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The inhibitors of apoptosis: there is more to life than Bcl2

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The inhibitor of apoptosis (IAP) genes constitute a highly conserved family found in organisms as diverse as insects and mammals. These genes encode proteins that directly bind and inhibit caspases, and thus play a critical role in deciding cell fate. The IAPs are in turn regulated by endogenous proteins (second mitochondrial activator of caspases and Omi) that are released from the mitochondria during apoptosis. Overexpression of the IAPs, particularly the X-chromosome-linked IAP, has been shown to be protective in a variety of experimental animal models of human neurodegenerative diseases. Furthermore, overexpression of one or more of the IAPs in cancer cell lines and primary tumor samples appears to be a frequent event. IAP gene amplification and translocation events provide genetic evidence that further strengthens the case for classifying the IAPs as oncogenes. Therapeutic strategies that interfere with IAP expression or function are under investigation as an adjuvant to conventional chemotherapy- and radiation-based cancer therapy. This paper reviews the structure and function of the IAP family members and their inhibitors, and surveys the available evidence for IAP dysregulation in cancer.
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Introduction

One of the central yet unresolved issues in apoptosis research is the determination of the cellular 'point-of-no-return'. Many experimental systems have suggested that the activation of the caspase cascade is irreversible, at least in part, due to the self-amplifying nature of the cascade. Caspase involvement in virtually all cell death paradigms can lead to the impression that caspase activation and apoptosis are synonymous, yet this is not the case. Limited activation has also been demonstrated to be essential for the proliferation of T and B lymphocytes (Alam *et al*., 1999; Kennedy *et al*., 1999; Mouhamad *et al*., 2002), and the differentiation of mature erythrocytes (Bratosin *et al*., 2001), monocytes (Pandey *et al*., 2000), and epidermal cells (Eckhart *et al*., 2000). Regulation of caspase activity is thus implicated in cell proliferation, apoptosis, and cellular differentiation (reviewed in Los *et al*., 2001). Although the understanding of the mechanisms regulating caspase function is far from complete, a major factor that has been shown to control their activity is the inhibitors of apoptosis (IAP) family of proteins.

The IAPs are a family of proteins that function as intrinsic regulators of the caspase cascade. Cellular proteins have been identified that inhibit specific 'upstream' or initiator caspases, but the IAPs are the only known endogenous proteins that regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7). As a result, the IAPs are being evaluated as potential key therapeutic targets in a wide range of human disease conditions. However, a critical issue in terms of their therapeutic utility is whether increased IAP activity can truly prevent apoptosis or merely delay the inevitable. Again, this raises the issue of the point-of-no-return: is it possible to rescue a cell that has been damaged to the extent that it will undergo apoptosis? Recent data using viral expression vectors and transgenic mice suggest that intervention downstream of mitochondrial permeabilization and caspase activation still allows a functional recovery. The IAPs have thus emerged as promising therapeutic targets on both sides of the apoptosis 'coin'; when overexpressed, cells become resistant to apoptotic triggers (e.g. for treatment of neurodegeneration), and when inhibited, cells become sensitized to cell death (e.g. for anticancer therapy).

IAP gene family

The IAPs are a family of proteins characterized by one or more 70–80 amino-acid baculoviral IAP repeat (BIR) domains. The BIR domain is a characteristic cysteine- and histidine-rich protein folding domain that chelates zinc and forms a compact globular structure consisting of four or five alpha helices and a variable number of antiparallel beta-pleated sheets. The core of a BIR domain consists of the variable consensus sequence, $C(X)_2C(X)_6W(X)_3D(X)_5H(X)_6C$, where X is any amino acid. The first IAPs were identified in baculoviruses (reviewed in Clem, 2001), and have since been found in *Drosophila* and numerous vertebrate species. The characterization of IAP proteins suggests that they function as an endogenous caspase inhibitors, as well as participate in cell cycle regulation and in the modulation of receptor-mediated signal transduction. The prototype

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Table 1 IAP nomenclature and activities

IAP	*Alternative names*	*Domains*	*Caspase specificity*	*Identified binding proteins*
NAIP	BIRC1	BIR(3) NOD LRR	Caspase-3 and -7	Hippocalcin
XIAP	BIRC4 API3 MIHA ILP-1	BIR(3) RING	Caspase-3, -7, and -9	Smac/DIABLO Omi/HtrA2 XAF1 TAB1 NRAGE
c-IAP1	BIRC2 API1 MIHB HIAP2	BIR(3) CARD RING	Caspase-3, -7, and -9	Smac/DIABLO Omi/HtrA2 TRAF1 TRAF2
c-IAP2	BIRC3 API2 MIHC HIAP2	BIR(3) CARD RING	Caspase-3, -7, and -9	Smac/DIABLO Omi/HtrA2 TRAF1 TRAF2 Bcl10
Survivin	BIRC5 API4 TIAP (mouse)	BIR Coiled coil	Caspase-3 and -7	β-tubulin Smac
Livin	BIRC7 KIAP ML-IAP	BIR RING	Caspase-3, -7, and -9	Smac
Ts-IAP	BIRC8 ILP-2	BIR RING	Caspase-9	

IAP family members identified in baculoviruses, as well as several of the mammalian IAPs, possess a carboxy-terminal RING zinc-finger (RZF). However, not all BIR-containing proteins possess this motif, as summarized in Table 1. The subsequent identification of BIR-containing proteins in unicellular organisms, such as yeast, which do not possess a cell death program, has necessitated a more strict definition of an IAP than the mere presence of a BIR domain. Members of the larger family are now termed BIRPs, for BIR-containing proteins, of which two major subfamilies have been described (reviewed in Miller, 1999). The IAP subfamily consists of those members possessing one or more BIRs, and to which have been ascribed an antiapoptotic activity. The other BIRP subfamily includes members that generally contain only a single BIR domain, and appear to function in cytokinesis and chromatin segregation, with possible secondary roles in apoptosis regulation (Miller, 1999). This review covers the structural and biochemical features of the IAPs, their regulation, their role in signal transduction pathways, and their potential as therapeutic targets in a variety of disease states.

Independent identification of the IAPs has led to multiple names ascribed to each family member, and is summarized in Table 1. The most frequently cited nomenclature has been used in this review. The first mammalian IAP homologue to be identified was neuronal apoptosis inhibitory protein (NAIP), which was isolated during a positional cloning effort to identify the causative gene for spinal muscular atrophy (SMA, Roy *et al*., 1995). In contrast to the baculoviral IAPs, which possess two BIR domains and a carboxy-terminal RZF, *naip* encodes three BIR domains and a very large and unique carboxy-terminus containing a nucleotide-binding oligomerization domain (NOD) (reviewed in Koonin and Aravind, 2000). SMA is a degenerative neuromuscular disease characterized by progressive loss of motor neurons leading to wasting of the voluntary muscles. Concurrent with the isolation of *naip*, a second candidate gene, survival motor neuron (*smn*; Lefebvre *et al*., 1995), was identified within the SMA critical region. Although *naip* deletion or mutation has since been ruled out as the primary causative event in SMA, deletions of the neighboring *smn* gene that encompass *naip* appear to lead to greater disease severity. The suggestion has therefore been made that NAIP deletion may have a modulating function (reviewed in Gendron and MacKenzie, 1999). The pattern of neuronal loss in SMA is consistent with the distribution of the NAIP protein within the central nervous system (Xu *et al*., 1999). Finally, NAIP's capacity to suppress cell death is compatible with its deletion playing a role in determining cell loss (Liston *et al*., 1996; Xu *et al*., 1997; Maier *et al*., 2002).

