Cellular Roles of the Inhibitor of Apoptosis Protein c-IAP1

by

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DEDICATION

To my mother & father…for everything.

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Chapter I

Introduction

Apoptosis in Homeostasis & Development

Homeostasis is a critical process for the normal development and survival of multicellular eukaryotic organisms. Regulation of cell growth and cell death is achieved by numerous complex cellular signaling pathways, which provide an equilibrium for the cell number by affecting the rate of growth and the rate of death in the complex organism (Jacobson et al., 1997; Kerr et al., 1972; Meier et al., 2000). Without a homeostatic balance, multicellular organisms can display severe pathological conditions such as cancer (Lowe and Lin, 2000; Thompson, 1995).

In 1972, the description by Kerr, Wyllie and Currie that cells can undergo an organized suicide program to achieve cell death was a major breakthrough in our understanding of how multicellular organisms develop and achieve homeostasis. Using electron micrographs, the process of cellular death termed apoptosis (Greek origin: apo = from, ptosis = falling) was shown to occur in a defined manner, where a cell dying through programmed cellular suicide displayed several hallmark features in contrast to a cell undergoing unorganized necrotic death. Kerr and colleagues described apoptosis as a non-inflammatory process with characteristic morphological changes including

chromatin condensation, nuclear and DNA fragmentation, and cellular breakdown into defined vesicular bodies that can bleb off and be engulfed by neighboring cells, thus minimizing the inflammatory response (Kerr et al., 1972).

Although the defining morphological events associated with apoptosis were described by Kerr and colleagues, Ellis and Horvitz later revealed that apoptosis was a genetically regulated process using the classical genetic model organism, the nematode worm, *Caenorhabditis elegans*. Using mutants of *C. elegans*, Ellis and Horvitz identified two genes, *ced-3* and *ced-4*, necessary for cell death. Loss of function mutation of *ced-3* or *ced-4* inhibited initiation of cell death, and cells harboring these mutations that normally underwent programmed cell death during development instead remained alive (Ellis and Horvitz, 1986). Subsequent to this finding, Horvitz and colleagues identified an additional *C. elegans* gene, *ced-9*, that functioned to protect cells from undergoing programmed cell death. *C. elegans* mutants deficient in *ced-9* were embryonic lethal, and an activating mutation of *ced-9* promoted survival of cells that normally underwent cell death during nematode development (Hengartner et al., 1992). The discovery that these genetic mutations impacted the development in *C. elegans* strengthened the hypothesis that cell death was critical for normal development in multicellular organisms.

Following the detailed characterization of the genetic regulation of programmed cell death in *C. elegans* by Horvitz and colleagues in the late 1980s, and the identification of the profound homeostatic and developmental implications associated with activation and regulation of cell death, research efforts to understand the genetic and molecular components of apoptosis in higher order species increased substantially. The critical effectors and regulators of cell death were subsequently identified in several organisms

during the 1990s, including the human and mouse orthologs of *ced-3*, *ced-4* and *ced-9*, and our understanding of the intricate intracellular mechanisms to initiate and regulate apoptosis has advanced considerably (Yuan et al., 1993; Tsujimoto et al., 1985; Liu et al., 1996; Zou et al., 1997).

The Apoptotic Pathways

In the current model of apoptosis, there are a variety of stimuli and cellular insults that can induce cell death, including death receptor signaling, ultraviolet light, DNA damage, cellular stress, and chemotherapy (Salvesen and Duckett, 2002). Two major cellular pathways that lead to apoptosis have been described, the extrinsic and the intrinsic pathway (Figure 1.1). Although two pathways exist which can lead to apoptosis, there is substantial overlap of the molecular components utilized in the extrinsic and intrinsic pathways, and these pathways eventually converge at caspase activation (Igney and Krammer, 2002). Furthermore, it has been proposed that the intrinsic or mitochondrial pathway can serve as an amplification loop when initiated following activation of the extrinsic pathway (Kuwana et al., 1998; Yin et al., 1999). However, for the purpose of demonstrating the mechanisms by which apoptosis can be induced, these pathways will be addressed separately, while noting overlapping components and converging points between the two pathways.

The extrinsic pathway involves responses to an intercellular signal or receptor ligand. The primary receptors that respond to intercellular signals to induce cell death are members of the tumor necrosis factor (TNF) receptor superfamily, including TNF

receptor-1 (TNFR-1) and the Fas receptor (also known as Apo-1 or CD95) (Beutler, 1999). Using biochemical analysis, it was determined that both of these TNF receptors contain a signature domain within their cytoplasmic tail. Termed the death domain (DD), and now used to define the apoptotic inducing subset of TNF receptors, this domain functions to provide a docking site for adaptor molecules, including Fas-associated death domain protein (FADD) and TNFR-1-associated death domain protein (TRADD) (Chinnaiyan et al., 1995; Boldin et al., 1995a; Boldin et al., 1995b; Hsu et al., 1996). FADD and TRADD facilitate the induction of receptor-mediated apoptosis by serving as bridges between TNF receptors and cysteine-aspartic acid proteases (caspases), key apoptotic machinery molecules that will be discussed more thoroughly below. The associations of Fas, FADD, and caspase-8 are collectively referred to as the death-inducing signaling complex (DISC), which is a central component of the Fas receptor-mediated cell death-signaling pathway, since association of the DISC complex promotes activation of caspases which leads to execution of cell death (Kischkel et al., 1995).

While the extrinsic pathway of cell death is activated by intercellular ligands and involves signal transduction by a subset of TNF receptors and their associated factors, the intrinsic pathway leading to cell death is activated by numerous intracellular signals. Cellular stress, viral infection and DNA damage result in activation of pro-apoptotic members of the B-cell lymphoma-2 (BCL-2) family BCL-2 homology 3 (BH3)-only proteins (Youle and Strasser, 2008). Activation of BH3-only proteins inhibits the activities of pro-survival members of the BCL-2 family (Willis et al., 2007), which in turn relieves the pro-apoptotic factors BCL-2 –antagonist/killer-1 (BAK) and BCL-2 –associated X protein (BAX) (Cheng et al., 2001). This results in the translocation of

BAX and BAK into the mitochondria and perturbation of the mitochondrial membranes, leading to release of mitochondrial resident pro-apoptotic molecules, including cytochrome *c* and second mitochondria-derived activator of caspase (Smac, DIABLO in mice) as shown in Figure 1.1 (Budihardjo et al., 1999; Du et al., 2000; Hao et al., 2005). Upon release into the cytosol, cytochrome *c* and Smac/DIABLO promote the activation of caspases by mechanisms discussed in more detail below (Du et al., 2000; Hao et al., 2005; Srinivasula et al., 2000; Brustugun et al., 1998; Cain et al., 1999).

Although the DISC is a central component of the Fas-mediated extrinsic pathway, and functions to induce cell death by activating caspases, the DISC can also promote cell death by activating components of the intrinsic pathway (Kuwana et al., 1998; Yin et al., 1999). The DISC promotes cleavage of the pro-apoptotic BH3-interacting domain death agonist (BID). Cleavage of BID to truncated BID (tBID) subsequently results in the derepression of BAK and BAX, activating the intrinsic pathway as outlined in the previous paragraph and shown in Figure 1.1 (Kuwana et al., 1998; Cheng et al., 2001; Li et al., 1998).

Central to the execution of cell death by both the extrinsic and the intrinsic apoptotic pathways, is the activity of caspases. Caspases are cysteine proteases that predominantly cleave their substrates after an aspartate residue within their target (Lavrik et al., 2005). Caspases are encoded as zymogen molecules, and proteolytic cleavage of the zymogen produces an enzymatically active caspase. The *ced-3* gene identified by Ellis and Horvitz was the first gene found to encode a caspase protein. However, caspases are evolutionarily conserved, and 14 mammalian caspases have since been identified (Stennicke and Salvesen, 1998; Nicholson, 1999; Budihardjo et al., 1999; Shi,

2004). Although subsequent biochemical analysis has shown that not all identified caspases have pro-apoptotic functions, the discussion below is limited to the activities of caspases involved in cell death.

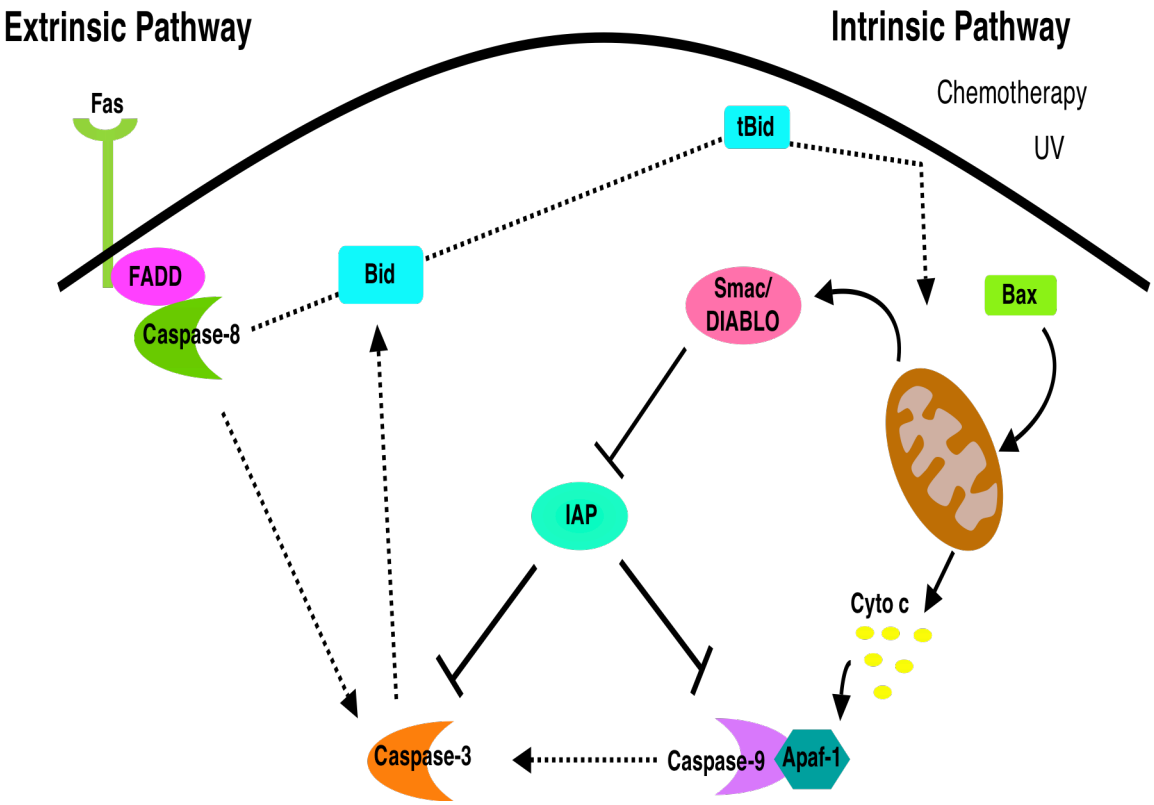

Figure 1.1: The extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is initiated by intercellular signals that activate a subset of pro-apoptotic TNF receptors, including the Fas receptor. Activation of Fas receptor results in the recruitment and activation of caspase-8 through the death-inducing signaling complex (DISC). Caspase-8 subsequently activates caspase-3. The intrinsic apoptotic pathway is initiated by intracellular insults such as chemotherapy or ultraviolet (UV) damage. Activation of the intrinsic pathway involves derepression and translocation of Bax or Bak, which triggers the release of cytochrome *c* and Smac/DIABLO from the mitochondria. Caspase-9 oligomerizes with Apaf-1, cytochrome *c*, and ATP to form the apoptosome, which activates caspase-9. Smac/DIABLO antagonizes IAP proteins, which function to inhibit caspases, thereby freeing caspases from IAP proteins. Caspase-3 is subsequently activated by caspase-9. Inhibitors of apoptosis (IAP) proteins are the most well described direct and indirect inhibitors of caspases and cell death.

Two subsets of caspases, initiator/apical caspases and effector/executioner caspases, have distinct temporal and spatial roles in the cell death pathway. Activation of caspases occurs in a hierarchical manner, whereby initiator caspases are activated first, and executioner caspases are sequentially cleaved by activated initiator caspases (Shi, 2002; Shi, 2004; Thornberry and Lazebnik, 1998). In mammals, caspase-8 and caspase-9 are the primary initiator caspases for the extrinsic and intrinsic pathways, respectively. Activation of caspase-8, occurs when this protein is recruited to the DISC, where the close proximity between caspase-8 molecules is thought to result in their enzymatic activation (Salvesen and Dixit, 1999; Medema et al., 1997; Chinnaiyan et al., 1995; Varfolomeev et al., 1998). Caspase-9 activation occurs in a slightly more complex fashion, through the formation of a holoenzyme, known as the apoptosome, that also includes an adaptor molecule, apoptotic protease-activating factor 1 (Apaf-1), cytochrome *c*, and ATP. The formation of the apoptosome is driven by the release of cytochrome *c* into the cytosol from the mitochondria. Association of procaspase-9 with this protein complex promotes oligomerization of procaspase-9, which produces active caspase-9 (Acehan et al., 2002; Zou et al., 1999; Cain et al., 2000; Cain et al., 1999; Rodriguez and Lazebnik, 1999).

