

Review

Inhibitor of apoptosis proteins and apoptosis

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Apoptosis is a physiological cell death process that plays a critical role in development, homeostasis, and immune defense of multicellular animals. Inhibitor of apoptosis proteins (IAPs) constitute a family of proteins that possess between one and three baculovirus IAP repeats. Some of them also have a really interesting new gene finger domain, and can prevent cell death by binding and inhibiting active caspases, but are regulated by IAP antagonists. Some evidence also indicates that IAP can modulate the cell cycle and signal transduction. The three main factors, IAPs, IAP antagonists, and caspases, are involved in regulating the progress of apoptosis in many species. Many studies and assumptions have been focused on the anfractuous interactions between these three main factors to explore their real functional model in order to develop potential anticancer drugs. In this review, we describe the classification, molecular structures, and properties of IAPs and discuss the mechanisms of apoptosis. We also discuss the promising significance of clinical applications of IAPs in the diagnosis and treatment of malignancy.

Keywords inhibitor of apoptosis proteins; apoptosis; baculovirus IAP repeat; IAP antagonist

Apoptosis, a crucial biological process, plays an essential role in regulating development, homeostasis, and immune defense by clearing redundant or abnormal cells in organisms. A delicate balance between pro-apoptotic and anti-apoptotic mechanisms determines whether a cell death signal can activate the execution of the apoptotic program. In this balance, pro-apoptotic proteins promote apoptosis and anti-apoptotic proteins inhibit apoptosis. As members

of the anti-apoptotic family of proteins, inhibitors of apoptosis proteins (IAPs) can inhibit the downstream components of the caspase activation pathways in the regulation of apoptosis and play important roles in regulating the progress of apoptosis in many species [1,2].

IAP Family Members

The *IAP* gene was first identified in insect SF-21 cells infected by baculovirus [3]. Encoded by a viral gene, this novel IAP can inhibit infected SF-21 cells from executing apoptosis. It has similar anti-apoptotic functions to p35 from *Autographa californica* multicapsid nucleopolyhedrovirus but shows no significant homology. This finding indicated that the *IAP* gene is 1.6 kb in size encoding a 31 kDa protein with a zinc finger-like motif. Subsequent studies identified anti-apoptotic proteins that can be grouped into the IAP family based on the presence of between one and three baculovirus IAP repeats (BIR) domains at the N-terminus. Some IAPs also have a really interesting new gene (RING) finger domain at the C-terminus. Many IAP family members have been identified in diverse species ranging from viruses to mammals (**Table 1**). In addition, eight human IAPs and three *Drosophila* IAPs have been studied extensively. Some newly identified IAPs or IAP-like proteins (ILPs) in new species have expanded the IAP family. In 2005, two novel ILPs, AtILP 1 and AtILP 2, were identified in plant *Arabidopsis thaliana* for the first time [4]. It was found that AtILPs have two conserved BIR-like domains, as in human ILP-1, that might play some roles in apoptosis. In *Xenopus* egg extracts, four maternal BIR family proteins have recently been identified [5]. The survivin-related *Xenopus* embryonic IAP, xEIAP/XLX, is inferior in apoptosis inhibition whereas xXIAP, a possible ortholog of X-chromosome-linked IAP (XIAP), greatly delays apoptotic initiation and is important for the survival of *Xenopus* eggs.

Molecular Structures of IAP Family Members

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Table 1 Inhibitor of apoptosis protein (IAP) family members

Abbreviation	Full name	Discoverer and time	Expression tissue or stage
Baculovirus			
cp-IAP	<i>Cydia pomonella</i> MNPV IAP	Crook <i>et al</i> , 1993	Entire virus
op-IAP	<i>Orgyia pseudotsugata</i> MNPV IAP	Birnbaum <i>et al</i> , 1994	
ac-IAP	<i>Autographa californica</i> MNPV IAP	Clem <i>et al</i> , 1994	
bm-IAP	<i>Bombyx mori</i> IAP	Huang <i>et al</i> , 2001	
HycuIAP	<i>Hyphantria cunea</i> NPV IAP	Ikeda <i>et al</i> , 2004	
Yeast			
SciIAP/Bir1p	<i>Saccharomyces cerevisiae</i> IAP	Uren <i>et al</i> , 1999 Yoon <i>et al</i> , 1999	Most parts of yeast
SpIAP/Bir1p	<i>Schizosaccharomyces pombe</i> IAP		
Plant (<i>Arabidopsis thaliana</i>)			
AtILP1	<i>A. thaliana</i> IAP-like protein 1	Higashi <i>et al</i> , 2005	Most plant tissue
AtILP2	<i>A. thaliana</i> IAP-like protein 2		
Nematode (<i>Caenorhabditis elegans</i>)			
CeIAP1/BIR-1	<i>C. elegans</i> BIRPs, BIR-1	Fraser <i>et al</i> , 1999	Highly expressed in embryogenesis
CeIAP2/BIR-2	<i>C. elegans</i> BIRPs, BIR-2		Only in adults and embryos
Fly (<i>Drosophila melanogaster</i>)			
DIAP1	<i>Drosophila</i> IAP 1	Hay <i>et al</i> , 1995	Most <i>Drosophila</i> tissue
DIAP2	<i>Drosophila</i> IAP 2		Most <i>Drosophila</i> tissue
Deterin	Deterin	Jones <i>et al</i> , 2000	Early stage embryos
Amphibian (<i>Xenopus</i>)			
xEIAP/XLX	<i>Xenopus</i> embryonic IAP	Tsuchiya <i>et al</i> , 2005	Highly expressed in oocyte and egg
xXIAP	<i>Xenopus</i> ortholog of XIAP		Oocyte and egg
Mammal			
XIAP/ILP-1/MIHA/BIRC4	XIAP	Duckett <i>et al</i> , 1996	Most human tissues
c-IAP1/hIAP1/MIHB/BIRC2	Cellular IAP 1	Rothe <i>et al</i> , 1995	Most human tissues; highly expressed in adult thymus, testis, ovary
c-IAP2/hIAP2/MIHC/BIRC3	Cellular IAP 2	Rothe <i>et al</i> , 1995	Most human tissues; highly expressed in adult spleen, thymus
ILP2/BIRC8/TS-IAP	IAP-like protein 2	Richter <i>et al</i> , 2001 Lagace <i>et al</i> , 2001	Adult testis
ML-IAP/livin/KIAP/BIRC7	Melanoma IAP	Vucic <i>et al</i> , 2000	Fetal liver, kidney; adult testis, thymus
NAIP/BIRC1	Neuronal apoptosis inhibitory protein	Roy <i>et al</i> , 1995	Adult liver, placenta, central nervous system
Survivin/TIAP/BIRC5	Survivin	Ambrosini <i>et al</i> , 1997	Fetal liver, kidney, lung, gastrointestinal tract; highly expressed in tumor
BRUCE/BIRC6/Apollon	BIR repeat-containing ubiquitin conjugating enzymesystem	Hauser <i>et al</i> , 1998	Golgi complex and the vesicular

