Review

Shinji Kamada*

Inhibitor of apoptosis proteins as E3 ligases for ubiquitin and NEDD8

Abstract: The inhibitors of apoptosis proteins (IAPs) are endogenous inhibitors for apoptosis. Apoptosis is carried out by caspases, which are the family of cystein proteases. IAPs regulate caspases through two conserved regions, the baculovirus IAP repeats (BIRs) and the really interesting new gene (RING) domains. Although the BIRs are responsible for binding to caspases, the RING domain can act as a ubiquitin-E3 ligase, leading to ubiquitylation of IAPs themselves and their pro-apoptotic IAP counterparts such as caspases. Recently, it is reported that another ubiquitin-like protein, neuronal precursor cell-expressed developmentally downregulated protein 8 (NEDD8), is also involved in the regulation of apoptosis through neddylation of caspases mediated by IAPs. On the contrary, the results against the function of IAPs as a NEDD8-E3 ligase are also suggested. This review presents the summary of IAPs, caspases, and the ubiquitin-proteasome system and how their interactions influence the regulation of apoptosis.

Keywords: apoptosis; caspases; inhibitor of apoptosis proteins; NEDD8; ubiquitin.

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Introduction

The inhibitors of apoptosis proteins (IAPs) have an ability to inhibit apoptosis, and eight and four IAPs have been thus far isolated in mammals and fruit flies, respectively (1, 2). They share a conserved region known as the baculovirus IAP repeat (BIR) domain, and some IAPs contain another conserved region called the really interesting new gene (RING) domain. In contrast, caspase is a family of cysteine proteases that are required for cytokine maturation and apoptosis execution (3–7). The apoptotic caspases are divided into two classes, the initiator caspases and the effector caspases. In addition, the ubiquitin-proteasome system (UPS) is a major mechanism for regulating protein function in eukaryotes. The covalent attachment of ubiquitin is mediated through at least three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (8). Some of the IAPs can inhibit the enzymatic activity of caspases through directly binding to caspases mediated with the region containing BIRs. The RING domain of IAPs can act as a ubiquitin-E3 ligase, leading to ubiquitylation and subsequent degradation of IAPs themselves and their counterparts (1, 2). Among the ubiquitin-like proteins (UBLs), neuronal precursor cell-expressed developmentally downregulated protein 8 (NEDD8) is most homologous to ubiquitin (9, 10). NEDD8 conjugation is mechanistically similar to ubiquitylation, in that NEDD8 is activated and transferred to substrates by E1, E2, and E3 enzymes. It is suggested that IAPs work as NEDD8-E3 ligases by themselves and for caspases in fruit flies and mammals (11). However, it is also pointed out that NEDD8 overexpression triggers NEDD8 activation mediated by the E1 enzymes for ubiquitin, leading to neddylation of target proteins (12). Furthermore, it is reported that X-linked inhibitor of apoptosis protein (XIAP) does not function as a NEDD8-E3 ligase for caspase-7 *in vivo* (13), and that overexpression of NEDD8 induces erroneous conjugation to ubiquitin substrates (14). This review will focus on the characteristic features of IAPs, caspases, and UPS, and the coordination of these proteins to regulate apoptosis (Figure 1).

IAPs – structure and function

The prototype IAP was originally described in baculoviral genomes through a genetic screen to identify regulators of host cell viability during virus infection (15, 16). Subsequently, the cellular orthologues in several species such as yeast, nematodes, flies, and humans have been identified (17–24). The defining feature of an IAP protein is the presence of the BIR domain, a zinc-binding motif of approximately ~70 amino acid residues that mediates protein-protein interactions (16, 25, 26) and, in most