Caspase-3 and caspase-7 are effector caspases common to both the intrinsic and extrinsic pathway. Once caspase-8 or caspase-9 has been activated, downstream executioner caspases, such as caspase-3 and -7, can be targeted and activated through cleavage by activated initiator caspases. Activation of executioner caspases ultimately lead to the gross morphological changes that characterize apoptosis, including DNA fragmentation and dissociation of the cytoskeletal structure. Executioner caspases have

diverse substrates with unique functions and their cleavage collectively compromise the integrity of the cell. For example, poly(ADP-ribose) polymerase (PARP), which is abundantly present in the nucleus and catalyzes poly(ADP-ribose) ligation following DNA strand breaks, is a substrate of caspase-3 and -7. Caspase-3 and -7 cleave PARP, which prevents ADP-ribose polymer synthesis in response to DNA damage (Kang et al., 2004). Additionally, cleavage by caspase-3 of the chaperone/inhibitor protein, inhibitor of caspase-activated deoxyribonuclease (ICAD), releases the nuclease, caspase-activated deoxyribonuclease (CAD), which is responsible for cleaving double-stranded DNA into oligonucleosomal fragments (Enari et al., 1998).

Inhibitors of Apoptosis

While there are many cellular insults and stimuli that activate cell death pathways, it was discovered that cells retain an intricate regulatory mechanism to control induction and execution of cell death. This is especially important, as unchecked cell death could be detrimental to the proper functioning of a complex multicellular organism (Salvesen and Duckett, 2002). Thus, it is not surprising that caspases are normally present in the inactive zymogen form. However, beyond regulating the activities of pro-apoptotic molecules such as caspases, it was hypothesized that the relative expression of effectors and regulators of cell death might also regulate the induction and execution of cell death. Thus, a cell that has acquired DNA damage or undergone cell cycle arrest might have an altered apoptotic threshold based on the presence and activity levels of apoptotic effectors and regulatory proteins, which would allow a damaged or stressed cell the ability to

recover, if cellular mechanisms exist to repair the damage or properly remove the insult. Based on this hypothesis, the stoichiometry of active pro- and anti-apoptotic molecules would also be a critical factor in determining the apoptotic threshold, and the activation of pro-apoptotic molecules might not be sufficient to induce and/or execute apoptosis (Salvesen and Duckett, 2002).

Supporting the hypothesis that cellular mechanisms exist to regulate the induction and execution of cell death, two major classes of anti-apoptotic proteins were identified, the BCL-2 protein family (introduced previously) and the inhibitor of apoptosis (IAP) protein family (Youle and Strasser, 2008; Salvesen and Duckett, 2002; Srinivasula and Ashwell, 2008). Members of these two protein families can regulate cell death through distinct mechanisms, and due to the context of this dissertation, the BCL-2 pro-survival family will only be discussed briefly.

The BCL-2 family, as reviewed by Youle & Strasser, contains at least 14 mammalian pro- and anti-apoptotic proteins that regulate cell death. There are five pro-survival BCL-2 family members, including BCL-2 and BCL-X_L, that function to repress the activities of pro-apoptotic BCL-2 family members, BAX and BAK, which in turn are capable of perturbing the outer mitochondrial membrane, as described above. Therefore, suppression of apoptosis by BCL-2 members occurs relatively early in the cell death signaling cascade, prior to formation of the apoptosome or activation of caspases (Youle and Strasser, 2008).

In 1993, Miller and colleagues identified a baculoviral gene, inhibitor of apoptosis (*iap*) that was capable of preventing cell death of infected insect cells (Crook et al., 1993). The observation that this viral gene encoded a protein that could inhibit apoptosis

supported that the intracellular mechanisms to achieve cell death are very tightly regulated, and that cellular insults might not always result in cell death, depending on the expression levels of cell death inhibitors. Following the identification of the baculovirus *iap* gene, *iap* genes were found to be highly conserved and present in the genomes of yeast, nematodes, fruit flies, mice, and humans (Duckett et al., 1996; Liston et al., 1996; Roy et al., 1995; Uren et al., 1996; Rothe et al., 1995a; Hay et al., 1995; Fraser et al., 1999; Uren et al., 1999). To date, eight mammalian IAP proteins have been identified (Figure 1.2). The best characterized of the mammalian IAP proteins are X-linked IAP (XIAP), cellular IAP 1 (c-IAP1) and cellular IAP 2 (c-IAP2).

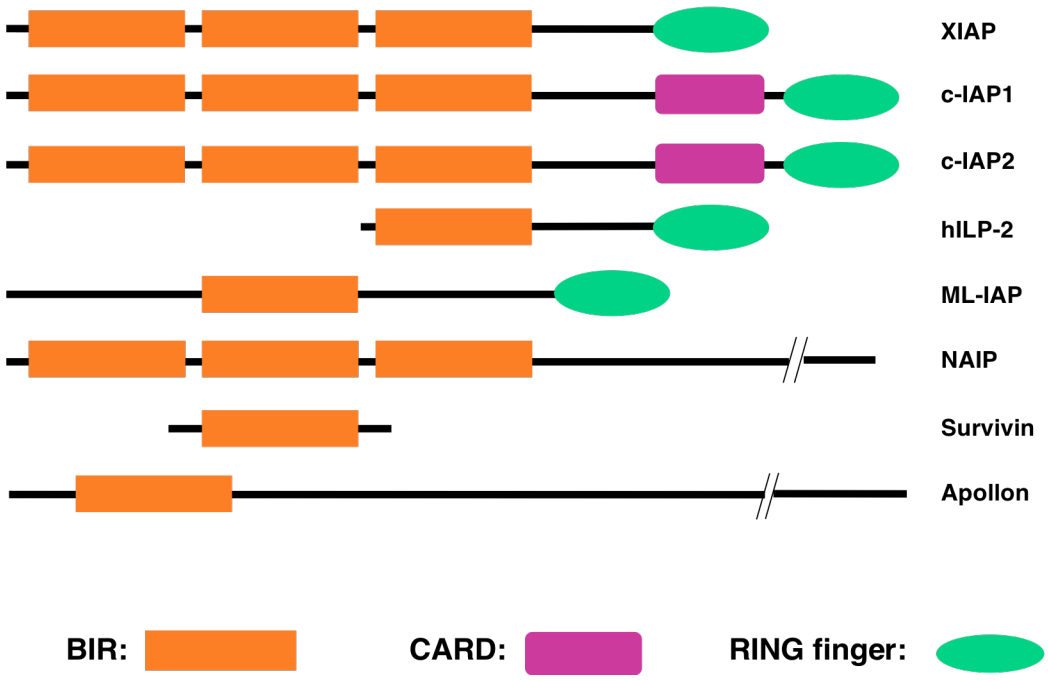

Figure 1.2: Schematic representation of mammalian Inhibitor of Apoptosis (IAP) proteins. BIR; baculovirus IAP repeat; CARD; caspase-recruitment domain; RING finger; really interesting new gene finger.

Inclusion in the IAP family is determined by the presence of a conserved ~70 amino acid zinc binding motif, the baculoviral IAP repeat (BIR) domain (Miller, 1999; Hinds et al., 1999; Sun et al., 1999). Each IAP protein has at least one BIR domain, and

many IAP proteins have two or three BIR domains in tandem at the amino-terminal region of the protein. The signature sequence in BIR domains $CX_2CX_{16}HX_6C$ (C = cysteine, H = histidine, X = any amino acid) coordinates a zinc ion at the core of the three β sheets formed by the BIR sequence (Hinds et al., 1999; Sun et al., 2000; Sun et al., 1999; Verdecia et al., 2000).

The principal function of the BIR domains in IAP proteins is to mediate protein-protein interactions. For example, the BIR2 domain region of XIAP coordinates interactions between XIAP and caspase-3, caspase-7, as well as another pro-apoptotic molecule released from the mitochondria, apoptosis-inducing factor (AIF) (Eckelman et al., 2006; Wilkinson et al., 2008). Meanwhile, BIR1 within c-IAP1 coordinates the association of c-IAP1 with the TNF signaling adaptor molecule, TNF-receptor-associated factor-2 (TRAF2) through the PXXER motif (P = proline, E = glutamic acid, and R = arginine) (Samuel et al., 2006; Varfolomeev et al., 2007). Interestingly, a correlation was noted between the number of BIR domains present in an IAP protein, and the affinity of that IAP protein for a given interaction partner or the number of potential interaction partners (Srinivasula and Ashwell, 2008). As the number of BIR domains increases, the affinity for associated proteins increases and/or the number of potential binding partners increases.

Although the presence of a BIR domain is the defining structural characteristic of an IAP protein, many IAP proteins contain additional domains. A really interesting new gene (RING) domain is present at the carboxy-termini of several IAP proteins, including XIAP, c-IAP1 and c-IAP2. The RING domains in IAP proteins are zinc-binding motifs contain six to seven cysteines and one or two histidines, which coordinate the binding of

two zinc ions to the RING domain (Joazeiro and Weissman, 2000). RING domains harbor E3 ubiquitin ligase activity that catalyzes the ubiquitination and degradation of target proteins through a sequential enzymatic reaction that also involves an ubiquitin activating enzyme (E1) and an ubiquitin conjugating enzyme (E2) (Joazeiro and Weissman, 2000; Lorick et al., 1999; Weissman, 2001). IAP proteins can undergo autoubiquitination and several targets of ubiquitination by IAP proteins have also been reported (Dohi et al., 2004; Conze et al., 2005; Silke et al., 2005). The role of ubiquitination in IAP protein stability and the consequences of interactions with intracellular signaling molecules is currently a major area of research.

Finally, c-IAP1 and c-IAP2 also contain a caspase-associated recruitment domain (CARD). The single CARD present in both c-IAP1 and c-IAP2 is located between the BIR3 and RING domains. CARDs are generally thought to function in heterodimerization between adaptor molecules and procaspases. However, the CARDs of c-IAP1 and c-IAP2 have not been as fully characterized as the BIR and RING domains, and the functions are currently unknown (Verhagen et al., 2001).

Anti-Apoptotic Functions of IAP Proteins

Since the discovery in 1993 that the presence of the baculoviral gene *iap* inhibited cell death in infected insect cells tremendous efforts have been made to determine the mechanisms by which IAP proteins protect against cell death. Presently, a significant collection of structural, biochemical, and *in vivo* research from numerous laboratories provides great insight into how different IAP proteins inhibit apoptosis (Shi, 2004). In

the current functional model, there are several mechanisms, both caspase-dependent and caspase-independent, that IAP proteins utilize to inhibit cell death.

XIAP is the prototypical IAP protein that binds directly to caspases through its BIR domains and inhibits the enzymatic activities of several caspases, including caspase-3, -7, and -9 (Eckelman et al., 2006; Shiozaki and Shi, 2004; Takahashi et al., 1998). Because of its ability to directly inhibit several chief caspases, XIAP is considered a potent cell death inhibitor. XIAP protective activitiy against cell death was found to be over 1000 fold greater than that of c-IAP1 or c-IAP2 (Deveraux et al., 1998).

Whereas XIAP binds to and inhibits the enzymatic activities of several caspases, c-IAP1 and c-IAP2 have subsequently been shown to bind to but not inhibit the enzymatic activities of caspases (Eckelman and Salvesen, 2006). Therefore, it was hypothesized that c-IAP1 and c-IAP2 might instead function to inhibit cell death by alternative mechanisms. The prototypical baculoviral IAP expressed by *Orgyia pseudotsugata* nucleopolyhedrosis virus (Op*M*NPV, termed OpIAP) was also found to be incapable of binding all tested caspases, but still inhibited cell death (Wilkinson et al., 2004; Wright and Clem, 2001). These findings supported the hypothesis that not all IAP proteins protect against cell death by directly inhibiting caspases.

Subsequent to the discoveries that several IAP proteins did not inhibit the enzymatic functions of caspases, but could still provide protection against cell death, biochemical studies identified IAP interacting proteins, second mitochondrial activator of caspases (Smac) in humans and direct IAP binding protein with low pI (DIABLO) in mice (Du et al., 2000; Verhagen et al., 2000). Smac/DIABLO is a 239 amino acid protein that is nuclear encoded, and contains a 55 amino acid mitochondrial localization

signal (MLS) at its amino-terminus. Therefore, Smac/DIABLO is shuttled into the inter-membrane space of the mitochondria after translation. Upon arrival in the mitochondria, the MLS is cleaved from Smac/DIABLO, revealing an IAP-binding motif (IBM) at the amino-terminus. The IBM of Smac/DIABLO is an evolutionarily conserved tetrapeptide also present in *Drosophila melanogaster* orthologs of Smac/DIABLO (Wing et al., 2001; Wright and Clem, 2001; Wu et al., 2001).

