IAP family proteins—suppressors of apoptosis

Quinn L. Deveraux and John C. Reed

The Burnham Institute, Program on Apoptosis & Cell Death Research, La Jolla, California 92037 USA

Apoptosis is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. Dysregulation of cell death pathways occur in cancer, autoimmune and immunodeficiency diseases, reperfusion injury after ischemic episodes, and in neurodegenerative disorders. Thus, proteins involved in apoptosis regulation are of intense biological interest and many are attractive therapeutic targets.

This review discusses the Inhibitor of Apoptosis [IAP] family of proteins. First discovered in baculoviruses, IAPs were shown to be involved in suppressing the host cell death response to viral infection. Interestingly, ectopic expression of some baculoviral IAPs blocks apoptosis in mammalian cells, suggesting conservation of the cell death program among diverse species and commonalities in the mechanism used by the IAPs to inhibit apoptosis. Although the mechanism used by the IAPs to suppress cell death remains debated, several studies have provided insights into the biochemical functions of these intriguing proteins. Moreover, a variety of reports have suggested an important role for the IAPs in some human diseases.

Structure of IAP family proteins

IAP family proteins are characterized by a novel domain of ~70 amino acids termed the baculoviral IAP repeat [BIR], the name of which derives from the original discovery of these apoptosis suppressors in the genomes of baculoviruses by Lois Miller and her colleagues [Crook et al. 1993; Birnbaum et al. 1994]. Up to three tandem copies of the BIR domain can occur within the known IAP family proteins of viruses and animal species [Fig. 1]. The conserved presence and spacing of cysteine and histidine residues observed within BIR domains (Cx5Cx3Wx2 Dx1Hx2C) suggests that this structure represents a novel zinc-binding fold, but formal proof of this has yet to be obtained.

Proteins containing BIR domains have been identified in a wide range of eukaryotic species, including the fission yeast Schizosaccharomyces pombe, the budding yeast Saccharomyces cerevisiae, the nematode Caenorhabditis elegans, the fly Drosophila melanogaster, and several mammalian species including mice, rats, chickens, pigs, and humans [Fig. 2]. Strictly speaking, however, membership in the IAP family of proteins requires both the presence of a BIR domain and the ability to suppress apoptosis. In this regard, many of these BIR-containing proteins are untested with respect to apoptosis suppression. Moreover, as it is debatable as to whether yeast possess an apoptosis program [Zha et al. 1996; Jürgensmeier et al. 1997; Madeo et al. 1997; Fraser and James 1998; Shaham et al. 1998], the presence of BIR-containing proteins in S. pombe and S. cerevisiae raises the possibility that BIR domains are not devoted exclusively to apoptosis suppression. Rather, they probably function as protein interaction domains that may have evolved to suit a variety of purposes.

Structure–function studies of IAP family proteins performed to date have uniformly demonstrated a requirement for at least one BIR domain for suppression of apoptosis, although other domains found within some IAPs may also be required under certain circumstances. For example, several of the mammalian, fly, and viral IAPs have a RING domain located near their carboxyl termini [Fig. 1]. The necessity for the RING domain for suppression of apoptosis appears to depend on cellular context. Some reports have indicated that the baculoviral IAPs require both amino-terminal BIR domains and the carboxy-terminal RING domain for their anti-apoptotic function in insect cells [Clem and Miller 1994; Harvey et al. 1997b]. However, a region encompassing a single BIR (BIR2) of baculovirus Op-IAP and Drosophila D-IAP1 was found to be sufficient for inhibition of apoptosis induced by the fly apoptosis protein HID in insect cells [Vucic et al. 1998b]. In a separate study, cell death suppressing activity mapped to the amino-terminal BIR domains in the Drosophila IAPs D-IAP1 or D-IAP2 and removal of the carboxy-terminal RING domain actually enhanced their ability to suppress developmental programmed cell death and cell death induced by ectopic expression of the fly apoptosis gene RPR (see below) in the developing fly eye [Hay et al. 1995]. Similarly, the human IAP family proteins c-IAP1, c-IAP2, and XIAP have been reported to retain anti-apoptotic function in the absence of their carboxy-terminal RING domains [Deveraux et al. 1997; Roy et al. 1997; Takahashi et al. 1998].

1Corresponding author.
E-MAIL jreed@burnham-inst.org; FAX (619) 646-3194.
The human c-IAP1 and c-IAP2 proteins contain a caspase recruitment domain (CARD) located between the BIR and RING domains [Fig. 1]. The functional significance of this domain for the anti-apoptotic function of IAPs is largely untested, but amino-terminal fragments of human c-IAP1 and c-IAP2 that retain only the BIR domains are sufficient to block apoptosis and therefore do not yet qualify to be considered IAPs, including Ac-IAP, BRUCE, the porcine pIAP, and the nematode and yeast IAPs. Amino acid length is indicated to the right of each protein. The mammalian proteins are based on human cDNA cloning results.

Expression and functions of human IAPs

To date, six IAP relatives have been identified in humans: NAIP, c-IAP1/HIAP-2, c-IAP2/HIAP-1, XIAP/hILP, Survivin, and BRUCE [Rothe et al. 1995; Duckett et al. 1996; Liston et al. 1996; Ambrosini et al. 1998]. Interestingly, the human c-IAP1 and c-IAP2 genes are located within ∼528-kD BIR-containing genes have been assigned to chromosomal locations Xq25, 11q22-23, 11q22-23, 5q13.1, and 17q25, respectively [Roy et al. 1995; Liston et al. 1996; Rajcan-Separovic et al. 1996; Ambrosini et al. 1998]. Mouse orthologs of most human IAPs have also been identified, implying conservation of the IAP gene family in mammals [Fig. 2]. The human XIAP, c-IAP1, c-IAP2, NAIP, and Survivin genes have been found to be expressed in opposing directions, control expression of ERP-1 and Survivin in an exclusionary fashion, wherein transcripts produced for one of these appear to inhibit the expression of the other through an antisense RNA-based mechanism [Ambrosini et al. 1998]. It remains to be deter-
mined whether any of these gene loci are directly involved in genetic alterations found in tumors.