Subsequent to the identification of NAIP, the IAP family expanded with the identification of cellular IAP1 (c-IAP1, a.k.a. HIAP2), c-IAP2 (a.k.a. HIAP1), and X-chromosome-linked IAP (XIAP), all of which contain three BIR domains and a carboxy-terminal RING finger (Rothe *et al*., 1995; Duckett *et al*., 1996; Liston *et al*., 1996; Uren *et al*., 1996). The family continued to expand with the identification of Survivin (single BIR,

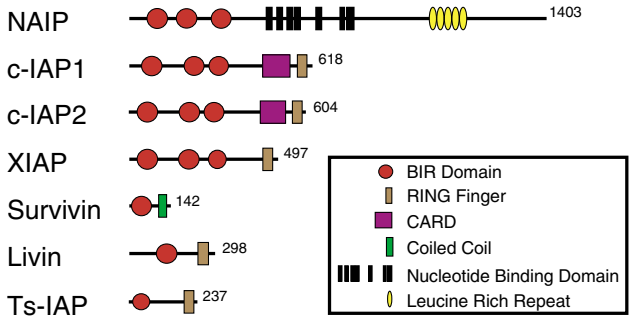

Figure 1 Domain structure of the IAP family. Individual domains are drawn to scale. The abbreviations are as follows: BIR: baculoviral IAP repeat; CARD: caspase recruitment domain; RING: RING zinc-finger; NOD: nucleotide-binding oligomerization domain; and LRR: leucine-rich repeats

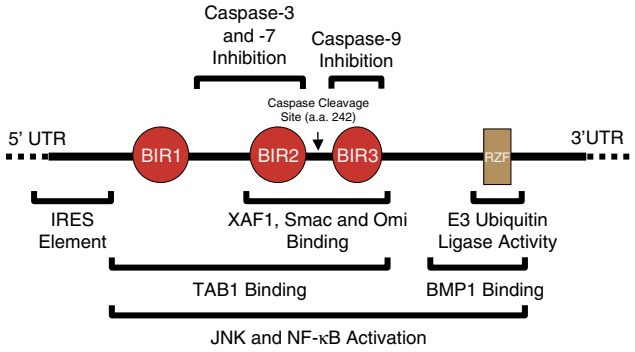

Figure 2 Functional map of XIAP activities and interactions. Some interactions have not been mapped to individual domains within the protein, and are indicated as requiring the entire protein. The 5′ and 3′ UTR regions are not shown to scale. IRES: internal ribosome entry site, UTR: untranslated region

carboxy-terminal coiled-coil domain; Ambrosini *et al*., 1997), Livin (single BIR and carboxy-terminal RING finger; Lin *et al*., 2000; Vucic *et al*., 2000; Kasof and Gomes, 2001), and testis-specific IAP (Ts-IAP; single BIR and carboxy-terminal RING finger; Lagace *et al*., 2001; Richter *et al*., 2001). Table 1 summarizes the names, domain structure and interactions of the IAP family members, with the protein domains illustrated schematically in Figure 1.

IAP BIR domains bind and inhibit caspases

Immediately following their identification, the mammalian IAPs were documented to suppress cell death in a variety of tissue culture systems, including triggers of both the endogenous and exogenous pathways of apoptosis (reviewed in LaCasse *et al*., 1998; Deveraux and Reed, 1999). It was subsequently shown that the IAPs could directly inhibit the activity of several recombinant caspases *in vitro* (Deveraux *et al*., 1997; Roy *et al*., 1997; Maier *et al*., 2002), and that isolated BIR domains possessed this activity (Takahashi *et al*., 1998).

The BIR domains of the IAPs are the most fully characterized functional units of the IAPs. Each BIR domain folds into a functionally independent structure that chelates a zinc ion and consists of a globular head and an unstructured tail derived from the amino-terminal 'linker' region located upstream of the individual BIR domains. The majority of IAP interactions have been mapped to the BIR domains, including inhibition of caspases, binding of c-IAP1 and two to TNF receptor-associated factors (TRAFs), and XIAP interaction with the TAB1 protein. Specific interactions with initiator (caspase-9) and effector (caspase-3 and -7) caspases have been mapped to individual BIR domains (Figure 2). As a general rule, the IAPs containing multiple BIRs employ the third BIR domain to inhibit caspase-9, and the second BIR domain functions to inhibit caspase-3 and -7 (Roy *et al*., 1997; Takahashi *et al*., 1998; Maier *et al*., 2002). Of the single BIR-containing proteins, Ts-IAP (ILP-2) inhibits caspase-9 (Richter *et al*., 2001), while the single Survivin BIR domain inhibits caspase-3 and -7 (Shin *et al*., 2001). Interestingly, the single BIR domain in Livin has been reported to inhibit caspase-3, -7, and -9 (Lin *et al*., 2000; Vucic *et al*., 2000; Kasof and Gomes, 2001), and thus appears to have a broader range of activity than any other single BIR domain.

Although the majority of the structural studies on the IAP–caspase interaction have focused on XIAP, the general conclusions are believed to hold true for most of the other IAPs as well. Furthermore, NMR and crystallography studies have used isolated BIR domains rather than the full-length proteins. Fortunately, the individual BIR domains appear to fold correctly, and their activities accurately reflect the physiological attributes of the full-length protein.

Despite the overall structural similarity of the BIR domains, the mechanisms of caspase inhibition differ significantly between BIR2 and BIR3. Crystallography (Chai *et al*., 2001; Huang *et al*., 2001; Riedl *et al*., 2001) as well as mutagenesis studies (Sun *et al*., 1999, 2000) on XIAP BIR 2 complexed with caspase-3 or -7 revealed that the linker region proximal to BIR2 (i.e. between BIR1 and BIR2) is far more important than the BIR domain itself. The linker region has been shown to stretch across the active site of caspase-3 and -7 and preclude substrate entry. Interestingly, the peptide is positioned in the reverse orientation relative to peptide inhibitors and natural substrates of the caspases, and is thus very distinct from other known protease inhibitors. Although caspase-3 and -7 are very closely related, and the linker region inhibits both enzyme catalytic sites, there are important differences. The linker region accounts entirely for the inhibition of caspases-3, and BIR2 can be replaced by an irrelevant protein such as GST (Chai *et al*., 2001; Huang *et al*., 2001). In contrast, the XIAP BIR2 domain makes contact with the amino terminus of the small subunit of caspase-7 and stabilizes the interaction of the linker in the catalytic groove (Suzuki *et al* 2001b). As a consequence, XIAP-mediated inhibition of caspase-3 is competitive, whereas inhibition of caspase-7 occurs by both a competitive and noncompetitive mechanism (see Figure 2).