As described earlier, following perturbation of mitochondria induced by cellular stress or damage, mature Smac/DIABLO is released into the cytosol, along with cytochrome *c*, where it functions as a direct IAP inhibitor by binding to IAP proteins through the IBM motif (Vaux and Silke, 2003; Yang and Du, 2004). Therefore, Smac/DIABLO functions to negatively regulate the inhibitory properties of IAP proteins by displacing IAP proteins bound to caspases, freeing caspases to become activated, and targeting IAP proteins for ubiquitination and proteasome-mediated degradation (Liu et al., 2000; Wu et al., 2000; Srinivasula et al., 2000; Srinivasula et al., 2001; Chai et al., 2000). Since its identification, Smac/DIABLO has been shown to associate with several IAP proteins, including XIAP, c-IAP1, c-IAP2, and OpIAP (Yang and Du, 2004; Wilkinson et al., 2004; Liu et al., 2000; Wu et al., 2000). Mutation or deletion of residue one of the IBM motif, alanine, abrogates binding of Smac/DIABLO to IAP proteins and prevents caspase-3 activation (Liu et al., 2000; Wu et al., 2000). In addition to the direct inhibitory activity of Smac/DIABLO it has been shown that Smac/DIABLO can induce the autoubiquitination of several IAP proteins. However, the converse has also been observed, and XIAP, c-IAP1 and c-IAP2 have all been shown to target Smac/DIABLO

for ubiquitination and proteasome-mediated degradation (MacFarlane et al., 2002; Hu and Yang, 2003).

Given that Smac/DIABLO associates with several IAP proteins that do not bind to or inhibit the activities of caspases, it has been proposed that these IAP proteins function to sequester mature Smac/DIABLO after release from the mitochondria. Sequestering Smac/DIABLO may serve as a regulatory mechanism to allow damaged cells to recover, even when pro-apoptotic proteins have been released or activated, and signifies a dynamic regulatory process. It remains to be tested whether by preventing Smac/DIABLO from antagonizing direct caspase inhibitors such as XIAP these other IAP proteins are displaying an alternative mechanism of cellular protection through sequestration and saturation of mature Smac/DIABLO.

Surprisingly, given its negative regulatory effects on IAP proteins, Smac-deficient cells did not display significant defects in cell death when challenged with apoptotic stimuli (Okada et al., 2002). A splice variant of Smac has also been identified that lacks the IBM and is localized to the cytosol (Roberts et al., 2001). Although this variant of Smac lacks the IBM, it can still potentiate cell death. This finding suggests that although full-length Smac/DIABLO functions to negatively regulate IAP proteins through the IBM, other regions of Smac/DIABLO might serve important cellular functions. One such possibility is that the interaction between Smac/DIABLO and IAP proteins could alter the function of IAP proteins in alternative signaling pathways, since it has now been well established that several IAP proteins, including c-IAP1 and c-IAP2, function in additional or alternative pathways (Rothe et al., 1995a; Srinivasula and Ashwell, 2008).

Non-Apoptotic Functions of IAP Proteins

While many IAP proteins have been shown to function to inhibit cell death, not all members of the IAP family inhibit apoptosis (Srinivasula and Ashwell, 2008; Verhagen et al., 2001). Several IAP proteins are instead referred to as BIR-containing proteins (BIRPs), because although they contain the signature IAP domain, they do not protect against cell death (Srinivasula and Ashwell, 2008; Verhagen et al., 2001). These IAP/BIRPs are present in yeast and *C. elegans*, and instead function in alternative cellular processes, such as cell division or cytokinesis (Fraser et al., 1999).

Furthermore, as reviewed extensively by Srinivasula and Ashwell, it is now clear that many members of the IAP family that can function as cell death inhibitors are critical to additional, non-apoptotic signaling pathways. While these proteins were categorized in the IAP family because they contain the signature BIR domain and their given name implies that their primary function is to protect against death-inducing stimuli, an increasing number of IAP proteins play critical roles in additional intracellular pathways, many of which are unrelated to apoptosis. IAP proteins have now been shown to function in copper homeostasis, cell division, innate immunity, and other intracellular signaling pathways (Burstein et al., 2004; Srinivasula and Ashwell, 2008; Conte et al., 2006; Varfolomeev et al., 2007; Vince et al., 2007; Tang et al., 2003; Petersen et al., 2007; Altieri, 2003).

The potent apoptosis suppressor, XIAP, has been shown to function in additional signaling pathways. Burstein et al. discovered that XIAP functions in copper homeostasis, when XIAP was found to associate with copper metabolism gene MURR1

domain containing 1 (COMMD1), a factor required for proper copper excretion. XIAP was subsequently shown to target COMMD1 for ubiquitination and degradation, resulting in a predicted increase in intracellular copper levels. Although it was found that BIR3 of XIAP mediated the interaction between XIAP and COMMD1, the E3 ubiquitin ligase activity in the RING domain in XIAP was found to catalyze the ubiquitination and proteasomal degradation of COMMD1 (Burstein et al., 2004).

The cellular IAP proteins, c-IAP1 and c-IAP2, were identified in a biochemical screen for type-2 TNF receptor (TNFR2) associated proteins (Rothe et al., 1995a). The association of c-IAP1 and c-IAP2 with TNFR2 was found to be mediated by the TNF receptor associated factors 1 and 2 (TRAF1 and TRAF2) . The interaction between c-IAP1/2 and TRAF2, as noted previously, is mediated by BIR1 in c-IAP1/2 (Samuel et al., 2006). However, at the time of the discovery that c-IAP1 and TRAF2 associate, the consequence of this association was thought to only affect transcriptional activation, not inhibition of apoptosis, since activation of TNFR2 regulates induction of nuclear factor-κB (NF-κB) transcription factors (Li et al., 2002; Rothe et al., 1995a; Rothe et al., 1995b).

NF-κB activation is critical for immune responses, cell survival and proliferation, and has been extensively reviewed by Hayden and Ghosh. NF-κB subunits are normally sequestered in the cytosol but upon a variety of stimuli, including ligation of TNF receptor family members, NF-κB subunits can translocate into the nucleus and activate transcription of NF-κB responsive genes. Two major NF-κB activation pathways have been described, the canonical and the alternative pathway (Figure 1.3). In the canonical pathway, phosphorylation and subsequent degradation of inhibitors of NF-κB (IκBs)

leads to translocation of NF-κB subunits into the nucleus. In the alternative pathway, following activation of NF-κB inducing kinase (NIK), p100/NF-κB2 is proteolytically processed to p52, which translocates into the nucleus (Hayden and Ghosh, 2004; Hayden et al., 2006; Hayden and Ghosh, 2008).

While the TRAF2-c-IAP1/2 interaction was identified over a decade ago, the precise role of this complex in TNF signaling has remained unclear. Initial reports suggested that c-IAP1 might have a pro-apoptotic role in TNFR2 signaling by targeting TRAF2 for ubiquitination and degradation following TNFR2 activation by TNF (Li et al., 2002). Given the prior finding that TRAF2 mediates NF-κB activation by TNF receptors (Rothe et al., 1995b), it was also hypothesized that c-IAP1 might alternatively be targeting other NF-κB regulatory molecules, including the DD-containing serine/threonine kinase, receptor interacting protein kinase 1 (RIP1), NF-κB essential modulator (NEMO), or NIK. Furthermore, the consequences of the interaction between TRAF2 and c-IAP1 on c-IAP1 stability currently remain unclear. However, given that the RING domains of TRAF proteins have been found to modulate protein stability by ubiquitination, and association of c-IAP1 with TRAF2 might also redirect the autoubiquitination properties of c-IAP1, this association might be critical to understanding how c-IAP1 functions in apoptotic and non-apoptotic signaling cascades.

IAP Proteins & Cancer

The evasion of apoptosis has been shown to be a hallmark of tumorigenesis, and many cancer therapy strategies, including chemotherapy, function by inducing apoptosis

of cancer cells (Brown and Attardi, 2005). Because several IAP proteins were found to be oncogenic and highly expressed in numerous neoplasms, it was thought that IAP proteins might confer resistance to apoptotic induction for cancer cells by inhibiting caspase activity (Fulda, 2007; Zender et al., 2006; Hunter et al., 2007; Altieri, 2003; Duffy et al., 2007). Therefore, as described by Wright and Duckett, several labs pursued designing compounds to target cancer cells to undergo apoptosis. Specifically, RNA interference and antagonist compounds were designed to target several pro-survival molecules, including specific IAP proteins and anti-apoptotic BCL-2 family members (Wright and Duckett, 2005). Indeed, anti-sense oligonucleotides directed against XIAP can sensitize cancer cells to chemotherapy and ligand-induced death, and currently there are a host of IAP inhibitors that are in clinical trials (Fulda, 2007; Hunter et al., 2007; LaCasse et al., 2006; Cummings et al., 2006).

Although several IAP proteins were observed to be highly expressed in forms of neoplasia, a signature chromosomal translocation involving the gene that encodes c-IAP2 also occurs in at least 33% of extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphomas). MALT lymphomas usually arise in the stomach in the context of chronic *Helicobacter pylori* infections. A fusion protein between the amino-terminal region of c-IAP2 and the carboxy-terminal region of mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1) is typically observed (Dierlamm et al., 1999; Uren et al., 2000). This translocation event is associated with a more aggressive tumor that does not respond to *H. pylori* eradication. The fusion protein is a potent activator of NF-κB by inducing self-oligomerization and increasing the E3 ubiquitin ligase activity of MALT1 on NEMO, resulting in IKK

activation (Varfolomeev et al., 2006; Zhou et al., 2005). The MALT1 portion of the c-IAP2/MALT1 fusion was shown to trigger the cleavage of A20, a deubiquitinating enzyme that is an inhibitor of NEMO, thus promoting NF-κB activation (Coornaert et al., 2008). Therefore, this finding supported the idea that IAP proteins might be supporting tumorigenesis by alternative or additional mechanisms beyond inhibition of cell death, however many possibilities are currently untested.

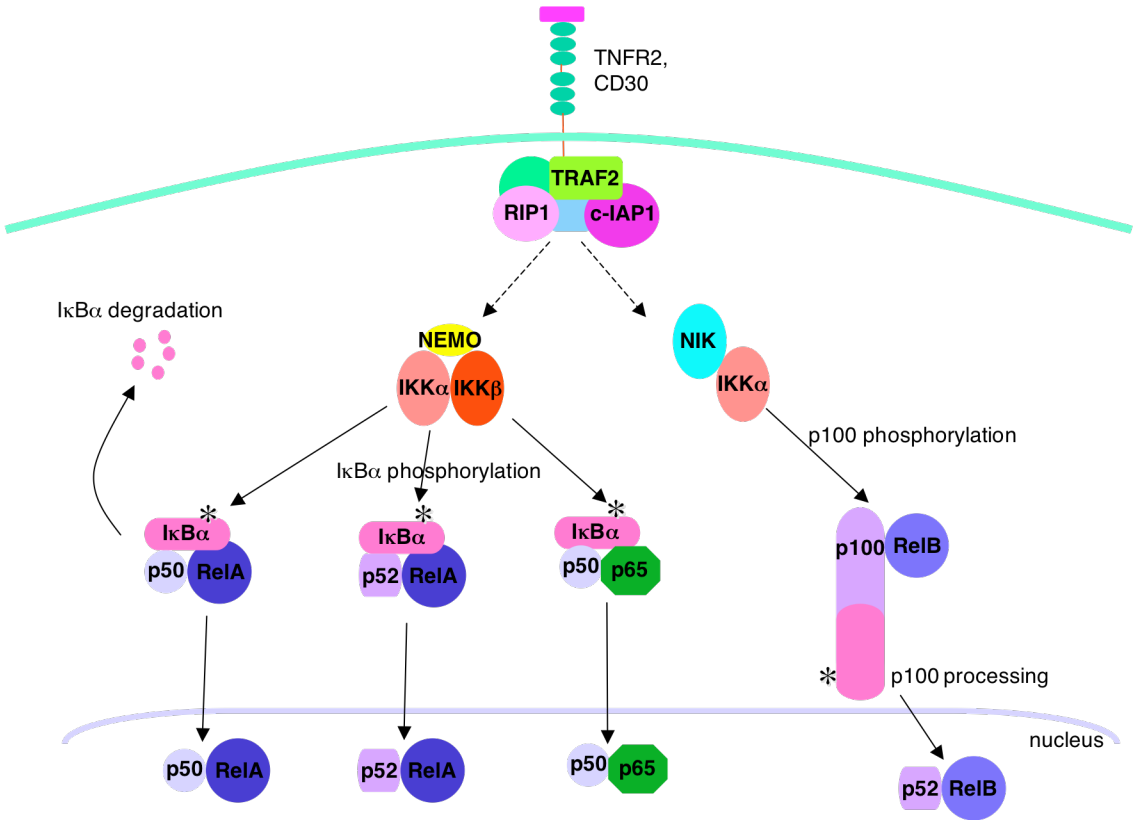

Figure 1.3: Activation of the canonical and alternative NF-κB pathways by TNF receptors. In the canonical NF-κB pathway, inhibitors of NF-κB (IκBs) are phosphorylated and degraded, freeing NF-κB subunits to translocate into the nucleus. In the alternative NF-κB pathway, activation of NIK leads to processing of p100/NF-κB2 to p52, which translocates into the nucleus. *= phosphorylation

An alternative to specific IAP targeted therapeutics was to design a compound that could interact with multiple IAP proteins. Therefore, numerous laboratories and pharmaceutical companies have designed synthetic, which could recapitulate the binding

properties of mature Smac/DIABLO. Several successful synthetic derivatives of Smac/DIABLO have been developed to target the IAP proteins and induce cell death by relieving IAP inhibition of caspases (Vucic and Fairbrother, 2007; Fischer and Schulze-Osthoff, 2005). Therefore, these tetrapeptide compounds were originally developed to promote apoptosis in cancer cells that may have elevated levels of IAP proteins, conferring a protective phenotype. However, the cellular impact of these IAP antagonist is much more profound than previously expected, since it was originally hypothesized that IAP proteins solely functioned to protect against cell death by inhibiting caspase activities. Additional novel roles in intracellular signaling pathways have been identified which can modulate sensitivity to cell death stimuli (Varfolomeev et al., 2007; Vince et al., 2007; Petersen et al., 2007).