BIR, baculovirus IAP repeats; MIH, multicapsid nucleopolyhedrovirus; MNPV, multicapsid nucleopolyhedrovirus.

IAP family members are characterized by the BIR domain, the name of which derives from the original discovery of these apoptosis suppressors in the genome of baculoviruses [3]. The BIR domains consist of approximately 70 amino acids that contain the characteristic sequence CX₂CX₁₆HX₆C.

With both hydrophobic and hydrophilic residues on its surface, the BIR core is theoretically capable of supporting protein-protein interactions. There are three subtypes of BIR domain, BIR1, BIR2, and BIR3, classified by their evolutionary relationship in phylogenesis. All the molecu-

lar structures of IAP family members are shown in **Fig. 1**. The RING finger domain (C₃HC₄) exists at the C-terminal in some IAPs. It contains one zinc atom chelated to three cysteines and one histidine and another zinc atom bound to four cysteines. Some IAP family members also contain other structures, such as the caspase activation recruitment domain, phosphate-loop and ubiquitin-conjugating (UBC). Although the BIR domain is required for the anti-apoptotic functions of the IAP family proteins, not all BIR-containing proteins have anti-apoptotic functions. All the IAPs are homologs with highly conserved sequences. The close relationship between baculoviral IAPs and insect IAPs suggests that baculoviral IAPs might have been acquired through gene transfer from host insect cells. Some baculoviral IAPs can even suppress apoptosis in mammalian cells [6].

Regulatory Mechanism of IAP in Apoptosis

The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. Three main factors, IAP, IAP antagonist, and caspase, are involved in regulating this progress. The detailed regulatory mechanism of IAP and the complicated interaction of the three main apoptotic factors are shown in **Fig. 2**.

IAP as inhibitor of caspase

IAP acts as endogenous inhibitor of caspases, the main executioners of apoptosis [7]. There are four pathways that have been described for caspase activation in apoptosis initiation: (1) the mitochondrial pathway (intrinsic pathway) where the release of cytochrome c from the mitochondria