Overexpression of XIAP, c-IAP1, c-IAP2, NAIP, or Survivin has been shown to suppress apoptosis induced by a variety of stimuli including tumour necrosis factor (TNF), Fas, menadione, staurosporin, etoposide (VP16), Taxol, and growth factor withdrawal [Duckett et al. 1996; Liston et al. 1996; Ambrosini et al. 1997; J. Li et al. 1998]. Although all these IAP family proteins are capable of suppressing cell death, their expression in vivo appears to be regulated differentially, thus presumably providing mechanisms for controlling the presence or absence of IAPs in response to particular cellular or environmental signals.

XIAP mRNA was observed in all adult and fetal tissues examined except peripheral blood leukocytes, indicating that it is a ubiquitously expressed member of the family [Liston et al. 1996]. However, analysis of XIAP expression at the single cell level by immunohistochemistry or in situ hybridization is needed to determine whether differentiation or cell lineage-specific expression occurs. Expression of c-IAP1 and c-IAP2 is highest in the kidney, small intestine, liver, and lung and lowest in the central nervous system [Young et al. 1999].

**Figure 2.** Comparison of BIR domains. (A) An alignment of the predicted amino acid sequences of BIR domains is presented. The second BIR domain of IAPs, which contain multiple BIRs, was chosen for alignment based on evidence that BIR2 of XIAP and OpIAP are required for suppression of apoptosis. Sequences were identified by a PSI-Blast search of the nonredundant database at NCBI [Altschul et al. 1997] and aligned using the MegAlign program (DNASTAR) with the CLUSTAL method. The following is a list of the species designations used for the above alignment and primary accession number or database entry number for each sequence. Human BIR domain proteins: hXIAP, P98170; hIAP1, Q13489; hIAP2, Q13490; hNAIP, 1737213; hSurvivin, 2315863; and BRUCE, Y17267. Pig: pIAP, 2957175. Chick: Q90660. Mouse: mXIAP, Q60989; mNAIP, 2352685; mIAP1, Q13489; mIAP2, Q62210; mSurvivin [TIAP], d1029206. Drosophila: dIAP1, Q24306; dIAP2, Q24307. Chilo iridescent virus: CIIAP, 2738454. Orgyia pseudotsugata nuclear polyhedrosis virus: OpIAP, P41436. Autographa california nuclear polyhedrosis virus: AcIAP, D36828. African swine fever virus: Asf–vIAP, 011452. Caenorhabditis elegans IAP proteins: CeIAP1, e249029; CeIAP2, e348121. Schizosaccharomyces pombe IAP protein: SpIAP, e339290. Saccharomyces cerevisiae: ScIAP, P47134. (B) Phylogenetic relationship of the same BIR domains is presented based on the sequences displayed in the MegAlign [DNASTAR] document using the CLUSTAL method [Higgins and Sharp 1989]. The phylogenies are rooted assuming a biological clock.
though the tissue distributions of c-IAP1 and c-IAP2 are reportedly similar, the relative expression of c-IAP1 is generally higher [Young et al. 1999]. NAIP mRNA appears to be expressed at levels sufficient for detection by Northern blot analysis only in adult liver and in placenta but can be detected in brain by RT–PCR [Roy et al. 1995].

Although most tissues have not been examined in detail, IAP expression has been investigated in the ovary, where apoptosis is thought to play an important role in ovulation. In granulosa cells from preantral and early antral follicles, extensive apoptosis was associated with reduced protein levels of c-IAP2 and XIAP [J. Li et al. 1998]. Gonadotropin treatment increased c-IAP2 and XIAP protein content and suppressed apoptosis in granulosa cells, resulting in the development of follicles to the antral and preovulatory stages. Thus, these IAPs may play an important role in determining the fate of the granulosa cells, and thus, the eventual follicular destiny [J. Li et al. 1998].

Survivin exhibits the most restricted expression of an IAP family member in adult tissues. Survivin mRNA is found by Northern blotting only in occasional normal adult human or mouse tissues [Ambrosini et al. 1997]. The Survivin promoter contains four copies of G1 repressor elements that have been implicated in controlling cell cycle periodicity in some G1/M-regulated genes [F. Li et al. 1998]. Moreover, when studied in reporter gene assays, the Survivin promoter exhibits typical M phase inducible transactivation, suggesting that Survivin is a bona fide cell cycle regulated gene and raising the possibility that Survivin expression may be induced in dividing cells [F. Li et al. 1998]. In HeLa cells, Survivin mRNA is up-regulated ~40-fold at G1/M [F. Li et al. 1998]. Immunohistochemical analysis and in situ hybridization studies have demonstrated expression of Survivin in several apoptosis-regulated fetal tissues [Adida et al. 1998]. In mouse embryo, prominent and nearly ubiquitous distribution of Survivin was found at embryonic day (E) 11.5, whereas at E15–E21, Survivin expression was restricted to only a few locations. The investigators of this work have suggested that expression of Survivin in embryonic and fetal development may contribute to tissue homeostasis and differentiation, with the gene then becoming quiescent in most normal adult tissue medulla [Adida et al. 1998]. However, given the cell cycle-dependent regulation of the Survivin gene, it remains to be determined whether Survivin expression in developing tissues is merely a reflection of rapid cell division in fetal tissues.

IAPs and neuronal cell death

Because gene knockout experiments have yet to be reported, the in vivo physiological roles of individual IAP family genes are presently unclear. The NAIP gene, however, was first identified because of its apparent deletion in patients with spinal muscular atrophy (SMA), a hereditary motoneuron degenerative disease [Roy et al. 1995; Liston et al. 1996]. Although the primary genetic defect in SMA has been ascribed to an adjacent gene [Liu et al. 1997], SMN, rather than NAIP, patients with the most severe forms of this disease appear to harbor deletions at 5q13.1 that encompass both the SMN and NAIP genes. Intriguingly, the SMN protein has been reported to bind Bcl-2 and enhance Bcl-2-mediated protection from apoptosis [Iwahashi et al. 1997], raising the possibility that two survival genes may be lost in more severely affected individuals and suggesting that NAIP may be required for the survival in vivo of some specific types of compromised neurons.