Dissertation Objectives

The goal of this dissertation was to characterize the cellular roles of c-IAP1 and its regulation by Smac/DIABLO or through signaling of TNF receptors. I investigated how association of c-IAP1 with TRAF2 and Smac/DIABLO affected the stability of c-IAP1, and the consequence of these events on cell death and NF-κB activation with the following specific aims:

Specific Aim I (Chapter 2): Determine the mechanism of c-IAP1 stabilization by TRAF2, and identify the cytoprotective effects of TRAF2-stabilized c-IAP1.

Specific Aim II (Chapter 3): Describe the two distinct signaling cascades that target the NF-κB regulatory factor c-IAP1 for degradation.

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Chapter II

Enhanced Cytoprotective Effects of the IAP Protein, c-IAP1, Through Stabilization with TRAF2

Summary

Inhibitor of apoptosis (IAP) proteins are key regulators of intracellular signaling that interact with tumor necrosis factor (TNF) receptor superfamily members as well as pro-apoptotic molecules such as Smac/DIABLO and caspases. While the X-linked IAP (XIAP) is an established caspase inhibitor, the protective mechanisms utilized by the cellular IAP (c-IAP) proteins are less clear, since c-IAPs bind to but do not inhibit the enzymatic activities of caspases. In this study, c-IAPs are shown to be highly unstable molecules that undergo autoubiquitination. The autoubiquitination of c-IAP1 is blocked upon association with TNF receptor-associated factor TRAF2, and this is achieved by inhibition of the E3 ubiquitin ligase activity intrinsic to the RING of c-IAP1. Consistent with these observations, loss of TRAF2 results in a decrease in c-IAP1 levels. Stabilized c-IAP1 was found to sequester and prevent Smac/DIABLO from antagonizing XIAP, and protect against cell death. Therefore, this study describes an intriguing cytoprotective mechanism utilized by c-IAP1, and provides critical insight into how IAP proteins function to alter the apoptotic threshold.

Introduction

The *Inhibitors of Apoptosis* (IAPs) are an evolutionarily conserved gene family originally described as encoding cell death inhibitors. IAP proteins have subsequently been found to participate in a variety of additional intracellular signaling processes (Srinivasula and Ashwell, 2008), and it has become evident that IAP proteins are versatile molecules playing numerous distinct roles within the cell. While a more complete understanding of these additional functions for IAP proteins is emerging, the distinct mechanisms utilized by some IAP proteins to function in their originally defined roles as cell death inhibitors remain unclear.

Members of the IAP family are characterized by the presence of one to three tandem repeats of an ~70 residue baculovirus IAP repeat (BIR) domain (Eckelman and Salvesen, 2006). The BIR domains of many IAP proteins have been shown to be the region within IAP proteins that associates with caspases and other pro-apoptotic molecules (Sun et al., 1999; Deveraux et al., 1999). IAP proteins have remarkably different apoptotic inhibitory abilities. For example, X-linked IAP (XIAP) is a highly potent cell death inhibitor (Deveraux and Reed, 1999), and is thought to be the only mammalian IAP protein that directly inhibits the enzymatic activities of caspases (Eckelman and Salvesen, 2006; Sun et al., 1999; Deveraux et al., 1999; Tenev et al., 2004). While cellular IAP1 and 2 (c-IAP1 and c-IAP2) are anti-apoptotic proteins that can bind to caspase-7 and -9, they do not inhibit the enzymatic activities of these caspases (Eckelman and Salvesen, 2006; Tenev et al., 2004).

Many IAP proteins, including c-IAP1 and c-IAP2, contain a carboxy-terminal RING domain that can function as an E3 ubiquitin ligase (Yang et al., 2000). The E3 ubiquitin ligase activity of the RING domain in c-IAP1 and c-IAP2 was previously shown to negatively regulate the apoptotic inhibitory properties of the c-IAP proteins, and to promote autoubiquitination and degradation of c-IAP1 (Clem et al., 2001; Yang and Du, 2004), thus hindering attempts to define the cellular properties of this protein.

A specialized property of the c-IAP proteins is their involvement in tumor necrosis family (TNF) signaling (Shu et al., 1996; Rothe et al., 1995a; Li et al., 2002). Both c-IAP1 and c-IAP2 were discovered in a biochemical screen for factors associated with the type-2 TNF receptor (TNFR2). This association was found to be indirect and bridged by interactions with TNF receptor-associated factors (TRAFs), most notably TRAF1 and TRAF2 (Rothe et al., 1995a). Though the consequences of the association between TRAF2 and c-IAP1 on TNF-mediated signaling have been investigated (Li et al., 2002), less is known about the functional significance of the association between TRAF2 and c-IAP1 on cell death inhibition. Since both c-IAP1 and TRAF2 possess E3 ubiquitin ligase activity in their respective RING domains, it seemed that the association between these molecules might impact the protective properties of c-IAP1 and alter the apoptotic threshold.

In this study, the role of TRAF2 in c-IAP1 stability and how the association of TRAF2 with c-IAP1 affects the apoptotic inhibitory properties of c-IAP1 were examined. The presence of TRAF2 greatly enhanced the stability of c-IAP1, and these data suggest that the interaction between TRAF2 and c-IAP1 inhibits the E3 ubiquitin ligase activity intrinsic to the RING domain of c-IAP1. Using stabilized c-IAP1, the anti-apoptotic

activity of c-IAP1 was characterized, and it was found that c-IAP1 suppresses apoptosis to a degree comparable to XIAP. Furthermore, we show that c-IAP1 functions to prevent the IAP antagonist, Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000), from interfering with XIAP inhibition of caspases. Together, this study demonstrates that although c-IAP1 does not directly inhibit caspase activity, stabilized c-IAP1 can sequester Smac/DIABLO, prevent Smac/DIABLO from antagonizing XIAP, and inhibit cell death.

Experimental Procedures

Materials: Reagents were obtained from the following sources: MG-132 (Sigma), protein G-coupled agarose, L-glutamine, and PBS (Invitrogen), DMSO (Sigma Aldrich), Dulbecco's modified Eagle's medium and fetal bovine serum (Mediatech), siRNA oligonucleotides (Xeragon/Qiagen), Caspase assay kit (BIOSOURCE), DEVD-AFC (BioMol), protease inhibitor mixture tablets (Complete mini) (Roche), and QuikChange site-directed mutagenesis kit (Stratagene). Dr. Tak Mak (University of Toronto) kindly provided TRAF2-deficient MEFs (Yeh et al., 1997). Antibodies were obtained from the following sources: HA (Covance), β-actin and peroxidase conjugated anti-HA (Sigma), TRAF2 (BD Pharmingen), XIAP (BD Transduction), and Smac/DIABLO (Calbiochem), peroxidase conjugated anti-mouse, anti-rabbit, and anti-rat (GE Healthcare). Dr. John Silke (La Trobe University, Melbourne) kindly provided anti-c-IAP1 (Vince et al., 2007).

Plasmids: pEBB HA-c-IAP1 and pEBB HA-c-IAP2 were subcloned from pEBG c-IAP1 and pEBG c-IAP2, respectively (Duckett et al., 1998). Site-directed mutagenesis of c-IAP1 and c-IAP2 to generate c-IAP1 H588A and c-IAP2 H574A was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Unless otherwise noted, plasmids and siRNA oligonucleotides used for this study have been described previously (Duckett et al., 1998; Duckett and Thompson, 1997; Wilkinson et al., 2004).

Cell Culture, Transfections and MG-132 Treatment: HEK293 cells and MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine and maintained at 37 ˚C in an atmosphere of 95% air, 5% CO_2. Cells were transfected with plasmids and siRNA oligonucleotides using a standard calcium phosphate transfection protocol. HEK293 cells were treated with 35 μM MG-132 for 4 hours. Following treatment with MG-132, cells were harvested and immunoblot analysis was carried out as described below.

Caspase Activity Assays: Adherent and floating cells were harvested and caspase-3 assays were performed using the Caspase assay kit (BIOSOURCE) according to the manufacturer's instructions. AFC released was measured overtime at 37 ˚C using a Cytoflour 4000 multi-well plate reader (Applied Biosystems). A total of 20 measurements at 90-sec intervals were taken for AFC release with an excitation wavelength = 400 nm and an emission wavelength = 508 nm.

Cell Lysate Preparation and Immunoblot Analysis: Cell lysates were prepared with RIPA lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors, for 30 min on ice to ensure complete lysis unless noted otherwise. Protein quantification was determined by Bradford assay (Bio-Rad). RIPA cell lysates of equal protein concentrations were prepared in LDS sample buffer, separated on denaturing NuPAGE 4-12% polyacrylamide gradient gels, and transferred to 0.45 μm nitrocellulose membranes (Invitrogen). Membranes were blocked in 5% milk in Tris buffered saline (TBS) with 0.02 - 0.2% Tween 20 (Bio-Rad) depending on the antibody requirements, followed by incubation with the indicated antibodies in 2.5% milk for 1 hour at room temperature or overnight at 4 ˚C. After washing with TBS containing 0.02 - 0.2% Tween, membranes were incubated with secondary antibodies for 1 hour at room temperature. Enhanced chemiluminescence (GE Healthcare) was used to visualize the blots on Kodak XAR film.

Immunoprecipitations: Cell lysates were prepared in RIPA lysis buffer, normalized for protein content, and incubated with HA or XIAP antibodies for 2 hours at 4 ˚C. Protein G-coupled agarose beads were then added to the lysates and incubated for an additional 1 hour. Centrifugation was performed to recover agarose beads, followed by washing in RIPA lysis buffer. Precipitated proteins were eluted by adding LDS sample buffer (Invitrogen) and heating the samples for 10 min at 95 ˚C. Recovered proteins were subsequently separated by electrophoresis, and immunoblot analysis was performed as described above.

Real Time Reverse Transcription-PCR: HEK293 cells were transfected with plasmids, and twenty-four hours following transfection total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer's instructions. Reverse transcription with random hexamer primers and MultiScribe™ Reverse Transcriptase (Applied Biosystems) was performed on 100 ng of total RNA. The indicated target assays were performed on 1 μL of resulting cDNA. Each target assay was performed in triplicate and normalized to glyceraldehyde 3-phosphate dehydrogenase.

Results

c-IAP1 levels are reduced in TRAF2-deficient cells

Previous studies have shown that the cellular signaling regulators, c-IAP1 and c-IAP2, can interact with TNF receptor superfamily members, and that an association with TRAF proteins, primarily TRAF1 and TRAF2, mediates this interaction (Li et al., 2002; Rothe et al., 1995a; Shu et al., 1996). Although the interaction between c-IAP1 and TRAF2 was subsequently described to result in c-IAP1-mediated ubiquitination and degradation of TRAF2 (Li et al., 2002), less is known about the functional consequences of the TRAF2:c-IAP1 interaction on c-IAP1 expression and cellular properties of c-IAP1.