The BIR3 domains of XIAP, c-IAP1, c-IAP2 (Deveraux *et al*., 1998; Bratton *et al*., 2001), and the single BIR domains in Livin (Vucic *et al*., 2000) and Ts-IAP (Richter *et al*., 2001) have been demonstrated to bind and inhibit caspase-9. The mechanism of caspase-9 inhibition has again been best characterized for the XIAP protein, and is very distinct from the binding to caspase-3 or -7. Caspase-9 becomes catalytically active through a conformational change when bound by apoptosis protease-activating factor-1 (Apaf1), and thus appears to be unique among the caspases in that there is no absolute requirement for proteolytic activation. In addition to its ability to proteolytically process caspase-3, caspase-9 can undergo a self-cleavage event in the linker region between the p20 and p10 subunits at Asp_{315}. The XIAP BIR3 domain directly binds caspase-9 via this newly exposed amino terminus and the interaction is stabilized through additional contacts with the enzyme. Using purified caspase-9 and XIAP BIR3, in the absence of Apaf-1 and cytochrome *c*, it was recently demonstrated that this interaction prevents caspase-9 homodimerization and stabilizes the enzyme in an inactive state similar to its monomeric form (Shiozaki *et al*., 2003). It is proposed that a similar change in caspase-9 morphology occurs within the apoptosome complex as a consequence of XIAP binding. Caspase-9 can also undergo further cleavage catalysed by caspase-3 at Asp_{330}, 15 amino acids from the initial, autocatalytic site. This proteolytic event further increases the enzymatic activity of caspase-9, and was proposed to remove the peptide sequence that binds XIAP (Srinivasula *et al*., 2001). However, recent work suggests that XIAP BIR3 can still interact with and inhibit the enzyme activity of processed caspase-9 in the apoptosome complex (Zou *et al*., 2003).

Thus, there are distinctly different mechanisms of inhibition for each of the three known caspase targets of the IAPs; caspase-3 is inhibited by the BIR1-2 linker region exclusively; caspase-7 is inhibited by a combination of the BIR1-2 linker and the BIR2 domain; and caspase-9 is inhibited by BIR3, without the apparent contribution of the upstream linker region. Furthermore, the inhibition of caspase-3 and -7 is brought about by steric hindrance in which substrate entry is blocked, whereas caspase-9 inhibition is mediated by a conformational shift that inactivates the enzyme. Finally, it should be noted that the BIR1 domains of XIAP, c-IAP1, c-IAP2, and NAIP display no caspase inhibiting activity. Significantly, the BIR1 segment is the least conserved of the three BIR domains, but its very existence implies that some unknown unique function remains to be identified.

RING fingers: targeting IAPs and their binding partners for degradation

RZFs are a subclass of zinc-finger domains that chelate two zinc ions in a characteristic crossbrace arrangement. Typically, RZFs are usually found at the amino terminus of proteins that function as E3 ubiquitin ligases. RZF proteins perform as adapters, recruiting target proteins to a multicomponent complex containing an E2 enzyme and provide specificity for ubiquitin-conjugating activity, and hence proteosomal degradation (reviewed in Pickart, 2001). Initial studies on the RZF domains of XIAP and c-IAP1 suggest that this motif provokes the ubiquitination and degradation of IAP proteins in response to apoptotic stimuli. Treatment of cells with glucocorticoids or etoposide results in the rapid degradation of these IAPs, which can be blocked by proteosome inhibitors (Yang *et al*., 2000). Later work established that c-IAP2 and XIAP can trigger the ubiquitination of caspase-3 and -7 (Huang *et al*., 2000; Suzuki *et al*., 2001c), suggesting that targeting of caspases to the proteosome may be one of the antiapoptotic mechanisms of the IAPs. In addition, XIAP, c-IAP1, and c-IAP2 have been demonstrated to ubiquitinate the second mitochondrial activator of caspases (Smac, a.k.a. DIABLO) protein (MacFarlane *et al*., 2002; Hu and Yang, 2003), an antagonist of IAP function (see below).

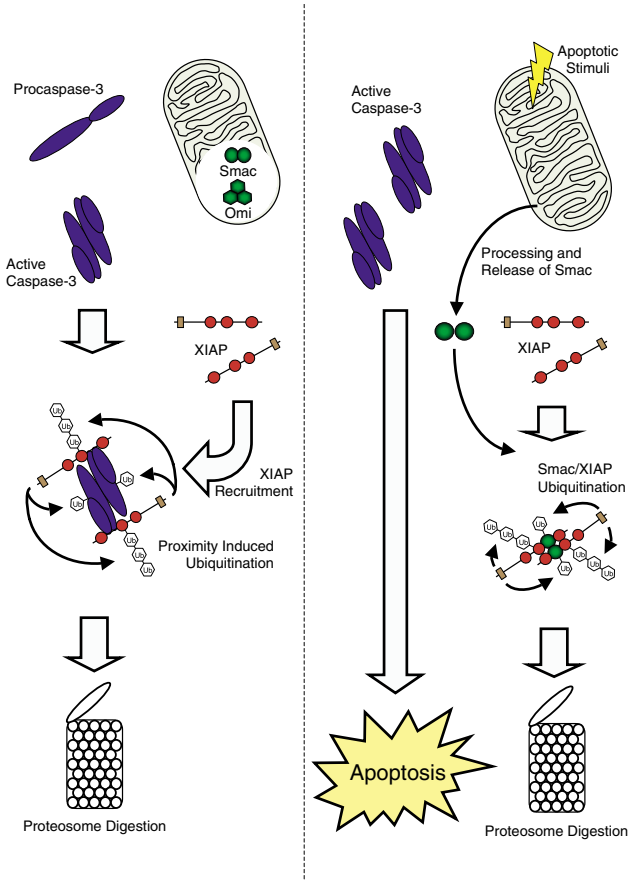

Figure 3 An induced proximity model for XIAP's ubiquitin ligase activity. On the left is a healthy cell or one that has received a sublethal apoptotic stress. Caspases are initially inhibited by XIAP binding. The proximity induced by caspase–XIAP interaction triggers autoubiquitination of XIAP, as well as ubiquitination of the caspase itself. The entire complex is degraded in the apoptosome. On the right side of the figure, the cell has received a lethal apoptotic stress. Endogenous XIAP (and the other IAPs) are saturated by interactions with Smac and/or Omi. Proteosome-mediated degradation of XIAP/Smac and XIAP/Omi complexes results in significant XIAP depletion and allows unrestricted caspase activation

