generated small-subunit amino terminus constitutes an IBM. To achieve potent inhibition, the BIR domain binds to at least the newly generated IBM. For the caspase-9 and BIR3 complex, this interaction was first suggested by biochemical studies (Srinivasula *et al*, 2001) and later observed in a crystal structure of the complex (Shiozaki *et al*, 2003). However, although it was appreciated that the BIR2 domain itself contributed to caspase-3 and caspase-7 inhibition (Huang *et al*, 2001; Sun *et al*, 1999),

A

XIAP-BIR2   207LKNWEPCDRAWSEHRRHFPNCFF229
cIAP1-BIR2   227LSNWEPKDDAMSEHRRHFPNCPF249
cIAP2-BIR2   212LSNWEPKDNAMSEHLRHFPKCPF234
NAIP-BIR2   204LGNWEEGDDPWKEHAKWFPKCEF226
Bruce-BIR   307LVCWEPTDEPWSEHERESPNCPF329
Survivin-BIR 064LEGWEPDDDPIEEHKKHSSGCAF086

XIAP-BIR3   307LTDWKPSEDPWEQHAKWYPGCKY329
cIAP1-BIR3   313LRCWESGDDPWVEHAKWFPRCEF335
cIAP2-BIR3   299LRCWESGDDPWVQHAKWFPRCEY321
MLIAP-BIR   131LQSWKRGDDPWTEHAKWFPSCQF153
ILP2-BIR   46LANWKPKEDPWEQHAKWYPGCKY 68
NAIP-BIR3   322LEKWQEGDDPLDDHTRCFPNCPF344

XIAP-BIR1   70VDRWQYGDSAVGRHRKVSPNCRF 92

B

XIAP-BIR2   140LLRTGQVVDISDT-IYPRNPAMYSEEARL167
cIAP1-BIR2   160PLNSRAVEDISSSRTNPYSYAMSTEEARF188
cIAP2-BIR2   145PVNSRANQDFSALMRSSYHCAMNNENARL173
NAIP-BIR2   135IAKYDIRVKNLKSRLRGGKMRYQEEEARL163
Bruce-BIR   234LLPSARPELGVGPGRSVDRSLMYSEANRR262
Survivin-BIR   1----MGAPTLPPAWQPFLDHRISTFKNWP 26

C

XIAP-BIR3   317WEQHAKWYPGCKYLLEQKGQEYINNIHLTHS347
cIAP1-BIR3   323WVEHAKWFPRCEFLIRMKGQEFVDEIQGRYP353
cIAP2-BIR3   309WVQHAKWFPRCEYLIRIKGQEFIRQVQASYP339
NAIP-BIR3   332DDHTRCFPNCPFLQNMKSSAEVTPDLQSRG362
MLIAP-BIR   141WTEHAKWFPSCQFLLRSKGRDFVHSVQETHS171
ILP2-BIR   56WEQHAKWYPGCKYLLEEKGHEYINNIHLTRS 86
Bruce-BIR   317WSEHERHSPNCPFVKGEHTQNVPLSVTLATS347
Survivin-BIR 74IEEHKKHSSGCAFLSVKKQFEELTLGEFLKL104