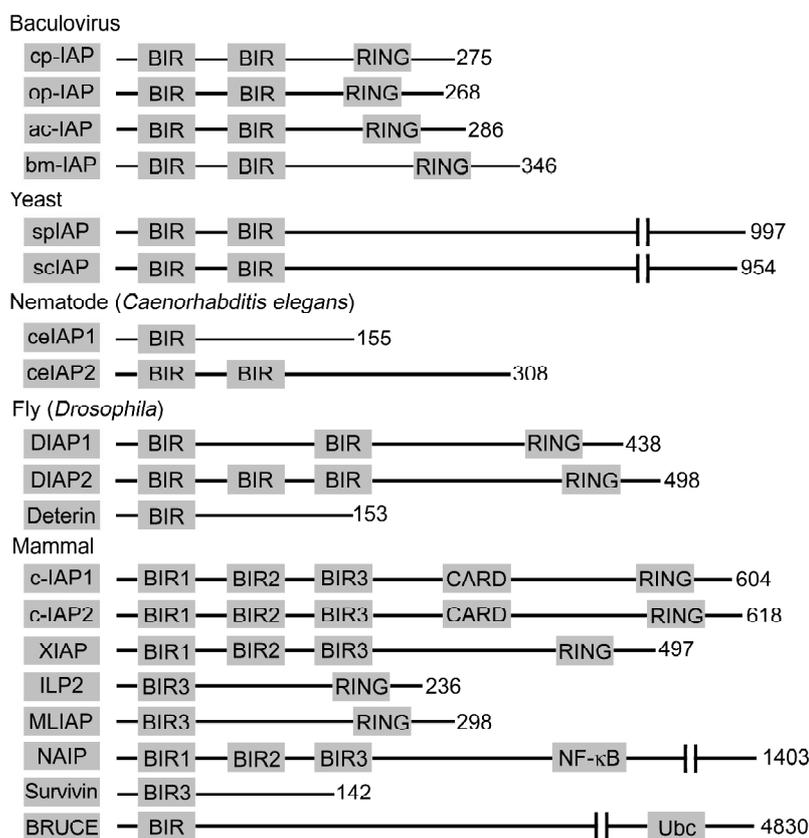


Fig.1 Molecular structures of inhibitor of apoptosis protein (IAP) family members According to the homology of baculovirus IAP repeats (BIR) domains and the presence or absence of a really interesting new gene (RING) finger domain, eight mammalian IAPs can be grouped into three classes. Class 1: cellular apoptosis inhibitory protein 1 (c-IAP1); cellular IAP 2 (c-IAP2); IAP-like protein 2 (ILP2); melanoma IAP (ML-IAP); X-chromosome-linked IAP (XIAP). Class 2: neuronal apoptosis inhibitory protein (NAIP). Class 3: BIR repeat-containing ubiquitin conjugating enzyme (BRUCE); survivin. ac-IAP, *Autographa californica* multicapsid nucleopolyhedrovirus (MNPV) IAP; bm-IAP, *Bombyx mori* IAP; CARD, caspase activation recruitment domain; CeIAP1/BIR-1, *C. elegans* BIRPs, BIR-1; CeIAP2/BIR-2, *C. elegans* BIRPs, BIR-2; cp-IAP, *Cydia pomonella* MNPV IAP; DIAP1, *Drosophila* IAP 1; DIAP2, *Drosophila* IAP 2; *HycuIAP*, *Hyphantria cunea* nucleopolyhedrovirus IAP; NF-κB, nuclear factor κB; op-IAP, *Orgyia pseudotsugata* MNPV IAP; ScIAP/Bir1p, *Saccharomyces cerevisiae* IAP; SpIAP/Bir1p, *Schizosaccharomyces pombe* IAP; UBC, ubiquitin-conjugating.

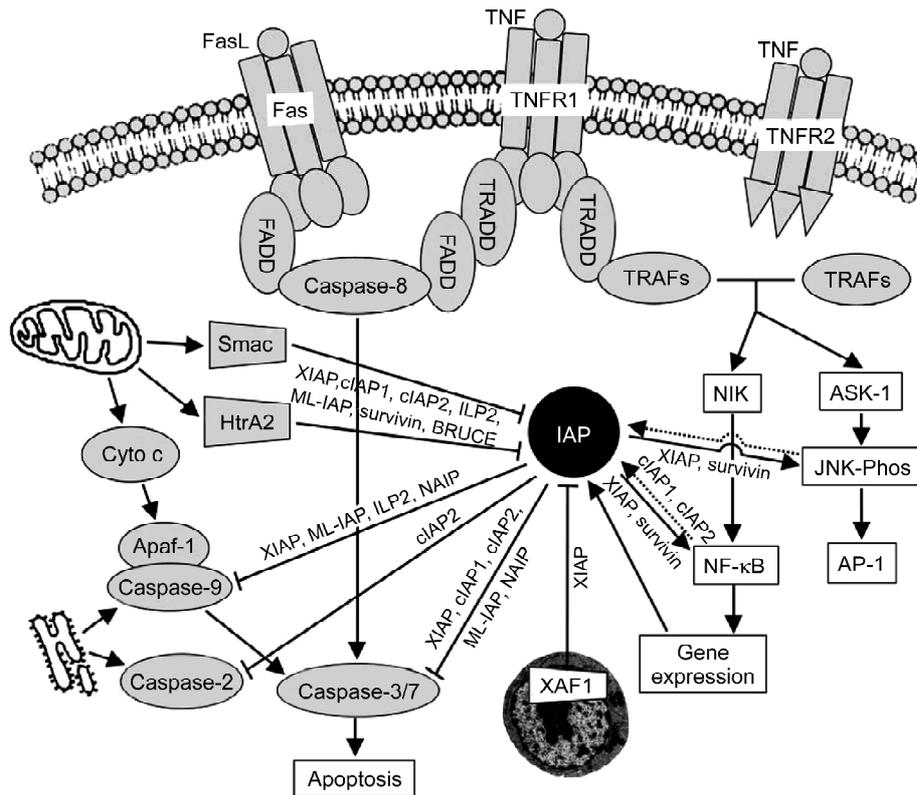


Fig. 2 Regulatory mechanism of inhibitor of apoptosis protein (IAP) in apoptosis This figure describes the complexity of the signaling network that is regulated by different members of the IAP family. It shows that specific IAP proteins inhibit certain caspases. IAPs are negatively regulated by IAP antagonists like second mitochondrial activator of caspases (Smac), high-temperature-regulated A2 (HtrA2), and X-linked IAP-associated factor 1 (XAF1), and executing positive feedback mechanisms with nuclear factor κ B (NF- κ B), and c-Jun N-terminal kinases (JNK) pathways. AP-1, activator protein-1; Apaf-1, apoptotic protease activating factor 1; ASK-1, apoptosis signal-regulating kinase 1; BRUCE, BIR repeat-containing ubiquitin conjugating enzyme; c-IAP1, cellular IAP 1; c-IAP2, cellular IAP 2; Cyto C, cytochrome c; FADD, Fas Associated Death Domain; FasL, Fas ligand; ILP2, IAP-like protein 2; ML-IAP, melanoma IAP; NAIP, neuronal apoptosis inhibitory protein; NIK, NF- κ B-inducing kinase; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2; TRADD, tumour necrosis factor receptor-associated death domain; TRAFs, The tumor necrosis factor receptor associated factors; XIAP, X-chromosome-linked IAP.