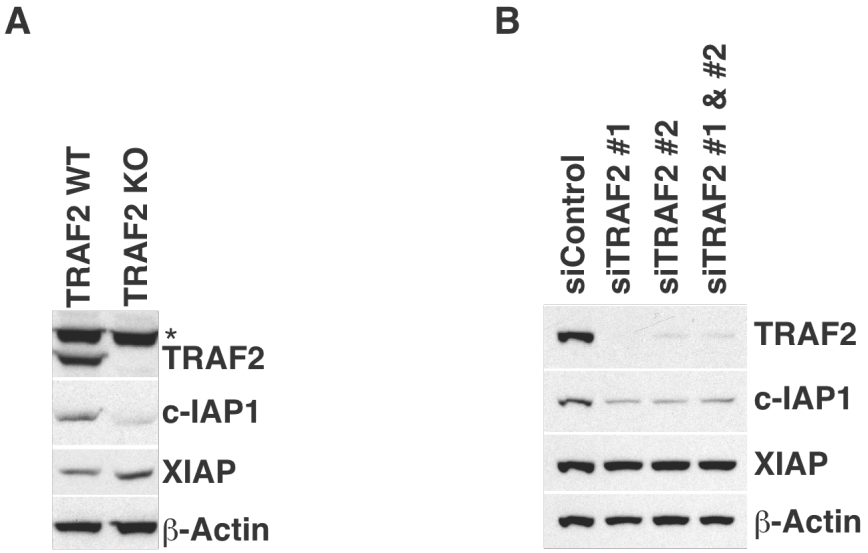

Figure 2.1: Reduction of c-IAP1 in TRAF2-deficient cells. (A) TRAF2-deficient MEFs were harvested and immunoblot analysis was performed for endogenous TRAF2, c-IAP1 and XIAP. The *asterisk* indicates a nonspecific protein recognized by the TRAF2 antibody. (B) HEK293 cells were transfected with double-stranded scrambled siRNA oligonucleotides or two different siRNA oligonucleotides targeting TRAF2. Forty-eight hours following transfection, cells were harvested and immunoblot analysis was performed for endogenous TRAF2, c-IAP1 and XIAP. Equivalent protein loading was confirmed in A and B by immunoblotting for β-actin.

To further characterize the interaction between c-IAP1 and TRAF2, we examined how loss of TRAF2 might affect c-IAP1 expression. Interestingly, in TRAF2-deficient murine embryonic-derived fibroblasts (MEFs) a notable and specific reduction in endogenous c-IAP1 protein levels was observed, while levels of other proteins, including XIAP, remained unchanged (Figure 2.1A). This finding suggested that TRAF2 might play a role in the regulation of c-IAP1 protein levels.

Given the observed reduction in endogenous c-IAP1 protein levels in TRAF2-deficient fibroblasts, we sought to determine whether a reduction of TRAF2 in human

cells would affect c-IAP1 using an RNA interference (RNAi) approach. Two small interfering RNA (siRNA) oligonucleotides for TRAF2 were used individually or in combination to suppress TRAF2 protein levels in human embryonic kidney (HEK) 293 cells (Figure 2.1B). Examination of c-IAP1 protein levels in the TRAF2 suppressed HEK293 cells revealed also a reduction in endogenous c-IAP1 levels. Similarly, other IAP proteins, such as XIAP, were unaffected by a reduction in TRAF2 levels (Figure 2.1B). These data suggest that the interaction between c-IAP1 and TRAF2 alters the regulation of c-IAP1 protein levels.

c-IAP1 and c-IAP2 undergo continuous basal autoubiquitination

Since the stability of c-IAP1 appeared to be dependent on TRAF2, we sought to compare the relative expression levels of several IAP proteins. Plasmids encoding XIAP, c-IAP1 and c-IAP2 with the same parental vector and epitope tag with no extraneous 5' or 3' sequences were transfected into HEK293 cells. Upon examination of cell lysates normalized for protein content, XIAP expression was consistently found to be significantly greater than c-IAP1 or c-IAP2 expression (Figure 2.2A), suggesting that c-IAP1 and c-IAP2 were unstable proteins.

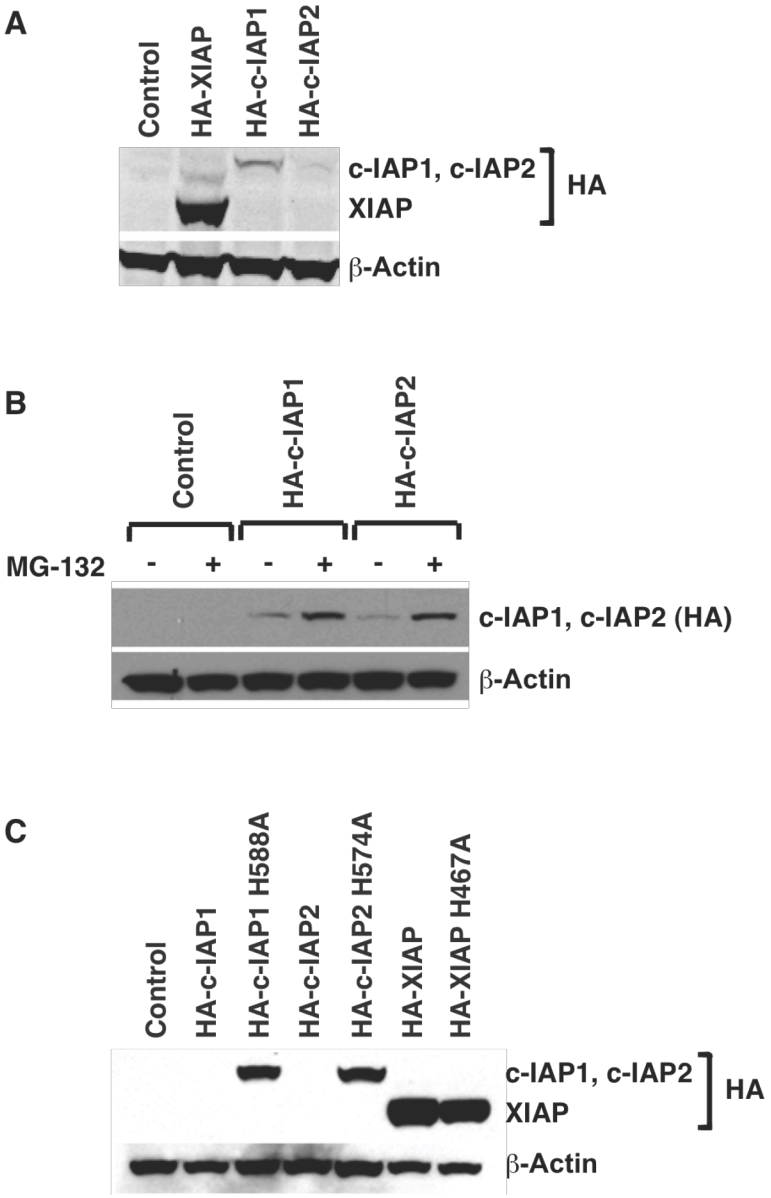

Figure 2.2: c-IAP proteins undergo autoubiquitination mediated by their RING domains. (A) HEK293 cells were transfected with amino-terminally HA epitope tagged c-IAP1, c-IAP2 and XIAP. Twenty-four hours following transfection, cells were lysed and immunoblotted for HA (c-IAP1, c-IAP2 and XIAP). (B) HEK293 cells were transfected with plasmids encoding HA-c-IAP1 and HA-c-IAP2. Eighteen hours after transfection, cells were left untreated or treated with 35 μM MG-132 for 4 hours. Following treatment with MG-132, cells were harvested and immunoblotted for HA (c-IAP1, c-IAP2, and XIAP). (C) Site-directed mutagenesis was performed to convert the coding sequence corresponding to histidine residues 588 in c-IAP1, 574 in c-IAP2, and 467 in XIAP located within the RING domains of these IAP proteins to alanines in order to generate E3 ubiquitin ligase deficient proteins. HEK293 cells were then transfected with plasmids encoding HA-c-IAP1, HA-c-IAP2, and HA-XIAP, or the corresponding HA-tagged RING mutants. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed. Equivalent protein loading was verified in A, B and C by immunoblotting for β-actin.

Since c-IAP1 and c-IAP2 were poorly expressed under these conditions, we examined whether these proteins were undergoing ubiquitination and proteasome-mediated degradation. HEK293 cells were treated with MG-132 to inhibit proteasomal activity, and to determine if the c-IAP proteins undergo ubiquitination and degradation. c-IAP1 and c-IAP2 protein levels were increased after MG-132 treatment in comparison to untreated cells that were transfected with c-IAP1 or c-IAP2 (Figure 2.2B) suggesting that the c-IAP proteins were sensitive to ubiquitination and proteasomal degradation.

In order to determine if the ubiquitination and degradation of c-IAP1 and c-IAP2 was due to the E3 ubiquitin ligase activity in their RING domains, site-directed mutagenesis was performed to replace critical histidines within their RING domains (H588A for c-IAP1 and H574A for c-IAP2). Interestingly, the expression of c-IAP1 and c-IAP2 variants deficient in their E3 ubiquitin ligase activities was greatly enhanced relative to their wild type counterparts and was comparable to that of wild type XIAP (Figure 2.2C). While loss of the E3 ubiquitin ligase activity of the RING domains resulted in stable forms of c-IAP1 and c-IAP2, modification of the corresponding critical histidine in the RING domain of XIAP did not result in a change in the expression level of XIAP H467A compared to wild type XIAP. Collectively, these data indicate that when expressed alone in culture, both c-IAP1 and c-IAP2 undergo RING-dependent autoubiquitination and degradation.

TRAF2 enhances the expression of c-IAP1 post-translationally

Since a reduction in endogenous c-IAP1 protein levels was observed that corresponded to reduced TRAF2 protein levels, and the c-IAP proteins were observed to

undergo autoubiquitination mediated by their E3 ubiquitin ligase activity when expressed alone in culture, we sought to determine if overexpression of any TRAF proteins might alter IAP protein levels. As shown in Figure 2.3, c-IAP1, c-IAP2, and XIAP were expressed with TRAF1 through 6. Surprisingly, expression of TRAF2 caused a marked enhancement in the expression of c-IAP1, whereas no other TRAF proteins significantly enhanced the expression of c-IAP proteins (Figures 2.3A and 2.3B). TRAF1 slightly enhanced the expression of c-IAP1, however the possibility that this effect could be due to heterodimerization of TRAF1 with TRAF2 remains untested. While enhanced expression of the c-IAP proteins, most notably c-IAP1, was observed in the presence of TRAF2, the expression of XIAP was unchanged by all tested TRAF proteins (Figure 2.3C).

These data indicated that TRAF2 was capable of stabilizing c-IAP1. We therefore examined whether the enhanced expression of c-IAP1 in the presence of TRAF2 was associated with a decrease in c-IAP1 autoubiquitination (Figure 2.4A). To investigate this hypothesis, the RING mutant of c-IAP1 was expressed with TRAF2, since the RING mutant of c-IAP1 is stable and does not undergo autoubiquitination. Coexpression of TRAF2 and the RING mutant of c-IAP1 (H588A) did not lead to additional enhancement of c-IAP1 H588A protein levels (Figure 2.4A). These data suggest that the association of TRAF2 with c-IAP1 might impairs the ability of c-IAP1 to undergo autoubiquination.

