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ORIGINAL PAPER

The inhibitors of apoptosis (IAPs) as cancer targets

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Abstract Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer, in addition to other human diseases including neurodegeneration, coronary disease and diabetes. The origin of cancer involves deregulated cellular proliferation and the suppression of apoptotic processes, ultimately leading to tumor establishment and growth. Several lines of evidence point toward the IAP family of proteins playing a role in oncogenesis, via their effective suppression of apoptosis. The central mechanisms of IAP apoptotic suppression appear to be through direct caspase and pro-caspase inhibition (primarily caspase 3 and 7) and modulation of, and by, the transcription factor NF-kappaB. Thus, when the IAPs are over-expressed or over-active, as is the case in many cancers, cells are no longer able to die in a physiologically programmed fashion and become increasingly resistant to standard chemo- and radiation therapies. To date several approaches have been taken to target and eliminate IAP function in an attempt to re-establish sensitivity, reduce toxicity, and improve efficacy of cancer treatment. In this review, we address IAP proteins as therapeutic targets for the treatment of cancer and emphasize the importance of novel therapeutic approaches for cancer therapy. Novel targets of IAP function are being identified and include gene therapy strategies and small molecule inhibitors that are based on endogenous IAP antagonists. As well, molecular mechanistic approaches, such as RNAi to deplete IAP expression, are in development.

Keywords Apoptosis · Cancer · IAPs · Caspases · Smac · NFkB · TNFR · TRAF · Knock out mice

Apoptosis and cancer

The development of cancer results when there is a failure in the control of the fundamental mechanisms that govern cell growth and cell death. The processes that regulate both cell proliferation and the destruction of potentially harmful cells are so closely linked that the inappropriate activation of growth-promoting oncogenes often initiates cell death. In a healthy organism, cells typically die via apoptosis; however, the uninhibited cell proliferation characteristic of most cancer cells requires that apoptosis be suppressed. As solid tumors continue to grow, the resulting microenvironment often becomes deficient in growth factors, and lacks an adequate oxygen supply. Although these conditions would trigger apoptosis in normal cells, cancer cells continue to proliferate [1, 2]. Most approaches used in cancer treatment, such as chemotherapy and radiation therapy, kill cancer cells by inducing apoptosis; however, cancer cells often develop resistance to these types of therapies. Furthermore, many cancer therapies indirectly activate apoptosis by chemically or physically damaging DNA. This may have the unintended effect of generating a pool of heavily mutated cancer cells which increases the chances of developing resistance [1–3]. Insight into how apoptotic pathways are deregulated in cancer is therefore

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critical both to the understanding of how this disease develops and progresses, and for the development of effective and reliable anti-cancer treatments. Thus, therapies that directly and specifically activate apoptosis would be predicted to be both safer and more effective than existing therapies.

At the molecular level, the evolving descriptions of mitochondrial dysfunction and caspase activation as key elements of apoptosis have provided significant insight into the understanding of the molecular basis of cancer. In normal cells, limited activation of the caspase cascade may be essential in cellular differentiation and/or replication [4], thus making the preservation of caspase control essential; however, in cancer cells it is often observed that caspase activation pathways are impaired or absent, thereby preventing cells from dying when they should [5]. Since the loss of caspase activation appears to be central to the prevention of most cell death events in cancer, finding strategies to overcome caspase inhibition will be of value for the development of novel cancer treatments.

Control of apoptotic pathways

Caspases

Caspases are a class of cysteine–aspartyl proteases that are synthesized as inactive precursor enzymes, or proenzymes. These proteases typically lie dormant in the healthy cell and in response to cell death stimuli are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. Once activated, caspases cleave their substrates typically after conserved aspartate residues and are responsible for most of the biochemical and morphological features of apoptotic cell death. The fundamental role of caspases in the apoptotic process was first realized when CED-3, a cysteine protease required for apoptosis, was discovered in the nematode *Caenorhabditis elegans*. CED-3 was subsequently identified as a member of a highly conserved family of proteins present in not only *C. elegans*, but in a range of species from *D. melanogaster* to all vertebrates [6]. To date 14 distinct mammalian caspases have been identified, 7 of which are central to the regulation of the apoptotic process.

Caspases in their proenzyme form contain three domains: an amino-terminal prodomain; a large subunit containing the active site cysteine within a conserved QACXG motif; and a carboxy-terminal small subunit. The prodomains are separated from the large subunit by an aspartate cleavage site and one or more aspartate cleavage sites are present in the linker region between the large and small subunits. Two cleavage events are required to activate caspases into functional proteases. The first proteolytic cleavage divides the proenzyme into large and small caspase subunits. A second cleavage event removes the amino-terminal prodomain. The resultant, functional caspase is a tetramer of two large p20 subunits and two small p10 subunits [7] (Fig. 1).

The presence of aspartate at the maturation cleavage sites is consistent with the ability of caspases to autoactivate by proximity-induced aggregation or be activated by other caspases as part of an amplification cascade [8, 9]. For example, in the mitochondrial or intrinsic cascade, a multi-protein complex (the apoptosome) assembles in response to death stimuli that includes recruitment of the procaspases. In this complex an adaptor molecule, apoptosis protease activating factor-1 (Apaf-1), binds to pro-caspase-9. Pro-caspase-9 is then observed to undergo intrachain cleavage. This cleavage appears to only have a moderate effect on its catalytic activity in that fully processed caspase-9 in isolation is only marginally active, similar to that of unprocessed caspase-9 [10, 11]. When caspase-9 is in association with the apoptosome, there is a substantial increase in the catalytic activity of the processed, as well as the unprocessed caspase-9 [11]. Intrachain cleavage therefore appears to be relatively inconsequential to caspase-9 activation, but rather its activation depends to a greater extent on conformational changes induced by association with the apoptosome [12].

Activation of caspases

The mammalian apoptotic caspases have been classified as either initiator caspases (caspases-2, -8, -9, and -10) or effector caspases (caspases-3, -6, and -7) based on their structure and function. Initiator caspases differ from the effector caspases in that they have long N-terminal domains that allow them to interact with death effector domains (DED) or caspase recruitment domains (CARD)

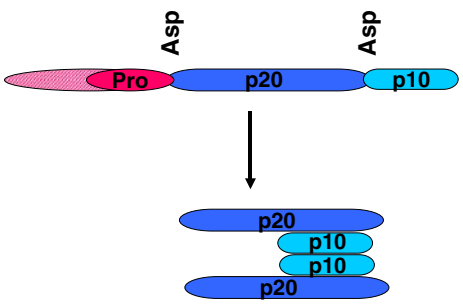

Fig. 1 Schematic of caspase structure. Caspases are synthesized as inactive zymogens. The prodomain varies in length between initiator caspases (long prodomains) (shaded) and effector caspases (short prodomains) (solid). Cleavage at aspartate (Asp) sites results in an active tetramer consisting of two large (p20) and two small (p10) subunits

present in adaptor proteins such as FADD (Fas-associated protein with death domain) and Apaf-1, respectively. The activation of initiator caspases results from cell death signaling through one of two pathways: (1) the extrinsic, death receptor pathway or (2) the intrinsic, mitochondrial pathway (Fig. 2). An example of signaling through the extrinsic pathway occurs when there is binding of a so-called ''death ligand'' to a cell surface receptor, such as Fas/CD95/Apo-1, a member of the tumor necrosis factor (TNF) family of apoptosis-inducing receptors. Binding of trimeric Fas ligand to the Fas receptor triggers oligomerization and formation of a death-inducing signaling complex (DISC) comprised of Fas, the adaptor molecule FADD and pro-caspase-8. The aggregation of pro-caspase-8 in this complex results in its auto-activation and subsequently activation of downstream effector caspases [13, 14].

Apoptotic signals that trigger the activation of the intrinsic pathway result in the release of cytochrome c from the inner-mitochondrial membrane space into the cytosol. Binding of cytochrome c and dATP causes the adaptor molecule, Apaf-1, to form a large multimeric complex called the apoptosome. Apaf-1 in the apoptosome recruits pro-caspase-9 through the interaction of their respective CARD domains. Pro-caspase-9 is then processed by auto-catalysis [15–17]. Recent data suggests that following this proteolytic activation, there is a subsequent recruitment of caspase-3 to the apoptosome [12]. Upon activation of the downstream effector caspases, including caspase-3 and -7, the disassembly of the cell occurs in what is known as the execution phase of apoptosis.

Bcl-2 family of proteins

The release of cytochrome c from the mitochondria into the cytosol is fundamental to apoptosome formation and subsequent downstream caspase activation, in the intrinsic cell death pathway. The mechanism by which this release of cytochrome c comes about is controversial. Uncoupling of mitochondrial oxidative phosphorylation is observed during apoptosis, resulting in the loss of mitochondrial transmembrane potential [18]. The opening of mitochondrial permeability transition pores (PTPs) is associated with this

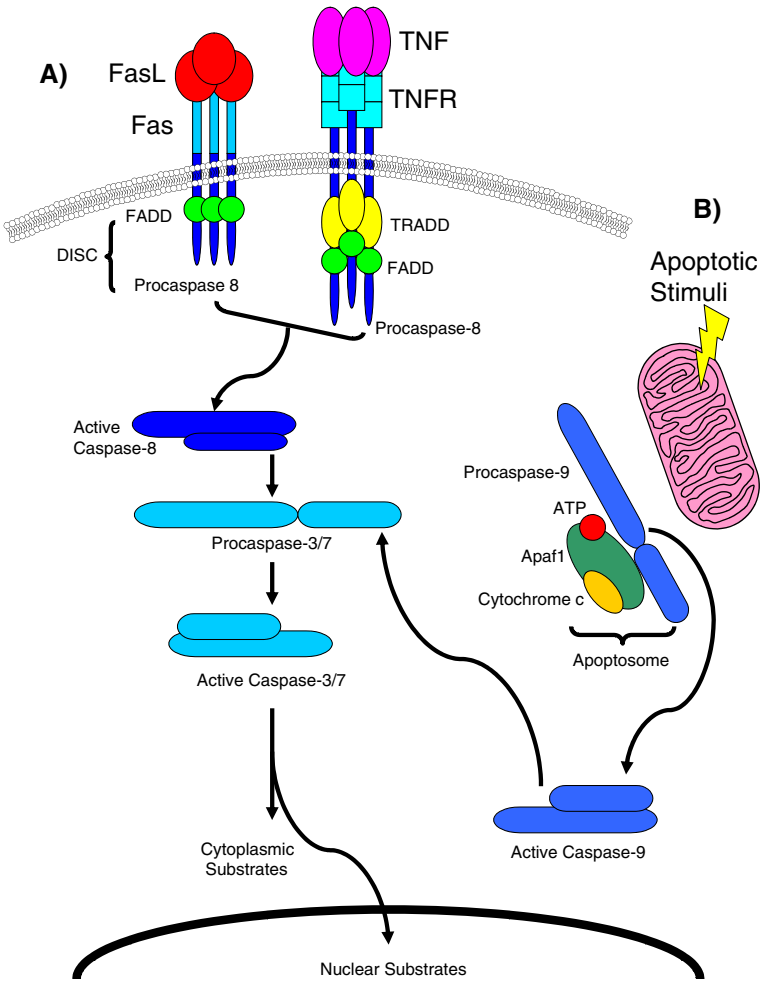

Fig. 2 Summary of intrinsic and extrinsic apoptosis signaling pathways. Death receptor-mediated signaling of apoptosis results from Fas ligand binding to the Fas/CD95/Apo-1 receptor or from TNFα binding to TNFR1 (**A**). Recruitment of TRADD and/or FADD results in pro-caspase-8 activation and subsequent downstream caspase-3 activation. Mitochondrial-mediated apoptotic signaling results in cytochrome c release and in formation of the apoptosome complex consisting of Apaf1 and caspase-9 (**B**). Following subsequent capase-3 activation, disassembly of the cell occurs

loss of potential and provides a mechanism by which cytochrome c might escape from the intermembrane space [19]. Other studies suggest that PTP opening is a secondary, caspase-dependent event and that cytochrome c release precedes PTP opening [20]. Therefore, the release of cytochrome c appears to be a consequence of a specific permeabilization event, which is independent of the generic membrane depolarization observed in a dying cell. In fact, there is evidence that members of the Bcl-2 protein family are key mediators of cytochrome c release in the context of apoptotic stimuli [21–23].