and the formation of apoptosomes activate caspase-9 and in turn caspase-3; (2) the death receptor pathway (extrinsic pathway), initiated by the ligand binding of extracellular signals and death receptors FasL (Fas ligand)/Fas, tumor necrosis factor (TNF)/TNF receptor on cell membrane; (3) the endoplasmic reticulum (ER) stress-induced apoptosis pathway that leads to the activation of caspase-2 and caspase-9; and (4) the activation by granzyme B of effector caspases by injecting cytolytic T cells and natural killer cells to target cells. All of these pathways converge on the activation of caspases, such as caspases 3, 6, 7, and 9. Four of the pathways are not distinct, in that the activation of one usually involves another.

Recent studies showed that IAP can inhibit the activity of caspases by binding of their conserved BIR domains to the active sites of caspases *in vitro* and *in vivo*. IAPs

inhibit caspases by promoting the degradation of active caspases, or by sequestering the caspases away from their substrates [8]. In insect SF-21 cells, *Drosophila* IAP 1 (DIAP1) can block apoptosis induced by the *Drosophila* caspase *Drosophila melanogaster* Interleukin-1 β -Converting Enzyme, insect sf-caspase-1, and mammalian caspase-3. In mammals, different IAPs execute various ways to regulate the function of caspases, and multiple BIR domains, even in the same IAP, use distinctly different functions to inhibit different caspases. In Fas/caspase-8-induced apoptosis, IAPs do not bind caspase-8 but inhibit its substrate caspase-3 to execute their anti-apoptotic roles. In contrast, IAPs carry out their caspase-suppression roles in three ways in the mitochondrial pathway of caspase activation: (1) competitively bind pro-caspase-9 and therefore interfere with the formation of apoptosome between

pro-caspase-9 and apoptotic protease activating factor 1; (2) directly bind caspase-9; and (3) directly bind caspase-3. Overexpression of IAP is induced by Bax and other pro-apoptotic Bcl-2 family proteins, known for their ability to target mitochondria and induce cytochrome c release. In the ER stress-induced apoptosis pathway, IAPs inhibit caspase-2 and caspase-9 by their BIR domain binding to suppress apoptosis [9].

XIAP, the best characterized IAP so far, is generally recognized as the most potent endogenous caspase inhibitor. XIAP has three BIR domains, BIR1, BIR2, and BIR3, which have high affinity but unequal functions to caspases. The BIR2 domain and the linker region between BIR1 and BIR2 specifically bind and inhibit caspase-3 and caspase-7. The BIR2 domain lies across the active site of caspase-3 and inhibits the activity of caspase-3 by blocking its substrate-binding pocket. Overexpression of the BIR1-2 fragment plays key roles in Fas/caspase-8-induced apoptosis. The caspase-9 inhibitory activity of IAP requires both the BIR3 and RING domain in the mitochondrial pathway. Without physically binding to the active site of caspase-9, the BIR3 domain forms a heterodimer with monomeric caspase-9, thereby preventing the dimerization and activation of caspase-9. As well as the trapping of caspase-9 in a monomeric form, the BIR3 domain can keep the active site of caspase-9 in an inactive conformation. Surprisingly, the BIR2, BIR3, or RING domains show no caspase inhibitory activity alone [10].

An emerging area of study in apoptosis is the critical contribution of the ER in both mitochondrial and ER-specific apoptosis pathways. Caspase-2, which is localized to the ER, is the proximal mediator in the ER stress-induced apoptosis pathway with caspase-9. They activate caspase-3 and caspase-7 and in turn cleave the caspase substrates. Although some IAPs are capable of binding to and inhibiting caspases 3, 7, and 9, only cellular IAP 2 (c-IAP2) directly binds and inhibits caspase-2 by its BIR2 domain. This result provides a novel mechanism in the early stages of ER stress-induced apoptosis [9].

IAP could block the convergence point of multiple caspase activation pathways and thus inhibit apoptosis. Caspases 2, 3, 7, and 9 can be suppressed by some kinds of IAPs, whereas other mammalian caspases (1, 6, 8, and 10) are known to be resistant to the inhibition. Different members of the IAP family vary more or less in regulatory mechanisms. Cellular IAP 1 (c-IAP1) and c-IAP2 can also bind caspases 3, 7, and 9, but not with as tight an affinity as XIAP. Melanoma IAP (ML-IAP) and ILP-2 are significantly inferior to XIAP in caspase inhibition, and their BIR domain is most homologous to the BIR3 domain. Research

on neuronal apoptosis inhibitory protein (NAIP) has shown its anti-apoptotic functions both *in vivo* and *in vitro*. In addition to its inhibitory activities on caspase-3 and caspase-7, like XIAP, NAIP can also use its BIR3 domain to interact with caspase-9, surprisingly in an ATP-dependent pathway [11].