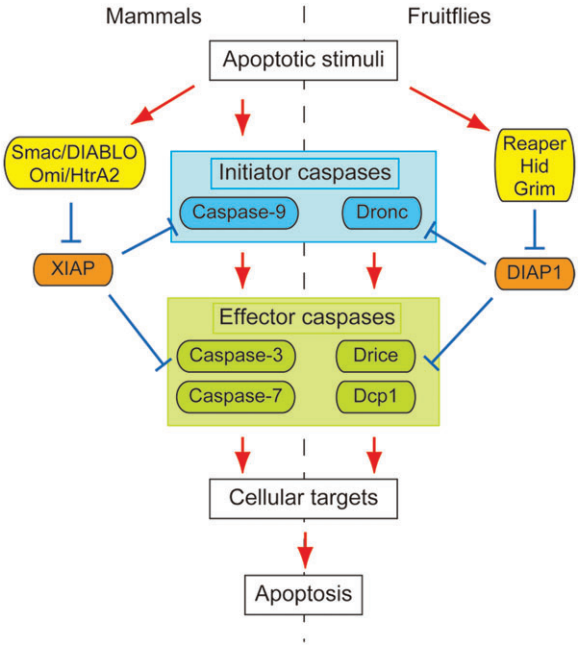

Figure 1 A conserved apoptotic pathway in mammals and fruit flies. Caspases and caspase regulators as described in the text are shown. Caspase-9 and Dronc are initiator caspases, whereas caspase-3, caspase-7, Drice, and Dcp1 belong to the class of effector caspases. XIAP and DIAP1 suppress apoptosis by negatively regulating caspases through direct binding and ubiquitylation. IAP antagonists such as Smac/DIABLO, Omi/HtrA2, Reaper, Hid, and Grim can remove the IAP-mediated negative regulation of caspases.

cases, binds to IAP-binding motifs (IBMs), which have the consensus sequence A-(V/T/I)-(P/A)-(F/Y/I/V/S) (27). Several proteins, including caspase-3, caspase-7, caspase-9, Smac (second mitochondria derived activator of caspase)/DIABLO (direct IAP-binding protein with low pI), Omi/HtrA2, and GSPT1/eRF3, have been shown to possess functional IBMs that are normally hidden but must be exposed and unblocked upon the proteolytic processing of these proteins (1). All known IBMs interact with the peptide-binding groove on the surface of the BIRs. In addition to the BIRs, some IAPs contain another more widely distributed region called the RING domain. The consensus sequence of RING domains is Cys-X2-Cys-X(9–39)-Cys-X(1–3)-His-X(2–3)-Cys/His-X2-Cys-X(4–48)-Cys-X2-Cys (where X is any amino acid), which forms a cross-brace structure and coordinates two zinc ions (28, 29). RING domains often function as modules that confer ubiquitin protein ligase (E3) activity and, in combination with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), catalyze the transfer of ubiquitin to target proteins (30). All known RING-containing IAPs have E3 activity, and the range of substrates includes molecules involved in apoptosis and signaling.

There are eight mammalian IAPs, which include XIAP (X-linked IAP)/BIRC4 (baculoviral IAP repeat containing protein 4), cIAP1/BIRC2, cIAP2/BIRC3, NAIP (neuronal apoptosis-inhibitory protein)/BIRC1, Survivin/BIRC5, Bruce/Apolon/BIRC6, ML-IAP (melanoma IAP)/Livin/BIRC7, and ILP2 (IAP-like protein-2)/BIRC8; and four fruit fly IAPs, which include DIAP1, DIAP2, Deterin, and Dbruce (31). The mammalian IAPs, XIAP, cIAP1, and cIAP2, contain three BIR domains and a RING finger domain in their amino-terminal and carboxy-terminal portions, respectively (Figure 2). The fruit fly IAPs, DIAP1 and DIAP2, bear a carboxy-terminal RING domain, and two and three BIR domains in their amino-terminal region, respectively (31).

Caspases – structure and function

Caspases, a family of cysteine proteases, are required for apoptosis execution and cytokine maturation (3–7). Caspases are zymogens that consist of three regions, an N-terminal prodomain, a large subunit, and a small subunit. The caspase subtypes are divided into two groups, inflammatory caspases and apoptotic caspases. The apoptotic

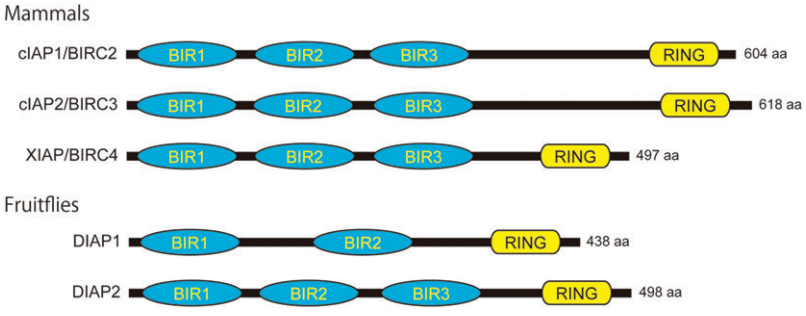

Figure 2 IAPs in mammals and fruit flies.
Several of the IAP proteins that are discussed in this review are drawn schematically. BIR, baculovirus IAP repeat; RING, really interesting new gene.