D

E

XIAP-BIR3   251PNSTNLPRNPSMADYEARIFTFGTWIYSVNKEQLARAGFY290
ILP2-BIR   1-----------MTGYEARLITFGTWMYSVNKEQLARAGFY 29

Fig 2 | The caspase-binding elements of human inhibitor of apoptosis proteins. Alignment of baculoviral IAP repeat (BIR) domains with the highest identity. (**A**) IAP-binding motif (IBM)-interacting groove exosite. The residues that line this groove are highlighted; those identical to XIAP BIR2 or XIAP BIR3 are shown in purple and cyan, respectively. The BIR3 of cIAPs, the BIR2 of neuronal apoptosis-inhibitory protein (NAIP) and the single BIR of ML-IAP share some features of both BIR2 and BIR3 of XIAP. (**B**) Executioner caspase-inhibitory peptide strand of XIAP that lies at the amino terminus of the BIR2 domain. Crucial residues are shown in green. (**C**) The helix distal to XIAP BIR3 targets the dimer interface of caspase-9. Crucial residues are shown in red. (**D**) Crystal structure of BIR2 (right, from Protein Data Bank (PDB) 1I3O) and BIR3 (left, from PDB 1NW9) from XIAP. The executioner caspase-inhibitory element is shown in green, whereas the caspase-9-inhibitory distal helix is shown in red. The IBM-interacting grooves of BIR2 and BIR3 are shown in purple and cyan, respectively. (**E**) Alignment of XIAP BIR3 with ILP2 showing the truncated amino terminus of the latter. cIAP, cellular IAP; IAP, inhibitor of apoptosis protein; ILP2, IAP-like protein 2; ML-IAP, melanoma IAP; XIAP, X-linked IAP.

three independently derived crystal structures of the caspase-7/ BIR2 complex failed to reveal any electron density for the BIR2 domain (Chai *et al*, 2001; Huang *et al*, 2001). By contrast, the caspase-3/BIR2 complex not only showed the entire BIR2 domain, but also revealed that the caspase small-subunit amino terminus docks in the IBM-interacting groove (Riedl *et al*, 2001). Although this interaction probably results from crystal packing, it is nevertheless required for potent caspase-3 and caspase-7 inhibition in solution (Scott *et al*, 2005). Therefore, the IBM-interacting groove of the BIR domain functions as an exosite, which is a term used for a functionally important region outside the active site of the enzyme. In a similar manner to the carboxy-terminal tail of hirudin binding to exosite I of the coagulation protease thrombin, the IBM-interacting groove acts as an anchor, strengthening inhibitor binding (Fig 3; Rydel *et al*, 1990).

Although this anchoring interaction in BIR2 and BIR3 of XIAP is conserved, the mechanism of enzyme inhibition is not. Early biochemical studies identified residues in XIAP that are crucial for caspase-3 inhibition (Fig 2B,D). Surprisingly, these residues are not in the BIR2 domain, but rather in the preceding peptide strand, with Leu 141 and Asp 148 being important for tight inhibition (Sun *et al*,

1999). After structural data became available, the importance of these residues was clear. All structures of a BIR2/caspase complex show that this peptide strand (residues 140–156) stretches across the catalytic-binding cleft of the enzyme (Fig 3). Asp 148 forms hydrogen bonds, whereas Leu 141 makes hydrophobic contacts with the caspase. Thus, tight inhibition of the executioner caspases, caspase-3 and caspase-7, by XIAP requires two surfaces: an exosite-anchoring motif and an active site-directed interaction.