NAIP may also be involved in adaptive responses to ischemia. Transient forebrain ischemia selectively elevates levels of NAIP in rat neurons that are resistant to ischemia reperfusion [Xu et al. 1997]. Up-regulation of endogenous NAIP expression or intracerebral injection of NAIP-encoding adenoviruses reportedly reduces ischemic damage in vivo in the rat hippocampus, suggesting that NAIP may play a role in conferring resistance to ischemia-induced cell death [Xu et al. 1997]. However, in cell culture experiments, transfection of primary cerebellar granule cell neurons with adenoviruses encoding NAIP, XIAP, c-IAP1, or c-IAP2 delayed but did not prevent apoptosis induced by K+ depolarization and serum deprivation. Nonapoptotic cell death induced by L-glutamate was unimpaired by these IAP family proteins [Simons et al. 1999]. Thus, IAPs are apparently insufficient to protect some types of neurons from insults often associated with ischemia.

IAP family gene expression in cancers

Although our knowledge of the role of IAPs in cancer is presently scant, these regulators of cell death are likely to become increasingly prevalent in topics concerning neoplasia as further investigations are undertaken. The oncoprotein v-Rel, a member of the Rel/NF-κB family of transcription factors, induces malignant transformation and inhibits apoptosis. The chicken homolog of c-IAP1 (ch-IAP1) was found to be up-regulated after expression of v-Rel in fibroblasts, a B-cell line, and in spleen cells [You et al. 1997]. ch-IAP1 reportedly suppresses mammalian cell apoptosis induced by the overexpression of caspase-1. Expression of exogenous ch-IAP1 in temperature-sensitive v-Rel-transformed spleen cells also inhibited apoptosis of these cells at the nonpermissive temperature. On the basis of these results, it appears that ch-IAP1 is induced and functions as a suppressor of apoptosis in the v-Rel-mediated transformation process [You et al. 1997].

More is known about the expression of Survivin in cancer than any other IAP family member. Reportedly, Survivin is expressed in a high proportion of the most common human cancers but not in normal, terminally differentiated adult tissues, thus making Survivin an exciting new tumor marker [Ambrosini et al. 1997]. The assessment of Survivin expression in human tumor specimens included both in situ RNA hybridization and immunohistochemical analysis, confirming expression in tumor cells but not admixed stromal cells or adjacent normal tissues [Ambrosini et al. 1998]. Thus, altered ex-
pression of Survivin appears to define a common event associated with the pathogenesis of most human cancers. Moreover, reductions in Survivin expression achieved using antisense strategies cause apoptosis and sensitization to anticancer drugs, at least in some tumor cell lines, implying that Survivin expression can be important for cell survival or chemoresistance in carcinomas (Ambrosini et al. 1998).

Not all tumors, however, express Survivin. For example, low grade non-Hodgkin's lymphomas, which are known for their activation of another type of anti-apoptotic gene, Bcl-2 (Tsujimoto et al. 1985), rarely express this IAP family protein (Ambrosini et al. 1997). These lymphomas are also tumors with very low growth fractions, a characteristic that could have bearing on the apparent cell cycle-dependent expression of Survivin. Moreover, even within a given type of cancer, heterogeneity in Survivin expression may be observed. Immunohistochemical assessments of Survivin expression in tumors where immunointensity, percentage immunopositivity, or both have been measured for purposes of segregating Survivin-negative from -positive (Survivin low from high) tumors suggest that expression of Survivin (or higher levels of Survivin expression) is associated with worse clinical outcome or other unfavorable prognostic features in neuroblastomas, colon and gastric cancers (Ambrosini et al. 1997, 1998; Adida et al. 1998; Lu et al. 1998). Although preliminary, assessments of Survivin expression in tumors where immunointensity, percentage immunopositivity, or both have been measured for purposes of segregating Survivin-negative from -positive (Survivin low from high) tumors suggest that expression of Survivin (or higher levels of Survivin expression) is associated with worse clinical outcome or other unfavorable prognostic features in neuroblastomas, colon and gastric cancers (Ambrosini et al. 1997, 1998; Adida et al. 1998; Lu et al. 1998). Although preliminary, assessments of Survivin expression, therefore, may be of prognostic significance for patients with some types of cancer.

Survivin may have relevance to cancer for other reasons as well. Not only is the expression of the Survivin gene enhanced at the G2/M phase of the cell cycle but the Survivin protein reportedly binds tightly (Kd ~5-7 μM) with polymerized microtubules (F. Li et al. 1998). Deletion of a predicted carboxy-terminal coiled-coil region (Δ100-142) from Survivin-impaired microtubule binding and abrogated Survivin’s ability to protect against taxol-induced apoptosis, implying that association with microtubules is critical for Survivin function. In this regard, gene transfer-mediated overexpression of wild-type Survivin failed to protect tumor cell lines against apoptosis induced by microtubule-disrupting agents such as nocodazole or vinblastine, further implicating binding of Survivin to polymerized microtubules as a necessary requirement for activating the anti-apoptotic function of Survivin or for targeting it to critical locations that require its protection (F. Li et al. 1998). Interestingly, mutation of a conserved cysteine in the Survivin BIR domain [Cys84-Ala] also abolished Survivin’s cytoprotective abilities. However, the BIR [Cys84-Ala] mutant retained the ability to associate with microtubules similar to wild-type Survivin and, indeed, appeared to interfere with the function of endogenous Survivin by competing for microtubule binding (F. Li et al. 1998). Overexpression of the BIR mutant also resulted in a progressive increase in caspase activity, occurring predominantly at the G2/M border. On the basis of these collective results, the researchers suggest that Survivin may counteract an apoptotic pathway that becomes active during a G2/M checkpoint (F. Li et al. 1998). Thus, these findings further underscore the interdependence of cell-cycle regulation and programmed cell death.

IAPs as inhibitors of caspase family cell death proteases

How do the IAPs suppress apoptosis? One possible explanation comes from recent studies demonstrating that several of the human IAPs (XIAP, c-IAP1, and c-IAP2) inhibit caspases directly (Deveraux et al. 1997; Roy et al. 1997). The caspases are a family of cysteine proteases with substrate specificity for aspartic acid. Caspases represent highly conserved components of apoptotic programs throughout the animal kingdom (for review, see Alnemri et al. 1996; Salvesen and Dixit 1997). XIAP, c-IAP1, and c-IAP2 were shown to bind and potently inhibit caspases 3, 7, and 9, but not caspases 1, 6, 8, or 10 or CED3 (Deveraux et al. 1997, 1998; Roy et al. 1997). Survivin also can be coimmunoprecipitated with caspases 3, 7, and 9 and it suppresses apoptosis induced by overexpression of these caspases, implying that Survivin also is a caspase inhibitor (Tamm et al. 1998). Thus, IAPs appear to represent the first identified family of endogenous cellular inhibitors of caspases in mammals.