It thus remains unclear whether the RZF contributes overall to the antiapoptotic activity of the IAPs, or whether it in fact antagonizes this activity, since conflicting results have been obtained when comparing RZF deletion mutants with the full-length protein (Yang *et al*., 2000; Clem *et al*., 2001; Suzuki *et al*., 2001c). We propose that the RZF functions to suppress apoptosis in conditions of low apoptotic stress, and that autoubiquitination dominates in situations of high apoptotic stress to target IAP complexes for disposal. In this model, which is presented in Figure 3, the mitochondrial release of limited amounts of cytochrome *c*, or the 'accidental' activation of small amounts of effector caspases would not have catastrophic effects. The IAPs would function to inhibit the caspases until they could be safely disposed via the proteosome. If the IAPs are consumed in this disposal event rather than recycled for subsequent use, then the observed drop in IAP levels in an apoptotic cell, although dramatic, would still be a consequence, rather than a trigger of apoptosis. In these circumstances, the cumulative load of caspase–IAP and Smac–IAP complexes would result in the significant depletion of cellular stores of available IAP protein. As yet, there have been no published reports of a specific mechanism regulating IAP autoubiquitination, which appears to occur only in apoptotic cells. Perhaps not coincidentally, the IAP ubiquitination targets are dimers (caspase-3, caspase-7, Smac) or higher-order complexes (caspase-9). We suggest an 'induced proximity' mechanism, analogous to caspase recruitment and activation. The formation of complexes containing more than one molecule of the IAPs may serve as the trigger for IAP crossubiquitination and subsequent degradation of the entire complex (see Figure 3).

CARD and NOD domains

The caspase recruitment domain (CARD) is a type of protein fold that typically mediates oligomerization with other CARD-containing proteins, as well as promotes homodimerization (reviewed in Weber and Vincenz, 2001). Both c-IAP1 and c-IAP2 have a CARD domain between the BIR domains and the carboxy-terminal RZF (Figure 1). The location of the CARD domain in the middle of these proteins is unusual. CARDs, death domains, and death effector domains, which are all structurally related, are almost universally located in the extreme amino terminus of the protein. To date, no function has been ascribed to the CARD domains of either c-IAP1 or c-IAP2.

The unique carboxy terminus of NAIP consists of an NOD followed by a distal cluster of 14 leucine-rich repeats (LRRs) of 20–29 amino acids each. Many NOD-containing proteins are organized and function in a similar manner: the centrally located NOD domain mediates self-association, triggering induced proximity of proteins bound by the amino-terminal domain (reviewed in Inohara and Nunez, 2001; Tschopp *et al*., 2003). The activation of the NOD cassette is controlled by a carboxy-terminal 'sensor' domain. Apaf1 is the prototype of these proteins, in which cytochrome *c* binding to the WD40 repeats in the 'sensor' domain triggers NOD-mediated oligomerization and a conformational change that exposes the amino-terminal CARD domain for recruitment of caspase-9. Additional NOD proteins have been identified that recruit caspases (NOD1/CARD4, IPAF/CLAN/CARD12) and/or activate the NF-κB pathway (IPAF, NOD2/CARD15). Like NAIP, the 'sensor' domains of NOD1, NOD2, and IPAF are LRRs, and are structurally related to a series of plant pathogen resistance (R) proteins. All of these LRR proteins bind intracellular LPS secreted by bacterial pathogens, and are involved in host responses such as cytokine production and NF-κB activation. The mouse *naip* gene cluster, which consists of at least six copies of *naip* (Yaraghi *et al*., 1998), maps to the *legionella* susceptibility locus (Growney and Dietrich, 2000), again providing intriguing indications that NAIP may be unique among the IAPs in playing a role in the host cell response to intracellular bacterial infection. We propose that NAIP binds LPS via the LRR domain, which triggers NOD oligomerization and exposure/activation of the BIR domains. Given the structural similarity between NAIP and IPAF, both proteins may be simultaneously activated by intracellular pathogens. With the formation of the 'inflammasome' and activation of proinflammatory caspases such as caspase-1, perhaps concomitant NAIP activation is required to suppress apoptosis that would otherwise result from activation of effector caspases by the proinflammatory initiator caspases. Although this model accounts for the conservation of the carboxy-terminal domain of NAIP, oligomerization and BIR domain activation in response to LPS has yet to be demonstrated.

IAPs as caspase substrates

In addition to inhibiting caspases, XIAP and c-IAP1 can also serve as substrates for these proteases. Apoptosis triggered by a variety of mechanisms results in the appearance of proteolytic fragments of XIAP (Deveraux *et al*., 1999). *In vitro* protease reactions suggest that a number of caspases, including caspase-3, -6, -7, and -8 can cleave XIAP. The initial processing event occurs at Asp_{242}, located in the linker region between BIR2 and BIR3, and generates two products: a BIR1-2 fragment and a BIR3-RING fragment. The BIR1-2 product, when synthesized in bacteria, retains the ability to inhibit caspase-3, and the corresponding coding region expressed from a plasmid will inhibit Fas-induced apoptosis. This inhibition does not appear to be as effective as the full-length XIAP protein, and the BIR1-2 fragment is further degraded to small peptide fragments during apoptosis. The BIR3-RING fragment appears to be longer lived, and retains the ability to inhibit caspase-9- and Bax-induced apoptosis. The physiological significance of XIAP processing remains uncertain. A model was proposed in which XIAP cleavage results in the separation of two functional

regions of the protein, allowing independent targeting of caspases (Deveraux *et al*., 1999). However, in most instances, the proteolytic fragments have only been observed in apoptotic cells (Deveraux *et al*., 1999; Johnson *et al*., 2000; Levkau *et al*., 2001), suggesting that XIAP cleavage is a marker of cell death, rather than a mechanism of protecting cells. It is worth noting that the XIAP fragments appear late in the apoptotic process, as much as 8 h after the initiation of rapid cell death triggers (e.g. anti-Fas antibody). At this point, all of the morphological and biochemical characteristics of apoptosis are well progressed, and the cell is past the point of no return.

c-IAP1 can also be cleaved by caspase-3, at Asp_{351}, just distal to the BIR3 domain. The evidence to date suggests that the carboxy-terminal fragment, consisting of the CARD domain and RING finger, is proapoptotic (Clem *et al*., 2001), while the amino-terminal fragment consisting of the three BIR domains is rapidly degraded. Precedent for caspase cleavage converting an antiapoptotic protein into a proapoptotic protein is well established with the Bcl-2 and Bcl-X_L proteins (reviewed in Karran and Dyer, 2001). However, both the mechanism by which the CARD-RING fragment of c-IAP1 triggers apoptosis and the physiological significance of caspase cleavage is as yet unknown.