The *Bcl-2* gene was first identified as a proto-oncogene that is over-expressed by an immunoglobulin enhancer when chromosome translocations occur in follicular B-cell lymphoma. Unlike all other previously identified oncogenes, *Bcl-2* was found to inhibit cell death, rather than promote cell proliferation [24]. Subsequently, CED-9, a functional homologue of Bcl-2, was identified as an apoptotic repressor in *C. elegans* [25]. The Bcl-2 family members appear to be responsible for determining whether or not the pro-caspase/apoptosome complex can assemble. Evidence for this is based on the observation that anti-apoptotic members such as Bcl-2, and its nematode counterpart CED-9, prevent apoptosome formation, but their pro-death family members can over-ride this anti-apoptotic activity [26]. At least 20 other Bcl-2 family members have been identified in mammals. The key determinant of a Bcl-2 related protein is the presence of at least one of four conserved Bcl-2 homology (BH1–BH4) domains. The Bcl-2 family members are divided into three categories of proteins according to their structure and function and are defined as: anti-apoptotic members, multi-domain pro-apoptotic members, and BH3-only pro-apoptotic members (Fig. 3) [27].

The anti-apoptotic members of the Bcl-2 family usually contain all four BH domains and include: Bcl-2, Bcl-X_L, Bcl-W, A1, and Mcl-1. These proteins potently inhibit apoptosis in response to many cytotoxic insults. They possess a carboxy-terminal transmembrane domain (TM) that targets them to the outer mitochondrial membrane, endoplasmic reticulum, and the nuclear envelope. In healthy cells Bcl-2 resides as an integral membrane protein, whereas Bcl-X_L and Bcl-W require an apoptotic signal to become tightly associated with the membrane [28].

The multi-domain pro-apoptotic members of the Bcl-2 family containing BH1, BH2, BH3, and TM domains, such as Bax and Bak, are essential for apoptosis in many cell types and this is supported by the fact that mice deficient in both of these genes show a severe developmental phenotype [29]. In healthy cells Bax exists as a cytosolic monomer, or is loosely attached to membranes. Following a death stimulus, Bax undergoes a conformational change and integrates into the outer mitochondrial membrane

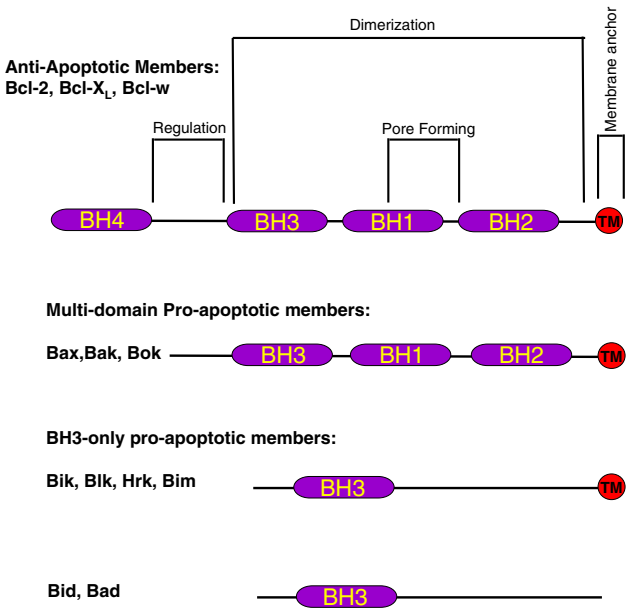

Fig. 3 Bcl-2 family members. The Bcl-2 family members can be divided into three groups of proteins according to their structure and function. The anti-apoptotic family members usually contain all four BH domains and include Bcl-2, Bcl-X_L, and Bcl-w. The multi-domain pro-apoptotic members are lacking the BH4 domain and include Bax, Bak, and Bok. Finally, the BH3-only pro-apoptotic family members only contain the BH3 homology domain and include Bid, Bad, Bim, Bik, Blk, and Noxa

where it oligomerizes [28, 30]. The oligomerization of Bax and Bak are thought to permeabilize the outer mitochondrial membrane, thus permitting the efflux of apoptogenic proteins, such as cytochrome c, into the cytosol. The mechanism by which they become activated and over-ride Bcl-2 is still a topic of debate [28]. There is however, one hypothesis that suggests that Bax and Bak directly form channels in the mitochondrial membrane. Consistent with this idea is the fact that Bax oligomers can form pores in liposomes that allow passage of cytochrome c [31, 32].

The BH3-only proteins appear to be the initiators of apoptosis in response to developmental cues or intracellular damage [33, 34]. In healthy cells BH3-only proteins are held in check by a variety of strategies. For example, Bim and Bmf are sequestered to the microtubule or actin components of the cytoskeleton by interactions with a dynein light chain. Also, Bad is maintained via phosphorylation and subsequent interaction with a scaffold protein, 14-3-3, and Bid is synthesized as a precursor protein and remains inactive until proteolytically cleaved by caspase-8. As a final example, Noxa is controlled at the transcriptional level where expression is induced by p53 in response to DNA damage. In response to a cell death signal these pro-apoptotic family members are activated and translocate to intracellular membranes where they neutralize the anti-apoptotic Bcl-2 homologues. The BH3-only proteins,

however, cannot kill in the absence of Bax and Bak, and consequently function upstream in the same pathway [35, 36].

Inhibition of caspase function

All apoptotic signaling pathways converge on the caspases making these proteases the ultimate effectors of apoptotic cell death. It is absolutely critical to the development and survival of an organism that the caspases are tightly regulated, as the inappropriate activation of these enzymes can have severe consequences with respect to the development of various disease conditions. Over-activation of caspases has been implicated in certain neurodegenerative disorders, whereas inappropriate suppression is implicated in the development of many cancers. The first level of caspase regulation is seen in their structure and activation. As described, caspases are synthesized as inactive zymogens and are only activated via signaling through strictly controlled pathways. A second level of regulation involves the specific inhibition of active caspases by naturally occurring inhibitors.

Cellular proteins have been identified that inhibit specific initiator caspases. There is one family in particular, the inhibitors of apoptosis (IAP) proteins that function as intrinsic regulators of the caspase cascade. The IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector caspases. Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts and is believed to have important consequences with respect to human cancer [3].

IAPs

The IAP family

The IAPs effectively suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, and genotoxic damage. The first IAP genes identified were the baculoviral genes *CpIAP* (from *Cydia pomonella* granulosis virus CpGV) and *OpIAP* (from *Orgyia pseudosugata* nuclear polyhedrosis virus OpNPV), where they were found to compensate for the loss of function of p35, a baculoviral pan-caspase inhibitor protein [37, 38]. The IAPs are believed to be used by baculoviruses to allow for viral propagation by preventing a defensive apoptotic response in host insect cells [39, 40]. Since the discovery of the baculoviral IAPs, numerous cellular homologues have been identified in a range of species from *Drosophila* to vertebrates. The first mammalian IAP, NAIP (Neuronal Apoptosis Inhibitory Protein), was identified during a positional cloning effort to identify the causative gene for Spinal Muscular Atrophy (SMA) [41]. Although deletions within the *naip* gene were initially believed to be the genetic defect in SMA, it was later shown that deletions of the neighboring *survival motor neuron* (*SMN*) gene caused the disease [42]. The loss of functional NAIP has however, been suggested to be a factor in determining the severity of the disease [43]. Since the discovery of NAIP (BIR containing gene 1/BIRC1), the IAP family has expanded to include seven other members: X-linked inhibitor of apoptosis (XIAP/MIHA/hILP/BIRC4/ILP-1); cellular IAP1/Human IAP2 (c-IAP1/HIAP2/MIHB/BIRC2); cellular IAP2/Human IAP1 (c-IAP2/HIAP1/MIHC/API2/BIRC3) [44–47]; Testis-specific IAP (Ts-IAP//hILP2/BIRC8/ILP-2) [48, 49]; BIR-containing ubiquitin conjugating enzyme (BRUCE/Apollon/BIRC6) [50, 51]; Survivin (TIAP/BIRC5) [52]; and Livin (KIAP/ML-IAP/BIRC7) [53–55] (Fig. 4).

IAP expression

Human *XIAP* mRNA appears to be ubiquitously expressed in all adult and fetal tissues. XIAP has been reported to inhibit apoptosis in response to a large variety of apoptotic stimuli including ultraviolet radiation, TNFα, Fas Ligand, menadione (a free radical inducer), and etoposide (a topoisomerase II inhibitor) [3, 45]. Central to the ability of

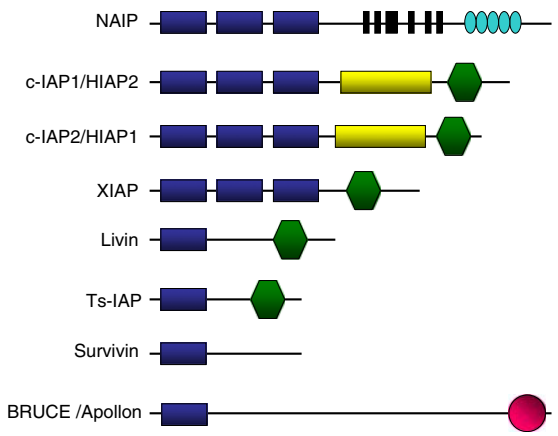

Fig. 4 Domain structure of the IAP family. The presence of at least one BIR domain is the defining characteristic of the IAP family. The human IAPs possess either one (Survivin, Livin, TsIAP, BRUCE) or three tandem BIR domains (XIAP, HIAP1, HIAP2, NAIP) (indicated by blue boxes). Along with the BIR domains, several IAPs contain a RING-zinc finger domain at the carboxy terminus (indicated by green hexagon). HIAP1/c-IAP2 and HIAP2/c-IAP1 both possess a caspase recruitment domain (CARD)(indicated by yellow box) in the linker region between the BIR domains and the RING domain. NAIP possesses a nucleotide binding site domain (indicated by black stripes), as well as a leucine-rich repeat domain (indicated by aqua ovals). BRUCE contains a UBC, ubiquitin-conjugation, domain (indicated by red circle)

XIAP to block apoptosis are the BIR domains which have been shown to directly inhibit both initiator and effector caspases. The extensiveness of *XIAP* mRNA expression has led to the suggestion that XIAP may be the IAP family member that provides a ''housekeeping'' function in the prevention of apoptosis in healthy cells. Expression of *XIAP* appears elevated in many cancer cell lines [45, 56], reinforcing the idea that *XIAP* expression level may control the apoptotic threshold of cells.

Interestingly, the XIAP transcript has long 5′ and 3′ UTRs that could provide a means for regulating the levels of XIAP protein independent of transcriptional control of the mRNA. Long 5′ UTRs are rarely found in eukaryotic cells and interfere with the normal ribosome scanning method of translation [57]. To overcome this, eukaryotic genes with long 5′ UTRs have often been shown to translate their proteins through a cap-independent, internal ribosome entry site (IRES). IRES elements permit ribosome binding near the translational start site of the message. An IRES element has been identified in the human XIAP 5′ UTR and permits XIAP expression in cells under stress conditions that typically block cap-dependent translation [58, 59]. Thus, XIAP protein may be preferentially up-regulated relative to other cellular proteins under the same stress conditions. In fact, it has been observed that under conditions of low dose γ-irradiation, XIAP translation is up-regulated in an IRES-dependent manner [58].

The closely related IAP family members, c-IAP1 and c-IAP2, have also been shown to inhibit the initiator and effector caspases, although likely by indirect means. They were first identified both by homology screening [45] and by their association in a complex with TNFα receptor 2 [46], where they interact with TNFα-receptor-associated factors -1 and -2 (TRAFs). The recruitment of these IAPs to the TNFR2 complex requires the presence of both TRAF1 and TRAF2 and is mediated by the interaction between the BIR domains of the IAPs and the TRAF domains [46, 60]. The TNFR1 complex is also able to recruit c-IAP1 through its interaction with TRAF2 via the adaptor molecule TNFα-receptor associated death domain protein (TRADD). The TNFα receptor (TNFR) can mediate both survival and death signals. For example, pro-survival signals result from an observed increase in the expression of c-IAP1 and c-IAP2 following activation of the NF-κB transcription factor in response to TNFR signaling. In this context, c-IAP1 and c-IAP2 have been proposed to play a specialized role in protecting cells from TNFα-induced apoptosis by reducing the level of caspase-8 activation [61, 62]. Whereas the survival signals are transduced by TRAF2/c-IAP1 interactions, pro-death signals are mediated through TRADD/FADD binding to caspase-8 [63].