Caspases are synthesized initially as single polypeptide chains representing latent precursors (zymogens) that undergo proteolytic processing at specific aspartic acid residues to produce subunits that form the active heterotetrameric protease. In mammalian cells, activation of the caspase zymogens has been reported to occur through at least three independent mechanisms: (1) cleavage by upstream active caspases; (2) cleavage by granzyme B, an aspartate-specific serine protease found in the granules of cytolytic T-cells; and (3) autoprocessing of zymogens with assistance from other caspase-interacting proteins that can occur in either a cis- or trans-acting manner (for review, see Salvesen and Dixit 1997; Stennicke and Salvesen 1998).

One mechanism for triggering autoprocessing and activation of pro-caspase-8 entails its recruitment to plasma membrane receptor complexes, such as the Fas, a member of the TNF family of cell death receptors. Pro-caspase-8 zymogens possess ~1% the activity of the processed fully active protease. When brought into close apposition by oligimerization around Fas receptor complexes, these zymogens trans-process each other, yielding autonomous, active proteases (Juo et al. 1998; Martin et al. 1998; Stennicke et al. 1998). Once activated, caspase-8 can then activate directly or indirectly pro-caspase-3 and other downstream caspases, which function as the ultimate effectors of apoptosis by cleaving a variety of substrate proteins in cells.

Another identified mechanism for initiating caspase activation requires the participation of mitochondria and involves a protein known as the apoptosis protease activating factor-1 [ApaF-1] (Liu et al. 1996; Li et al. 1997; Reed 1997; Zou et al. 1997). ApaF-1 is a cytosolic protein that rests in a latent state until bound by cytochrome c. Cytochrome c is commonly released from the mitochon-
dria during apoptosis induced by many, but probably not all, cell death stimuli (for review, see Reed 1997; Green and Reed 1998). The resulting Apaf-1/cytochrome c complex associates with the zymogen form of caspase-9 in the presence of dATP or ATP, promoting the autocatalytic activation of caspase-9. Once activated, caspase-9 can then cleave and activate procaspase-3 directly, resulting in a cascade of additional caspase activation and apoptosis.

Therefore, caspases 8 and 9 represent the pinnacl caspases in the Fas/TNF family death receptor and cytochrome c/Apaf-1 pathways, respectively (Fig. 3). Studies with cells derived from caspase-8 and caspase-9 knock-out mice indicate that caspase-8 is absolutely required for Fas-induced apoptosis, whereas caspase-9 is necessary for apoptosis induced by multiple stimuli known to trigger cytochrome c release from mitochondria (Hakem et al. 1998; Joo et al. 1998; Kuida et al. 1998; Varfolomeev et al. 1998). Although IAPs do not bind or inhibit caspase-8, they do bind to and inhibit its substrate caspase-3, thus arresting the cascade of proteolysis and providing protection from Fas/caspase-8-induced apoptosis (Deveraux et al. 1997, 1998; Roy et al. 1997). In contrast, in the mitochondrial pathway for caspase activation, XIAP, c-IAP1, and c-IAP2 bind directly to the pinnacl caspase pro-caspase-9, and prevent its processing and activation induced by cytochrome c, both in intact cells and in cell extracts where caspase activation is induced by addition of exogenous cytochrome c (Deveraux et al. 1998). Because they can also bind directly to and potently inhibit the next caspase in the cytochrome c/Apaf-1-induced cascade, caspase-3 (Li et al. 1997), these IAP family proteins also presumably interfere with a reported amplification loop in which active caspase-3 cleaves and activates additional pro-caspase-9 molecules (Srinivasula et al. 1998). These observations are consistent with reports that overexpression of IAP family proteins inhibits apoptosis induced by Bax and other pro-apoptotic Bcl-2 family proteins, which are known for their ability to target mitochondria and induce cytochrome c release (Deveraux et al. 1997, 1998; Wolter et al. 1997; Bossy-Wetzel et al. 1998; Jurgesmeier et al. 1998; Mahajan et al. 1998). The IAPs, however, do not interfere with Bax-mediated release of cytochrome c in vitro using isolated mitochondria as well as intact cells (Finucane et al. 1999; Jurgesmeier et al. 1998), an observation that is consistent with other data indicating that the human IAPs (at least XIAP, c-IAP1, c-IAP2, and Survivin) block caspase activation and apoptosis downstream of Bax, Bik, Bak, and cytochrome c (Deveraux et al. 1997, 1998; Orth and Dixit 1997; Roy et al. 1997; Duckett et al. 1998; Tamm et al. 1998). The failure of IAPs to prevent cell death stimuli from triggering cytochrome c release has important implications for determining whether cell death will be prevented in the long term versus merely delayed (see below).

Apoptosis induced by overexpression of pro-caspases 3, 7, or 9 is also suppressible by coexpression of XIAP, c-IAP1, c-IAP2, or Survivin (Deveraux et al. 1997; Roy et al. 1997; Tamm et al. 1998). The overexpression of these zymogens presumably results in a few molecules becoming active proteases, resulting in feed-forward amplification in which the active caspases cleave and activate more of their zymogens or other caspase zymogens. Consequently, in cotransfection experiments, IAPs generally prevent the appearance of cleaved caspases, most likely by squelching this amplification loop (Fig. 3). In this regard, the ratio of caspase to IAP is likely to be critical in determining whether apoptosis is prevented successfully in such experiments, possibly explaining the reported failure of IAPs to inhibit cell death induced by overexpression of caspase-7 in transient transfection studies in which the relative levels of protein production were not determined (Hawkins et al. 1996).

Interestingly, binding of XIAP, c-IAP1, c-IAP2, and Survivin to caspases 3 and 7 requires the proteolytic processing and activation of these caspases. In contrast, the IAPs bind both the unprocessed and processed forms of their target caspases.