Interestingly, some recent research seems to prove that only a few IAP proteins, like DIAP1 in *Drosophila* and XIAP in mammal, possess the ability to inhibit caspase [12]. Eckelman and Salvesen suggested that c-IAP1 and c-IAP2, which can also bind caspases 3, 7, and 9 but with not as tight an affinity as XIAP, lost or never acquired the caspase inhibitory ability. It was also shown that neither of the BIR domains (BIR2 and BIR3) in c-IAP1 or c-IAP2 can inhibit caspases because of critical substitutions in the regions targeting caspase inhibition in XIAP. The BIR domains of c-IAPs can be converted to tight binding caspase inhibitors by substituting these critical residues with XIAP residues. Thus, c-IAP1 and c-IAP2 could only execute their binding function to caspase, rather than caspase inhibition [13].

Some IAPs that have only one or two BIR domains are found in many species, like survivin and the BIR repeat-containing ubiquitin conjugating enzyme (BRUCE) in mammal, *Schizosaccharomyces pombe* IAP and *Saccharomyces cerevisiae* IAP in yeast, *Caenorhabditis elegans* IAP 1 (CeIAP1) and CeIAP2 in nematode, and deterin in fly, and these IAPs are relatively weak caspase inhibitors or play no roles in binding or inhibiting caspases. In some studies, they were even distinguished from the forenamed IAPs as BIR-domain-containing proteins, because of the differences in both the functions and structural features of their BIR domains. The mechanism of these IAPs functioning as mitotic regulators will be discussed.

IAP and the ubiquitination process

As well as binding with the BIR domains, IAP can also inhibit the activity of caspase in the ubiquitination process. Ubiquitination is a post-translational protein modification procedure that plays important roles in apoptosis and signal transduction. By operating the processes of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3), target proteins are attached by ubiquitins. They are in turn recognized and degraded by some proteasomes. The C-terminus RING domain of IAP has been identified as the essential motif for the activity of ubiquitin ligase (E3) that is sufficient to cause ubiquitylation and subsequent proteasome-mediated proteolysis [14].

The RING domain in mammalian IAPs, including c-IAP1, c-IAP2, XIAP, ML-IAP, as well as *Drosophila*

DIAP1 and DIAP2, possesses E3 ligase activities. The giant BIR-containing protein BRUCE is a unique IAP family member with dual roles. Its N-terminal BIR domain can mediate substrate binding, whereas the C-terminal UBC domain provides E2 activities. BRUCE has an unusual ubiquitin conjugation system in that it could combine in a single polypeptide ubiquitin conjugating (E2) with ubiquitin ligase (E3) activity, forming a chimeric E2/E3 ubiquitin ligase [15]. Survivin is degraded by ubiquitin-targeted proteolysis at the end of mitosis [16]. Even more recently, XIAP has also been reported to ubiquitylate caspase-9 by itself [17].

If certain IAPs can negatively regulate their own activity, autoubiquitination will work and degrade itself. This process can lower the effect of IAP and allow the cell to undergo apoptosis. In c-IAP2, either full-length protein or its RING domain alone could execute E3 ligase activity *in vitro* to promote autoubiquitination, as well as monoubiquitylation of caspase-3 and caspase-7. A recent study also indicated that the C-terminal RING domain of c-IAP1 is required for binding to XIAP and promoting XIAP degradation in several cells [18]. c-IAP1 can heterodimerize with XIAP through a RING-RING interaction so as to regulate the endogenous XIAP abundance and reduce it in a proteasome-dependent pathway. By doing so, these two mechanisms (ubiquitination and autoubiquitination) seem to work in counteraction to keep a fine balance. IAPs appear to enhance the degradation of themselves or their targets in an unclear regulatory mechanism.

IAP and IAP antagonists

IAP, which is at the center of virtually all apoptotic pathways, is also subject to strict regulation through feedback mechanisms. A number of important inhibitory factors that could counteract the anti-apoptotic activity of IAP have been discovered. They are several kinds of endogenous pro-apoptotic proteins that function on almost every kind of IAP [1]. Recently, neutralization between IAP and IAP antagonists becomes to be a researching mania for the treatment of cancers.

These endogenous IAP binding proteins were first identified in *Drosophila*, named Reaper, Grim, Hid, and Sickie and were shown to bind and inhibit DIAP1. They were defined as critical inhibitors of IAP activity. Later, mammalian counterparts of the IAP antagonists were identified, named second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO) [19] and high-temperature-regulated A2/Omi (HtrA2/Omi) [20], that are mitochondrial-derived proteins, as well as X-linked IAP-associated factor 1 (XAF1) [21], a nuclear protein. In re-

cent studies, G1 to S phase transition protein/ polypeptide chain release factor 3 (GSPT1/eRF3) was also found to associate with the ER in mammal [22], and Nma111p, a nuclear protein, plays a crucial role in yeast apoptosis like its mammalian counterpart HtrA2/Omi [23]. The functional execution of these proteins seems to require normal induction of apoptosis. Structural studies have shown the physical interactions between these IAP binding proteins and IAP. The IAP binding proteins in *Drosophila* and mammal all have a highly conserved homologous sequence, named the IAP-binding motif domain, at the N-terminus that can bind IAP BIR domains so as to mediate their proapoptotic function, at least in part, by competing for interaction with IAP, thus displacing the bound caspases that are then free to amplify the caspase cascade continuously.