The other IAP members, Livin and Survivin, have a distinctly different pattern of expression and function compared to XIAP, c-IAP1 and c-IAP2. Livin, also called ML-IAP (melanoma-IAP) or KIAP (kidney IAP), is expressed at high levels in embryonic tissues and in transformed cells [54]. There are two splice variants of Livin, Livin α and Livin β. The two splice variants display different tissue distribution and anti-apoptotic characteristics; however, high levels of both splice variants are observed, specifically, in melanoma and colon cancer. Over-expression of either Livin isoform blocks apoptosis induced via the extrinsic, death receptor pathway. Interestingly, the two isoforms behave differently in the presence of the apoptosis-inducing drugs staurosporine and etoposide; Livin α protects against staurosporine and Livin β protects against etoposide, but not vice versa [64]. The anti-apoptotic properties of Livin cannot be solely attributed to its BIR domain due to the fact that there is no difference between the BIR domains of the two Livin isoforms, yet they still respond differently to apoptotic triggers [64].

Survivin is most highly expressed during fetal development, and is transcriptionally restricted to expression during the G2/M phase of the cell cycle where it is proposed to function as a mitotic spindle checkpoint protein [65–67]. Survivin appears to associate with caspase-3 during mitosis and seems to suppress caspase-mediated cleavage of centrosome-associated p21 Waf1 [67]. The distinct localization of Survivin to the mitotic spindles and its critical function in cytokinesis suggests that this protein functions in cell cycle rather than apoptosis regulation [65]. This is similar to Survivin orthologues found in yeast [68], *C. elegans* [68], and in the fly [69]. Interestingly, the BIR domain in Survivin is most similar to the three-dimensional structure of XIAP BIR3 and it is predicted that the anti-apoptotic action of this IAP involves the inhibition of caspase-9 [70].

IAP structure and function

The IAPs are structurally similar in that they all contain one or more 70–80 amino acid Baculovirus IAP Repeat (BIR) domains in which the core of the domain consists of the variable sequence $C(X)_2C(X)_6W(X)_3D(X)_5H(X)_6C$, where X is any amino acid. The cysteine- and histidine-rich BIR domain chelates zinc and forms a globular structure that consists of four or five alpha helices and a variable number of anti-parallel β-pleated sheets [71]. As summarized in Fig. 4, the prototypical mammalian IAP family members contain a carboxy-terminal RING (Really Interesting New Gene) zinc finger. In XIAP, c-IAP1 and c-IAP2, this RING domain has been shown to possess E3 ubiquitin ligase activity, directly regulating self-ubiquitination and protein degradation [72].

Unique to c-IAP1 and c-IAP2 is the presence of a CARD domain (Fig. 4). CARD domains are a subfamily of

a larger group of related protein folds found in cell death proteins. CARDs typically mediate oligomerization with other CARD containing proteins. This is similar to other proteins with related fold structures called the death domains (DD) and the DED (reviewed in Martin [73]). The function of CARDs in the IAPs is not yet known but they undoubtedly form protein–protein interactions, possibly with Apaf-1, and with some of the DD or DED-containing proteins [73].

IAP inhibition of caspases

Following the discovery of the IAPs was the immediate observation that the expression of these proteins, in a variety of cell lines, effectively inhibits the apoptotic process when mediated by either the intrinsic or extrinsic pathways (Fig. 5). The mechanism by which the IAPs inhibit apoptosis was first identified by Deveraux et al. [74]. In these studies, XIAP was shown to be ineffective at inhibiting the initial cleavage of caspase-3 by caspase-8 signaling, but was able to prevent the subsequent processing of caspase-3 into its mature subunits. Therefore, XIAP inhibits extrinsic apoptotic signaling, not by interfering directly with caspase-8 activation, but rather via inhibition of the downstream effector caspases [74, 75]. Furthermore, XIAP was shown by protein–protein interaction studies to bind specifically to caspase-3 and -7, but not to caspases-1,-6, or -8 [74]. In vitro assays later confirmed, by blocking cytochrome c-induced activation of pro-caspase-9, that XIAP, c-IAP1 and c-IAP2 could prevent downstream proteolytic processing of pro-caspases-3, -6, and -7 (Fig. 6) [75].

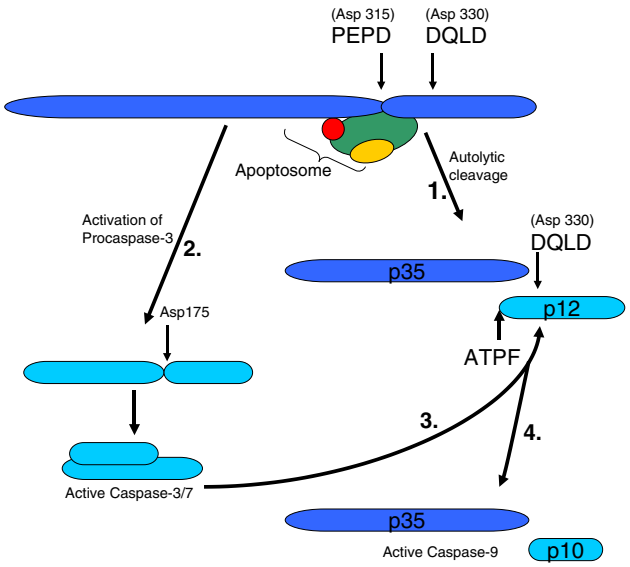

Fig. 5 Catalytic cleavage of caspase-9. Cleavage at Asp^{315} results in large (p35) and small (p12) caspase-9 subunits. At the amino terminus of p12 is a XIAP BIR3 recognition site, with the sequence ATPF (**1**). Cleavage of caspase-9 results in activation of pro-caspase-3 (**2**). In a feedback mechanism active caspase-3 is then able to further cleave caspase-9 at the Asp^{330} site in the p12 subunit (**3**), generating a p10 subunit that lacks the XIAP binding motif (**4**). As a result, the enzymatic activity of caspase-9 is effectively increased

To further dissect the exact mechanism of IAP-mediated caspase inhibition, the ability of various fragments of XIAP to suppress both in vitro caspase activation and apoptosis in intact cells was examined. Utilizing recombinant proteins composed of the BIR1 + BIR2 domains and the BIR3 + RING domains, more precise interactions were observed in which BIR2 specifically inhibited caspases-3 and -7 and BIR3 inhibited caspase-9 activity [76, 77]. In favor of the idea that the IAPs may potentially regulate the caspases through distinct domains was the observation that following Fas-induced apoptosis XIAP itself becomes cleaved into two distinct fragments; one consisting of BIR1 and BIR2 and the other consisting of BIR3 and the RING domain [77]. The cleavage of XIAP though, has so far only been observed in cells that are in the late stages of apoptosis and may merely be a function of general protein degradation during the apoptotic process, rather than the consequence of an anti-apoptotic mechanism [77, 78].

Crystallography and mutagenesis studies established that individual BIR domains of the IAPs do indeed have different mechanisms of caspase inhibition. Complexes of XIAP BIR2 with caspases-3 and -7 indicate that the linker region between BIR1 and BIR2 is the only IAP element which has contact with caspases-3 and -7, whereas the BIR2 domain is hidden in the crystal structure [79]. Using GST-fusions with the XIAP linker region alone, it was observed that caspase-3 is inhibited and the BIR2 domain is most likely irrelevant in this interaction [79]; however, the interaction with caspase-7 is slightly different in that the BIR2 domain is required to stabilize the linker interaction with this caspase [80]. In essence, the effector caspases appear to be inhibited simply by the steric hindrance due to BIR2 and/or its linker blocking the substrate entry site.

Two recent reports from the Salvesen laboratory [81, 82] suggest that only XIAP is a true direct inhibitor of caspases, and that other IAPs simply bind caspases but do not inhibit. This suggestion is based on the fact that XIAP is stable and exhibits the greatest potency for caspase inhibition compared to the other IAPs. Importantly, while c-IAP1 and 2 can bind caspases, their ability to inhibit caspases in vitro has been attributed to an artifact of the GST-fusion moiety, which allows aggregation and steric inhibition of the caspases by the c-IAP fusions [81]. The one thing that is certain, is that c-IAP1 and c-IAP2 are true antagonists of caspases. An example is the viability afforded by c-IAP1 and c-IAP2 when co-expressed with caspases in yeast [83], a simple genetic system devoid of

many complicating factors [84] and more physiologically relevant compared to in vitro enzyme systems. However, this yeast system does not address the issue of 'direct' caspase inhibition, as c-IAPs may inactivate caspase through the ubiquitin-proteosomal system or by other means. The question of direct inhibition is more difficult to prove and still open to debate. It may be that recombinantly expressed c-IAPs and other IAPs lack the correct conformation to directly inhibit caspases owing to missing post-translational modifications, or because a lack of necessary cellular co-factors that are not present in *Escherichia coli* or that are lost during purification. In contrast, XIAP would appear to be functionally active under these circumstances.

The BIR3 domain of XIAP, c-IAP1 and c-IAP2 [15, 75] and the single BIR domain in Livin [54] and Ts-IAP [48] have been shown to directly bind to and inhibit caspase-9 (although with the potential caveats discussed above for c-IAP1, c-IAP2, livin and Ts-IAP, [82]). Furthermore, the mechanism of XIAP interaction with caspase-9 differs from the interactions with caspases-3 and -7. Catalytic cleavage of caspase-9 occurs at Asp^{315} to yield large (p35) and small (p12) caspase-9 subunits. The newly generated amino-terminus of the p12 subunit starts with amino acid sequence ATPF. This peptide sequence binds to a pocket on the surface of XIAP BIR3. Fifteen amino acids downstream is a caspase-3 cleavage site at Asp^{330} (Fig. 5). The co-crystal structure of caspase-9 complexed with XIAP BIR3 reveals that the BIR3 domain forms a heterodimer with caspase-9 [85]. In effect, XIAP BIR3 stabilizes caspase-9 in an inactive state, by preventing caspase-9 homodimerization, which is essential for its autocatalytic activity. Interestingly, further cleavage by caspase-3 at Asp^{330} in the p12 subunit of caspase-9 generates a p10 subunit that lacks the XIAP binding motif. Therefore, a feedback proteolytic mechanism was proposed as follows: caspase-9 activation in the apoptosome results in pro-caspase-3 cleavage, thereby activating caspase-3. Caspase-3 feedback cleavage at the Asp^{330} in the p12 caspase-9 subunit removes the XIAP BIR3 recognition motif of caspase-9, thereby preventing IAP inhibition and increasing the enzymatic activity of caspase-9 (Fig. 5) [86, 87]. Although this is an attractive model, there is evidence to suggest that removal of the IAP-binding motif may not prevent XIAP association, and that surface contacts between XIAP and the caspase-3-generated p10 fragment of caspase 9 can still maintain this interaction [87].

One recent study describes a ''two-site interaction mechanism'' to illustrate how BIR domains are able to achieve specificity and potent inhibition of their target caspases. This model describes a conserved IAP Binding Motif (IBM) interacting groove that participates in inhibition by binding neoepitopes that are revealed once caspase activation occurs. These IBM interacting grooves are the most conserved surface feature of BIRs and are found on many BIR domains including BIR2 and BIR3 of XIAP and BIR1 and BIR2 of DIAP1, a functional orthologue in *Drosophila melanogaster* [88, 89]. In spite of their sequence conservation, there is convincing evidence for the differential roles of the BIR domains in the regulation of caspase activity. To summarize, caspase-3 is inhibited exclusively by the linker region between BIR1 and BIR2, whereas caspase-7 inhibition requires both the linker region and the BIR2 domain of XIAP. It is evident that caspase-9 is inhibited in direct response to BIR3 binding, thereby preventing further caspase-9 activation as well as suppressing downstream effector caspase activity. Thus far, none of the IAP BIR1 domains have been shown to have any caspase inhibiting activity; however, Akt/PKB (protein kinase B)-mediated phosphorylation at serine 87 within BIR1 reduces auto-ubiquitination and stabilizes XIAP [90]. BIR1 therefore appears to play a regulatory role rather than directly participating in apoptosis suppression. For example, the BIR 1 domains of c-IAP1 and c-IAP2 are proposed to play a role in TRAF interactions and ubiquitination reaction [91, 92].