Figure 3. Possible points of IAP interaction with cell death pathways. Apoptosis pathways activated by Fas (CD95), mitochondria, or TNFR1 (CD120a) are depicted. Several human IAP family proteins have been reported to block caspases 3, 7, and 9 directly. Possible involvement of IAPs in NF-kB and JNK signal transduction pathway activated by TRAFs through TNFR1- and TNRF2-type cytokine receptors is also indicated. Many of these assigned functions for IAPs shown in the figure remain speculative to date.
caspase-9 [Deveraux et al. 1997, 1998; Roy et al. 1997]. Recent data indicate that significant differences exist between the activation mechanism of caspase-9 compared to other caspases. Activation of caspase-9 does not absolutely require its cleavage, but does require association with active Apaf-1 (Stennicke et al. 1999). The apparent ability of unprocessed caspase-9 to assume an active conformation may, therefore, explain why IAPs can bind to it.

The BIR regions [BIR1, BIR2, and BIR3] of human XIAP, c-IAP1, and c-IAP2 were found to be necessary and sufficient for their caspase inhibitory and anti-apoptotic activities [Deveraux et al. 1997; Roy et al. 1997]. Subsequently, the caspase inhibitory activity and anti-apoptotic activity of XIAP was localized specifically to the BIR1 domain [BIR2] of its three tandem BIR domains [Takahashi et al. 1998], indicating that a single BIR domain can be sufficient to potently inhibit caspases ($K_i < 2 \text{ nM}$ for XIAP–BIR2 measured against caspases 3 and 7). Similarly, the smallest of the known IAP family proteins, Survivin, which contains a single BIR domain, can bind caspases and prevent caspase-induced apoptosis [Tamm et al. 1998]. Surprisingly, the BIR1 and BIR3 domains of XIAP apparently lack caspase-binding capability, despite their striking amino acid similarity to BIR2 [42% for BIR1; 32% for BIR3]. Assuming these results cannot be ascribed to trivial explanations such as misfolding of protein fragments taken out of their normal context of the intact protein, these observations suggest that not all BIR domains are created equal. Thus, it is plausible that BIR domains within the same protein may have distinct functions.

The IAPs appear to bind and inhibit their caspase targets at either a 1:1 or 2:1 molar ratio, possibly reflecting the presence of two active sites per enzyme [Deveraux et al. 1997; Roy et al. 1997]. The inhibitory constants ($K_i$) for XIAP, c-IAP1, and c-IAP2 measured against caspases 3 and 7 range from ∼0.2–10 nM, indicating that the IAPs are quite potent protease inhibitors (Table 1). Although the mechanism of caspase inhibition by the human IAPs is currently unknown, one might suspect they function like other caspase inhibitors, such as the viral proteins CrmA or p35 [discussed below]. However, the inhibitory mechanism of the IAPs does not appear to involve peptide bond hydrolysis [Deveraux et al. 1997; Roy et al. 1997]. In contrast, p35 is hydrolyzed by its caspase targets and remains tightly bound to the active site [Zhou et al. 1998]. IAPs, however, may still function as competitive inhibitors of caspases—similar to the Kunitz, Kazal, and Eglin families of serine protease inhibitors that possess loops that conform to the catalytic pocket of their target proteases [Bode and Huber 1991]. Likewise, the cystatins contain loops that adapt to active sites of the papain family of cysteine proteases [Turk and Bode 1991]. In these mechanisms, the loop region binds tightly to the catalytic groove of the protease, yet no peptide bond hydrolysis is observed. Caspases use cysteine and histidine residues as part of their catalytic mechanism and can be inhibited by metals such as zinc—a potent inhibitor of caspases [Perry et al. 1997; Stennicke and Salvesen 1997]. Thus, it is also possible that metal binding by the BIR domain may play a role in the caspase inhibitory mechanism used by the IAP-family proteins.

### IAP involvement in signal transduction

A number of studies have linked the IAP family of proteins to signal transduction pathways used by members of the TNF receptor [TNFR] family. Although the extracellular domains of all members of the TNFR family share amino acid sequence homology and a characteristic spacing of cysteines involved in disulfide bond formation, their intracellular domains can vary. TNFR1 and TNFR2 are the prototypical representatives of the two major branches of this receptor family. The cytosolic domain of TNFR1 contains a Death Domain (DD) that has been linked to pro-caspase-8 activation by way of the adaptor protein Tradd. The Tradd protein also contains a DD and interacts not only with TNFR1 and itself, but also with Fadd, another DD-containing protein that in turn binds pro-caspase-8 through a homophilic interaction motif called the Death Effector Domain (DED) (for review, see Singh et al. 1998). In this way, TNFR1 can trigger caspase-8 activation and apoptosis, analogous to Fas.

Tradd, however, also binds another type of signaling protein that induces NF-κB activation and promotes cell survival. These signaling proteins are called TRAFs, for TNF receptor-associated factors [for review, see Baker and Reddy 1996; Arch et al. 1998]. Several TRAF family proteins have been identified and at least three of them, TRAF-2, TRAF-5, and TRAF-6, shown to activate NF-κB apparently through their ability to bind a NF-κB-inducing kinase NIK. Through the concerted actions of additional kinases, the ultimate result is phosphorylation of I-κB on serine 32 and 36, thus targeting this NF-κB inhibitor for polyubiquitination and degradation by the 26S proteasome [Mercurio et al. 1997; Regnier et al. 1997; Zandi et al. 1998]. Unlike TNFR1, which requires an adapter protein, the cytosolic domains of TNFR2 and

### Table 1. Comparison of caspase inhibitors

| Caspase | 1 | 3 | 6 | 7 | 8 |
|---------|---|---|---|---|---|
| Human   |   |   |   |   |   |
| XIAP    | N.S. | 0.7 | N.S. | 0.2 | N.S. |
| c-IAP1  | N.S. | 108 | N.S. | 42  | N.S. |
| c-IAP2  | N.S. | 35  | N.S. | 29  | N.S. |
| Viral   |   |   |   |   |   |
| CrmA    | 0.01 | 500 | 110 | N.S. | 0.95 |
| p35     | 9.0 | 0.11 | 0.38 | 1.8 | 0.48 |
| Synthetic |   |   |   |   |   |
| Ac–DEVD–CHO | 15 | 0.5 | N.D. | 35  | N.D. |
| Ac–YVAD–CHO | 0.7 | 5 x 10⁶ | N.D. | 5 x 10⁶ | N.D. |

Inhibitory constants ($K_i$ in nM) were determined as described previously [for review, see Stennicke and Salvesen 1998]. [N.S.] No significant inhibition; [N.D.] not determined.
a variety of other TNF family cytokine receptors, including CD27, CD30, and CD40, directly bind TRAFs involved in NF-κB activation. However, these TNFR2-like family members do not bind Tradd-like adaptors proteins and do not recruit cell death proteases. Thus, although TNFR1 triggers directly caspase-8 recruitment and activation, the TNFR2-like subgroup of receptors does not. Moreover, the TNFR2 branch of the TNF family receptors typically stimulates cell proliferation and survival rather than death.