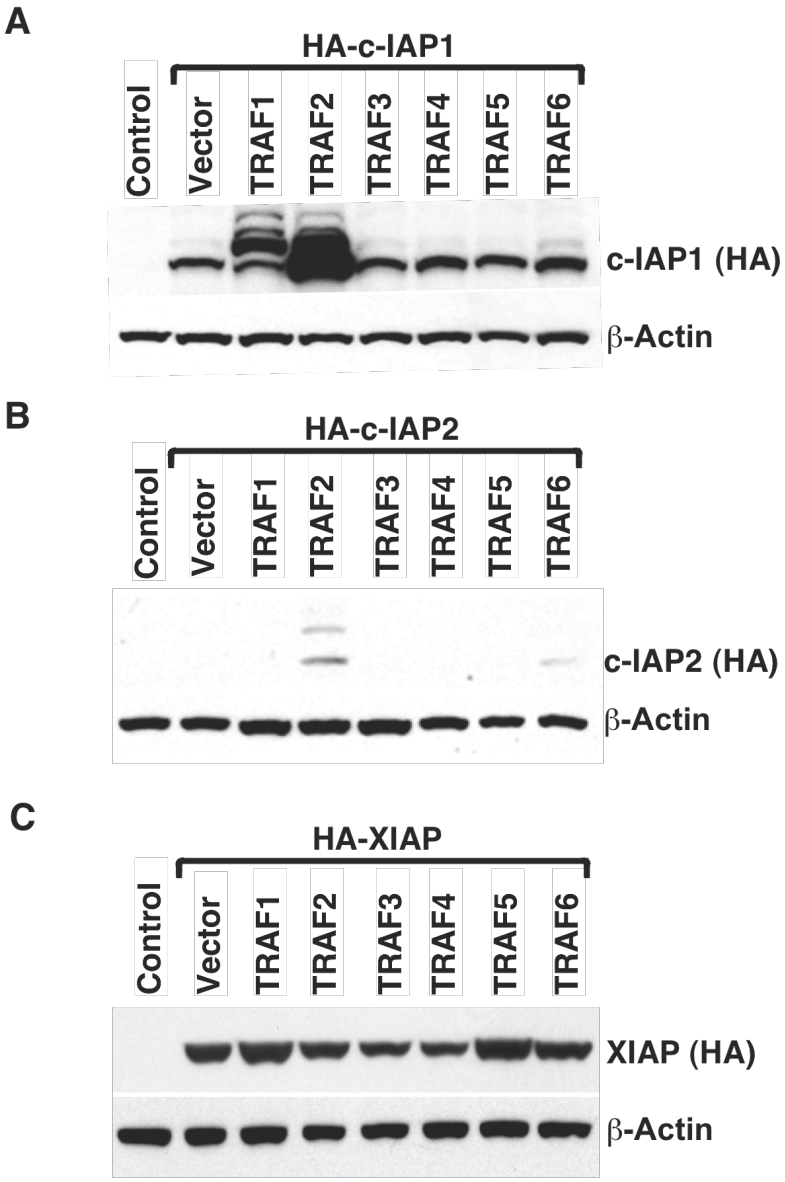

Figure 2.3: TRAF2 selectively stabilizes c-IAP1. (A) HEK293 cells were transfected with a plasmid encoding HA-c-IAP1 in the presence each of the six TRAFs. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for HA-c-IAP1. (B) HEK293 cells were transfected with a plasmid encoding HA-c-IAP2 in the presence each of the six TRAFs. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for HA-c-IAP2. (C) HEK293 cells were transfected with a plasmid encoding HA-XIAP in the presence each of the six TRAFs. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for HA-XIAP. Immunoblot analysis was performed to confirm equivalent expression of all six TRAF proteins in A-C, and equivalent protein loading was verified in A-C by immunoblotting for β-actin.

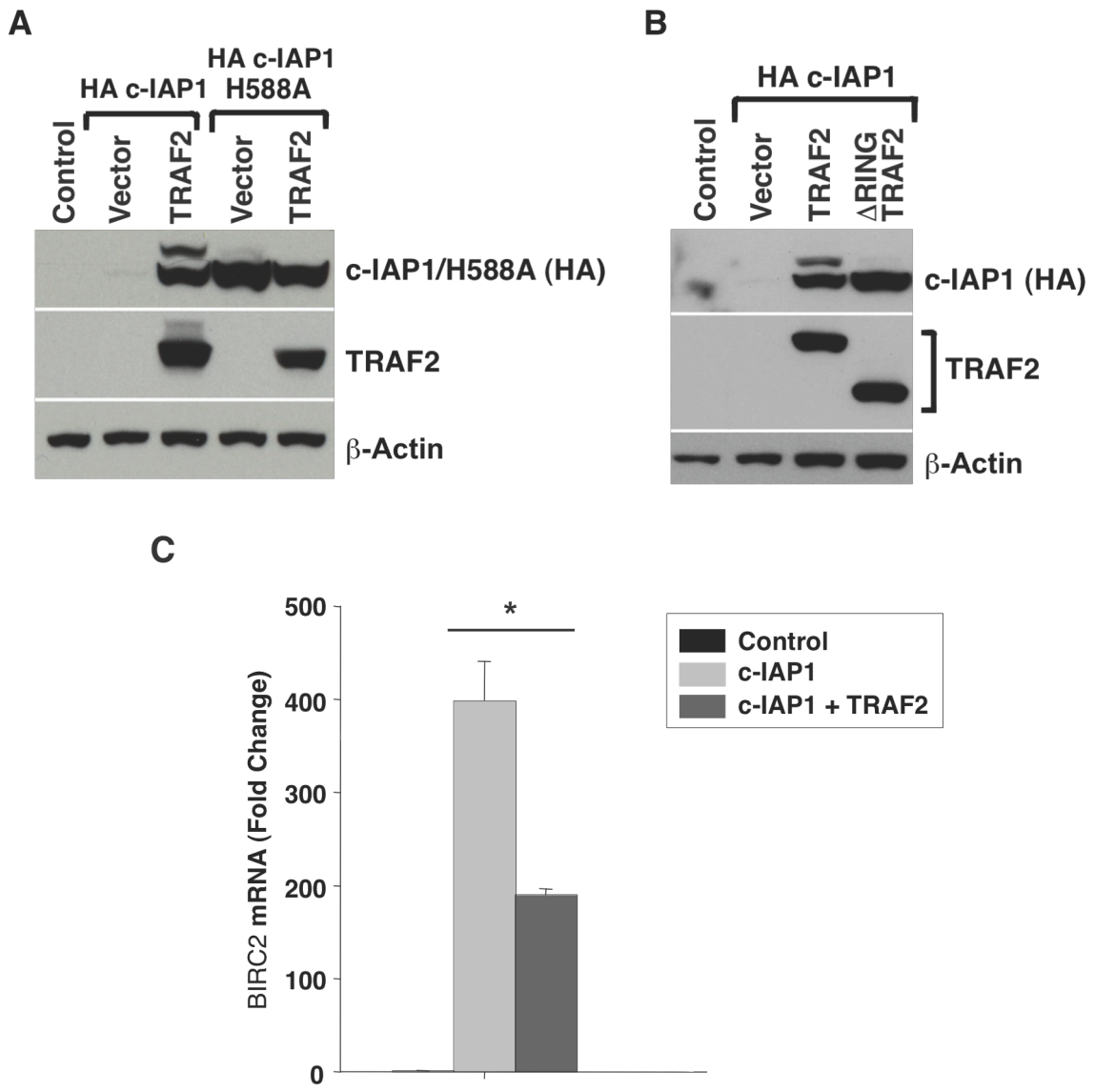

Figure 2.4: TRAF2 mediated c-IAP1 stabilization occurs post-translationally and does not require the RING of TRAF2. (A) HEK293 cells were transfected with HA-c-IAP1 or HA-c-IAP1 H588A, the c-IAP1 RING mutant, in the presence of TRAF2. Twenty-four hours following transfection, cells were harvested and immunoblot analysis was performed for HA-c-IAP1/H588A and TRAF2. (B) HEK293 cells were transfected with HA-c-IAP1 in the presence of TRAF2 or ΔRING TRAF2, a variant of TRAF2 which lacks the RING domain (residues 1-86). Twenty-four hours following transfection, cell lysates were prepared and immunoblotted for HA-c-IAP1 and TRAF2/ΔRING TRAF2. Equivalent protein loading was verified in A and B by immunoblotting for β-actin. (C) HEK293 cells were transfected with expression plasmids encoding HA-c-IAP1 in the absence or presence of TRAF2, or an empty control plasmid, as indicated. Twenty-four hours following transfection, quantitative analysis by real time reverse transcription was performed. Total RNA was extracted, reverse transcribed, and cDNA was analyzed by real-time PCR using a Taqman probe for the *BIRC2* gene. The data shown in C are the averages ± S.D. for multiple independent samples. T-test analysis was performed to calculate significance, and p-values of less than 0.01 are indicated with an asterisk (*).

TRAF2 has previously been reported to participate in the activation of several NF-κB signaling pathways (Wang et al., 1998), which in turn leads to the transcriptional activation of numerous genes involved in the acute phase of the inflammatory response, including *BIRC2* (c-IAP1) and *BIRC3* (c-IAP2) (Wang et al., 1998). To determine whether TRAF2 might be affecting transcription of *BIRC* genes, a variant of TRAF2 lacking the amino-terminal RING domain of TRAF2 (ΔRING TRAF2) was utilized. This variant of TRAF2 has previously been shown to block NF-κB activation by various members of the TNF receptor superfamily, including TNFR2, CD40 and CD30 (Rothe et al., 1995b; Duckett et al., 1997). The ΔRING TRAF2 variant was able to stabilize c-IAP1 protein levels (Figure 2.4B) indicating that the RING domain of TRAF2 is not required for stabilization of c-IAP1. Furthermore, the increase in c-IAP1 protein levels observed in the presence of TRAF2 was not the result of NF-κB activation, since ΔRING TRAF2 blocks NF-κB activation (Rothe et al., 1995b).

To further examine whether TRAF2 was enhancing c-IAP1 post-translationally or by transcriptional upregulation, RNA transcript levels of c-IAP1 were examined in the absence and presence of TRAF2 by quantitative analysis using real time reverse transcription-PCR. No increase in c-IAP1 transcript levels was observed when TRAF2 was expressed with c-IAP1 (Figure 2.4C). Collectively, these results support that TRAF2-mediated c-IAP1 stabilization occurs not through NF-κB activation and transcriptional upregulation, but through a post-translational interaction between TRAF2 and c-IAP1.

Stabilized c-IAP1 is cytoprotective

Since either coexpression of c-IAP1 with TRAF2 or mutation of the RING domain within c-IAP1 produced a stabilized c-IAP1, we sought to determine whether stabilized c-IAP1 might be capable of conferring a cytoprotective effect. To activate a well-described, caspase-dependent apoptotic program, Bax was ectopically expressed in HEK293 cells (Bratton et al., 2002). Prior studies have shown that XIAP can suppress this Bax-induced apoptotic process (Deveraux et al., 1999). Expression of c-IAP1, stabilized by coexpression of TRAF2, induced a significant reduction in caspase activation in comparison to Bax expressed alone or c-IAP1 expressed without TRAF2. The level of protection by c-IAP1 stabilized by TRAF2 was comparable to the level of protection observed for XIAP (Figure 2.5A). The ubiquitin ligase-deficient variant of c-IAP1 (H588A) was also capable of robustly protecting cells from undergoing Bax induced apoptosis (Figure 2.5B). Additionally, c-IAP1 stabilized by the ΔRING TRAF2 variant was capable of protecting against Bax induced cell death to a level comparable to c-IAP1 stabilized by TRAF2 (data not shown), suggesting that TRAF2 was not providing protection from cell death by activating the NF-κB signaling pathways. Taken together, these data suggest that stabilized c-IAP1 is a potent inhibitor of cell death capable of protecting cells to a level observed with XIAP.

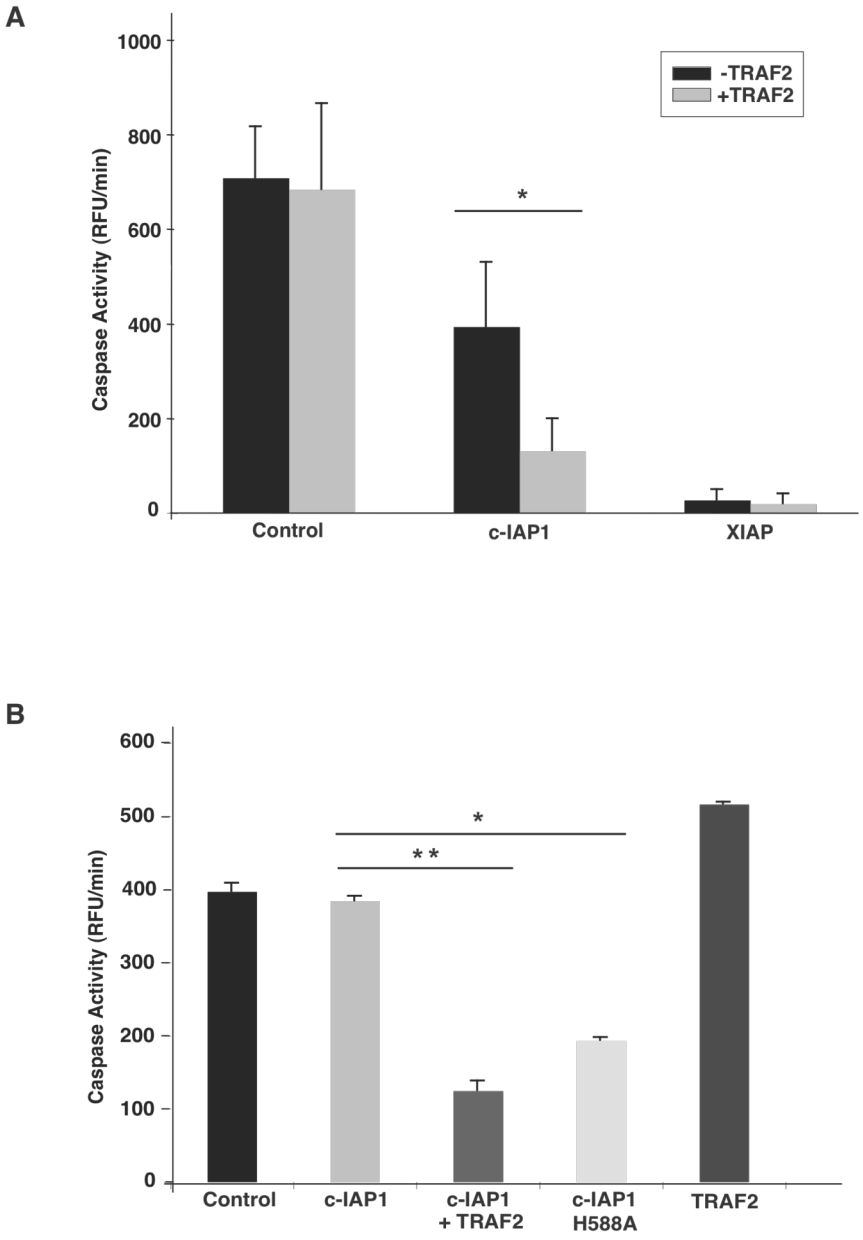

Figure 2.5: TRAF2 stabilized c-IAP1 and the c-IAP1 RING mutant suppress Bax-mediated cell death. (A) HEK293 cells were transfected with c-IAP1 or XIAP plasmids in the absence or presence of TRAF2, along with Bax. Sixteen hours following transfection, the presence of caspase-3 activity was determined by incubation with the fluorogenic substrate, DEVD-AFC. (B) HEK293 cells were transfected with c-IAP1 in the absence or presence of TRAF2, and the c-IAP1 RING mutant H588A in the presence of Bax. Sixteen hours following transfection, the presence of caspase-3 activity was determined by incubation with the fluorogenic substrate, DEVD-AFC. The data shown in A and B are the averages ± S.D. for multiple independent samples, and are the result of at least three independent experiments. T-test analysis was performed to calculate significance, p-values of less than 0.01 are indicated with an asterisk (*) and p-values of less than 0.05 are indicated with two asterisks (**).