Negative regulators of IAP function

Currently, there are three proteins that have been identified that bind IAPs and suppress their activity. These proteins have been termed XAF1 (XIAP-associated factor1), Smac, and Omi (a.k.a HtrA2). The XAF1 protein was identified by two-hybrid screening with XIAP, and encoded a novel, zinc-finger-rich protein. *In vitro* experiments using purified, recombinant proteins demonstrated that XAF1 can directly bind XIAP and interfered with XIAP-mediated caspase-3 inhibition (Liston *et al*., 2001). Cell culture experiments using recombinant adenoviruses demonstrate that XAF1 reverses XIAP-mediated protection against chemotherapeutic drugs, such as etoposide or cisplatin. XAF1 protein accumulates in the nucleus, whereas XIAP is predominantly cytosolic. However, XAF1 can trigger the relocalization of XIAP from the cytosol to the nucleus, perhaps as a means of sequestering XIAP. Interestingly, XAF1 is ubiquitously expressed in normal tissues, but is found at extremely low levels (less than 1% of control normal tissues) in the majority of the NCI 60 cell line panel of cancer cells (Fong *et al*., 2000). This loss of XAF1 expression in transformed cells has been proposed to contribute to apoptosis suppression by allowing unrestricted IAP activity.

Unlike XAF1, the Smac protein resides in the mitochondria of healthy cells, and is released upon apoptotic stress with similar kinetics to cytochrome *c*. In the process of being released from the mitochondria, the 55 amino-acid mitochondrial localization signal peptide is proteolytically removed (Du *et al*., 2000; Verhagen *et al*., 2000). The mechanism of Smac release has not been entirely resolved. Treatment of apoptotic cells with caspase inhibitors allows for cytochrome *c* discharge, but blocks release of Smac, suggesting mitochondrial release by separate mechanisms (Adrian *et al*., 2001). The relatively small cytochrome *c* ($\sim$12 kDa) protein has been proposed to escape through Bax or Bak formed membrane pores. By comparison, the Smac dimer ($\sim$100 kDa) may require the formation of the permeability transition pore subsequent to caspase activation.

Smac has been demonstrated to bind all of the IAPs tested to date, including XIAP, c-IAP1, c-IAP2, Survivin (Du *et al*., 2000), and Livin (Vucic *et al*., 2002). Although Smac can bind to either BIR2 or BIR3 of XIAP, thereby interfering with either caspase-3/-7 or caspase-9 inhibition, the binding is considerably stronger with BIR3 (Liu *et al*., 2000; Srinivasula *et al*., 2000). The crystal structure of Smac revealed that the protein forms a long, bridge-like structure consisting of three extended alpha helices bundled together, and an unstructured amino terminus (Wu *et al*., 2000). Smac homodimers form via a large hydrophobic interface, and this homodimerization appears to be required for activity (Chai *et al*., 2000). The unstructured, newly generated amino terminus of Smac makes critical contacts with XIAP BIR3 and mediates XIAP inhibition. Additional contacts with the helical bundles of Smac are predicted from the crystal structure, and are more significant in interactions with the BIR2 domain (Srinivasula *et al*., 2000). Cocrystallization of Smac and XIAP BIR3 established that the amino-terminal tetrapeptide sequence of Smac (Ala-Val-Pro-Ile) fits within a surface groove of the BIR3 domain, with the alanine residue bound within a hydrophobic pocket. Some, but not all of the ability of Smac to inhibit XIAP BIR3 function can be reconstituted with short peptides. The surface contacts that Smac makes with XIAP BIR3 overlapped completely with a surface map of BIR3/caspase-9 contacts (Sun *et al*., 2000). Furthermore, the similarity to the cleavage site in the linker region of caspase-9 (Ala_{316}-Thr-Pro-Phe) suggests a competition model in which Smac competes for or displaces XIAP from caspase-9. A key component of this model was established when Asp_{316} cleavage site mutants of caspase-9 were established. Mutant caspase-9 is fully active, but cannot undergo proteolytic processing between the large and small subunits. XIAP cannot inhibit this enzyme, demonstrating that a critical feature of XIAP-mediated inhibition is interaction with the amino terminus of the linker region of partially processed caspase-9 (Ekert *et al*., 2001; Srinivasula *et al*., 2001).

The identification of Smac also provided a critical insight into the function of the proapoptotic *Drosophila* proteins Reaper, Hid, Grim, and Sickle. These proteins control virtually all cell death in the fly and can all be antagonized by the *Drosophila* IAP DIAP1, but share little or no sequence conservation (reviewed in Song *et al*., 2000). However, the amino termini of these proteins all share homology with the Smac tetrapeptide motif, and have been shown to bind a similar groove on

	Caspase-9	(P20) 315	A	T	P	F	Q	E	G	L	R	T
	Smac/DIABLO	(Mito) 56	A	V	P	I	A	Q	K	S	E	P
Mammalian (human)	Omi/HtrA2	(Mito) 156	A	V	P	S	P	P	P	A	S	P
Insect (*Drosophila*)	Jafrac2	(ER) 18	A	K	P	E	D	N	E	S	C	Y
	Reaper		M	A	V	A	F	Y	I	P	D	Q	A
	Hid		M	A	V	P	F	Y	L	P	E	G	G
	Grim		M	A	I	A	Y	F	I	P	D	Q	A
	Sickle		M	A	I	P	F	F	E	E	E	H	A

Figure 4 IBM-containing proteins in humans and *Drosophila*. The human IBM-containing proteins undergo proteolytic processing to expose the IBM tetrapeptide. The upstream peptide encodes either the large subunit of caspase 9 (P20) or a mitochondrial targeting sequence (Mito). In *Drosophila*, most IBMs are found at the amino terminus of the protein and are believed to be exposed by constitutive methionine aminopeptidase activity. The Jafrac2 protein is unique in *Drosophila* and more closely resembles the mammalian situation in which a localization signal (ER) is removed in order to expose the IBM. The amino-acid position of the alanine is indicated in those proteins that require processing

the surface of BIR2 of DIAP1 (Wu *et al*., 2001). These short consensus sequences have been termed IAP-binding motifs (IBMs) or Reaper/Hid/Grim (RHG) motifs. The alignment of various *Drosophila* and mammalian IBMs is shown in Figure 4.

Subsequent to the identification of Smac, a second mitochondrial IAP-binding protein, called Omi or HtrA2, was identified by several groups (Suzuki *et al*., 2001a; Hegde *et al*., 2002; Martins *et al*., 2002; Van Loo *et al*., 2002; Verhagen *et al*., 2002). Like Smac, the trimeric Omi protein is released from the mitochondria of apoptotic cells and is processed to generate a Smac-like tetrapeptide motif at the amino terminus. Direct binding to XIAP and inhibition of the XIAP–caspase interaction appears to be only one of the proapoptotic activities of Omi. The serine protease activity of Omi also contributes to cell death in a noncaspase-dependent manner, although the cellular targets of the protease activity have not been identified. By analogy to the *Escherichia coli* HtrA2 protein, it was predicted that the human Omi/HtrA2 protein would be involved in the proteolytic degradation of misfolded proteins under conditions of cellular stress. Although no cytosolic targets have been identified, proteolytic removal of the 155 amino-acid mitochondrial targeting peptide of Omi is a self-catalysed event (Savopoulos *et al*., 2000), raising the possibility that other mitochondrial leader sequences, including that of Smac, may be processed by Omi.