How are the IAPs relevant to these TNF family receptor signaling complexes? The human c-IAP1 and c-IAP2 proteins were first discovered by virtue of their association with TNFR2 receptor complexes [Rothe et al. 1995]. c-IAP1 and c-IAP2 do not contact TNFR2 directly, but are recruited to the receptor by binding to TRAF-1/ TRAF-2 heterocomplexes [Rothe et al. 1995]. The amino-terminal BIR-containing region of these IAPs is required for interactions with TRAFs. The interaction of c-IAP1 and c-IAP2 with TRAF-1 and TRAF-2 appears to be specific, in that these IAPs do not bind to TRAF-3, TRAF-4, TRAF-5, or TRAF-6 and other IAPs [XIAP, NAIP] reportedly fail to bind TRAFs altogether [Roy et al. 1997]. Thus, TRAF binding is not a universal feature of IAP family proteins.

c-IAP2 has been functionally implicated in TNF induction of NF-κB and protection from apoptosis [Chu et al. 1997]. First, TNFα has been shown to induce expression of c-IAP2 through stimulation of NF-κB. Second, overexpression of c-IAP2 reportedly can also lead to NF-κB activation. Third, c-IAP2 expression suppresses cell death induced by TNFα through the receptor TNFR1. These c-IAP2 activities are reportedly blocked in cells by coexpressing a dominant form of IκB that is resistant to TNF-induced degradation, implying that c-IAP2 participates in a positive feedback mechanism regulating NF-κB activation by targeting IκB degradation. Moreover, a mutant of c-IAP2 lacking the carboxy-terminal RING domain inhibited NF-κB induction by TNF and enhanced TNF killing. On the basis of these findings, the researchers suggested that c-IAP2 is critically involved in TNF signaling events that induce NF-κB and that are required for suppression of TNF-induced apoptosis [Chu et al. 1997].

In this regard, despite the ability of TNFR1 to induce activation of caspase-8, TNFα is not cytotoxic in most types of cells unless inhibitors of macromolecular synthesis are also applied, thus preventing gene expression. This paradox has been attributed to parallel activation of the aforementioned NF-κB pathway that induces the expression of several anti-apoptotic genes including certain IAPs. The question that remains is whether induction of IAP family genes is critical for the anti-apoptotic effect of NF-κB. Studies of the effects of TNFα on IAP family gene expression in endothelial cells suggests the answer to the first of these questions may be difficult to obtain because of redundancy in IAP family genes. Transcription of c-IAP1, c-IAP2, and XIAP genes was found to be strongly up-regulated with treatment of endothelial cells with the TNFα, interleukin-1β, and lipopolysaccharide—reagents that lead to NF-κB activation [Stehlik et al. 1998]. In these studies, overexpression of IκB suppressed NF-κB activation and prevented the induction of all these IAP family genes. IκB overexpression also sensitized endothelial cells to TNFα-induced apoptosis. Ectopic expression of at least one of the IAPs, XIAP, suppressed the IκB effect, thereby protecting endothelial cells from TNFα-induced apoptosis, suggesting that XIAP represents one of the NF-κB-regulated genes that can counteract the apoptotic signals caused by TNFα-induced activation of caspase-8 [Stehlik et al. 1998]. Thus, although we do not know whether IAP expression is necessary for NF-κB-mediated protection against TNFα, it is sufficient. These findings also indicate that it may be worth considering whether dysfunctional regulation of the IAPs occurs in sepsis and some inflammatory conditions, where cytokine-induced endothelial cell death occurs.

At what point or points in the apoptotic cascade do the IAPs interfere with TNF-induced apoptosis? Recently, NF-κB was reported to block TNFα-induced activation of pro-caspase-8 [Wang et al. 1998]. Under conditions in which NF-κB activation was prevented with dominant-negative IκB, gene transfection studies suggested that the combination of TRAF-1, TRAF-2, c-IAP1, and c-IAP2 was needed to substitute for NF-κB and fully suppress TNFα-induced apoptosis. In the same cells, however, either c-IAP1 or c-IAP2 alone was sufficient to suppress apoptosis induced by etoposide—a stimulus that appears to enter the apoptosis pathway primarily at the level of mitochondria [Wang et al. 1998]. The implication is that c-IAP1 and c-IAP2 require TRAF-1 and TRAF-2 to interfere with the upstream cell death protease caspase-8, but not for inhibiting caspases that operate downstream of mitochondria [Fig. 3]. Moreover, these and other data [Deveraux et al. 1998; McCarthy and Dixit 1998] suggest that the IAPs have the ability to regulate different apoptotic pathways or distinct steps within the same apoptotic pathway, possibly depending on what additional proteins the IAPs bind, such as TRAFs.