IAP protein regulation

Ubiquitination and degradation

The ubiquitination and subsequent proteasomal degradation of the IAPs may be a key regulatory event in the apoptotic program. Furthermore, the carboxy-terminal RING domain of the IAPs is believed to be the component that mediates both the ubiquitination and degradation of the IAPs, as well as that of their substrates [72, 93]. RING domain-containing proteins possess E3 ubiquitin ligase activity and function as specific adapters by recruiting target proteins to a multi-component complex containing an E2 enzyme. Earlier studies on the RING domains of XIAP and c-IAP1 showed that this domain is involved in the ubiquitination and degradation of the IAPs in response to apoptotic triggers. Following treatment with glucocorticoids or etoposide, XIAP and c-IAP1 were rapidly degraded in a proteasome-dependent manner that could be blocked in the presence of proteasome inhibitors [72]. Other studies showed that XIAP and c-IAP1 were able to promote the ubiquitination and proteasomal degradation of caspases-3 and -7, thereby enhancing the anti-apoptotic effect of the IAPs [94]. Although the RING domain has been clearly shown to be involved in the ubiquitination and degradation of the IAPs, it remains unclear whether this activity enhances the anti-apoptotic activity of the IAPs or actually antagonizes the activity. Studies using RING-deletion mutants have provided evidence for both scenarios. In one study,

over-expression of RING-deleted XIAP and c-IAP1 mutants in cell culture resulted in the loss of auto-ubiquitination and proteasomal degradation, and conferred better protection against apoptotic stressors than wild-type XIAP and c-IAP1 [72]; however, in a separate study, RING-deletion mutants of XIAP were found to be less effective than wild-type XIAP at preventing apoptosis induced by the over-expression of caspase-3 or Fas [94].

XIAP ubiquitination sites have been identified and the role of IAP ubiquitination was examined by site directed mutagenesis of key lysine residues. Over-expression of wild-type or XIAP mutant protein in cultured cells did not reveal any differences in their ability to protect against Bax or Fas-induced apoptosis [95]. These findings suggest that ubiquitin mediated destruction of the IAPs may not be as significant as believed, at least in the context of over-expressed proteins [95]. Alternatively, there is a possibility that RING domains may function to suppress apoptosis in conditions of low apoptotic stimulus by the ubiquitination of caspases, whereas high levels of apoptotic stress trigger self-degradation of the IAPs leading to cell death.

While c-IAP1 and c-IAP2 are typically present in low abundance in the cell, it has been proposed that c-IAP2 levels are maintained at low levels via constitutive ubiquitination and subsequent degradation by c-IAP1 [96]. Insight into c-IAP1 E3 ligase function was determined with the generation of c-IAP1 knockout mice. Although these mice appeared to be both viable and fertile, they had significantly elevated c-IAP2 protein levels with normal mRNA levels, suggesting that in a normal setting, c-IAP1 ubiquitination of c-IAP2 leads to low protein levels of c-IAP2 [96]. Furthermore, co-expression and in vitro binding studies of c-IAP1 and c-IAP2 along with TRAF1/2, demonstrated that the c-IAPs and TRAF proteins form a multimeric complex. In this complex TRAF1 and TRAF2 appear to function as adaptors, bringing c-IAP1/2 together, allowing c-IAP1 to ubiquitinate c-IAP2 [96]. In a previous study c-IAP1 was observed to have a pro-apoptotic function by causing the ubiquitination and proteasome-mediated degradation of TRAF2 in response to TNFR2 signaling and thus sensitize cells to TNFα-induced cell death [97]. Thus it appears that c-IAP family members function as ubiquitin protein ligases and are capable of regulating one another as well as components of the TNFR2 signaling complex.

Recent studies demonstrate that the ubiquitination and degradation of XIAP depends on phosphorylation status. More specifically, XIAP has been identified as a substrate for Akt/PKB. Akt/PKB is a serine/threonine kinase, known to promote cell survival and suppress apoptosis in a number of cell lines when induced by a variety of cell death stimuli. Protein sequence analysis shows that the Akt/PKB phosporylation sequence RXRXXS/T is found within the BIR1 domain of XIAP [90]. Phosphorylation of XIAP by Akt/PKB inhibits cisplatin-induced auto-ubiquitination, thereby reducing XIAP degradation. Furthermore, increased levels of XIAP are associated with decreases in cisplatin-stimulated caspase-3 activity and apoptosis [90].

Negative regulators of the IAPs

Several proteins play critical roles in maintaining an adequate balance between too much and too little apoptosis. To help maintain this balance are classes of proteins which act as negative regulators of the IAPs, and specifically interact with and relieve their caspase-inhibitory effects.

XAF1

XAF1 (XIAP-Associated Factor 1) is a zinc finger-rich protein that was first identified by a yeast two-hybrid screen with XIAP as bait. The ability of XAF1 to bind with XIAP was confirmed by in vitro experiments. Using purified, recombinant proteins it was shown that XAF1 directly associates with XIAP to antagonize XIAP-mediated caspase-3 inhibition [98]. Using recombinant adenoviruses to over-express XAF1 in cell cultures, XAF1 was shown to reverse XIAP-mediated protection against chemotherapeutic drugs such as cisplatin and etoposide. In comparison to the cytosolic localization of XIAP, XAF1 protein appears to be localized in the nucleus. Following apoptotic stress, XAF1 is able to elicit XIAP re-localization from the cytosol to the nucleus, perhaps as a means of sequestering XIAP [98]. In contrast, two reports found no evidence of interaction between XAF1 and XIAP under the conditions tested [99, 100]. Furthermore, one of those studies demonstrated that XAF1 could still exert pro-apoptotic effects in cells devoid of XIAP [100]. The conclusion was that XAF1 can activate the mitochondrial apoptotic pathway to facilitate cytochrome c release, and that XAF1 direct antagonism of XIAP was not required. However, these above findings are most likely explained by the fact that XAF1 also binds c-IAP1 and c-IAP2 with at least equal, if not greater affinity than XIAP (S. Plenchette, V. Arora, H. Cheung, and R.G. Korneluk, unpublished observations). XAF1, therefore, is likely a pan-IAP inhibitor, not just a XIAP antagonist.

XAF1 is ubiquitously expressed in normal tissues, but is found at extremely low levels in the majority of the NCI 60 cell-line panel of cancer cells [56]. Studies show that restoring XAF1 expression increases the sensitivity of tumor cell types to apoptotic triggers. Gene array analysis revealed that *xaf1* mRNA can be up-regulated in response to interferon-β (IFN-β) treatment [101]. Although the melanoma cell lines used in this study were found to be

resistant to Apo2L/TRAIL (Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand), pre-treatment with IFN-β sensitized the cells to Apo2L/TRAIL-induced apoptosis, ostensibly due to the up-regulation of XAF1 protein. XAF1 over-expression via transient transfection indicated that XAF1 was able to sensitize the cells to Apo2L/TRAIL to the same degree as IFN pre-treatment [101]. When other studies examined the expression levels of XAF1 in human colon cancer tissues, low levels of *xaf1* mRNA, compared to adjacent healthy tissues of the same individuals, were found. Furthermore, XAF1 over-expression in cultured colon carcinoma cell lines displayed an increase in Apo-2L/TRAIL and drug-induced apoptosis [102, 103]. These studies show that the over-expression of XAF1 strongly influences cellular sensitivity to the pro-apoptotic effects of Apo2L/TRAIL. In summary, therefore, the down-regulation or loss of *XAF1* expression in cancer cells may contribute to apoptosis suppression through unrestricted IAP activity.

Smac/DIABLO

Smac/DIABLO (Second mitochondrial activator of caspases, or Direct IAP Binding protein with Low PI) is a 25-kDa mitochondrial protein that promotes apoptosis via its ability to eliminate IAP-mediated caspase inhibition. The ability of Smac to prevent IAP inhibition of caspases makes this protein a functional human equivalent to the *Drosophila* death proteins Reaper, Grim and Hid [99, 104].

Initial mitochondrial targeting of Smac is dependent upon a 55 amino acid sequence found at its amino-terminus. This sequence is proteolytically cleaved within the mitochondria, enabling the eventual release of mature Smac into the cytosol via Bax/Bak channels. Upon receiving an apoptotic signal via the intrinsic mitochondial stress pathway, both Smac and cytochrome c are released with similar, but not necessarily identical, kinetics. Although the exact mechanism of Smac release from the mitochondria is not entirely known, one study suggests that, despite their significant size difference, the Smac dimer (100 kDa) and cytochrome c (12 kDa) are released through the same Bax- or Bak-formed membrane pores. This mechanism is supported by the fact that Bcl-2 over-expression can inhibit both cytochrome c and Smac release [105]; whereas Bid-induced permeabilization of the outer-mitochondrial membrane induces the rapid and complete release of cytochrome c and Smac from the intermembrane space [106]. Bax has been suggested to be involved in this process since Smac release can be prevented by inhibiting m-calpain, which is considered to be a proteolytic activator of Bax [107].

Other studies, however, suggest that Smac and cytochrome c are released through different mechanisms. TRAIL was found to result in cytochrome c release and apoptosis in wildtype, Bid (–/–), Bax (–/–) or Bak (–/–) mouse embryonic fibroblasts (MEFs), but not in Bax/Bak (–/–) double knockout MEFs [108]. In contrast, TRAIL induced mitochondrial Smac release was blocked in all of the single knockout and the double knockout MEFs. Therefore, it was concluded from these experiments that the release of Smac and cytochrome c from mitochondria is, in fact, differentially regulated in receptor-mediated pathways of apoptosis [108]. Differences in the mechanism of Smac and cytochrome c release were also observed in the presence of caspase inhibitors. Under these conditions, Smac release is prevented whereas cytochrome c is permitted, suggesting that Smac efflux from the mitochondria is a caspase-catalyzed event [105]. It may be that Smac release simply requires further membrane permeability due to increased or persistent mitochondrial damage.

Smac isoforms

Several Smac isoforms have recently been identified and studies using these proteins have suggested that the pro-apoptotic function of Smac may be mediated by additional, non-IAP mechanisms. Smacβ, an alternatively spliced isoform of Smac, lacks the mitochondrial targeting sequence found in full length Smac. In vitro experiments show that Smacβ interacts with purified XIAP protein; however, in intact cells, XIAP binding is not observed [109]. Smacβ is still considered to be pro-apoptotic due to its ability to potentiate apoptosis following death receptor and chemical stimuli [109]. Unlike Smacβ, Smac3, a more recently identified isoform of Smac, contains both an NH_2-terminal mitochondrial targeting sequence and an IAP binding motif. Following an apoptotic stress, Smac3, like Smac, is released from the mitochondria into the cytosol where it interacts with the BIR2 and BIR3 domains of XIAP. There are some reports that the Smac3 isoform is unique in that it is able to induce the acceleration of XIAP auto-ubiquitination and destruction, whereas Smac only seems to have this effect on c-IAP1 and c-IAP2 [110, 111]; however, another study describes the ability of Smac to antagonize both XIAP auto-ubiquitination as well as XIAP-dependent ubiquitination of caspase-7 [112]. By disrupting IAP-caspase interactions and repressing the ubiquitin ligase activities of the IAPs, Smac may effectively prevent caspase demise via ubiquitination [112]. Although Smac and its isoforms may differ in how they potentiate apoptosis, the involvement of all Smac proteins with the IAPs, at multiple levels, is an effective measure to ensure that the apoptotic program can proceed.

Omi/HtrA2

Following the identification of Smac was the discovery of another mitochondrial IAP binding protein called Omi, or HtrA2 [113–115]. Omi/HtrA2 is a mammalian homologue of the *E. coli* bacterium heat-inducible serine protease, known as HtrA. Omi was predicted to have a function similar to that found in bacteria where HtrA behaves as a chaperone or protease that determines whether misfolded proteins are refolded or targeted for destruction [114]. Similar to Smac, Omi contains an amino-terminal mitochondrial targeting sequence that is cleaved upon import into the mitochondria. Proteolytic cleavage of the targeting sequences exposes a conserved IAP-binding motif at the amino-terminus with the tetrapeptide sequence AVPS. Following an apoptotic stress, mature Omi/HtrA2 is released together with mature Smac from the mitochondria into the cytosol where it interacts with the IAPs and promotes apoptotic cell death [113, 114]. Omi, however, exerts its pro-apoptotic activity both by its ability to disrupt caspase-IAP interaction, as well as its serine protease activity. Furthermore, Omi has been shown to potentiate apoptosis through the direct proteolytic processing of its IAP protein targets [116, 117]. Studies have demonstrated that when Omi is over-expressed in cells, there is an observed increase in degradation products of XIAP and cleavage products of c-IAP1. Additionally, when endogenous Omi levels are suppressed by RNA interference, IAP degradation and/or cleavage is not observed and cells become desensitized to TRAIL and etoposide treatment [116, 117]. A recent study indicates that as well as potentiating apoptosis via proteolysis of the IAPs, Omi may also enhance caspase activation [118]. In this study, when Omi was over-expressed in the cytosol as an ubiquitin fusion protein, there was an indirect induction of outer mitochondrial membrane permeabilization, followed by cytochrome c-dependent caspase activation.

The serine protease activity of Omi may also induce apoptosis in a caspase-independent manner through the proteolysis of unidentified cellular targets [113, 114]. Although cytosolic targets for Omi have not been identified, the proteolytic removal of its 133 amino acid mitochondrial targeting sequence is a self-catalyzed event [119]. This raises the possibility that other mitochondrial targeting sequences, including that of Smac, may be processed by Omi, and therefore Omi could be required to generate pro-apoptotic IBM-exposed sequences.