In a simplified version of the overall process of cell death as it relates to caspase activation and the IAPs, the following summary (Figure 5) is presented. Apoptotic stresses acting through the endogenous pathway triggers the expression and/or activation of proapoptotic Bcl-2 family members. The balance of pro- and antiapoptotic Bcl-2 proteins constitutes the first apoptotic checkpoint. Given sufficient activation, the proapoptotic Bcl-2 proteins trigger the release of cytochrome *c*, presumably

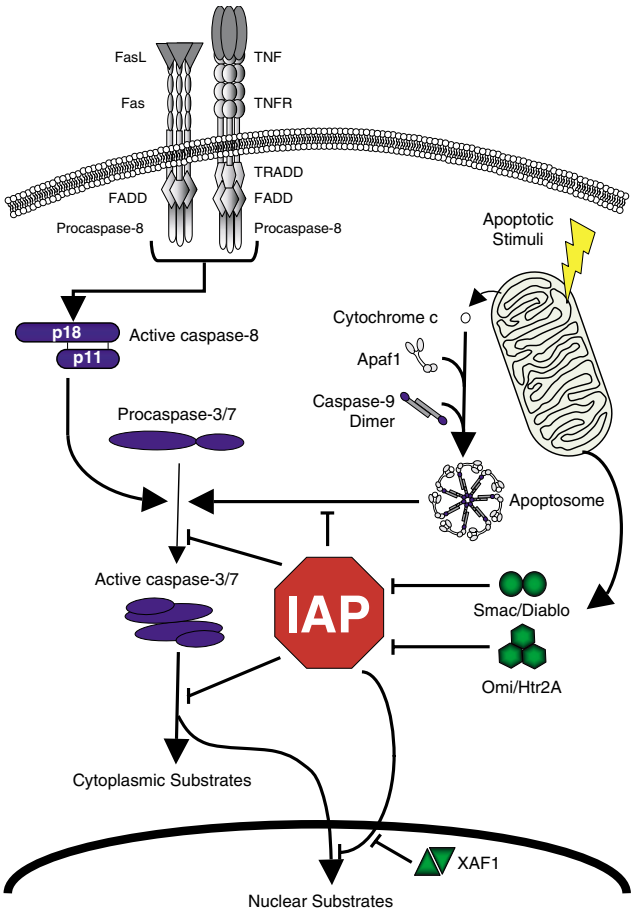

Figure 5 Role of the IAPs in regulating both the endogenous and exogenous pathways. Inhibition of both initiator (caspase-9) and effector (caspase-3 and -7) caspases uniquely situates the IAPs at the junction of both major pathways. See text for details

via channel formation in the outer mitochondrial membrane. The release of cytochrome *c* triggers a second checkpoint, in which levels of the IAPs and XAF1 determine the outcome. XAF1 is proposed to titer the amount of functional IAP protein in the cell. Pre-existing high levels of IAP (or conversely, low levels of XAF1) expression may suppress newly activated caspase-9 and any effector caspases that have become activated, and the ubiquitin ligase activity triggers the disposal of activated caspases via the proteosome. However, if sufficient caspase-9 activation occurs, caspase-3 and/or -7 are activated and caspase-9 is further processed to its most active state. In addition, activated caspases trigger the further permeabilization of the mitochondrial membranes through the activation of the permeability transition pore, which allows the release of apoptosis-inducing factor (AIF), Smac, and Omi. AIF transits to the nucleus and triggers chromatin condensation, while Smac and Omi bind and inhibit any further participation of the IAPs, thereby allowing unrestricted caspase activity to proceed. In addition, the IAPs are themselves degraded by the caspases (i.e. XIAP) or processed in such a way as to promote the continued release of proapoptotic factors (i.e. c-IAP1).

An additional negative regulator of IAP function in *Drosophila* has been reported recently. Jafrac2 is a thioredoxin peroxidase that resides in the endoplasmic reticulum (ER) of healthy cells. The 17 amino-acid ER leader peptide is proteolytically removed after synthesis, but the mature protein is only released into the cytosol after an apoptotic stimulus (Tenev *et al*., 2002). Although the mammalian equivalent to Jafrac2 has not yet been reported, there are likely to be unique IBM-containing IAP inhibitors in various subcellular compartments responsible for executing organelle-specific apoptotic pathways.

Specialization and regulation: the IAPs are not as redundant as they appear

With the discovery that XIAP could inhibit only a subset of the caspases, the logical prediction was that the other IAPs would display high affinities for the remaining members of the caspase family. Quite the reverse holds true, and all IAPs appear to be restricted to inhibiting caspase-3, -7, and/or -9, although their respective affinities do vary. This apparent redundancy ignores critical aspects of IAP expression and subcellular localization that make each IAP distinct. Some of the unique attributes of individual IAPs have been defined, while others no doubt remain to be discovered.

The most distantly related IAP, Survivin, is restricted to expression at the G2/M point in the cell cycle, and is the only IAP to associate with chromatin structures (Li *et al*., 1998). Survivin thus appears to play a unique role in monitoring the success of chromosome replication and the suppression of caspase activity in the nucleus. Livin has also been reported to localize in the nucleus, but does not appear to be cell cycle regulated nor does it associate with particular structures within the nucleus (Kasof and Gomes, 2001). One possibility is that Livin serves a 'housekeeping' function and prevents accidental caspase activation within the nucleus (Figure 6).

Ts-IAP is an autosomal, retrotransposed, intronless copy of XIAP, and is expressed solely in the testis (Lagace *et al*., 2001; Richter *et al*., 2001). There are examples of other X-chromosome-linked genes having retrotransposed autosomal copies. The phosphoglycerate kinase (PGK) gene is one such example, in which the chromosome 19 copy contains no introns and is expressed under the control of a promoter unrelated to the X-linked ancestral gene. Like Ts-IAP, the PGK-2 gene is active only in the testis, where it is required in order to compensate for X-chromosome inactivation during spermatogenesis (McCarrey and Thomas, 1987). The distribution of Ts-IAP within the testis has not been characterized, and it will be interesting to determine if it is indeed expressed in spermatogonia.