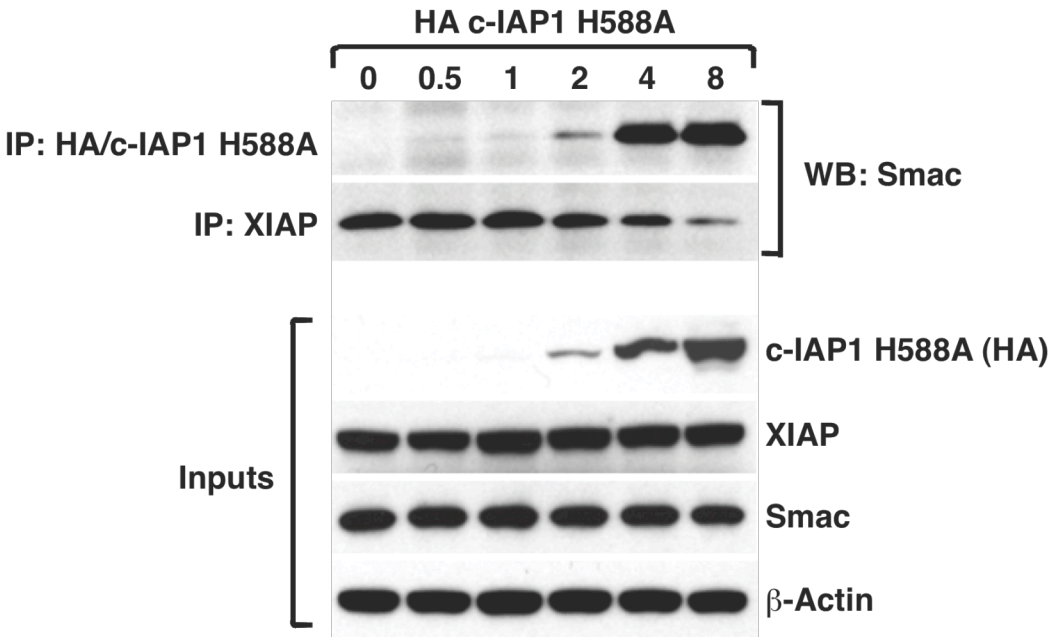

Figure 2.6: c-IAP1 competitively associates with Smac. HEK293 cells were transfected with increasing amounts of the expression plasmid, HA-c-IAP1 H588A (0-8 μg/10 cm^2 plate). Twenty-four hours following transfection, lysates were precipitated with XIAP or HA antibodies. Immunoblot analysis was then performed to evaluate the presence of Smac in the precipitate. Immunoblot analysis was also performed on the input lysates to verify the protein levels of HA c-IAP1, and endogenous XIAP and Smac. Equivalent protein loading was verified by immunoblotting for β-actin.

c-IAP1 competitively inhibits the interaction between Smac/DIABLO and XIAP

Prior reports suggest that c-IAP proteins function to inhibit cell death without directly inhibiting the enzymatic activity of caspases (Eckelman and Salvesen, 2006; Tenev et al., 2004; Suzuki et al., 2001). Based on these findings and our observations that stabilized c-IAP1 can protect against Bax induced cell death, the binding interaction between c-IAP1 and Smac/DIABLO was examined to determine if c-IAP1 was capable of protecting cells indirectly, for example by sequestering Smac/DIABLO away from endogenous XIAP. This hypothesis was tested by transfecting increasing amounts of a plasmid encoding c-IAP1 H588A, immunoprecipitating either XIAP or c-IAP1 from cell lysates, and immunoblotting for Smac/DIABLO in precipitated complexes (Figure 2.6).

As the expression level of c-IAP1 increased, the amount of endogenous Smac/DIABLO that was precipitated with c-IAP1 also increased. Interestingly, at the same time, the amount of endogenous Smac/DIABLO that was precipitated with endogenous XIAP was reduced (Figure 2.6). These data indicate that stabilized c-IAP1 can competitively inhibit the interaction between Smac/DIABLO and XIAP.

Discussion

In this study, we investigated the mechanisms by which c-IAP proteins are regulated, and found that TRAF2 stabilized c-IAP1 (Figure 2.3). We demonstrated that stabilized c-IAP1 was capable of inhibiting cell death to a level comparable to the potent cell death inhibitor, XIAP (Figure 2.5). While XIAP is considered the major mammalian cell death inhibitor because it is capable of binding to and inhibiting caspases, c-IAP1 and c-IAP2 were shown to bind but not inhibit the enzymatic activity of caspases (Eckelman and Salvesen, 2006; Tenev et al., 2004). Therefore, it has been hypothesized that c-IAP1 might instead interfere with the binding of the XIAP antagonist, Smac/DIABLO, and function to sequester Smac/DIABLO from neutralizing the apoptotic inhibition of XIAP (Eckelman and Salvesen, 2006; Tenev et al., 2004). Using stabilized c-IAP1, the anti-apoptotic properties of c-IAP1 were examined. As demonstrated here, c-IAP1 can sequester Smac/DIABLO and competitively interfere with Smac/DIABLO binding to XIAP, releasing XIAP to inhibit caspases (Figure 2.6). Therefore, these findings provide evidence that stabilized c-IAP1 is capable of protecting against cell death, however the mechanism by which stabilized c-IAP1 protects against cell death is

distinct from the protective mechanism utilized by XIAP. Our studies describe a mechanism by which c-IAP1 inhibits cell death and clarify how IAP proteins function to promote cell survival.

Although c-IAP1 and c-IAP2 are members of the IAP family because they both contain three BIR domains, the structural motif used to define IAP proteins, it is now evident that these molecules participate in additional signaling pathways. The observation that c-IAP1 can function as a putative oncogene through synergizing with c-MYC (Xu et al., 2007) and the presence of c-IAP1 in an amplicon associated with squamous cell carcinoma and hepatocellular carcinoma, where c-IAP1 expression correlates with resistance to radiotherapy (Imoto et al., 2001; Zender et al., 2006), provide important indications of the significant but not yet fully defined cellular functions of c-IAP1. Recently, much interest has been placed on understanding the non-apoptotic role of c-IAP proteins in TNF receptor signaling to NF-κB because of their association with TRAFs (Vince et al., 2007; Varfolomeev et al., 2007; Petersen et al., 2007; Gaither et al., 2007). c-IAP1 and c-IAP2 play an important role in TNF receptor-mediated cellular proliferation through the activation of NF-κB (Vince et al., 2007; Varfolomeev et al., 2007; Petersen et al., 2007; Tang et al., 2003; Gaither et al., 2007). Furthermore, the discoveries of compounds that selectively target c-IAP proteins are being thoroughly investigated because of their potential therapeutic effects (Vince et al., 2007; Varfolomeev et al., 2007; Petersen et al., 2007; Gaither et al., 2007).

Since it is clear that c-IAP proteins may be an important target for cancer therapeutics, it is critical that all cellular roles of c-IAP proteins - both non-apoptotic and apoptotic – are thoroughly investigated, because it is evident that c-IAP1 and c-IAP2 are

multifunctional proteins that play important roles in cell survival and proliferation, as well as other potential signaling cascades. Given that c-IAP1 and c-IAP2 have the potential to regulate signaling to NF-κB through interactions with TRAFs, as well as associate with Smac/DIABLO, which can regulate caspase-mediated apoptosis and caspase-independent signaling, it is not surprising that numerous forms of cancer may utilize c-IAP1 to drive oncogenesis. While the anti-apoptotic and NF-κB mediated cell proliferation roles for c-IAP1 and c-IAP2 are now more defined, it will be imperative to differentiate which of these roles are critical for the oncogenic function.

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Chapter III

Two Distinct Signaling Cascades Target the NF-κB Regulatory Factor c-IAP1 for Degradation

Summary

The cellular Inhibitor of Apoptosis factor c-IAP1 has recently emerged as a negative regulator of the non-canonical NF-κB signaling cascade. While synthetic IAP inhibitors have been shown to trigger the autoubiquitination and degradation of c-IAP1, less is known about the physiological mechanisms by which c-IAP1 stability is regulated. Here we describe two distinct cellular processes that lead to the targeted loss of c-IAP1. Recruitment of a TRAF2:c-IAP1 complex to the cytoplasmic domain of specific members of the TNF receptor superfamily leads to the targeting and degradation of the TRAF2:c-IAP1 heteromer through a mechanism requiring the RING domain of TRAF2, but not c-IAP1. In contrast, the induced autoubiquitination of c-IAP1 by IAP antagonists causes the selective loss of c-IAP1, but not TRAF2, thereby releasing TRAF2. Thus, c-IAP1 can be targeted for degradation by two distinct processes, revealing the critical importance of this molecule as a regulator of numerous intracellular signaling cascades.

Introduction

Inhibitor of apoptosis (IAP) proteins are a broadly expressed group of intracellular factors that function in a wide range of cellular processes in addition to their initially described role in the suppression of programmed cell death (Srinivasula and Ashwell, 2008). A growing body of work is revealing alterations in both expression and activity of numerous IAP proteins in neoplastic, lymphoproliferative and metabolic disorders, although given the diverse range of functions attributed to these proteins, the specific contributions of the IAP proteins to these disorders are still being elucidated (Hunter et al., 2007; Mufti et al., 2006; Rigaud et al., 2006).

IAP proteins contain one to three tandem repeats of an ~70 residue baculovirus IAP repeat (BIR) domain (Eckelman et al., 2006). In addition, several members of the IAP family, including the cellular IAP proteins (c-IAP1 & c-IAP2), contain a carboxy-terminal RING domain that possesses E3 ubiquitin ligase activity (Vaux and Silke, 2005; Yang et al., 2000). Several IAP proteins have been implicated in the regulation of cell death through their ability to bind and enzymatically inhibit certain members of the caspase family, which are the primary effectors of the apoptotic program. However, IAP proteins differ remarkably in their caspase-inhibiting ability, with X-linked IAP (XIAP) being the best described caspase inhibitor, while c-IAP1 and c-IAP2 can bind caspases but do not inhibit their enzymatic activity (Eckelman and Salvesen, 2006; Deveraux et al., 1998). Originally identified through their association with the tumor necrosis factor receptor 2 (TNFR2), c-IAP1 and c-IAP2 are recruited to tumor necrosis factor (TNF)

receptors by tumor necrosis factor receptor-associated factors (TRAFs), primarily TRAF2 (Rothe et al., 1995).