GSTP1/eRF3

The discovery of proteins that function as IAP antagonists via a tailor-made sequence such as those found in Smac and Omi, has made way for the identification of other IBM containing proteins that are found not only in the mitochondria but in other organelles. One recently identified IBM-containing protein is an isoform of the polypeptide chain-releasing factor GSPT1/eRF3 protein that is localized to the endoplasmic reticulum (ER). GSPT1 typically functions via the interaction with poly(A)-binding protein (PABP) and acts as a polypeptide chain release factor in translation. As with other IAP-binding proteins, GSPT1 is proteolytically processed following an apoptotic stress to reveal a conserved NH_2-terminal IBM with the amino acid sequence AKPF [120]. Processed GSPT1 has been shown to interact with the IAPs, promote caspase activation, ubiquitinate IAPs, and induce apoptosis [120]. The fact that GSPT1 is in the ER emphasizes the importance of cross-talk between organelles during both the initiation and execution phases of apoptosis.

Chk1

Checkpoint kinase 1 (Chk1) is a dual function kinase that is active during the S-M phase transition of the cell cycle, regulating Cdc25A function, and prevents mitotic progression in the presence of DNA damage. From a domain-based databank search for IBM-containing protein homologues, Chk1 was identified as a candidate that contains an N-terminal motif, MAVPF, identical to that present in the *Drosophila* Hid protein and would require removal of the amino-terminal methionyl residue by an aminopeptidase in order to be active. This Chk1 motif is also homologous to the N-terminus of processed caspase-9, Smac, and the *Drosophila* homologues Grim and Reaper [121]. Chk1 and XIAP reportedly form complexes in association with condensed chromosomes aligned at the metaphase plate during mitosis. To date Chk1 is the only identified IAP-interacting protein that is not implicated directly in apoptotic processes; rather, the interaction between Chk1 and XIAP may involve cross-talk between the mechanisms that control cell cycle and apoptosis [121].

Other IBM-containing proteins

Still more IBM-containing proteins, all mitochondria-derived, have been recently identified [122], and potentially more exist. If we extrapolate from the situation in *Drosophila*, with four known IAPs (DIAP1/thread, DIAP2, deterin, dBruce) versus eight human IAPs, and five known *Drosophila* IBM-containing antagonists (reaper, hid, grim, sickle, jafrac), then we would predict that more mammalian IBM-containing proteins remain to be discovered. It has been suggested that of all the mitochondrial apoptogenic proteins, SMAC (an IBM protein) and cytochrome c remain as the only likely killer proteins [123].

The IAPs and apoptosis

In this review, the significance of caspase-IAP interactions in mediating apoptosis has been established. The complexity of IAP regulation that is required to keep apoptotic pathways in check, either by ubiquitination and degradation pathways, or via protein–protein interactions with negative regulators such as XAF1, Smac and/or Omi, has also been presented. How these various regulatory mechanisms function together in a general model of apoptotic cell death is summarized in Fig. 6.

Apoptotic cell death can be triggered either via the intrinsic or the extrinsic pathway where eventually all signaling converges on the effector caspases-3 or -7. Apoptotic stresses acting through the intrinsic pathway result in the release of cytochrome c (most likely via Bax- or Bak-channel formation in the outer mitochondrial membrane) resulting in caspase-9 activation. The consequences of cytochrome c release are determined by the activity of the IAPs. Pre-exposure to apoptotic stresses or the presence of high levels of IAPs may inhibit activated caspase-9 and any subsequent effector caspase activation (Fig. 5). The ubiquitin ligase activity of the IAPs may target the bound caspases for proteasome degradation, thereby aborting the apoptotic process. This type of regulation could be considered a safety mechanism to prevent transient or incidental cytochrome c leakage from eliciting an unnecessary, full-blown apoptotic response. In the case where sufficient caspase-9 activation occurs, the effector caspases-3 and-7 will become activated, triggering more significant or persistent changes in mitochondrial membrane permeability. This in turn releases more cytochrome c, as well as Apoptosis Inducing Factor (AIF), Smac, and Omi. AIF translocates to the nucleus where it is involved in chromatin condensation and degradation. Meanwhile, Smac and Omi are able to bind to and sequester IAPs so that unrestricted caspase activation can proceed (Fig. 7).

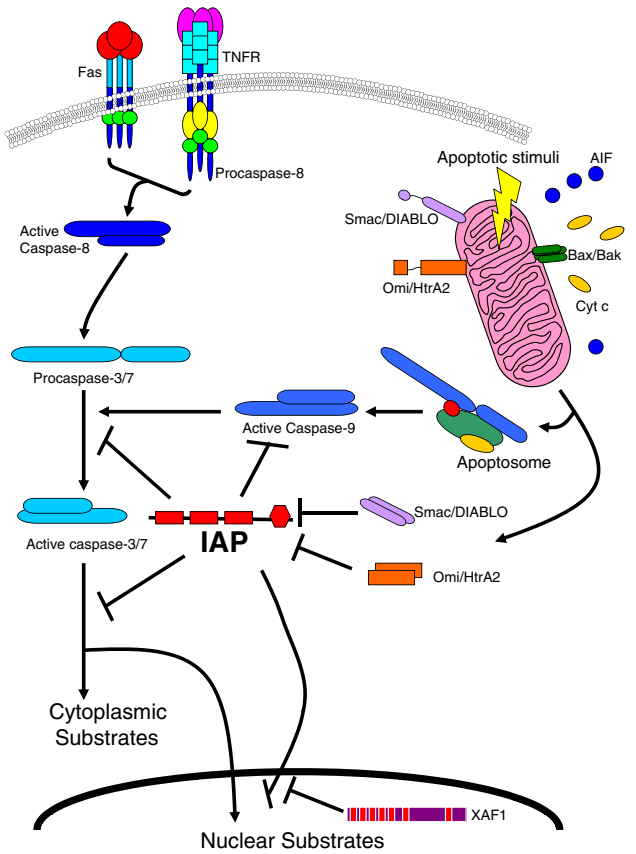

Fig. 6 IAPs effectively inhibit the apoptotic process when mediated via the intrinsic or extrinsic pathways. Caspase-8 signaling induces the initial downstream caspase-3 cleavage. Consequently, the IAPs effectively block any subsequent processing of caspase-3 into its mature, active form. The IAPs are not only able to effectively block caspase-3 processing, but are also able to inhibit cytochrome c-induced activation of caspase-9 as occurs in the intrinsic death pathways

With Smac and Omi positioned in the mitochondria, their inhibition of IAP-caspase interactions should predictably occur only when there is apoptotic signaling via the intrinsic pathway; however, Smac has also been shown to exert its pro-apoptotic effects on apoptotic processing through the extrinsic or death receptor pathway by functioning at the level of the effector caspases. Death receptors and their ligands such as Fas-FasL and TRAIL-DR4, -DR5 receptors are able to initiate caspase activation independently of the mitochondria. Since both the intrinsic and extrinsic pathways converge on the effector caspases-3 and -7, high levels of IAPs are able to prevent caspase activation at this distal point, thereby terminating the receptor pathway via the inhibition of caspase-3. Given that the mitochondria can be by-passed in the death receptor pathway, the mechanism of Smac release may not be intuitively obvious; however, it seems that in some cell types, stimulation of death receptors results in caspase-8 cleavage and the subsequent activation of the pro-apoptotic Bcl-2 family member Bid [30, 124]. When Bid becomes cleaved its truncated form is able to stimulate the formation of Bax- or Bak- pores in the outer mitochondrial membrane. This in turn induces the release of cytochrome c and Smac, and formation of the Apaf-1/caspase-9 apoptosome. Since caspase-8 is not sensitive to direct IAP inhibition, persistent activation of the death receptor pathway should result in the relief of inhibition on downstream effector caspases by the release of Smac via the secondary activation of the mitochondrial pathway [125]. Thus, there is cross-talk between several signaling pathways to ensure that once an appropriate cell death signal is given, the apoptotic pathway is fully engaged.

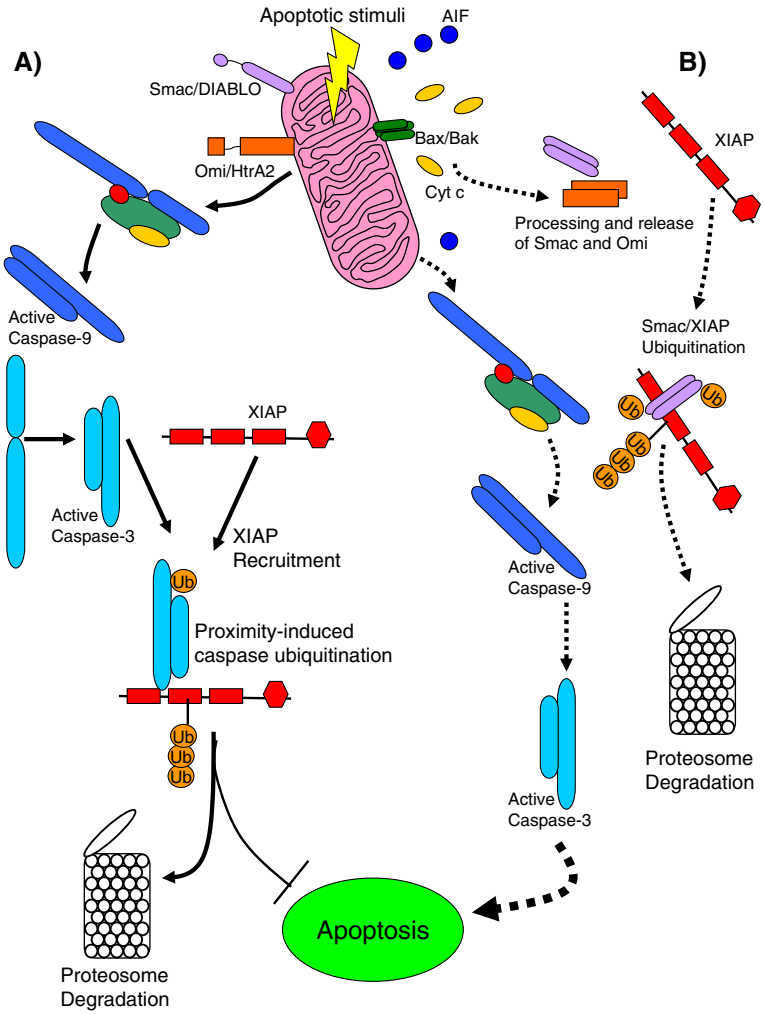

Fig. 7 Mechanisms that regulate apoptotic cell death. The presence of high levels of the IAPs results in IAP-induced caspase inhibition. Caspase-IAP interaction triggers autoubiquitination of both the IAP and of the caspase itself. The entire complex is degraded and the apoptotic process is aborted **(A)** (solid line). Alternatively, if a cell receives a sublethal apoptotic stress, endogenous IAPs are saturated by interactions with Smac/DIABLO and/or Omi/HtrA2. Proteosome-mediated degradation of IAP-Smac and or IAP-Omi complexes results in IAP depletion, allowing for unrestricted caspase activation **(B)** (dotted line)

IAPs and cancer

A variety of cancer cell lines and primary tumor biopsy samples show elevated IAP expression levels [3, 52, 54, 56, 126, 127]. The most dramatic example of IAP over-expression in tumors was observed with Survivin. As discussed earlier, Survivin expression is limited to embryonic tissues and many different tumor types, but is absent in most healthy, adult tissues [52, 54]. Furthermore, the presence of Survivin in patient tumor biopsy samples correlates with poor prognosis, increased rates of treatment failure, and relapse [128]. The prognostic significance of IAP over-expression is less clear for some of the other IAPs. For example, XIAP protein levels correlate with disease severity and prognosis in acute myelogenous leukemia (AML) [126], but not in non-small cell lung carcinoma (NSCLC) [129, 130]. Another study indicated that XIAP is an independent prognostic marker for renal cell carcinoma [131]. Furthermore, an immunocytochemical survey of tumor tissue shows that XIAP immunostaining patterns allow for the ready distinction of malignant from benign populations [132]. In this survey, it was further observed that strong XIAP staining was most prevalent in ovarian carcinomas and less prevalent in mammary carcinomas. These results suggest that the expression levels of the IAPs could be expected to have a significant impact in the development and maintenance of cancer due to their central role in the regulation of apoptosis.