In addition to NF-κB, one study has provided evidence that some IAP family proteins can regulate the Jun amino [N]-terminal kinase [JNK] pathway [Sanna et al. 1998]. Specifically, JNK1 activation induced by overexpression of pro-caspase-1 is reportedly augmented by coexpression of XIAP. Moreover, XIAP also reportedly blocked cell death induced by overexpression of pro-caspase-1. Intriguingly, the ability of XIAP to suppress cell death in this context was abrogated by coexpression of a dominant-negative form of JNK1 [Sanna et al. 1998]. However, the mechanism by which activated JNK1 might suppress apoptosis in this or other systems has not been elucidated. Moreover, as JNK1 has been shown to be a pro-apoptotic component in several instances [Schulze-Osthoff et al. 1998], it seems unlikely that JNK activation is the predominant mechanism used by IAP family proteins to suppress cell death. Although unexplored to date, it is tempting to speculate that the functional connection of XIAP to pro-caspase-1 may somehow be related to the observation that at least some IAPs...
Insights into IAPs from insects

IAPs were first discovered in insect viruses (Crook et al. 1993; Birnbaum et al. 1994). Possibly one of the most ancient evolutionary pressures for a cell suicide program can be attributed to viruses. Death of infected host cells stymies viral propagation, thereby protecting uninfected neighboring cells. However, many viruses have co-evolved strategies for promoting cell survival by targeting conserved steps in the host cell death program (Komiyama et al. 1994; Bump et al. 1995; Zhou et al. 1997). An example of a viral regulator of apoptosis is the coxopox virus protein CrmA, a serpin family protease inhibitor that exhibits specificity for caspases 1 and 8, with inhibitory constants of 0.01 and 0.95 nM, respectively. Another viral inhibitor of caspases is the baculovirus protein p35, which is structurally distinct from CrmA and has no apparent cellular homolog. The p35 protein has broad inhibitory activity against most of the caspase family enzymes, with $K_i$’s typically of ~1 nM (Komiyama et al. 1994; Zhou and Salvesen 1997). In addition to p35, baculoviruses also encode IAPs (Clem and Miller 1994). The BIR, RING, and other sequences in the IAPs exhibit no similarity to p35. Despite their apparent lack of structural similarity to p35, the IAP proteins encoded by the Orgyia pseudotsugata (Op) and Cydia pomonella (Cp) baculoviruses have been shown by genetic complementation analysis to functionally overlap with p35 with respect to suppression of the insect cell death response to viral infection. Similar to p35, ectopic expression of baculoviral OpIAP or CpIAP protects both insect and mammalian cells from apoptosis induced by a variety of stimuli, including caspase overexpression—observations that are consistent with the idea that the IAPs block apoptosis at an evolutionary conserved point common to many apoptotic programs (Clem and Miller 1994; Hawkins et al. 1996).

Although IAPs can complement p35-deficient baculoviruses (Clem and Miller 1994), some studies suggest that IAPs and p35 suppress apoptosis by distinct mechanisms. For example, OpIAP and p35 were reported to exhibit differences in their abilities to protect PC12 cells from apoptosis induced by serum withdrawal, leading the investigators to conclude that they function by distinct mechanisms (Hawkins et al. 1998). Moreover, in insect cells from the fall armyworm Spodoptera frugiperda (Sf 21 cells), p35 reportedly blocks apoptosis induced by transfection of plasmids encoding active SF-caspase-1, an insect caspase that becomes activated during the course of baculoviral infection (Sheshagiri and Miller 1997). Although overexpression of OpIAP can prevent processing of the pro-form of SF-caspase-1 and suppress apoptosis induced by viral infection, it does not block cell death induced by direct gene transfer-mediated expression of active SF-caspase-1 (Sheshagiri and Miller 1998). This observation implies that OpIAP can interfere with upstream mechanisms responsible for activation of SF-pro-caspase-1 but, unlike p35, it is not a direct inhibitor of this particular protease. These results can be interpreted at least two ways: either OpIAP is not a caspase inhibitor or OpIAP is a caspase inhibitor but SF-caspase-1 is downstream of the caspases it targets. This latter argument has its basis in the observation that the human IAP family proteins XIAP, c-IAP1, c-IAP2, Survivin reportedly bind and inhibit selected caspases but not all caspases, unlike p35, which is a broad specificity inhibitor of these enzymes (Fig. 4).

Additional insights into the mechanisms of IAP family proteins have come from genetic and biochemical analysis of the cell death pathway in Drosophila. The baculoviral OpIAP and CpIAP proteins and the Drosophila IAP family proteins DIAP-1 and DIAP-2 have been shown to bind the Drosophila cell death proteins Reaper (RPR), Hid, and Grim (Vucic et al. 1997, 1998a). Expression of RPR, Hid, or Grim in the fly or in cultured insect cells promotes apoptosis that can be suppressed by coexpression of viral OpIAP or CpIAP or of cellular IAPs, DIAP-1 and DIAP-2. The RPR, Hid, and GRIM proteins contain a conserved amino-terminal 14 amino acid sequence that
has been shown to be necessary and sufficient for inducing apoptosis and for binding to either the baculoviral or endogenous cellular IAPs [Vucic et al. 1998a]. Thus, binding to IAPs appears to be the common feature that unifies these *Drosophila* cell death proteins [Fig. 4]. The RPR, HID, and GRIM proteins, however, do not bind the baculovirus p35 protein, indicating that the IAPs and p35 either suppress death through distinct mechanisms or are subject to differential regulation, at least with respect to HID, GRIM, and RPR. In this regard, it was shown recently that DIAP-1 can block apoptosis induced by the *Drosophila* caspase drICE [Kaiser et al. 1998]. Moreover, DIAP-1 reportedly binds directly to the active form of drICE but not itszymogen form. These observations, therefore, provide further evidence that caspase inhibition is an evolutionarily conserved mechanism by which the IAPs block cell death [Deveraux et al. 1997].

Another more recently described cell death gene identified in the fly, DOOM, also encodes an IAP-binding protein [Harvey et al. 1997a], suggesting that the IAPs define a major point of convergence for regulation of the cell death pathway in the fly [Fig. 4]. It is unclear, however, whether the various *Drosophila* proteins described above are cell death inducers that are inhibited by the IAPs, or conversely whether they are trans-dominant inhibitors of the IAPs, which free IAP inhibitable proteins so that they can induce apoptosis [e.g., a caspase].