The regulatory mechanism of Smac/DIABLO and HtrA2/Omi has been extensively characterized. During the disruption of the mitochondria, more than 40 regulators or executors involved in mammalian apoptosis might be released simultaneously, including cytochrome c, endonuclease G, Apoptosis-inducing factor IAP, and Smac/DIABLO and HtrA2/Omi. On release, Smac/DIABLO and HtrA2/Omi are cleaved to become their activated forms that exist as arc-shaped dimers and pyramid-shaped homotrimers, respectively. The structural study of molecular recognition between Smac and IAP shows that the IAP-binding motif domain in active Smac, which is only the first four residues (56–59) of the sequence, binds across the third β -strand of the BIR3 domain and competitively inhibits the BIR3 domain from binding caspase-9, which also has an overlapped binding site the same as Smac. HtrA2 also inhibits the function of XIAP by directly binding the BIR3 domain, but with less affinity than Smac. The full-length protein of Smac and its NH₂-terminal peptides can also bind the BIR2 domain of XIAP to disrupt the association between BIR2 and caspase-3 with slightly weaker affinity. This mechanism might be related more to steric hindrance than competitive binding [24].

The isoform of Smac/DIABLO, named Smac3, which is generated by alternative splicing of exon 4, can also interact with the second and third BIR domains of XIAP, just as Smac/DIABLO. Strikingly, only Smac3 can accelerate XIAP autoubiquitination and destruction. Smac3-accelerated XIAP ubiquitination is contingent on the physical association of XIAP with Smac3 and an intact RING domain of XIAP. Smac3-stimulated XIAP destabilization is partly attributed to its ability to enhance XIAP ubiquitination [25].

In TNF-mediated up-regulation of *IAP* gene expression, TNF α can increase mRNA and protein levels of c-IAP1,

c-IAP2, and XIAP, but not ML-IAP or survivin in tumor cell lines. IAPs act synergistically with TNF family members to promote survival of tumor cells [26]. Correspondingly, IAP antagonists can induce the autoubiquitination activity and rapid proteasomal degradation of IAPs, like cIAP-1 and cIAP-2. Depending on TNF signaling and *de novo* protein biosynthesis, IAP antagonists can also induce NF- κ B-stimulated production of TNF α that kill cells in an autocrine fashion [27,28].

XAF1 is another IAP inhibitor that binds and sequesters XIAP in the nucleus. It is ubiquitously expressed in normal tissues, but is present at low or undetectable levels in many different cancer cell lines. Although XAF1 might play key roles in mediating the apoptosis of cancer cells, it is still unclear whether the inhibition of XIAP in the nucleus simply separates the XIAP from cytosolic caspases or whether there are additional effects from XIAP located in the nucleus [21].

As well as the endogenous IAP binding proteins, such as Smac or HtrA2, several additional potential IAP antagonists are reported in the development of targeted therapies directly against IAP in cancer. Some members of the IAP family, including c-IAP2 and survivin, can be regulated by survival cytokines, including interleukin-3, interleukin-5, and granulocyte/macrophage colony stimulating factor [29]. Mitochondrial proteins, including glutamate dehydrogenase, Nipsnap 3 and 4, caseinolytic peptidase X leucine-rich pentatricopeptide repeat motif-containing protein, and 3-hydroxyisobutyrate dehydrogenase, are newly described to interact with XIAP, mainly by way of BIR2. Through the interaction, they are able to antagonize XIAP inhibition of caspase-3 *in vitro* [30].

There are a lot of antisense oligonucleotides and small molecule chemical IAP inhibitors that have been generated to study the regulatory function of IAP, especially in tumor cells and therapies. In one of the classic therapeutic strategies, antisense oligonucleotides are used to decrease the target IAP mRNA and subsequently decrease the protein available for both XIAP and survivin. Recently, Wang *et al* discovered a phenylurea-based XIAP antagonist that can block the interaction of downstream effector caspases with XIAP, thus inducing apoptosis of tumor cell lines through a caspase-dependent, Bcl-2/Bax-independent mechanism [31].

Complicated interaction between IAPs, IAP antagonists, and caspases

Above all, the accepted regulatory model is that IAP can suppress cellular apoptosis through the inhibition of caspases, whereas, in contrast, some IAP antagonists like

Smac/DIABLO can directly bind and provide inhibitory activity to IAPs, particularly XIAP. However, the *in vitro* affinity that many IAPs possess for caspases is much lower when compared with XIAP, raising the possibility that the method by which they mediate cellular protection might involve mechanisms beyond direct caspase inhibition [12]. Recently, some interesting findings have emerged to suggest that this scheme might not be as simple as originally thought. If the binding of Smac/DIABLO to XIAP neutralizes the cytoprotective effects of XIAP, how could binding of XIAP to Smac/DIABLO block the death-promoting function of Smac/DIABLO? Which of these two opposite directions is more important [32]?

Do cytoprotective IAPs inhibit apoptosis through the neutralization of IAP antagonists rather than by directly inhibiting caspases? It is a very interesting hypothesis and much research has reached this conclusion. Green *et al* showed that the expression of *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus IAP (op-IAP) in mammalian cells can block the activation of caspase-3. But surprisingly, instead of inhibiting caspase-3 directly, op-IAP executes its protection by binding to Smac/DIABLO efficiently, thereby preventing Smac/Diablo-mediated inhibition of endogenous cellular IAP proteins (such as XIAP), which may then continue to directly inhibit caspases. Op-IAP also has the ability to ubiquitinate pro-apoptotic cellular proteins such as Hid using both the RING domain and BIR2, which might play an important role in the anti-apoptotic process [33].