Less clear is the need for multiple copies of the triple-BIR-containing IAPs, NAIP, c-IAP1, c-IAP2, and XIAP. The unique carboxy terminus of NAIP suggests that it may function in some way related to host defense against intracellular parasites, but much remains to be

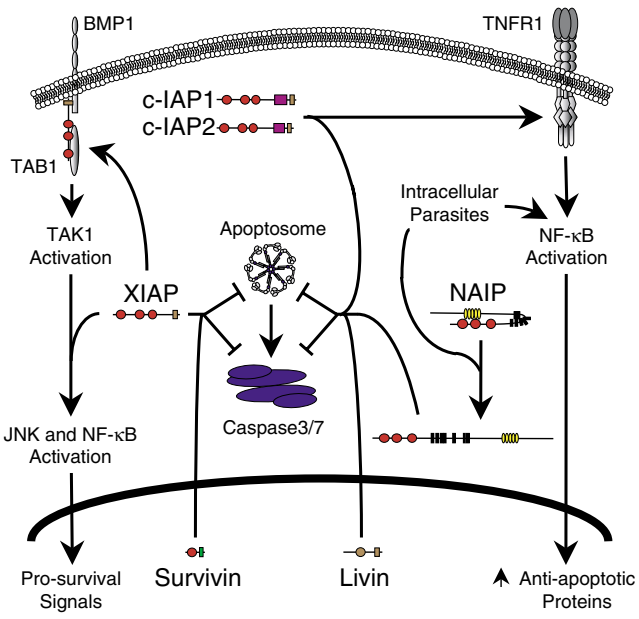

Figure 6 Summary of IAP activities. In addition to their caspase inhibiting activity, the IAPs have significant roles in signal transduction pathways. XIAP can function as an adapter protein, recruiting TAB1 and TAK1 to BMP receptors, resulting in the activation of both the JNK and NF-κB survival pathways. XIAP has been reported to bind and activate JNK1 directly. Two of the IAPs, c-IAP1 and c-IAP2, have been identified as components of the TNFR1 and TNFR2 complexes, and are believed to influence the outcome of TNF signaling by both inhibiting caspases and activating the NF-κB pathway. NAIP is shown in a folded conformation that conceals its BIR domains until activated by LPS. Livin and Survivin are shown as playing a role in preventing nuclear caspases from being activated

discovered. Although the remaining IAPs are all cytoplasmic and display similar activities, they have different tissue distribution. XIAP mRNA is expressed in all tissues at a relatively constant level, but protein synthesis is controlled by a unique mechanism. The XIAP transcript is ~9 kb, yet the coding region accounts for only 1.5 kb of this. The 5′ untranslated region (UTR) is at least 1.5 kb. Such long 5′ UTRs are relatively rare in eucaryotic mRNAs, and are predicted to constitute an insurmountable obstacle to normal scanning ribosome initiation. Subcloning of the XIAP 5′ UTR into bicistronic expression vectors has demonstrated that it contains an internal ribosome initiation site or IRES element (Holcik *et al*., 1999).

IRES elements were first identified in picornaviruses, which shut down host protein synthesis by inactivating a key initiation factor necessary for cap-dependent translation. The IRES element recruits ribosomes internally at the beginning of the ORF and thereby allows viral polyprotein synthesis to continue. Cellular IRES elements are relatively rare, but have been identified in a number of oncogenes and growth factor genes (reviewed in Holcik *et al*., 2000). IRES-containing transcripts thus continue to direct protein synthesis under a number of cellular stress conditions in which cap-dependent translation is shut down. There are many cellular stresses that trigger shut down of scanning ribosome

initiation, including serum starvation, chemotherapeutic drugs, γ-irradiation, heat shock, viral infection, and stress in the ER (reviewed in Holcik *et al*., 2000). In some cases, the explanation is clear as to why the cell would do this. For example, in heat-shocked cells unfolded proteins are not exported to the golgi and accumulate in the ER. When this happens, ER stress triggers the shut down of scanning ribosome initiated protein synthesis, thereby halting the further accumulation of proteins in the ER. The cell then tries to use this 'time out' to correct the problem before resuming protein synthesis. Proteins that are initiated by an IRES element and are thus resistant to this shutdown include chaperone proteins like BiP, which helps in the refolding of misfolded proteins, and XIAP, which helps the cell to survive this period of cell stress. Thus, the regulation of XIAP is distinct from that of the other IAPs, and may at least partially explain the requirement for more than one IAP gene.

In addition to its caspase inhibition and E3 ubiquitination activities, XIAP has been shown to play a role in signal transduction pathways. TAK1 is mitogen-activated protein kinase kinase kinase (MAPKKK) that can activate both the NF-κB and JNK1 signal pathways. The XIAP BIR domains bind to the TAB1 adapter protein, while the carboxy-terminal RING finger bind to the cytoplasmic tail of bone morphogenic protein (BMP) receptors. TAB1 in turn recruits TAK1 to the receptor complex (Figure 6, Yamaguchi *et al*., 1999). Although it seems clear that XIAP is required for BMP signaling, the relative significance of JNK1 and NF-κB activation in XIAP-mediated cytoprotection has not been determined. The use of dominant-negative TAK1 and/or JNK1 strongly suggests that the prosurvival activity of overexpressed XIAP (and also NAIP and Livin) is highly dependent on these pathways (Hofer-Warbinek *et al*., 2000; Sanna *et al*., 2002a). Additional XIAP interacting factors, including ILPIP (Sanna *et al*., 2002b), are still being identified and characterized, with much yet to be discovered about these pathways. Nevertheless, there appears to be at least three major mechanisms by which XIAP can suppress cell death: inhibition of caspases, targeted degradation of proapoptotic proteins, and transcriptional activation of prosurvival pathways via TAK1 (see Figure 6).

Two of the IAPs, c-IAP1 and c-IAP2, are components of the protein complex that forms on the cytoplasmic tail of TNFα receptor 2. This binding is not direct, and is mediated by two additional proteins, TRAF1 and TRAF2 (Rothe *et al*., 1995). In addition, c-IAP1 can also complex with TRAF2 and TRADD on the TNFR1 cytoplasmic domain (Shu *et al*., 1996). Signal transduction from the TNF receptors is extremely complex, resulting in either apoptosis or proliferation depending on the cell type and environmental cues. In the simplest scenario, recruitment of TRADD and FADD results in the formation of a death-inducing signaling complex that recruits and activates caspase-8 by an induced proximity activation mechanism. The formation of alternative complexes involving the TRAF and IAP proteins favors NF-κB activation, which in turn transcriptionally activates several prosurvival genes, including c-IAP1, c-IAP2, and XIAP (Figure 6, and reviewed in Lee and Collins, 2001). In what appears to be a positive feedback loop, c-IAP2 and XIAP appear to be able to trigger the activation of NF-κB (Chu *et al*., 1997; Hofer-Warbinek *et al*., 2000; Levkau *et al*., 2001). The role of the IAPs in TNF receptor complexes is not fully understood, and may be unrelated to direct caspase inhibition. Interestingly, c-IAP1 and c-IAP2 are not as potent as XIAP in both biochemical assays of caspase inhibition (Roy *et al*., 1997), and in most cell death models. The one exception to this is TNFα-mediated cell death, in which c-IAP1 outperforms the other IAPs (Wright *et al*., 2000), again suggesting specialized roles for each of the IAPs.