Although targeting the active site is reminiscent of a standard 'lock and key' inhibitor mechanism, this is not the case for BIR2 and executioner caspases. If we compare caspase-3 inhibition by BIR2 with synthetic peptidyl inhibitors, such as DEVD-CHO, there are significant differences. Peptidyl inhibitors bind similarly to a substrate, occupying the specificity recognition sites of the protease. They exploit the catalytic function of the protease to trap the bound state, and act as covalent modifiers or transition-state analogues. Although the BIR2 peptide strand binds across the substrate-binding cleft, and Asp 148 occupies the S4-binding pocket, the S1, S2 and S3 pockets of the caspase remain unoccupied. In addition, all potential carbonyls are too far from the catalytic cysteine for nucleophilic attack. Equally important, the peptide strand binds in
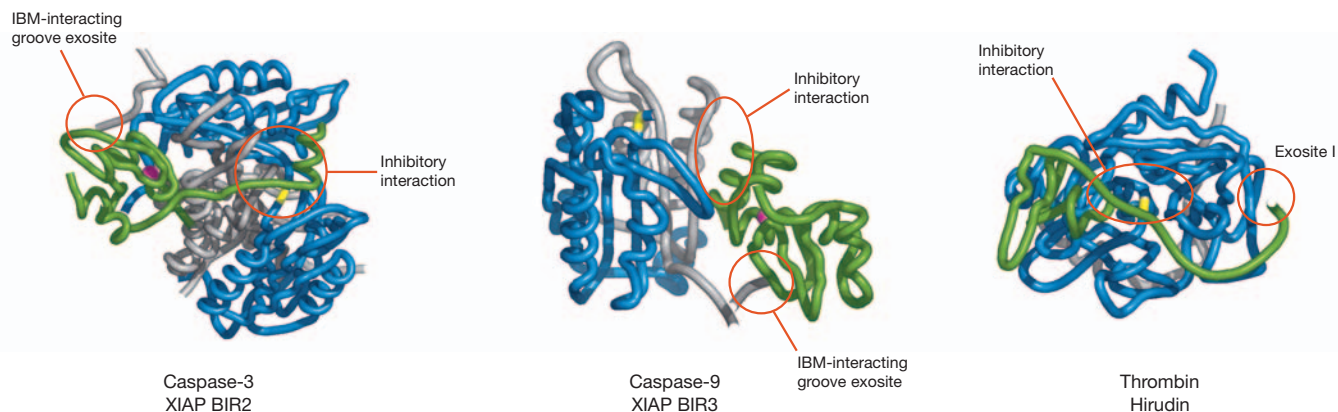
Fig 3 | Two-site binding mechanisms for protease inhibition. The colour coding is as follows: the inhibitors are shown in green with the co-ordinating zinc in purple, the protease large subunit is shown in blue with the catalytic residue in yellow and the small subunit is shown in grey. (**A**) X-linked IAP (XIAP) baculoviral IAP repeat 2 (BIR2) complex with caspase-3 (Protein Data Bank (PDB) 1I3O). The amino-terminal peptide strand preceding the BIR2 domain binds across the substrate-binding site, whereas the IAP-binding motif (IBM) of a caspase-3 small subunit, in an adjacent asymmetric unit, docks in the IBM-interacting groove. The mechanism of inhibition is steric occlusion of the active site. (**B**) XIAP BIR3 complex with a caspase-9 monomer (PDB 1NW9). The IBM of caspase-9 docks into the IBM-interacting groove of BIR3, facilitating interactions of the carboxy-terminal BIR3 helix with the dimer interface of caspase-9. This prevents dimerization or induces monomerization of caspase-9 with concomitant collapse of the active site. (**C**) Hirudin complex with thrombin (PDB 1HRT). The substrate-binding site is blocked, whereas the carboxy terminus docks to exosite I of thrombin.

a 'back to front' orientation. This unusual reverse-binding mechanism has been observed for members of the papain family of proteases (clan CA, family C1A). Here, the zymogen has a fully formed active site, but the propeptide of the enzyme spans the substrate-binding cleft in the reverse direction, keeping the enzyme silent until it reaches the lysosome (Coulombe *et al*, 1996). Cytotoxic T-lymphocyte antigen 2, which has sequence similarity with the propeptides of some papain-like enzymes, might inhibit cathepsin L through the same mechanism (Kurata *et al*, 2003).

As opposed to targeting the enzyme active site directly, the BIR3 domain of XIAP abolishes activity using a fundamentally different mechanism. The functional inhibitory surface—separate from the IBM-interacting groove—is a helix found immediately after the BIR3 domain (Fig 2C,D). Mutagenesis identified His 343 as crucial for caspase-9 inhibition (Sun *et al*, 2000). Structural analysis of a BIR3/caspase-9 complex (Fig 3) showed that this distal helix packs against the dimer interface and monomerizes caspase-9, forcing the protease into an inactive conformation with a collapsed active site (Shiozaki *et al*, 2003). Active caspases are obligate dimers, so this reverses the mechanism of caspase-9 activation. Another possibility is that BIR3 binds to monomeric caspase-9 that has been adventitiously cleaved, but not activated, through its proteolytically generated small-subunit IBM. This would facilitate the interaction at the BIR3 distal helix/caspase-9 interface, preventing dimerization and activation. Nonetheless, XIAP is the first example of a natural protease inhibitor that uses this kind of allosteric mechanism.