caspases are generally divided into two classes: the initiator caspases, which include caspase-2, caspase-8, caspase-9, and caspase-10 in mammals, and Dronc and Dredd in fruit flies; and the effector caspases, which include caspase-3, caspase-6, and caspase-7 in mammals, and Drice, Decay, Damm, Dcp1, and Strica in fruit flies. The activation of effector caspases is catalyzed through proteolytic processing by the initiator caspases, whereas the activation of initiator caspases containing large prodomains is suggested to occur in a protein complex generated by the binding through the prodomains. The large prodomains have characteristic structures, such as the death effector domain (DED) and the caspase recruitment domain (CARD). Caspase-8, caspase-10, and Dredd contain two tandem DEDs and are activated through binding to the adaptor proteins, FADD and dFADD. The CARD is found in caspase-2, caspase-9, and Dronc, and plays an essential role for the interaction with other CARD-containing proteins, leading to the activation of these caspases. Caspases discussed in the text, such as caspase-3, caspase-7, caspase-9, Drice, Dcp1, and Dronc, are drawn schematically in Figure 3.

IAPs as caspase inhibitors

IAPs can potently inhibit the enzymatic activity of active caspases (1, 32, 33). Although initial biochemical studies *in vitro* and overexpression analyses demonstrated that human survivin, XIAP, cIAP1, and cIAP2 bind and effectively inhibit caspase-3, caspase-7, and caspase-9, subsequent and more quantitative studies have made it clear that mammalian XIAP is the only IAP that functions as a direct caspase inhibitor (34). Other IAPs, such as cIAP1, cIAP2, DIAP1, and DIAP2, are inefficient in directly inhibiting caspases *in vitro*. Consequently, overexpression of XIAP efficiently inhibits caspase activation and apoptosis stimulated by the intrinsic and extrinsic pathways (35–40), and cells that lack XIAP are sensitized to apoptosis (41–44).

XIAP is able to inhibit caspase-3, caspase-7, and caspase-9 with nanomolar activity. Residues in the linker region between the BIR1 and BIR2 domains of XIAP bind to the active site pockets of caspase-3 and caspase-7, and thereby occlude substrate entry and inhibit the catalytic activity of the caspases (26, 45–49). As the linker region preceding the BIR2 domain plays an essential role in inhibitory binding, the BIR2 domain has little direct role in the inhibitory mechanism. However, the BIR2 domain is functionally important as it makes additional contact with an IBM motif of activated caspases (49, 50). The IBM motifs in pro-caspase-3 and pro-caspase-7 are hidden, but become exposed following cleavage-mediated activation of the caspases. The coordinated binding of XIAP to the catalytic pockets and IBMs of the caspases effectively enhances caspase binding, and is essential for XIAP to inhibit caspase activities. The mechanism by which XIAP binds to caspase-9 is entirely different to the manner in which it binds to caspase-3 and caspase-7. Caspase-9 requires a dimerization-induced conformational change to generate a productive catalytic pocket, and XIAP interferes with caspase-9 dimerization through the binding of the BIR3 domain to the homodimerization surface of caspase-9 (51, 52). Mammalian caspase-9 also has an IBM, so IAP antagonists will directly compete with not only caspase-3 and caspase-7 but also caspase-9 for binding to XIAP (51). Although XIAP-mediated inactivation of caspase-3, caspase-7, and caspase-9 does not require a functional RING finger under *in vitro* conditions or when overexpressed (32, 53), recent evidence indicates that endogenous XIAP requires a functional RING to exert its full anti-apoptotic potential *in vivo* (54).