There are more distinct conclusions on mammalian IAP that could efficiently bind Smac/Diablo so as to provide protection in cells that express other caspase-inhibiting IAP, such as XIAP. ML-IAP, which is inferior to XIAP in caspase inhibition, has a very high affinity for Smac. It can bind mature Smac to form an ML-IAP-Smac complex and disrupt the endogenous interaction between XIAP and mature Smac [34]. Survivin can manifest its cytoprotection by physically associating with XIAP and forming a survivin-XIAP complex, increasing XIAP stability against ubiquitination/proteasomal destruction and synergistic inhibition of caspase-9 activation *in vivo* and *in vitro* [35]. Ceballos-Cancino *et al* found that survivin could regulate the specific release of mitochondrial intermembrane protein Smac/DIABLO during apoptosis that is induced by etoposide. And survivin could also stabilize the cytosolic levels of released Smac/DIABLO by associating with Smac/DIABLO to delay its release [36].

The RING domain-bearing IAP can also mediate the polyubiquitination of Smac/DIABLO by ubiquitin ligase (E3) activity. For example, DIAP1 was reported to cause

ubiquitylation of the *Drosophila* IAP antagonists Grim, Hid, and Reaper. XIAP and c-IAP1 were found to mediate ubiquitylation of Smac/DIABLO *in vitro* [16]. BRUCE's activity to ubiquitylate Smac depends on the catalytically active UBC domain and the correctly folded BIR domain that binds the substrate Smac [15].

In addition to merely competing for binding sites, certain IAP antagonists can also destabilize IAP and cause IAP degradation by proteasomes. Reaper and UBCD1 in *Drosophila* caused degradation of DIAP1 in a RING-dependent manner, presumably by promoting DIAP1 autoubiquitination and degradation [37]. In another report, Hid was found to stimulate DIAP1 polyubiquitination and degradation, whereas Reaper and Grim could down-regulate DIAP1 through mechanisms that do not require DIAP1 function, such as a ubiquitin-protein ligase [37]. In contrast, Smac/DIABLO does not promote the ubiquitin ligase activity of XIAP in the same way as *Drosophila* IAP antagonists. It mainly potentiates apoptosis by simultaneously antagonizing caspase-IAP interactions and repressing IAP ubiquitin ligase activities [38].

A recent study even reached an alternative conclusion, that caspase-3 could attenuate the inhibition of caspase-9 mediated by XIAP. During the mitochondrion-mediated pathway, the initiator caspase-9 can be activated and in turn activate caspase-3 and caspase-7. The activated caspase-3 then activates caspase-9 by cleaving caspase-9 and forms a positive feedback amplification loop to accelerate apoptosis. The short peptide motif that cleaved at Asp³³⁰ in caspase-9 permits caspase-9 to interact with IAPs. This shows that cleavage by caspase-3 does not activate caspase-9, but enhances apoptosis by alleviating XIAP inhibition of this apical caspase at Asp³¹⁵ by autolytic cleavage [39].

IAP in Cell Cycle and Signal Transduction

Despite the suppression of the caspase pathway, IAP has been reported in a variety of cellular processes including the cell cycle and signal transduction.

IAPs with only one BIR domain mainly function as regulators of the cell cycle, such as: survivin in mammal; *Sacch. cerevisiae* IAP and *Schiz. pombe* IAP in yeast; CeIAP1 and CeIAP2 in *C. elegans*; and deterin in *Drosophila*. Survivin is a fascinating member of the IAP family, with its dual roles in mitosis and apoptosis. It is a relatively weak caspase inhibitor but serves critical roles in mitotic regulation. Survivin is expressed in most human tumors, but is rarely detected in fully differentiated normal cells. With the localization to mitotic spindles, survivin is necessary for the

assembly of metaphase spindles and promotes the stabilization of microtubule-chromatin interactions. The direct biochemical functional analysis in *Xenopus* egg extracts also justified this conclusion. Removal or inhibition of survivin could cause the disruption of spindles [40]. The roles of survivin make it a good prognostic marker and an attractive target for cancer therapy. Most interestingly, it has been shown that overexpressed c-IAP1, which localizes exclusively to nuclei in cells, can also modulate the cell cycle, possibly by interfering with the mitotic functions of survivin. These findings could have important implications for cancers in which c-IAP1 overexpression occurs [41].

Recently, several IAPs have been shown to be involved in many signaling cascades. However, the mechanisms involved in the regulation of the IAP genes are not fully understood. In the NF- κ B pathway, where it is potentially seminal for development of inflammation-associated tumors, c-IAP2-mucosa-associated lymphoid tissue 1 fusion protein could constitutively activate NF- κ B so as to contribute to human cancer. However, c-IAP2 and mucosa-associated lymphoid tissue 1 alone do not possess the same activation capacity. In the mitogen-activated protein (MAP) kinase pathway in human endothelial cells, TNF α could induce the expression of c-IAP1 and c-IAP2 at the transcriptional level. Thus, MAP kinases could participate in the inhibition of apoptosis by the induction of c-IAPs. It is a MAP kinase-dependent and NF- κ B-independent process [42]. In the extracellular signal-regulated kinase pathway in colon cancer, XAF1 expression was up-regulated by inhibition of the extracellular signal-regulated kinase 1/2 pathway through transcriptional regulation, so that the expression of XIAP would, conversely, be down-regulated [43]. In the phosphoinositide 3-kinase/Akt signal pathway, which is strongly activated by insulin, survivin is significantly decreased by wild-type Fragile Histidine Triad (Fhit). Then, overexpression of constitutively active Akt will inhibit Fhit-induced apoptosis by the loss of endogenous Fhit expression [44–46].