Are the other mammalian IAPs caspase inhibitors?
As a result of these studies on XIAP, IAPs have become attractive targets for drug design for the treatment of chronic and acute diseases that have a cell-death component (Schimmer & Dalili, 2005). So, are all IAPs caspase inhibitors?

Cellular IAP1 (cIAP1) and cIAP2 consist of three BIR domains and a really interesting new gene (RING) domain, and are the closest paralogues of XIAP (Fig 1). Similar to XIAP, overexpression of cIAPs protects cells from apoptosis. Together with their high sequence and domain conservation, it has been assumed that cIAPs are also caspase inhibitors and are redundant with XIAP. Indeed, one study found that mice deficient in XIAP showed elevated levels of cIAP1 and cIAP2 proteins (Harlin *et al*, 2001), although another independently derived line did not (Olayioye *et al*, 2005).

The BIR domains of cIAPs contain IBM-interacting grooves that are highly conserved with those in XIAP (Fig 2A). Furthermore, the peptide strand preceding BIR2 of cIAP1 has some of the residues that are crucial for XIAP-mediated inhibition of the executioner caspases. cIAP2 has the crucial Asp 153, but other residues in this peptide strand are less conserved (Fig 2B). Experimentally, recombinant cIAP BIR2 fragments that encompass the preceding peptide strand have no executioner caspase-inhibitory activity (Eckelman & Salvesen, 2005; Table 1). However, replacing the cIAP1 strand with that of XIAP can restore potent executioner caspase inhibition. Regarding caspase-9, the BIR3 domain of both cIAPs has only one of four dimer interface-interacting residues (Fig 2C), and so neither cIAP can demolish caspase-9 activity. It should be noted that cIAPs can bind caspases *in vitro*, in an active-site-independent manner, although the significance of this has yet to be established (Eckelman & Salvesen, 2005; Tenev *et al*, 2005). Consequently, although the cIAPs contain a caspase-binding scaffold, they either lost or never gained the specific residues required for caspase inhibition.

On the basis of structural requirements, a BIR must contain both of the caspase-binding elements that are found in XIAP—the IBM-interacting groove exosite and an inhibitory element—to inhibit caspase activity. We therefore predict that the other mammalian IAPs are not caspase inhibitors (Fig 2). Vucic and colleagues showed that melanoma IAP (ML-IAP) is not a tight inhibitor of caspase-9 (Table 1),

Table 1 | K_i values for mammalian baculoviral inhibitor of apoptosis repeat domains against human caspases

K_i (nM)	Caspase-3	Caspase-7	Caspase-9
XIAP full length	<0.8[a,b]	<0.07[a]	210[b]
XIAP BIR2[c]	0.7	0.2	NI
XIAP BIR3[d]	NI	NI	10
GST-cIAP1 full length[e]	108	42	ND
cIAP1 full length[f]	>2,000 (NI)[b,g]	>2,000	>2,000
cIAP1 BIR2[f]	>10,000	>10,000	NI
X-cIAP1-BIR2[f,h]	19	<0.2	NI
cIAP1 BIR3[f]	NI	NI	>5,000
cIAP1 BIR3 (R342G/Q349H/G350L)[f,i]	NI	NI	<80
GST-cIAP2 full length[e]	35	29	ND
cIAP2 full length[b,g]	NI	ND	ND
cIAP2 BIR2[f]	>5,000	>5,000	NI
cIAP2 BIR3[f]	NI	NI	>5,000
ML-IAP BIR[j]	ND	ND	3,200
ML-IAP-Q (S150G/Q167H/E168L)[j,k]	ND	ND	<0.01
GST-ILP2 full length[l]	NI	NI	752
ILP2 BIR[l]	NI	NI	472
GST-NAIP BIR123[m]	14	50	ND
GST-NAIP BIR3	185[m]	ND	33[n]
NAIP BIR2[o]	NI	NI	NI