The ubiquitin-proteasome system

The UPS is composed of two different steps: the covalent attachment of multiple ubiquitin molecules to the

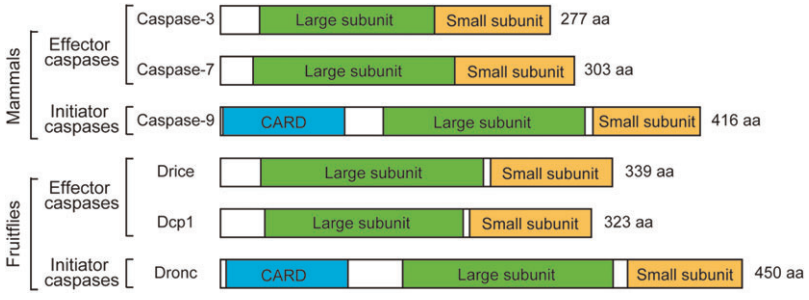

Figure 3 Caspases in mammals and fruit flies.
Several of the caspases that are discussed in this review are drawn schematically. CARD, caspase recruitment domain.

protein substrate and the degradation of polyubiquitylated protein by the 26S proteasome complex (8). The first step is mediated by at least three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The specific recognition of a large number of target proteins is mainly due to the multiplicity of E3 enzymes in the UPS (8). The existence of >1000 different E3 ligases confers specificity and versatility on multiple target proteins (55). E3 ligases are categorized into four major classes on the basis of their structural characteristics (56): HECT-type, U-box-type, PHD-finger-type, or RING-finger-type (Figure 4). RING-finger-type E3 ligases are further divided into two classes; one class comprises multiprotein complexes that contain a distinct substrate-binding protein and an E2 recruitment protein (which contains a RING domain); in the other class, the components are encoded in the same polypeptide (56, 57). The multisubunit RING-finger-type E3 ligases include APC (anaphase-promoting complex), SCF (SKP-cullin-F-box), SOCS (suppressor of cytokine signaling), and BTB (broad complex, tramtrack, and bric-a-brac) (2). Although there are various substrate recruitment domains in the F-box, SOCS box, and BTB proteins, these multiprotein E3 ligases all share the same RING-domain-containing protein, Rbx1/ROC1/HRT1. The substrate-binding proteins and Rbx1/ROC1/HRT1 are assembled onto a cullin scaffold (Figure 5A). The IAPs and other RING-domain-containing proteins, such as MDM2 and TRAF6 (tumor necrosis factor receptor-associated factor-6), belong in the other class of E3 ligases (2, 57) (Figure 4). In contrast to the multisubunit RING-finger-type E3 ligases, E3 ligases in IAPs combine a substrate-binding domain (the BIRs) and a RING domain in the same protein, and there is no recruitment for cullin scaffolds to ubiquitylate substrates (Figure 5B).

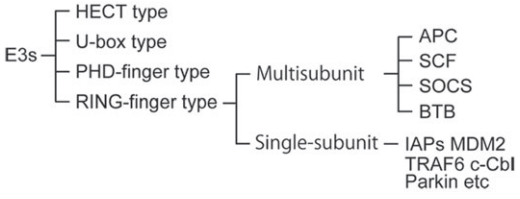

Figure 4 Subtype of ubiquitin-E3 ligases.
E3 ligases are divided into four major classes: HECT-type, U-box-type, PHD-finger-type, and RING-finger-type. RING-finger-type E3 ligases are further divided into two classes. One class contains APC, SCF, SOCS, and BTB, which are multiprotein complexes. Another class contains IAPs, MDM2, TRAF6, c-Cbl, BRCA1, and Parkin, in which a substrate-binding domain and an E2 recruitment domain (RING) are encoded in the same polypeptide.

Ubiquitin and ubiquitin-like proteins

The attachment of ubiquitin or UBLs through an isopeptide bond to the primary amino group of a target constitutes a major mechanism for regulating protein function in eukaryotes. Posttranslational modification by UBLs regulates a large number of processes, including cell division, cell death, immune responses, and embryonic development. Consequently, deficiencies in UBL pathways are associated with a variety of diseases, particularly cancer, neurodegenerative disorders, and muscle atrophy (58, 59).

Ubiquitin is a 76-amino-acid polypeptide that is conjugated through an isopeptide linkage to other proteins.