IAP-based therapeutics

Neuronal cell death in most neurodegenerative disorders, as well as in traumatic brain injury and spinal cord damage, exhibits most of the hallmarks of apoptosis (reviewed in Mattson, 2000). Limiting the extent of caspase activation in the target neuronal population may be therapeutically relevant in slowing the progression of Alzheimer's, Parkinson's, ALS, and Huntington's disease, as well as in retinal degenerations and in the injured CNS. Given that the IAPs have been demonstrated to suppress apoptosis initiated by virtually every trigger tested to date in tissue culture cells (reviewed in LaCasse *et al*., 1998), their utility has been explored in *in vivo* model systems. Stereotactic injection of adenoviral expression vectors has been used to determine the protective effect of NAIP and XIAP in the rat hippocampus in the four-vessel occlusion global ischemia model. Suppression of caspase activation shortly after the ischemic event, as well as long-term histological preservation of the vulnerable CA1 neurons was observed. Perhaps most significantly, functional rescue of memory and learning ability was demonstrated (Xu *et al*., 1997, 1999). Adenoviral vectors encoding NAIP, c-IAP1, and c-IAP2 have been shown to suppress apoptosis in the sciatic nerve axotomy model (Perrelet *et al*., 2000, 2002), and adeno-XIAP in an optic nerve axotomy model (Kugler *et al*., 2000). Additionally, NAIP overexpression has been shown to be protective both histologically and functionally in the 6-hydroxydopamine model of Parkinson's disease (Crocker *et al*., 2001). Finally, transgenic mice overexpressing XIAP in the brain are protected from the effects of MPTP toxicity (Crocker *et al*., 2003). These studies raise the hope that neurodegenerative disease intervention may be possible using more advanced gene therapy vectors encoding the IAPs.

Cancer is a disease that is extremely heterogeneous, in which many different tumor types can arise from virtually any tissue. Numerous proto-oncogenes have been identified, which, when mutated or aberrantly expressed, can contribute to the transformed phenotype. Despite the complexity of cancer genetics, there are

fundamental characteristics shared by all cancers, regardless of tumor origin. The deregulation of most growth-promoting oncogenes triggers apoptosis in an otherwise normal cell (reviewed in Evan and Vousden, 2001). As a consequence, the suppression of apoptosis is a fundamental and requisite change in all cancer cells, regardless of origin (Hanahan and Weinberg, 2000). Apoptosis is also the primary means by which radio- and chemotherapy modalities kill cancer cells (reviewed in Johnstone *et al*., 2002). The discovery that the bcl-2 oncogene functions as an apoptosis inhibitor revolutionized cancer biologists' concepts of tumorigenesis. Apoptotic inhibition emerged as a key component in cancer formation, progression, and resistance to therapy.

Given the central role of the IAPs in controlling apoptosis, it is not surprising that expression studies have revealed elevated IAP levels in a wide variety of cancer cell lines and primary tumor biopsy samples (LaCasse *et al*., 1998; Imoto *et al*., 2001, 2002; Dai *et al*., 2003; Yang *et al*., 2003). In addition to these correlative expression studies, direct genetic evidence is emerging that the IAPs can function as oncogenes. Chromosome amplification of the 11q21–q23 region, which encompasses both c-IAP1 and c-IAP2, has been observed in a variety of malignancies, including medulloblastomas, renal cell carcinomas, glioblastomas, gastric carcinomas, and non-small-cell lung carcinoma (Dai *et al*., 2003). Furthermore, esophageal squamous cell carcinomas frequently display this amplification, and transcriptional profiling has identified c-IAP1 as the sole target gene that is consistently overexpressed in these tumors (Imoto *et al*., 2001).

Additional direct genetic evidence for an oncogenic role of the IAPs is found in extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B-cell lymphomas. Two recurrent translocation events have been documented in MALT lymphomas: t(11;18)(q21;q21) and t(1;14)(p22;q32). The more frequent t(11;18) translocation occurs in up to 50% of extranodal MALT lymphomas (Baens *et al*., 2000), and are unusual in that they invariably encode an in-frame chimeric protein consisting of the c-IAP2 BIR domains (minus the CARD or RING domains), fused to the carboxy terminus of MALT1 (Uren *et al*., 2000). Significantly, the majority of gastric MALT lymphomas that do not respond to antibiotic therapy display the c-IAP2-MALT1 translocation (Liu *et al*., 2001). This appears to be due to a feedback mechanism, in which the c-IAP2-MALT1 fusion protein triggers NF-κB activation, which in turn transcriptionally upregulates the NF-κB-responsive *c-iap2* promoter. A positive feedback loop also explains why tumor regression does not occur with antibiotic therapy, since there is no longer a requirement for *H. pylori* infection and the resultant inflammatory response to achieve chronic NF-κB activation.

Inhibition of IAP expression or function has clear therapeutic potential in cancer therapy. In addition to tumor types with underlying genetic alterations to the IAPs, a generalized approach for increasing the apoptotic sensitivity of cancer cells using IAP inhibitors may prove effective. Adenoviral vectors encoding an antisense XIAP cDNA sensitize chemoresistant ovarian carcinoma cell lines (Sasaki *et al*., 2000; Li *et al*., 2001), as well as increasing the radiation sensitivity of non-small-cell lung carcinoma cells (Holcik *et al*., 2000). Antisense oligonucleotides that downregulate XIAP expression levels have been shown to chemosensitize cancer cells *in vitro* (Bilim *et al*., 2003) and in *in vivo* xenograft models (Hu *et al*., 2003). Injection of Smac peptides in combination with TRAIL ligand has also been demonstrated to trigger complete tumor regression in an orthotopic model of human glioma in nude mice (Fulda *et al*., 2002). Together, these lines of evidence indicate that targeting IAP expression and/or function, alone or in combination with conventional anticancer therapeutics, will be effective in human cancer therapy.

Conclusions

The process of apoptosis is controlled at multiple steps, each of which is influenced by both pro- and anti-apoptotic proteins. The equilibrium between the cell death-inducing caspase cascade and the IAPs constitutes one such fundamental decision point. An ongoing debate in apoptosis research concerns the so-called 'point-of-no-return', after which the cell cannot be rescued. Many researchers have proposed that mitochondrial permeability changes irreversibly commit the cell to die, and that inhibition of downstream events, including caspase activation, will only delay apoptosis or result in secondary necrotic cell death. However, there is evidence that some cell types can recover and resume proliferation despite the transient activation of the caspase cascade and the appearance of apoptotic morphology (reviewed in Vaughan *et al*., 2002). The recurrent upregulation of IAP expression in cancer cell lines and tumors also indicates that this decision point is crucial in determining overall cell fate. Experimentally, overexpression of the IAPs blocks apoptosis and results in functional recovery in a number of neurodegenerative model systems, again suggesting that the window for apoptosis intervention may be larger than was previously assumed.

The IAPs not only control cell death, but also influence signal transduction pathways and protein turnover. Clearly, many aspects of IAP function in all of these processes remain to be determined. With the recognition of apoptosis as a fundamental aspect of so many human disease states, IAPs and other antiapoptotic proteins are now acknowledged as being outstanding therapeutic targets.

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