ND, not determined; NI, not inhibited.
[a]Scott *et al*, 2005. [b]Half-maximal inhibitory concentration (IC50; Silke *et al*, 2001). [c]Deveraux *et al*, 1997. [d]IC50 (Sun *et al*, 2000). [e]Roy *et al*, 1997. [f]K_i apparent (Eckelman & Salvesen, 2005). [g]*In vitro* toxicity assay in yeast. [h]cIAP1 BIR2 domain with the caspase-3 and caspase-7 binding elements of XIAP fused to the amino terminus. [i]Crucial residues are mutated to those found in XIAP BIR3 IAP-binding motif (IBM)-interacting groove. [j]K_i apparent (Vucic *et al*, 2005). [k]ML-IAP truncated at the carboxy terminus to Gln 172 with mutations in the IBM-interacting groove to those found in XIAP BIR3. [l]Shin *et al*, 2005. [m]Maier *et al*, 2002. [n]Davoodi *et al*, 2004. [o]B.P.E., unpublished results. All recombinant proteins that did not contain a glutathione-*S*-transferase (GST) tag contained a histidine tag or no tag.
BIR, baculoviral IAP repeat domains; cIAP, cellular IAP; IAP, inhibitor of apoptosis; ILP2, IAP-like protein 2; ML-IAP, melanoma IAP; NAIP, neuronal apoptosis-inhibitory protein; XIAP, X-linked IAP.

and elegantly demonstrated that the mutation of three residues in the distal helix to those found in XIAP endowed ML-IAP with potent caspase-9-inhibitory function (Vucic *et al*, 2005). For neuronal apoptosis-inhibitory protein (NAIP) BIR2, Lys 143 replaces Asp 148 of XIAP (Fig 2B). This drastic substitution should preclude the inhibition of executioner caspases, and, on the basis of sequence-conservation arguments, the third BIR domain might not even contain an IBM-interacting groove. Shin and colleagues have shown that although IAP-like protein 2 (ILP2) contains all of the surface elements required for caspase-9 inhibition, the BIR domain is an intrinsically unstable domain owing to a natural amino-terminal truncation (Fig 2E) and is not a caspase inhibitor (Shin *et al*, 2005). There is still a possibility that ILP2 requires a binding partner to stabilize the polypeptide and to promote caspase inhibition. However, ILP2 messenger RNA has only been detected in testis, and the ILP2 protein has yet to be detected *in vivo*. Survivin does not directly inhibit caspases; however, together with hepatitis B X-interacting protein (HBXIP), this IAP might prevent caspase-9 activation at the apoptosome (Marusawa *et al*, 2003). Bruce might also prevent caspase-9 activation, although the mechanism is unclear (Qiu & Goldberg, 2005).

The problem with glutathione-*S*-transferase tags

On the basis of structural requirements, it seems clear that no other human IAP contains all the elements required for inhibition of caspase activity. However, in addition to the studies discussed above, others have suggested that some IAPs can inhibit caspases. A possible explanation for these conflicting results is the method used to purify the respective IAP protein. A recent study investigated the effect of the glutathione-*S*-transferase (GST)-purification tag on the caspase-inhibitory potential of XIAP. A chimeric protein containing the peptide strand of XIAP (residues 124–168) attached to green fluorescent protein in place of the BIR domain was fused to either a GST or a hexahistidine tag (Scott *et al*, 2005). As this chimaera has only the active site-occluding surface, and lacks the anchoring exosite interaction, this protein should be a poor caspase inhibitor. The chimaeras showed markedly different inhibitory properties depending on their oligomeric state. The hexahistidine-tagged chimaera was a monomer displaying weak inhibition of caspase-3, whereas the GST-tagged chimaera was a mixture of oligomeric species displaying 100-fold enhancement of inhibitory function. GST-XIAP BIR2 is also a mixture of oligomeric species, whereas XIAP BIR2 with a hexahistidine tag is a monomer in solution (F.L.S., unpublished data). Initial studies with GST-tagged cIAPs revealed a similar problem, with apparent inhibition of caspase-3 and caspase-7, albeit two to three orders of magnitude weaker than XIAP (Table 1). GST-tagged BIR domains from ML-IAP and NAIP were suggested to be potent caspase inhibitors (Davoodi *et al*, 2004; Maier *et al*, 2002; Vucic *et al*, 2000); however, more recent studies with hexahistidine-tagged BIRs have produced results that agree with structural and biochemical predictions. We eagerly await kinetic analyses of non-oligomeric versions of NAIP BIR domains to test their inhibitory properties, with preliminary results indicating that they are incompetent (Table 1). Consequently, the artefactual inhibition observed in IAPs that are fused to GST can be attributed to the generation of oligomeric species, warning against using GST constructs to characterize enzyme inhibition. There is a possibility that IAPs might form larger oligomeric complexes that could mimic the artefactual effect of GST on caspase inhibition, although we are unaware of any evidence for this. There is some evidence that IAPs can form hetero-complexes *in vitro*, but these seem to influence the susceptibility of IAPs to proteasomal degradation, rather than caspase inhibition (Dohi *et al*, 2004; Silke *et al*, 2005).