Promising Clinical Applications of IAP

Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. It is known that dysfunction of the apoptotic pathway and deregulated cellular proliferation will ultimately lead to carcinogenesis and tumor progression. Many different apoptosis regulators have been documented in rendering tumor cells resistant to apoptosis both *in vivo* and *in vitro*, especially the most potential IAP. When IAP family members are overexpressed,

cancer cells no longer proceed to apoptosis and become increasingly resistant to standard chemo- and radiation therapies [1,2].

Many studies have established a circumstantial association between IAPs and cancer. Pathological overexpression of several IAP family members has been detected in several classes of human cancers. For example, the overexpression of IAPs, such as XIAP, c-IAP1, c-IAP2, NAIP, and survivin, has been detected in prostate cancer [47] and breast cancer [48]. A preferential cytoplasmatic localization of c-IAP1 was observed in pancreatic carcinogenesis [49]. c-IAP2 is also overexpressed in pancreatic ductal adenocarcinomas [49]. The elevated expression of XIAP has been investigated in esophageal cancer tissues and cell lines, and compared with normal tissues. It raises the possibility that high levels of IAPs might confer an insensitivity to apoptosis induction by caspase-3 activation and promote tumorigenesis by keeping mutated cells alive [50].

In human pancreatic carcinogenesis, c-IAP1 expression is constantly high in pancreatic intraepithelial neoplasia lesions, as well as in a subset of primary and metastatic pancreatic ductal adenocarcinomas. c-IAP2 is also overexpressed and detectable in low- and high-grade pancreatic intraepithelial neoplasia lesions [49]. Zhu *et al* postulated that survivin plays an important role in the onset of gastric carcinoma and that high survivin expression is an early event of gastric carcinoma [51]. Kluger *et al* reported that overexpression of XIAP is up-regulated by pretreatment with phenoxodiol in metastatic melanoma [52]. As well as the central regulatory mechanisms of IAP suppression through direct caspase and pro-caspase inhibition, IAPs appear to regulate NF- κ B family transcriptional activators which have also been associated with malignancy. The cIAPs have been found to function as regulators of NF- κ B signaling. Through their ubiquitin E3 ligase activities, c-IAP1 and c-IAP2 promote proteasomal degradation of NF- κ B-inducing kinase, the central Ser/Thr kinase in the non-canonical NF- κ B pathway [27].

Due to the unique pathological overexpression of IAP that has been documented in cancer, a novel and promising strategy is suggested to develop targeted therapies directly against IAP for the treatment of malignancy. Compared to conventional cancer chemotherapies, the starting point for developing these more selective and less harmful anticancer drugs is to use various IAP inhibitors to suppress IAP activities. Several approaches have been taken to target and eliminate IAP functions, in order to attempt to re-establish sensitivity, reduce toxicity, and improve efficacy of cancer treatment. Recently, some small

molecules, like deguelin and D,L-sulforaphane, have been shown to down-regulate IAPs to release their inhibitory activity over pre-existing active caspases present in cancer cells [48,53]. These targeted therapies could better control the primary cancer by decreasing its chance of developing to secondary malignancies, and can also be used in tumor treatment in combination with standard cancer chemotherapies.

Another possible anti-IAP therapeutic strategy is a molecular method named RNA interference (RNAi). The core of the RNAi strategy is to deplete IAP expression. Recent evidence has suggested that XIAP is a key determinant in the chemoresistance of cancer cells. The small interfering RNA (siRNA) is constructed and transferred into cancer cells, in order to block the overexpression of IAPs and other proteins, like S-phase kinase-associated protein-2. These processes evaluate the effect of siRNA on cellular apoptosis [54,55]. Treatment with XIAP siRNA in combination with paclitaxel, cisplatin, fluorouracil, and etoposide could efficiently decrease XIAP expression and induce cellular apoptosis [50,56,57]. Survivin knockdown by RNAi leads to growth rate inhibition of myeloma cells related to apoptosis induction and deep cell-cycle disruption, and makes myeloma cells sensitive to conventional antimyeloma agents [58]. The vector-based short hairpin RNAs can effectively reduce the overexpression of survivin and Ki67 in renal cancer. They induce apoptosis and are used as a new agent in renal cancer gene therapy [59].

Interestingly, there are a number of publications showing IAP expression in normal tissues and cells. Although survivin is usually not expressed, c-IAP1, c-IAP2, and XIAP have been found broadly expressed at the mRNA level within normal cells [60]. In normal pancreatic tissues, c-IAP1 expression is constantly as high as that measured in pancreatic cancer cells in the same conditions. But a preferential cytoplasmatic localization of c-IAP1 has been observed in tumor tissues. This suggests that c-IAP1 might contribute to the regulation of the apoptotic process in the normal and the neoplastic pancreas, depending on its sub-cellular localization [49]. In normal tissues, IAPs could have some potential physiological roles, such as the regulation of the immune system [61], the response to cell damage [2,61], and cell survival and differentiation [62]. Further investigation is needed to help us understand the detailed physiological roles of IAPs in normal tissues.

In summary, research into the extensive characterization of the interaction between IAPs, caspases, and IAP antagonists in apoptosis is so important and indispensable that it will afford the necessary theoretical support in anti-cancer therapy.

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