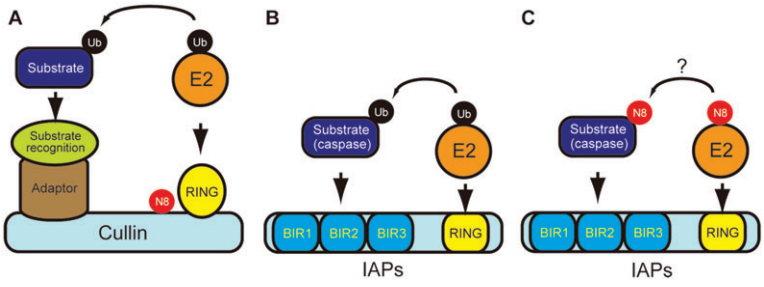

Figure 5 Schematic diagrams of RING-finger-type E3 ligases.
(A) Cullin-RING E3 ubiquitin ligase complexes (CRLs). CRLs contain SCF, SOCS, and BTB E3 ligases, which are assembled around a cullin scaffold. CRLs bind to substrate though adaptor proteins and substrate recognition proteins, such as F-box, SOCS-box, and BTB proteins, and recruit the ubiquitin-charged E2 through RING-finger proteins, such as Rbx1 and Rbx2, and the ubiquitin is transferred directly to the substrate. The assembly and activity of CRLs is regulated through reversible conjugation of NEDD8 to the cullin. (B) IAPs as ubiquitin-E3 ligases. IAPs bind substrates, including caspases, through BIR domains, and recruit the ubiquitin-charged E2 through the RING domain, and the ubiquitin is transferred directly to the substrate. (C) IAPs as NEDD8-E3 ligases. Analogous to a ubiquitin-E3 ligase, it is suggested that IAPs may function as a NEDD8-E3 ligase for caspases. However, whether IAPs modify caspases with NEDD8 *in vivo* is obscure, as discussed in the text. BIR, baculovirus IAP repeat; RING, really interesting new gene; Ub, ubiquitin; N8, NEDD8.

Although an important role of the ubiquitin system is to regulate the half-life of proteins by targeting them for degradation by the proteasome, there are many ubiquitin modifications that do not result in protein degradation but instead alter the activity of the modified protein (60). Ordinarily, monoubiquitylation or multi-monoubiquitylation (one ubiquitin on several lysine residues within the substrate) does not result in degradation of a substrate. Polyubiquitin chains of more than four ubiquitins, assembled through K48 linkage, are a potent signal for recruitment to the proteasome and subsequent degradation. It is unclear whether E3 ligases can determine if a substrate becomes mono- or polyubiquitylated, or whether E3 ligases can determine if ubiquitin is linked in a K48 manner.

There are 17 human UBLs from nine phylogenetic classes (61). Although all UBLs have been identified to conjugate to substrates in a manner analogous to ubiquitin, the different UBLs have their own discrete E1-E2-E3 cascades and have distinct effects on their targets (62–64).

NEDD8 – structure and function

Besides ubiquitin, another well-studied UBL is NEDD8, which shares approximately 60% amino acid identity with ubiquitin (10). Similar to ubiquitylation, neddylation, the NEDD8 conjugation cascade, involves E1, E2, E3, and deneddylating enzymes (65, 66). NEDD8 is conjugated through an isopeptide linkage between its carboxy-terminal glycine (Gly) 76 and a lysine of the target proteins. Like ubiquitin, NEDD8 is first synthesized as a precursor that contains one or more additional residues beyond Gly76, and is processed at the carboxy-terminal of Gly76 residue by deneddylating enzymes (10). Although ubiquitin carboxy-terminal hydrolase (UCH)-L3 can process NEDD8 precursors as well as ubiquitin precursors (67–71), NEDD8-specific deneddylase 1 (DEN1)/NEDP1/SENP8 has been shown to catalyze the processing of NEDD8 precursors with high specificity (72–75). The E1 NEDD8-activating enzyme (NAE), which is composed of the NAE1 (APP-BP1) and Uba3 heterodimer, mediates adenylation of the exposed carboxy-terminal glycine of NEDD8 in an ATP-dependent reaction, and transfers this residue to the E1 cysteine side chain through thiolester linkage (76). Activated NEDD8 is subsequently transferred to the E2 NEDD8-conjugating enzyme, Ubc12, forming another thiolester linkage. Ube2F is another NEDD8 E2 and preferentially transfers NEDD8 to cullin 5 (77). Contrary to ubiquitin, the NEDD8 E1 (NAE1) and E2s (Ubc12 and Ube2F) are specific for NEDD8 activation and conjugation. A NEDD8-E3 ligase then transfers NEDD8 to the ε-amino group of lysyl residue on substrates, resulting in the formation of an isopeptide bond. NEDD8 E3s contain RING finger domains, which include Rbx1/ROC1, Rbx2/ROC2, MDM2, c-CBL, SCF^{FBXO11}, defective in cullin neddylation 1 protein (DCN1), and DCN1-like (DCNL)1–3 (78–84).