Genetic models of IAP deficiency, mutation and disease

The fundamental role the IAPs and their antagonists play in apoptosis control, development, homeostasis, and disease progression has been addressed in part through the use of gene ablation strategies in mice, and through the genetic analysis of various human and mouse disorders. While the subject of IAP transgenesis and knock-out strategies is large, we will restrict our discussion to mouse knock-out models for the IAPs and antagonists (Table 1) well as diseases related to IAP and antagonist mutations (Table 2). For the sake of brevity, we will not discuss RNAi or antisense validation studies in vivo, nor present the seminal

work delineating the importance of these genes in *Drosophila*, which has been discussed elsewhere [9, 165].

Six different IAP genes have been deleted in mice through homologous recombination (Table 1), only two of which are embryonic lethal. The majority of these IAP knock-outs are in fact viable and develop normally, and at first glance do not demonstrate any overt phenotype. The argument has been made that the lack of phenotype is due to the high degree of redundancy seen in the IAP family, and that possible compensatory increases occur in other IAP genes. The two lethal phenotypes are also particular, in that the apollon/BRUCE knock-out late lethality (days E14.5 to the perinatal stage) is due to a placental development problem denoting a specific role for apollon in spongiotrophoblast proliferation [150, 151], while the early lethality (prior to E4.5) of survivin knock-out mice is most likely related to survivin's role in cytokinesis. Significantly, this phenotype is also seen in other knock-out and RNAi depletion studies of survivin orthologs in man [166, 167], *Drosophila* [69], *C. elegans* [68] and yeast [168, 169].

Although a gross phenotype was not initially apparent in many of the IAP knock-out models, these models all revealed specific attributes owing to the loss of a specific in vivo IAP. The models demonstrated increased sensitivity of certain cell types to specific death stimuli, with dramatic results in some cases, such as the rescue of c-IAP2 knock-outs to a lethal dose challenge of lipopolysaccharide due to the death of c-IAP2-null macrophages [134]. Furthermore, cells derived from these animals, such as MEFs, have provided valuable tools to experimentally dissect the roles of various molecules in apoptotic pathways (see Table 1 for references).

The importance of the role of IAPs in normal homeostasis and disease progression has come to light recently with evidence of loss-of-function mutations in various disorders (Table 2). NAIP (birc1), the first mammalian IAP identified, was discovered through its genetic association with the most severe cases of spinal muscular atrophy, and thought to represent a phenotypic modifier [41]. The NAIP locus has undergone expansion and specialization in the mouse with several functional copies expressed under different promoters. The NAIP5 (birc1e) gene is responsible for resistance to *Legionella pneumophila* infection of macrophages [158, 159]. It remains to be proven if NAIP performs a similar immunomodulatory function in man.

Recently, loss-of-function mutations in XIAP (birc4) have been found to underlie some cases of X-linked lymphoproliferative disorder, pointing to a direct role for this IAP in NK cell and lymphocyte homeostasis [163]. The IBM-containing protein, Omi, has been identified as the mnd2 mutation responsible for motor neuron degeneration in mutant mice [164]. These mutations identify physiological roles that the various IAPs and antagonists play in normal cells, and will help to guide the development of future drugs and delineate the possible side effects these compounds may have.

Genetics of IAPs in cancer

There is direct genetic evidence that IAPs are able to function as oncogenes. This is seen in the case of the c-IAPs where chromosome amplification of the 11q21-q23 region, encompassing both c-IAP1 and c-IAP2, has been observed in a variety of malignancies, including medulloblastomas, renal-cell carcinomas, glioblastomas, gastric carcinomas and non-small cell lung carcinomas. Esophageal squamous-cell carcinomas frequently display this amplification, and transcriptional profiling has identified c-IAP1 as the sole target that is consistently over-expressed in these tumors [170]. More recent findings have also shown amplification of 11q22 in primary tumors of non-small cell lung and small cell lung cancers. Analysis indicated that both c-IAP1 and c-IAP2 were over-expressed in the primary lung carcinomas [171]. A recent genomic comparison of human and mouse tumors revealed that c-IAP1 and c-IAP2 were also amplified in the corresponding 11q22 syntenic region at 9qA1 [172]. More importantly, when c-IAP1 and another gene from 9qA1, YAP, were introduced into murine ES cells and injected into mice, they formed hepatomas [172]. In addition, c-IAP2 could also transform p53-null hepatoblasts in combination with cMyc over-expression. This provides the first concrete proof of the role of c-IAP1 as an oncogene [172].

Further genetic evidence implicating the IAPs as potential oncogenes was found in extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B-cell lymphomas. The translocation events t(1;14)(p22;q32) and t(11; 18)(q21;q21) have been well documented in MALT lymphomas and involves both NF-κB activation and c-IAP2. The less frequently observed translocation t(1;14)(p22;q32) juxtaposes the Bcl-10 gene on chromosome 1p22 adjacent to the immunoglobulin heavy chain (IgH) gene on chromosome 14 where the IgH enhancer causes Bcl-10 over-expression [173].

The more common translocation event, present in approximately 50% of extranodal MALT lymphomas, involves a gene rearrangement of the *MALT1* locus 18q21 with the *c-IAP2* gene (also known as *API2*), located at 11q22 resulting in the c-IAP2-MALT fusion t(11;18)(q21;q21) [160, 174–176]. This gene fusion encodes a chimeric protein consisting of the c-IAP2 BIR domains fused in-frame to the carboxy-terminus of MALT1 [177] (Fig. 8). Typically, gastric MALT lymphomas are associated with chronic inflammation due to *Helicobacter pylori* infection, and can be treated with

Table 1 Summary of IAP and antagonist gene ablation strategies and phenotypes in mammals or derived cells

Gene	KO strategy	Phenotype and comments (references)
Birc1a (NAIP1)	Total ablation of NAIP1 in mice	Normal development presumably due to redundancy of NAIP genes in mice. However, hippocampal neurons of *naip1–/–* mice are more susceptible to seizure-induced cell death [133].
Birc2 (c-IAP1)	Total ablation of c-IAP1 in mice	Viable and no obvious sensitization to proapoptotic stimuli presumably due to upregulation of c-IAP2 protein. In vitro studies demonstrate that c-IAP2 is a ubiquitin-ligase target of c-IAP1 [96].
Birc3 (c-IAP2)	Total ablation of c-IAP2 in mice	Viable. C-IAP2–/– mice are resistant to LPS-induced sepsis, as macrophages die [134]. T-cells from these mice also die when treated with glucocorticoids even in the presence of survival factors [134]. The developmental expression of the knocked-in *lacZ* reporter gene was analyzed. C-IAP2 expression is not evident until day E11.5, showing discrete expression patterns in various structures, tissues and organs thereafter [135].
Birc4 (XIAP)	Total ablation of XIAP in mice	Viable. Originally no overt phenotype was detected, nor any increased sensitivity to apoptotic stimuli presumably due to compensatory increases in c-IAP1 and c-IAP2 expression [136]. However, additional investigations showed that XIAP-deficiency leads to delayed lobuloalveolar development in the mammary gland [137]. *Xiap*-null cardiomyocytes or sympathetic neurons revealed that either Smac, or XIAP-loss, to be the ''competence to die factor'', as cytochrome c alone is sufficient to kill these cells unlike wild-type cells [138, 139]. Furthermore, XIAP and survivin complexes show synergistic inhibition of apoptosis, which is abolished in XIAP–/– MEFs [140].
	Targeted disruption in human HCT-116 colon carcinoma cells	XIAP-null HCT-116 cells are remarkably sensitive to TRAIL in vitro [141] while XIAP-null HCT-116 tumor xenografts in mice are cured by adoptive cellular immunotherapy [142].
Birc5 (survivin)	Total ablation	Embryonic lethality prior to day E4.5 [143].
	Heterozygous deletion	Viable, however *survivin* -/+ mice are more sensitive to Fas-induced apoptosis of the liver [143]. In a mouse model of stroke, the extent of vascularization of the infarct was found to be dependent on the expression of survivin, since vessel density is significantly reduced in *survivin* -/+ mice [144].
	Conditional KO (T-cell)	Analysis of early deleted (lck-cre) survivin mice shows arrest at the pre-T-cell receptor proliferation checkpoint. Loss of survivin at a later stage (CD4-cre) results in normal thymic development, but peripheral T cells are immature and significantly reduced in number. Loss of survivin does not lead to increased apoptosis. However, thymocyte proliferation of survivin-deficient T cells is greatly impaired [145]. In another report, loss of survivin (lck-cre) blocked the transition from CD4-CD8- double negative thymocytes to CD4+ CD8+ double positive cells. In response to proliferative stimuli, cycling survivin-null double negative cells exhibit p53-mediated growth arrest and p53-independent cell death [146].
	Conditional KO (neuronal precursor cells)	Conditional deletion (nestin-cre) of survivin starting at day E10.5 leads to massive apoptosis of neuronal precursor cells in the CNS. Neonates die shortly after birth from respiratory insufficiency, and show a marked reduction in brain size associated with severe multifocal apoptosis [147].
	Conditional KO (endothelial cells)	Lack of endothelial cell (Tie1 expressing) survivin results in embryonic lethality due to hemorrhages observed from days E9.5–E13.5, and defects are seen in angiogenesis, cardiogenesis and neurogenesis [148].
Birc6 (apollon)	Total ablation of BRUCE/apollon in mice	Gene disruption causes embryonic and perinatal lethality [149]. Growth retardation is discernible after day E14, and is linked to impaired placental development [150]. There is a progressive loss of the placental spongiotrophoblast layer from days E11.5–14.5 due to a lack of proliferation of spongiotrophoblast cells in the developing placenta [151]. Smac induces apoptosis in apollon-null cells, but not in wild-type cells [149]. Apollon-null MEFs are sensitive to multiple mitochondrial death stimuli but resistant to TNF [152]. Serine protease, catalytically-inactive, mutants of Omi can induce apoptosis of apollon-null MEFs, presumably through Omi's IBM sequence [153].
Birc7 (livin)	Not yet tested	
Birc8 (TsIAP)	Not testable in mice	(TsIAP/ILP2 is only expressed in humans and the great apes [49]).

Table 1 continued

Gene	KO strategy	Phenotype and comments (references)
Smac	Total ablation of Smac/DIABLO in mice	Viable with no overt phenotype detected originally. Several Smac-deficient cells (ES, MEFs, T cell, B cell) were tested and responded normally to all apoptotic stimuli applied. The data suggest the existence of redundant or compensatory molecules capable of fulfilling Smac's role [154]. However, Smac-deficient oocytes are more prone to die upon cytochrome c injection [155].
	Targeted disruption of Smac gene in human HCT116 colon carcinoma cells	Interestingly, apoptosis induced by PUMA, a BH3-only protein, is abrogated in Smac-null HCT116 cells [156].
Omi/HtrA2	Total ablation of Omi/HtrA2 in mice	Deletion of Omi results in a neurological Parkinsonian phenotype, and post-natal death at 1 month [157].
	Double Omi and Smac knock-out	Simultaneous deletion of Smac does not alter phenotype of the Omi knock-out mice. Data suggests a role for Omi in protection against neuronal stress [157].

antibiotic therapy; however, the majority of these lymphomas that do not respond to antibiotics exhibit the c-IAP2-MALT1 translocation. The c-IAP2-MALT translocation establishes a positive feedback loop for c-IAP2 by activating NF-κB. This in turn transcriptionally activates the promoter for c-IAP2 [178, 179]. In this way, the c-IAP2-MALT1 fusion induces NF-κB activation and contributes to an anti-apoptotic phenomenon by upregulating transcription of anti-apoptotic genes such as c-IAP2, A20 and Mcl-1 [176]. As a result, the lymph cells are no longer dependent on the inflammatory mechanism of NF-κB activation and eliminating the *H. pylori* is no longer sufficient to shut down inappropriate cell proliferation.

A recent study involved the generation of an Eμ-API2-MALT1 transgenic mouse that further exemplifies the effect of NF-κB activation due to the API2-MALT fusion protein. In this study the Eμ-API2-MALT1 transgenic mouse showed that the expression of the API2-MALT1 fusion protein is not adequate for the formation of lymphomas during the lifespan of the mouse [161], unless chronic antigenic stimulation is provided [162] (Table 2). Expression of the MALT1 fusion did affect B-cell maturation in the bone marrow and triggered the expansion of splenic marginal zone B cells. The survival of B cells in this transgenic model, therefore was thought to be promoted via enhanced NF-κB activation [161]. Of note, the API2-MALT1 fusion in the transgenic model is under control of the Eμ promoter and not the NFkB-responsive promoter of API2/c-IAP2. This may explain the need for continued antigen stimulation for lymphomagenesis to occur in this mouse model.