Conclusion

The structural and biochemical requirements of caspase inhibition by XIAP have been thoroughly established. On the basis of these, it is probable that other human IAPs are not direct caspase inhibitors, unless they use a distinctly different mechanism. Earlier studies concluding that IAPs other than XIAP were direct caspase inhibitors have largely been revised, and it seems that XIAP is the only mammalian IAP that directly inhibits caspase activity. The mechanism by which other mammalian IAPs attenuate apoptosis is less clear and remains under intense investigation. One possibility is that IAPs bind proapoptotic molecules (including caspases and Smac/DIABLO, which is an IAP antagonist) through their IBM-interacting grooves, and target them for degradation by the ubiquitin-proteasome pathway (Vaux & Silke, 2005a,b). Targeting could occur through their own RING domains or by forming complexes with other RING-containing IAPs. There

is also some shared topography between the IBM-interacting grooves of different BIR domains in different IAPs, suggesting some overlap in binding specificity. Finally, the formation of IAP heterocomplexes might influence their stability and, hence, their ability to regulate apoptosis (Silke *et al*, 2005).

From an evolutionary perspective, it seems that XIAP has diverged from the other IAPs and acquired the ability to inhibit caspase activity directly. The implications are that XIAP is the first line of defence against inappropriate caspase activity, placing it at an important node in the mammalian apoptotic pathway. However, this assignment is contentious considering the fact that the XIAP-null mice have yet to display a significant phenotype (Harlin *et al*, 2001; Olayioye *et al*, 2005). It is therefore tempting to suggest that there is an evolutionarily conserved 'back-up system' that takes over, at least during development. This back-up might be IAP-mediated targeting of activated caspases for proteasomal degradation. It is far from clear which mammalian IAP could perform this function. cIAP2 can monoubiquitinate executioner caspases *in vitro*, but this modification is not normally associated with proteasome targeting and degradation (Huang *et al*, 2000). Monoubiquitination might sequester active caspases away from death substrates, but this has yet to be shown. There is also some evidence that Bruce (Apollon) can bind caspase-3, caspase-7 and caspase-9, and can polyubiquitinate caspase-9, when ectopically expressed *in vitro* (Bartke *et al*, 2004; Hao *et al*, 2004; Qiu & Goldberg, 2005).

Given the recent interest in the development of therapeutic small molecules for targeting IAPs in disease, and the evidence that XIAP is probably the only bona fide caspase inhibitor, we look forward to studies that delineate the mechanisms used by other IAPs to protect cells from apoptosis both *in vitro* and *in vivo* (Schimmer & Dalili, 2005).

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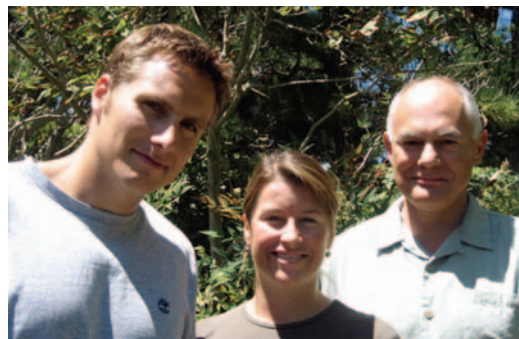
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