Linkage between ubiquitylation and neddylation

Cullin-RING E3 ubiquitin ligase complexes (CRLs) are the largest family of E3 ligases with >200 members (85). CRLs are assembled around a cullin scaffold that mediates the physical interaction between different subunits, such as BTB and F-box proteins, which are responsible for substrate recognition, and RING domain-containing proteins, necessary for E2 recruitment (Figure 5A). The assembly and activity of CRLs is regulated through the reversible conjugation of NEDD8, which is covalently attached to the cullin backbone, by at least two mechanisms (70). First, neddylation promotes the assembly of the cullin complex by dissociating the cullin inhibitor CAND1 (86). Second, cullin neddylation enhances the recruitment of the activated E2 to the RING domain of Rbx1 (87) by inducing a conformational change in its C-terminal domain (88, 89). While the RING-finger protein Rbx1 promotes cullin neddylation (78, 90, 91), it is suggested recently that DCN1 functions as an E3 ligase for cullin neddylation in yeast and *Caenorhabditis elegans* (80). In fact, in yeast, DCN1 directly binds to the cullin and the NEDD8 E2 enzyme, and promotes NEDD8 conjugation through the formation of this complex (83, 92). There are five members of DCN-like proteins, DCNL1–5, in humans, of which DCNL1–3 have been shown to mediate neddylation of cullin 3 (84). As described above, ubiquitylation is positively regulated through cullin neddylation, which affects a wide variety of biological processes.

IAPs as ubiquitin-E3 ligases for caspases

The importance of the RING finger domain of IAPs in the regulation of caspases and apoptosis comes from genetic studies in *Drosophila*, in which *DIAP1* has a pivotal role in cell viability (93). DIAP1 regulates apoptosis by directly binding to the caspase-9 homologue Dronc and the caspase-3-like effector caspases Drice and Dcp1 (94–96).

While the association of IAPs with caspases is a critical step in the regulation of apoptosis, the physical interaction between DIAP1 and caspases alone is insufficient to regulate caspases *in vivo* because mutant fruit flies harboring RING finger mutation in DIAP1 that abrogates its ubiquitin ligase activity, but not caspase binding, represent a severe loss-of-function phenotype (97, 98). Following binding, the RING domain is necessary for ubiquitylation and inactivation of the initiator caspase Dronc, and the effector caspases Drice and Dcp1 (98, 99). Although the molecular mechanisms through which polyubiquitylation inactivate Dronc remain obscure and might involve not only degradative but also non-degradative inactivation mechanisms (100), the functional consequence of ubiquitylation for Drice and Dcp1 is clear – the conjugated ubiquitin chain seems to interfere with substrate entry into the catalytic pocket of the caspases (99).

IAP-mediated ubiquitylation of caspases is not restricted to *Drosophila*, as mammalian IAPs, such as XIAP, cIAP1, and cIAP2, also reportedly ubiquitylate caspase-3 and caspase-7, targeting them for either monoubiquitylation (101) or polyubiquitylation (47, 102). Although the functional consequence of caspase monoubiquitylation remains obscure, polyubiquitylation of caspase-3 and caspase-7 has been reportedly linked to their degradation (47, 102). However, the non-degradative polyubiquitylation of caspase-3 is also pointed out recently as described below. XIAP, cIAP1, and cIAP2 catalyze their own ubiquitylation, leading to degradation through the activity of RING in cells subjected to a proapoptotic stimulus (103, 104). This autoubiquitylation and degradation is an important regulatory step because XIAP containing a RING domain mutation that lacks E3 activity is relatively stable and confers resistance to apoptotic stimuli in cells (103). While no significant apoptotic phenotypes in their knockout mice have been reported previously (105–107), mice with a disruption in *XIAP* gene deleting the RING finger domain, which lost the ubiquitin-E3 ligase activity, show the stabilization of XIAP-ΔRING protein in apoptotic thymocytes (54). Interestingly, the increased amounts of XIAP-ΔRING protein did not lead to attenuated, but rather increased, caspase activity and apoptosis, indicating that the BIR domains themselves are not sufficient to block caspase activity *in vivo*. Remarkably, reduced levels of caspase-3 polyubiquitylation and no increased levels of caspase-3 were observed in mutant cells. These results suggest a non-degradative mode of caspase ubiquitylation. XIAP also ubiquitylates caspase-9; however, the functional outcome of this modification has not been determined (108). These results demonstrate a physiological requirement of RING-finger domain in XIAP for the inhibition of caspases *in vivo*.