Targeting IAPs for cancer therapy

The issue of primary or acquired resistance to current chemotherapeutic-based treatments is a major impediment to effective cancer treatment. Although there are many genetic and biochemical alterations that occur in cancer cells, in vitro experiments demonstrate that the up-regulation of IAP expression increases resistance to chemotherapeutic and radiation resistance. The fundamental role of the IAPs in apoptosis regulation suggests that there is value in exploiting the inhibition of IAP expression and function as a direct therapeutic strategy.

IAP antagonist-based therapy

Antisense-oligonucleotides as IAP antagonists

Applications of antisense oligodeoxynucleotides (AS ODN) as selective inhibitors of gene expression are being studied for efficacy in treating particular genetic disorders. In addition, many AS ODNs are being assessed both preclinically and in early clinical trials for several cancer types. The AS ODNs are short stretches of DNA, approximately 12–30 nucleotides long and are complementary to a specific mRNA strand. Hybridization of the AS ODNs to the mRNA by Watson–Crick base pairing prevents the target gene from being translated into protein, thereby blocking the action of the gene and results in degradation of the mRNA [180–182]. The specificity in the AS ODNs approach is based on the fact that any sequence of approximately 13 bases in RNA and 17 bases in DNA is estimated to be represented only once in the human genome. While this is true in theory, in practice non-specific 'off-target' effects are often seen with AS ODNs or with small interfering RNA (siRNA). These non-specific effects must be determined empirically, such as through the use of RNA profiling by DNA microarray, and/or ruled out by the inclusion of numerous appropriate control sequences, and by multiple different sequences targeting the same mRNA to ensure specificity [183–185]. Whereas small-molecule

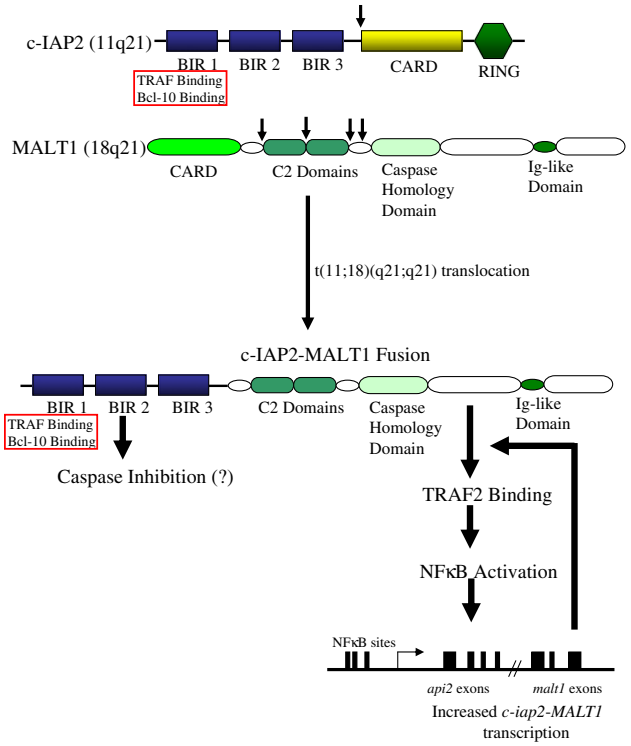

Fig. 8 c-IAP2 is involved in MALT lymphoma. The more frequent t(11;18)(q21;q21) translocation in MALT lymphoma disrupts the c-IAP2 gene (indicated by arrow), *api2*, on chromosome 11q22 and the MALT1 gene, *mlt*, on chromosome 18q21. The resulting novel protein, c-IAP2-MALT1, possesses the c-IAP2 BIR domain (arrow) fused to the carboxy-terminus of MALT1 at one of four different points (indicated by four arrows on MALT 1 protein diagram). A representative fusion protein is shown. These proteins are both anti-apoptotic and NFκB-activating, possibly through TRAF2/TRAF6 binding. A positive feedback amplification loop is established in which the fused gene is expressed under the control of the *c-iap2* promoter, which contains several NFκB sites

drugs target a protein on the basis of its molecular domain structure, AS ODNs recognize a target mRNA based on its sequence [181, 182]. To find suitable sequences for the treatment of a specific cancer, genes that are differentially expressed or regulated in diseased and normal tissues need to be identified. Determining the roles of cancer-related genes in tumor development is a rapidly progressing area in cancer research. A multitude of candidate genes that are involved in apoptosis have been found and represent potential targets for antisense-based therapies.

Recently published work shows that down-regulating XIAP gene expression via siRNA increased apoptosis in cultured MCF-7 breast cancer cells and enhanced the killing effects of etoposide and doxorubicin [186]. Another study used short hairpin RNAs as an RNAi approach directed against XIAP, and showed that XIAP mRNA could be reduced by as much as 85% in some breast carcinoma cell lines. This reduction in XIAP dramatically sensitized these cell lines to TRAIL and to taxane-induced killing [187]. The therapeutic approaches discussed in the following sections will focus on AS ODNs and why the alteration of IAP expression by these agents, represent viable therapies.

Antagonizing survivin

Survivin is expressed in fetal tissues, becomes restricted during development, and is absent in most healthy, differentiated adult tissues [188], with the exception of stem cells, thymus and testes. Significantly, Survivin is re-expressed during malignant transformation and is found in nearly all tumor types including neuroblastomas [189], pancreatic, prostate, gastric, colorectal, hepatocellular, and breast carcinomas, as well as lung and bladder cancers, melanomas, B-cell lymphomas and esophageal cancer [190]. The expression of Survivin in cancer is a predictor of both poor prognosis and decreased survival time, and is implicated in conferring chemo- and radio-resistance phenotypes to tumor cells.

Due to the ubiquitous nature of Survivin in human cancer, one therapeutic focus is the targeting of this IAP in the attempt to down-regulate and eliminate its expression. The therapeutic potential of using a survivin antisense approach was initially illustrated with the observation that EPR-1 (effector cell protease receptor-1) and Survivin were encoded by mRNAs transcribed from opposite strands of the same chromosome locus [191]. When *epr-1* cDNA was over-expressed in HeLa cells, Survivin was observed to be down-regulated, the rate of spontaneous apoptosis increased, and cell proliferation was inhibited [191]. These experiments indicated that identifying a method to effectively down-regulate Survivin could be therapeutically beneficial. Experiments were later performed to assess the ability of 20-mer AS ODNs to down-regulate Survivin expression in human lung adenocarcinoma cell lines. These AS ODNs were observed to decrease Survivin protein levels in a dose-dependent manner, induce apoptosis, increase levels of caspase-3 activation, and increase the sensitivity of cells to chemotherapeutics [66, 192]. Another study reported the use of different AS ODNs that spanned the *survivin* gene to inhibit Survivin expression in neuronal tumors. The results show that, depending on the tumor type, the AS ODN inhibition of Survivin could induce cell death through either a caspase-dependent or caspase-independent pathway [193]. In vivo models showing the effect of survivin downregulation identify this approach as a viable strategy to inhibit tumor growth [194–196]. Current phase I clinical trials of a Survivin AS ODN are underway by Eli Lilly and Company.

Table 2 Summary of IAP and antagonist natural mutations and deletions in mammals leading to disease

Gene	Disease	Phenotype and comments (references)
Birc1 (NAIP)	Type 1 Spinal Muscular Atrophy (SMA, type 1)	Approximately 2/3 of type 1 SMA individuals display a deletion in the NAIP gene in addition to the deletion of the causative adjacent gene, SMN. NAIP is thought to be a phenotypic modifier, with loss of function producing a worse prognosis [41].
Birc1e (NAIP5)	*Legionella* susceptibility (Legionnaire's disease)	The NAIP locus is expanded in mice to include multiple copies dependent on the strain. Mice lacking the *naip5/birc1e* gene are susceptible to *Legionella pneumophila* infection [158]. NAIP5 is a major regulator of intracellular replication of *Legionella* in macrophages [159].
Birc3 (c-IAP2)	MALT lymphoma	*c-IAP2* is translocated in t(11;18)(q21;q21)-bearing lymphomas [160]. The chromosomal translocation fuses the BIR domains of c-IAP2 with the paracaspase domain of MALT1. It is unknown if the transforming properties of the fusion results from c-IAP2 loss-of-function (e.g. deletion of E3 ligase function for bcl10) or MALT1 gain-of-function (e.g. NFkB-activating function) or both. Transgenic mice bearing the c-IAP2-MALT1 fusion under control of the Eμ promoter, demonstrate increased survival and expansion of B cells [161]. Continued immune stimulation by injection of complete Freund's adjuvant leads to lymphoma development in these mice [162].
Birc4 (XIAP)	X-linked lymphoproliferative disorder (XLP)	Mutations in the SAP signaling adaptor molecule underlie 60% of XLP cases. Recently, mutations in XIAP leading to loss of expression were discovered in three kindreds without SAP mutations. Apoptosis of lymphocytes from XIAP-deficient patients is enhanced in response to various stimuli including CD3, Fas and TRAIL. In addition, XIAP-deficient patients, like SAP-deficient patients, have low numbers of NK T-cells. By identifying an X-linked immunodeficiency that is caused by XIAP loss of function, XIAP is shown to be a potent regulator of lymphocyte homeostasis in vivo [163].
Omi/HtrA2	Motor neuron degeneration 2 (mnd2)	The mouse mutant mnd2 exhibits muscle wasting, neurodegeneration and juvenile lethality. A loss of function mutation in Omi is responsible. Loss of Omi protease activity increases the susceptibility of mitochondria to permeability transition, and increases the sensitivity of MEFs to stress-induced cell death [164]. These findings may appear paradoxical for Omi's role as a proapoptotic IAP antagonist but may relate to other essential functions that Omi carries out in the mitochondria.

Antagonizing XIAP

In contrast to Survivin, the importance of XIAP in cancer prognosis is less clear; however, XIAP is the most potent of the IAPs with respect to its ability to inhibit caspase activation and suppress apoptosis. Furthermore, XIAP is highly over-expressed in many tumor cell lines of the NCI panel [56, 126] and its expression has been correlated with poor prognosis in acute myelogenous leukemia (AML) [126]. These factors indicate that XIAP is an attractive target for improving treatment responses for a variety of tumor types.

One study examined the effects of XIAP down regulation using AS ODNs on human non-small cell lung cancer growth both in vitro and in vivo. *Xiap* AS ODNs were observed to effectively down-regulate both specific mRNA and protein levels. *Xiap* AS ODNs effectively induced apoptosis on their own and sensitized the tumor cells to the cytotoxic effects of several chemotherapeutics, including Taxol®, etoposide, and doxorubicin [197]. Furthermore, administering *xiap* AS ODNs in a xenograft model of human non-small cell lung cancer showed a significant down-regulation of XIAP protein. Tumor establishment was delayed when AS ODNs were combined with vinorelbine treatments [197]. Other studies showed that *xiap* AS ODNs enhanced tumor regression in conjunction with radiotherapy in a mouse model of lung cancer [195]. When XIAP was down-regulated in the pancreatic carcinoma cell line, Panc-1, with a second generation, mixed backbone AS ODN (AEG35156/GEM®640) these cancer cells were also sensitized to TRAIL-induced killing [187]. Similarly, TRAIL, in combination with another *xiap* AS ODN (in phosphorodiamidate morpholino chemistry) potentiated both cisplatin sensitivity and TRAIL killing in androgen-refractory prostate cancer cells [198]. Overall, there are promising data indicating that when XIAP protein expression levels are reduced via RNAi or antisense approaches, a decreased apoptotic threshold to an array of chemotherapeutics can be achieved [199]. Notably, a *xiap* AS ODN (AEG35156) is being tested for cancer treatment

and is in the third year of phase I/II clinical trials in the United Kingdom, Canada and the United States, and initial results appear promising [200, 201].

Adenoviral expression of dominant negative mutants of survivin

Adenoviral vectors targeting regulators of both cell cycle and/or apoptosis have been examined for potential applications in cancer therapy alone, or in combination with chemotherapy drugs [202]. One such application of adenoviral-based gene therapy is the intratumor adenoviral delivery of wild type p53 in patients with non-small cell lung cancer, thereby restoring checkpoint functions of apoptosis and/or cell cycle arrest [203]. Targeting the Survivin pathway via dominant negative mutants in an adenoviral gene therapy approach may also be beneficial for cancer treatment. When initial experiments were performed using DNA plasmids to transfect Survivin mutants into melanoma cell lines, one dominant negative mutant, carrying a cysteine 84-alanine (Cys^{84}-Ala) mutation in the BIR domain, significantly increased the number of apoptotic cells. Additional mutants of Survivin were later constructed, one of which carries a Thr^{34}-Ala mutation. This mutation destroys a phosphorylation site for the cyclin-dependent kinase $p34^{cdc2}$. Without phosphorylation at Thr^{34}, Survivin is no longer able to associate with procaspase-9 and inhibit apoptosis [204]. When the Thr^{34}-Ala dominant negative mutant was transfected into three different melanoma cell lines, spontaneous apoptosis and an increased sensitivity to cisplatin was observed. Conditional expression of Thr^{34}-Ala also prevented tumor formation in a subcutaneous xenograft model of melanoma [205].