Evolutionary conservation of the mechanisms used by RPR and GRIM to induce apoptosis has been suggested by recent studies indicating that these fly cell death proteins can induce apoptosis in mammalian cells [McCarthy and Dixit 1998]. RPR- or GRIM-induced apoptosis of mammalian cells was reportedly inhibited by a broad range of protein and peptidyl caspase inhibitors as well as by the human c-IAP1 and c-IAP2 proteins. In addition, in vivo binding studies demonstrated that both RPR and GRIM physically interacted with human IAPs, and required the homologous 14-amino-acid amino-terminal segment for IAP binding. When expressed in mammalian cells, RPR and GRIM colocalized with c-IAP1 to a perinuclear location. Deletion of the amino-terminal 15 amino acids of RPR or GRIM abolished colocalization with c-IAP1; however, these amino-terminal deletion mutants still promoted apoptosis, which was also still suppressed by coexpression of c-IAP1 or c-IAP2. The researchers suggested that although these RPR and HID mutants fail to bind IAPs, the c-IAPs are nevertheless able to attenuate death by inhibiting downstream active caspases [McCarthy and Dixit 1998]. Thus, the IAPs may possess mechanisms for inhibiting cell death pathways at several distinct steps [Fig. 4].

Interestingly, an effector (DREDD) of RPR-, HID-, and GRIM-mediated apoptosis was recently discovered and appears to be a caspase [P. Chen et al. 1998]. Heterozygosity at the *DREDD* locus suppressed RPR- and GRIM-induced apoptosis in transgenic flies, implying that the relative abundance of the DREDD protein can significantly influence the extent to which RPR and GRIM retain pro-apoptotic function. In cotransfection experiments, RPR, GRIM, and HID also trigger caspase-like processing of DREDD. On the basis of the observation that RPR is sufficient to initiate caspase activity in a cell-free system [Evans et al. 1997], the investigators suggested that RPR, HID, and GRIM might engage the apoptotic pathway through this novel caspase (P. Chen et al. 1998). It would be interesting to determine whether the viral and fly IAPs bind to and inhibit the caspase DREDD. Because it has not been shown that IAPs can bind directly HID, GRIM, and RPR using purified proteins, it is conceivable that the IAPs actually bind DREDD and that their reported association with HID, GRIM, and RPR is an indirect consequence of the binding of these death proteins to DREDD rather than the IAPs within a multiprotein complex.

Other mechanisms, however, may be involved in apoptosis induced by HID, GRIM, and RPR, including effects on voltage-gated potassium (K+) channels [Avdo- nin et al. 1998] and through interactions with another apoptosis-modulating protein called SCYTHE [Thress et al. 1998]. If RPR, HID, and GRIM do trigger apoptosis through effects on K+ channels or interactions with SCYTHE, these events must nonetheless result in caspase activation as p35 potently suppresses apoptosis induced by these *Drosophila* death proteins, both in cell culture models and in transgenic flies. Consequently, an IAP connection to caspase inhibition might still be involved.

**Summary**

IAP family proteins are conserved throughout animal evolution and can block apoptosis when expressed in cells derived from multiple species. In many instances IAP family proteins can suppress apoptosis across species barriers [for review, see Clem and Duckett 1998], implying that although the details of their regulation may vary, these proteins evidently target a common mechanism involved in programmed cell death. Although all IAP family proteins require at least one BIR domain for their anti-apoptotic function, it should be emphasized that not all BIR-containing proteins are necessarily involved in apoptosis regulation as indicated by the failure of the Ac-IAP protein to suppress apoptosis despite harboring a BIR domain. Until proven otherwise, the most likely explanation for how IAPs prevent apoptosis is by binding to and inhibiting caspases, as indicated by recent studies [Deveraux et al. 1997, 1998; Roy et al. 1997; Takahashi et al. 1998; Tamm et al. 1998]. Assuming that this biased viewpoint is correct, how important are IAP family proteins likely to be for ensuring cell survival in vivo? The answer to that question probably depends on the type of cell under investigation and the specific cell death stimulus involved. If IAPs function primarily as inhibitors of caspases, then we can anticipate from other experiments where artificial means were used to suppress these proteases that IAPs will be capable of rescuing cells from some cell death signals but not others [Green and Reed 1998]. Mitochondrial involvement appears to be one of the key variables that determines whether caspase inhibitors are sufficient to

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provide long-term protection and preservation of clonogenic potential, versus merely delaying death by converting an apoptotic stimulus into a necrotic one (Reed 1997; Green and Reed 1998). In many types of cells, loss of cytochrome c from mitochondria, for example, has two ways of killing cells: (1) activation of caspases through Apaf-1; or (2) cessation of mitochondrial electron chain transport with subsequent ATP depletion, generation of reactive oxygen species, and related sequelae. If the role of IAPs is relegated to caspase suppression, this may prevent cytochrome c-induced apoptosis, but not necessarily stop cell death induced by caspase-independent mechanisms. Recent studies in which release of cytochrome c was found to be a potentially reversible event suggest that whether cytochrome c loss from mitochondria defines a cell death commitment point will likely vary among cell types and depending on a variety of environmental factors (Q. Chen et al. 1998). These factors may include the extent to which cells are able to produce sufficient ATP from anaerobic glycolysis in the cytosol and the method by which mitochondrial membrane barrier function was altered to allow for exodus of cytochrome c (i.e., reversible versus irreversible/rupture). Defining the in vivo requirements for IAPs in the maintenance of cell survival may be difficult because of potential redundancy. Humans have at least five and possibly more IAP family genes and even lower organisms, such as *Drosophila*, appear to contain at least two IAP genes, implying evolutionary pressure to ensure adequate back-up if one of these genes were to become inactivated. If IAPs do indeed function predominantly as caspase inhibitors, then one could imagine a very important role for these endogenous protease inhibitors in ensuring that the small amounts of adventitial caspase activation, which must surely occur on a routine basis, do not amplify out of control, resulting in inappropriate cell death. In this regard, virtually every other protease system studied to date contains molecules whose sole function is directed toward dampening proteolysis through the cascade [for review, see Colman et al. 1994], thus ensuring that biologically appropriate triggering of the pathway only occurs when certain thresholds are surpassed. By analogy, it is attractive to consider IAP family proteins in the same way, as proteins that set thresholds for how much caspase activation is necessary to successfully trigger apoptosis. Through alterations in the levels of IAP family gene expression and interactions of IAPs with other proteins, this IAP-dependent threshold for caspase-induced apoptosis could be varied to suit various physiological needs. Dysregulation of these normal control mechanisms then could be a contributor to various diseases characterized by excessive [ischemia, AIDS, SMA] or inadequate [cancer, autoimmunity] cell death.

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