Are IAPs NEDD8-E3 ligases for caspases?

The covalent attachment of ubiquitin or UBLs to target proteins is reversibly regulated. Specialized deconjugating enzymes, which are referred to as deubiquitylating enzymes (DUBs), remove ubiquitin or UBLs from target proteins (109). Approximately 100 DUBs that are estimated to be encoded in the human genome are responsible for deconjugating ubiquitin, NEDD8, and SUMO from target proteins (110, 111). DUBs mediate not only the maturation of ubiquitin and UBLs from precursor peptides but also the removal of ubiquitin and UBLs from target proteins. In combination with E3 ligases, DUBs play essential roles in the regulation of many cellular processes (109).

Recently, it is suggested that neddylation of caspases plays essential roles in apoptosis (11). They identified three NEDD8-specific isopeptidases that, when knocked down, prevented apoptosis by using systemic *in vivo* RNAi analysis in *Drosophila*. In addition, they proved that null mutants of one of these genes, *DEN1*, suppressed cell death induced by IAP antagonists, Reaper and Hid (head involution defective). The report strongly suggested that the protein modifications with NEDD8 play essential roles in the regulation of apoptosis. Furthermore, they showed that DIAP1 and XIAP neddylated Drice and caspase-7, respectively, when NEDD8 was overexpressed in cells, thereby inhibiting the protease activities of caspases. However, it is also suggested that the elevation of free NEDD8 level relative to ubiquitin triggers the activation of NEDD8 by the ubiquitin E1 enzyme (12, 112, 113). Therefore, we set out to confirm whether XIAP targets caspase-7 for neddylation in cells (13). In the report, we examined the NEDD8-E3 ligase activities of XIAP in comparison with the ubiquitin-E3 ligase activities, in which XIAP was coexpressed with HA-NEDD8 or HA-ubiquitin under the control of the same promoter to assure the equivalent protein levels for exogenously expressed NEDD8 and ubiquitin. Although neddylation of XIAP mediated by itself was detected, the efficiency was far less than ubiquitylation. Furthermore, no neddylation of caspase-7 was observed under the condition in which caspase-7 could be modified with ubiquitin. These results may suggest that XIAP does not function as a NEDD8-E3 ligase for caspase-7 *in vivo* (Figure 5C). In addition, another group also reported that overexpression of NEDD8 erroneously conjugates NEDD8

to ubiquitin substrates such as caspase-7, p53, and HIF1α by the ubiquitin pathway (14). Therefore, it will be needed to reexamine whether IAPs function as NEDD8-E3 ligases for caspases at endogenous protein levels. However, the finding that genetic ablation of *DEN1* suppressed cell death induced by IAP antagonists in *Drosophila* is interesting apart from the function of IAP as a NEDD8-E3 ligase. DEN1 is the cystein protease that specifically processes the NEDD8 precursor and has been suggested to deconjugate NEDD8 from cullins (72–74). Chan et al. (114) originally reported the characterization of *DEN1* null mutants in *Drosophila* in which many cellular proteins are highly neddylated. As IAP antagonist-induced cell death is inhibited by *DEN1* loss (11), neddylated proteins observed in *DEN1* mutants would contribute to the inhibition of apoptosis. There are two possibilities that the neddylated proteins in *DEN1* mutants regulate the cell death pathways; one is that pro-apoptotic proteins such as caspases are conjugated with NEDD8 leading to the inactive states, and the other one is that anti-apoptotic proteins such as IAPs are conjugated with NEDD8 leading to the active states. Analyses of cellular proteins highly neddylated in *DEN1* mutants will be helpful in clarifying the molecular mechanisms of how neddylation contributes to the regulation of apoptosis. However, as *DEN1* null mutant flies were viable and fertile (11, 114), the elevation of neddylated proteins does not affect normal development, suggesting that the modification with NEDD8 appear to occur under non-apoptotic conditions. We should keep these results in mind together with the results that IAPs do not regulate caspases under apoptotic conditions because IAPs are neutralized by IAP antagonists under such conditions.