A replication-deficient adenovirus (pAd-T^{34}A) was later used to over-express the Thr^{34}-Ala Survivin dominant mutant. In one study it was observed that transduction with pAd-T^{34}A caused spontaneous apoptosis in breast, cervical, prostate, lung, and colorectal cancer cell lines. In contrast, pAdT^{34}A had no observable effects on the proliferation or viability of healthy cells that do not express Survivin [202]. Mitochondrial release of cytochrome c, cleavage of caspases-9 and -3, and the catalytic enhancement of caspase-3 activity were observed in the apoptotic pAd-T^{34}A-infected cells. In combination with chemotherapeutics such as Taxol®, the extent of apoptosis in HeLa and MCF-7 cells was also increased [202]. Experiments using the MCF-7 human breast cancer xenograft model showed that pAdT^{34}A transduction suppressed de novo tumor formation, inhibited the growth of pre-established tumors, reduced intraperitoneal tumor dissemination, and induced massive apoptosis in all transduced cells [202]. Thus, adenoviral targeting of the Survivin pathway using dominant negative mutants may be a powerful tool for selective cancer gene therapy.

Survivin immunotherapy

Cancer vaccines targeting cancer-restricted epitopes represent a novel approach to eradicate tumors through stimulation of the host's immune system. Survivin is a shared tumor-associated antigen expressed in a variety of malignancies. Sera from cancer patients contain antibodies and cytolytic T-cells (CTLs) against survivin. Approaches targeting survivin epitopes are currently undergoing pre-clinical and clinical evaluation (reviewed in [206]). Several phase I trials, involving either administration of survivin peptides or survivin-directed autologous CTLs, have concluded and progressed on to larger phase II trials in some cases.

Negative IAP regulators as therapeutics

XAF1

Studies have shown that restoring XAF1 expression levels increases the sensitivity of tumor cell types to apoptotic triggers. For example, one study using gene arrays observed that in the presence of interferon β (IFN- β), *xaf1* mRNA was up-regulated in several apoptosis-resistant cancer cell lines including melanoma, leukemia, and fibrosarcoma cells [101]. The melanoma cell lines used in this study were largely resistant to Apo2L/TRAIL, an important mediator of IFN-β-induced apoptosis. Pre-treatment with IFN-β sensitized the cells to Apo2L/TRAIL-induced apoptosis, potentially due to the up-regulation of XAF1. Forced over-expression of XAF1 sensitizes the cells to Apo2L/TRAIL to the same degree as IFN pre-treatment [101]. Other studies examined the expression levels of XAF1 in human gastric and colon cancers where it was observed that there were low levels of *xaf1* mRNA compared to adjacent healthy tissues presumably due to hypermethylation of the *xaf1* promoter [103]. From these studies it can be concluded, that Smac, or XAF1 over-expression strongly influences cellular sensitivity to the pro-apoptotic effects of Apo2L/TRAIL.

It has been recently shown that XAF1 is able to function differentially between cancer cells and healthy cells, making this protein a potentially powerful candidate as a cancer therapeutic [207]. Using xenograft nude mouse models in which gastric and colon cancers were established by subcutaneous injections, it was observed that XAF1 over-expression alone was able to suppress tumor formation. Further studies showed that when adenoviral-*xaf1* was delivered by intratumoral injections in combination with

Apo2L/TRAIL or chemotherapeutics, there was enhanced killing by apoptosis, and eventually a complete eradication of established tumors [207]. When tissues surrounding the tumors were examined for the presence of apoptotic cells via TUNEL staining, it was observed that apoptosis was not induced in the normal cells [207].

XAF1 has also shown selective killing potential in other human tumor cells including pancreatic and breast cancer cell lines. When these human tumor cell lines and normal cell lines were transduced with adenoviral *xaf1*, approximately 80% of the tumor cells underwent apoptosis compared to 5–10% in the normal cell lines [208]. The ability of XAF1 to selectively induce apoptosis in cancer cells leaving healthy cells unaffected makes this protein potentially effective for gene therapy.

Smac

Smac/DIABLO appears to function as a general IAP inhibitor in that it has been shown to bind to XIAP, c-IAP1, c-IAP2, survivin, livin, apollon, with the exception of NAIP [99, 104, 149, 209–211]. The first four amino-terminal residues of mature Smac/DIABLO, Ala-Val-Pro-Ile, are absolutely required for Smac/DIABLO function and deletion of this sequence will abolish Smac/DIABLO-IAP interaction [212–214]. The crystal structure of Smac/DIABLO indicates that the protein consists of three extended alpha helices bundled together to form an arc-shaped structure, exposing an unstructured amino terminus. Smac/DIABLO homodimerizes through an extensive hydrophobic interface and this homodimerization is essential for its activity [213]. The newly generated amino terminus in mature Smac/DIABLO makes critical contacts with XIAP BIR3 and mediates XIAP inhibition. The co-crystal structure of XIAP BIR3 and Smac/DIABLO indicate that the amino-terminal four residues Ala-Val-Pro-Ile in Smac/DIABLO recognize a surface groove on BIR3, with the Ala residue bound within a hydrophobic pocket [212]. The amino-terminal four residues of the caspase-9 linker peptide (Ala_{316}-Thr-Pro-Phe) share significant homology to the amino-terminal tetrapeptide in mature Smac/DIABLO. Initially it was believed that binding of the caspase-9 linker peptide and Smac/DIABLO to the BIR3 domain of XIAP is mutually exclusive, suggesting a competition model in which Smac/DIABLO displaces XIAP from caspase-9 [11]. Smac/DIABLO was also predicted to bind XIAP BIR 2 and disrupt caspase-3 and -7 inhibition, possibly by steric hindrance [213]. More recent experiments suggest that Smac/DIABLO is unable to remove caspase-3 and -7 inhibition by the linker-BIR2 domains of XIAP and inefficiently relieves caspase-9 inhibition by BIR3. However, when constructs were used that express the XIAP-BIR2 and -BIR3 domains in tandem, Smac/DIABLO was shown to effectively prevent IAP inhibition of both initiator and effector caspases. Furthermore, the affinities of the BIR2 and BIR3 domains of XIAP for Smac/DIABLO were shown to be almost identical with each Smac dimer interacting with the BIR2 and BIR3 domains of one XIAP molecule to form a 2:1 stoichiometric complex [215]. Although individual BIR domains of XIAP are adequate for inhibiting in vitro caspase activity, these findings suggest that both BIR2 and BIR3 are required not only for XIAP-Smac/DIABLO interaction but also for subsequent liberation of caspase inhibition [215]. To date there has not been any observable interaction of BIR1 from any of the IAPs with Smac/DIABLO.

The structural analysis of Smac binding to XIAP indicates that the amino-terminal tetra-peptide recognizes a surface groove on the BIR3 domain, implying that peptides or small molecules modeled on this binding motif might serve as prototypical drugs whose activity might complement that of Smac [216]. When Smac peptides composed of the first 4–8 amino-terminal residues are delivered into MCF-7 breast cancer cells, they are capable of interacting with XIAP, c-IAP1, [217, 218] and the single BIR domain of ML-IAP [209]. As well as being fully competent in interacting with the IAPs, these peptides enhance the induction of apoptosis and long term anti-proliferative effects of a range of chemotherapeutics including paclitaxel, etoposide, and doxorubicin [217, 218].

Smac-like peptides delivered in combination with previously used cancer therapeutics such as Apo2L/TRAIL appears to be a promising method to reduce tumor burden [217, 219]. It was observed in intracranial malignant glioma xenograft models that the in vivo delivery of Smac peptide, in conjunction with Apo2L/TRAIL, completely eradicated established tumors and increased the survival time of treated mice [219]. Apo2L/TRAIL induces apoptosis via the extrinsic/death receptor pathway by binding to the transmembrane receptors TRAIL-R1/DR4 and TRAIL-R2/DR5. Although Apo2L/TRAIL was once a promising cytotoxic agent in the treatment of cancer, recent studies have shown that when administered alone, Apo2L/TRAIL over time becomes ineffective at killing cancer cells, likely due to the development of tumor cell resistance [220]. Thus the delivery of Smac peptides in the presence of Apo2L/TRAIL appears to have a significant impact on sensitizing otherwise resistant cells to apoptosis [218, 219, 221, 222].

Some studies have shown that N-terminal Smac tetrapeptides bind only to the BIR3 domain of XIAP with low affinity, are sensitive to proteolytic degradation, and have a poor capacity to penetrate cells [223]. Efforts to circumvent the limitations of Smac peptides led to the discovery of small molecule Smac mimetics, compounds that inhibit the IAPs with higher affinities than Smac peptides. Such

submicromolar, small-molecule, non-peptidic antagonists were initially reported by Park et al. [224]. These compounds were created by individually replacing several amino acid residues while still maintaining the essential interactions necessary for binding in the Smac binding site on XIAP BIR3 [224]. Another identified class of compounds is a series of proteolytically stable, capped tripeptides consisting of unnatural amino acids that bind to XIAP BIR3 with high nanomolar affinities. These compounds are cytotoxic in cancer cells and delay tumor growth in breast cancer xenograft models [225].

An additional compound class, known as a modified oxazoline molecule was shown, after several modifications, to bind to the IAPs with a higher affinity than Smac tetrapeptides, and block IAP interaction with caspase-9. This compound also acted synergistically with Apo2L/TRAIL and various chemotherapeutic agents [201]. Additional studies using a high IAP-expressing breast cancer cell line, MDA-MB-231 showed that at low nanomolar concentrations, this Smac-mimetic compound significantly sensitized these cells to both Apo2L/TRAIL- and etoposide-induced apoptosis via caspase-3 activation [226]. The effectiveness of this compound is due to its ability to mimic the dimeric structure of Smac protein, which, as mentioned previously, acts at the IAP BIR2-linker and BIR3 regions to liberate caspases-3, -7, and -9 [201, 226].

Other potentially effective XIAP inhibitors include a class of compounds known as polyphenylureas that not only possess a high binding affinity for the BIR2 domain of XIAP, but also appear to have the ability to reverse caspase-3 inhibition by XIAP [227]. Furthermore, these compounds cause XIAP to dissociate from effector caspase-3 in vitro, and also restore caspase-3 enzymatic activity [228]. Thus these polyphenylureas, but not their structural analogs, interact with XIAP in a mechanism distinct from that of Smac and Smac-peptide. Unlike the polyphenylurea-based compounds, Smac peptides are not able to remove caspase-3 from XIAP because they do not interact with the linker region of XIAP.

The polyphenylurea XIAP antagonist compounds may be considered a significant improvement to Smac-peptide approaches in regulating XIAP inhibition. Although both the Smac peptide- and polyphenylurea antagonists are able to act synergistically with chemotherapeutics and Apo2L/TRAIL to sensitize tumor cells, only the latter class of compounds has the intrinsic ability to reduce tumor viability. The polyphenylurea compounds were shown to reduce clonogenic survival of cancer cells, and suppress the growth of established tumors in xenograft models in mice [229]. Furthermore, the capacity of these compounds to induce apoptosis was not affected by genetic alterations in the expression of any of the pro- or anti-apoptotic Bcl-2 family members, which are frequently observed in many cancers [228]. These compounds also displayed a broad spectrum of activity in many cancer cell lines and elicited little toxicity in normal cells [227, 229]. The observations from these studies further confirm that alleviating IAP suppression of caspases greatly sensitizes cancer cells to apoptosis, making this family of proteins a promising target for the development of cancer therapeutics.

Summary

Apoptosis is controlled at multiple steps, each of which is influenced by pro- and anti-apoptotic proteins. The equilibrium between the cell death that induces the caspase cascade and inhibition of that process by the IAPs constitutes a fundamental decision point. The recurrent up-regulation of IAP expression in cancer cell lines and tumors indicates that this decision point is important in determining cell fate. The IAPs not only control cell death, but also influence signal transduction pathways, protein turnover, and progression through cell cycle. Still, many aspects of IAP function in all these processes remain to be discovered. With the recognition of apoptosis suppression as a fundamental aspect of human cancer, the IAPs and other anti-apoptotic proteins are now acknowledged as being outstanding therapeutic targets.

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