Expert opinion

IAPs prevent apoptosis through the modulation of caspases in two different ways. One is the inhibition of enzymatic activity of caspases by direct binding through the region containing BIRs. However, it is suggested that mammalian XIAP is the only IAP that functions as a direct caspase inhibitor *in vivo*. The other one is the modification of caspases with ubiquitin mediated by RING. Although the degradation of caspases and IAPs themselves by RING-mediated ubiquitylation is reported, non-degradative inactivation mechanisms are also suggested, in which the conjugated ubiquitin chain interferes with substrate entry into the catalytic pocket of caspases. In addition to the modification with ubiquitin, neddylation of caspases by IAPs, which prevents protease activities of caspases, is reported. Although it is still unclear whether IAPs function as NEDD8-E3 ligases for caspases at endogenous protein levels as discussed in the text, the finding that genetic ablation of deneddylase in *Drosophila* suppresses apoptosis induced by IAP antagonist is interesting. As the covalent attachment of NEDD8 to target proteins is reversibly regulated, the metabolic abnormalities of NEDD8 may affect protein functions such as degradation, conformation, and localization in a wide variety of substrates. The precise mechanisms by which the modification with NEDD8 regulates apoptosis will need to be clarified.

Outlook

Oncogenic mutations often inactivate signaling pathways for apoptosis or activate pro-survival pathways, and impaired apoptosis is the hallmark of most, if not all, cancers. As inhibition of apoptotic programs is a key factor in tumor formation, cancer therapies based on the activation of apoptotic pathways are an attractive intervention. Several IAPs become overexpressed in human cancers, such as XIAP, cIAP1, and cIAP2, which modulate apoptosis by inhibiting caspase activity either directly or indirectly as discussed above. In contrast, induction of apoptosis results in the release of Smac/DIABLO protein complex from mitochondria, which binds BIRs of IAPs, including XIAP, cIAP1, and cIAP2, and neutralizes their anti-apoptotic activity (115). Interestingly, Smac binding triggers autoubiquitylation of cIAP1 and cIAP2, but not XIAP, leading to degradation. These results suggest that Smac could promote apoptosis by at least two mechanisms: preventing caspase inhibition mediated by XIAP and targeting of cIAP1 and cIAP2 for degradation (116). Small compounds or peptides that mimic Smac function are designed from the crystallization of XIAP BIR3 in a complex with Smac (115, 117). Only four amino acids located at the N-terminal region of Smac are required for binding to the IAPs; thus, small molecule compounds could act to antagonize IAP activity. Smac peptides sensitized primary cancer cells to apoptosis (118, 119) and strongly enhanced antitumor activity in a mouse model (119). More than 50 patents have been heretofore filed that are aimed at blocking IAPs and pushing cancer cells into apoptosis. Several studies using Smac mimetics have revealed a potential for therapeutic applications to prevent cancer development. IAP antagonists are now entering clinical trials and may proceed to clinical application in the future.

Highlights

- IAPs are endogenous inhibitors for apoptosis.
- IAPs regulate caspases through two conserved regions, BIRs and the RING domain.
- The region containing BIRs binds directly to caspases and IAP antagonists.
- The RING domain can act as a ubiquitin-E3 ligase, leading to ubiquitylation and subsequent degradation of IAPs themselves and their pro-apoptotic IAP counterparts.
- A group suggests that NEDD8, which is most homologous to ubiquitin, is also involved in the regulation of apoptosis through neddylation of caspases mediated by IAPs.
- Other groups point out the possibility that overexpression of NEDD8 erroneously conjugates NEDD8 to ubiquitin substrates through the ubiquitin pathway.
- Although careful examinations are necessary to explore the NEDD8-E3 ligase activities of IAPs, the finding that genetic ablation of deneddylase in *Drosophila* suppresses apoptosis induced by IAP antagonist is interesting.
- The precise mechanisms by which the modification with NEDD8 regulates apoptosis are expected to be clarified.

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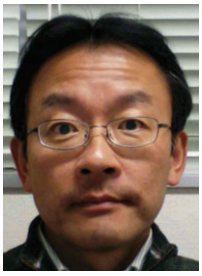

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