CD30, a member of the tumor necrosis factor receptor superfamily, has been the subject of much investigation because of its greatly elevated expression in several types of leukemia and lymphoma cells, including anaplastic large cell lymphoma (ALCL) and Hodgkin's lymphoma (HL) (Stein et al., 2000; Younes and Carbone, 1999). Normally, CD30 expression is restricted to a small subset of activated B- and T-cells, and has been shown to induce downstream signaling events through TRAFs, similarly to other members of the TNF receptor family such as TNFR2 and CD40. In particular, the activated CD30 receptor leads to the activation of the nuclear factor κB (NF-κB) transcription factor cascade through a mechanism that is largely TRAF-dependent (Duckett et al., 1997; Lee et al., 1996; Aizawa et al., 1997; Boucher et al., 1997). Induction of NF-κB-responsive genes is critical for immune responses, cell survival and proliferation, and the upregulation of CD30 in leukemia and lymphoma cells promotes aberrant NF-κB activation driving lymphomagenesis (Horie et al., 2004; Horie et al., 2002). Two major NF-κB pathways have been described both of which are activated in response to CD30 stimulation (Wright et al., 2007). The canonical pathway involves phosphorylation of IκBα, and in the alternative pathway p100/NF-κB2 is proteolytically processed to p52 (Hayden and Ghosh, 2008).

c-IAP1 and c-IAP2 were originally identified as TRAF associated proteins (Rothe et al., 1995), and c-IAP1 was identified in amplicons associated with squamous cell carcinoma and hepatocellular carcinoma, in which elevated expression correlates with resistance to radiotherapy (Imoto et al., 2001; Zender et al., 2006). More recently, c-

IAP1 was identified as a putative oncogene, functioning synergistically with c-MYC, in a murine hepatocellular carcinoma genetic screen (Zender et al., 2006), and independently c-IAP1 was also shown to target the c-MYC regulatory factor, MAD1, for proteasome-mediated degradation (Xu et al., 2007).

Previous reports have revealed several elegant mechanisms by which the activity of IAP proteins can be regulated. For instance, a number of mitochondrial proteins have been identified that are released into the cytosol during apoptosis, along with cytochrome *c*, which can bind to and antagonize IAP proteins (Du et al., 2000; Suzuki et al., 2001). The mammalian prototype of these factors that contain the so-called IAP binding motif (IBM) is second mitochondrial-derived activator of caspase (Smac, DIABLO in mice), which was originally identified as an XIAP antagonist, but which has been subsequently shown to also recognize other IAP proteins, including c-IAP1. Many of the binding properties of Smac can be recapitulated by a conserved tetrapeptide sequence, and small molecule IAP antagonists that mimic the action of Smac have been designed to potentiate apoptosis in cancer cells (Arnt et al., 2002; Wu et al., 2007). Additionally, Smac can be selectively released from mitochondria in the absence of an apoptotic stimulus, and indeed, in healthy cells cytosolic Smac has been described, produced either as an alternatively spliced variant whose maturation has been shown to bypass mitochondria, or through other less well-defined processes (Deng et al., 2003; Roberts et al., 2001). These findings suggest that the regulation of IAP proteins may be of central importance in the homeostatic control of a number of physiological processes in addition to apoptosis.

Here we examine the functional consequences reflected by the cellular interactions between c-IAP1, TRAF2 and Smac. We describe two apparently

independent mechanisms by which c-IAP1 can be targeted for degradation. Receptor-mediated cellular activation induces the degradation of a TRAF2:c-IAP1 complex through the E3 ubiquitin ligase activity of TRAF2 which is mediated by its RING. In contrast, Smac or a synthetic IAP antagonist triggers the selective degradation of c-IAP1, but not TRAF2, through the RING of c-IAP1. The consequence of c-IAP1 targeting by either of these processes is the activation of the non-canonical NF-κB pathway, consistent with previously proposed mechanisms for the role of c-IAP1 in this signaling cascade. Since NF-κB activation has frequently been associated with the transcriptional induction of gene signatures involved in cytoprotection, these findings indicate that the c-IAP proteins and IBM-containing proteins such as Smac can function in pro-apoptotic and anti-apoptotic signaling pathways.

Experimental Procedures

Materials: Reagents were obtained from the following sources: Ni-NTA-agarose (Invitrogen), DMSO (Sigma Aldrich), Cell culture media (Mediatech), Ficoll-paque PLUS (GE Healthcare), and Quik-Change site-directed mutagenesis kit (Stratagene). AEG40730 was a kind gift from Aegera Therapeutics. Antibodies were obtained from the following sources: TRAF2 (BD Pharmingen), COX IV (Invitrogen), Smac/DIABLO (Calbiochem), GST (Santa Cruz), peroxidase conjugated anti-HA, peroxidase conjugated anti-FLAG, β-actin (Sigma Aldrich) and p52/p100 (Upstate). Peroxidase-conjugated anti-mouse, anti-rabbit and anti-rat (GE Healthcare). Anti-c-IAP1 was kindly provided by Dr. John Silke (La Trobe University, Melbourne) (Vince et al., 2007).

Plasmids: pEBB-HA c-IAP1 was subcloned from pEBG c-IAP1 (Duckett et al., 1998). Site-directed mutagenesis of c-IAP1 to generate c-IAP1 H588A was performed using the Quikchange Mutagenesis Kit (Stratagene). pEBB His_6-Ub was a kind gift of Dr. Ezra Burstein (University of Michigan). Unless otherwise noted, plasmids used for this study have been described previously (Duckett and Thompson, 1997; Wilkinson et al., 2004b; Wilkinson et al., 2008).

Cell Lines: HEK293 cells were cultured in Dulbecco's modified Eagle's medium, Karpas 299 and L428 cells were cultured in RPMI medium, and CHO cells were cultured in F-12 nutrient medium. All media was supplemented with 10% fetal bovine serum and 2 mM L-glutamine and maintained at 37 ˚C in an atmosphere of 95% air, 5% CO_2.

Transfections: HEK293 cells were transfected with plasmids using a standard calcium phosphate transfection protocol. Both plasmids and siRNA oligonucleotides were transfected into Karpas 299 cells using a Bio-Rad Gene Pulser II electroporator set on infinite resistance, 300 V and 950 microfarads.

Physiological CD30 Stimulation and Treatment with AEG40730: Stimulation of Karpas 299 cells with $CD30L^+$ CHO cells was performed as previously described (Wright et al., 2007). Briefly, Karpas 299 cells were resuspended in a 1:1 mixture of RPMI and F-12 nutrient media at a final concentration of 10^6 cells/mL. CHO cells (negative control) or $CD30L^+$ CHO cells were seeded 24 hours prior at 0.8 x 10^6 cells/well, and 1 mL of Karpas 299 cells were layered on CHO cells in 6-well plates for 2

hours. Karpas 299 cells were then removed from the CHO cells by gentle pipetting and collected by centrifugation for 5 min at 200 x g. Cells were washed with 1 mL PBS, and subsequently centrifuged for 5min at 200 x g, and resuspended in RIPA lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors. HEK293 or Karpas 299 cells were treated with 0-25 nM AEG40730 for 24 hours, or treated for 0-48 hours with 25 nM AEG40730, as described in results. DMSO was used as a control for all AEG40730 treatments.

Cell Lysate Preparation and Immunoblot Analysis: Cell lysates were prepared with RIPA lysis buffer, supplemented with protease inhibitors, for 30 min on ice to ensure complete lysis unless noted otherwise. Protein quantitation was determined by Bradford assay (Bio-Rad). RIPA cell lysates of equal protein concentrations were prepared in LDS sample buffer, separated on denaturing NuPAGE 4-12% polyacrylamide gradient gels, and transferred to 0.45 μM nitrocellulose membranes (Invitrogen). Membranes were blocked in 5% milk in Tris buffered saline (TBS) with 0.02 - 0.2% Tween 20 (Bio-Rad) depending on the antibody requirements, followed by incubation with the indicated antibodies in 2.5% milk for 1 hour at room temperature or overnight at 4 ˚C. After washing with TBS containing 0.02-0.2% Tween, membranes were incubated with secondary antibodies for 1 hour at room temperature. Enhanced chemiluminescence (GE Healthcare) was used to visualize the blots.

Cellular Fractionation Preparation: Karpas 299 or L428 cells (10^6 cells/treatment) were prepared as described previously (Wilkinson et al., 2004a), with minor protocol

modifications. Cells were harvested, washed with PBS, resuspended at 3 x 10^7 cells/mL in digitonin extraction buffer (PBS containing 250 mM sucrose, 70 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 200 μg digitonin/mL) supplemented with additional protease inhibitors. Following incubation on ice for 5 min, samples were centrifuged for 5 min at 1,000 x g. RIPA lysis buffer was used to lyse sample pellets, as described above. Supernatants (cytoplasmic extracts) were collected and centrifuged again at 1,000 x g for 5 min to remove any residual debris. Extract protein concentrations were normalized using a Bradford assay (Bio-Rad).

RNA Interference: Cells (10^7 cells/ transfection) were transfected with 16 μg of short interfering RNA oligonucleotides (Xeragon/Qiagen) by electroporation. Gene-specific targeting of Smac was performed using a previously described oligonucleotide corresponding to nucleotides 156-176 of the coding sequence of Smac (Wilkinson et al., 2004b). As a negative control, a previously described oligonucleotide corresponding to nucleotides 322-342 of GFP was utilized (Wilkinson et al., 2004b). 24 hours following electroporation, dead cells were removed by centrifugation the cells at 400 x g for 20 min on a Ficoll-Pique PLUS density cushion. Forty-eight hours post-transfection, CD30 was stimulated on the transfected cells for 2 hours as described above. Following CD30 stimulation, total RNA was isolated from half of the cells and subjected to real time reverse transcription-PCR. The remaining fraction of cells was used for whole cell lysate preparation and Western blot analysis.

Real Time Reverse Transcription-PCR: Karpas 299 cells were transfected with plasmids or oligonucleotides, stimulated with CD30L for 2 hours as described above and then washed with PBS. Total RNA was isolated using the RNeasy minikit (Qiagen) according the manufacturer's instructions. Reverse transcription with random hexamer primers and MultiScribeTM Reverse Transcriptase (Applied Biosystems) was performed on 100 ng of total RNA. The indicated target assays were performed on 1 μL of resulting cDNA. Each target assay was performed in triplicate and normalized to glyceraldehydes 3-phosphate dehydrogenase.

Ubiquitin Precipitations: To detect His-Ubiquitin conjugated proteins, cell lysates were prepared in 8 M Urea lysis buffer (300 mM NaCl, 0.5% Nonident P-40, 50 mM $NaPO_4$, 50 mM Tris pH 8.0, and 1 mM phenylmethylsulfonyl fluoride) followed by sonication (25 pulses at output control 2.5, 75% duty cycle), normalized for protein content, and incubated with Ni-Agarose beads for 2 hours at 4°C. Agarose beads were recovered by centrifugation, washed in 8 M Urea lysis buffer, and precipitated proteins were eluted by adding LDS sample buffer (Invitrogen) and heating samples for 10 min at 95°C. Recovered proteins were subsequently separated by electrophoresis, and immunoblot analysis was performed.

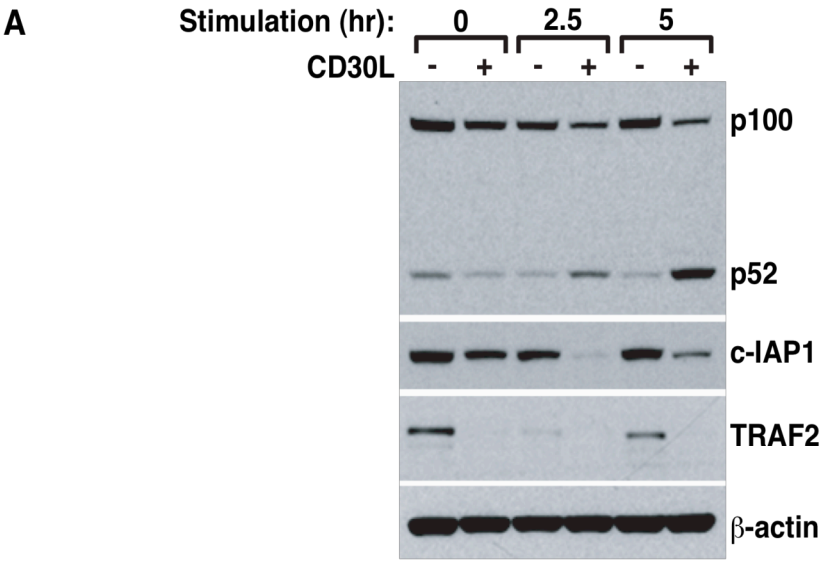
Results

CD30 signaling activates the non-canonical NF-κB pathway and the degradation of both c-IAP1 and TRAF2

Ligand-mediated activation of many members of the TNF receptor superfamily, including CD30, initiates a series of intracellular signal transduction cascades (Baud and Karin, 2001). The non-canonical NF-κB signaling pathway is activated by CD30, and is thought to utilize the signaling intermediate, TRAF2, which binds directly to the cytoplasmic tail of CD30 (Duckett et al., 1997; Lee et al., 1996; Aizawa et al., 1997; Boucher et al., 1997). In earlier studies, we found that receptor activation rapidly induced the degradation of TRAF2 (Duckett and Thompson, 1997; Wright et al., 2007), and we proposed a model in which the depletion of TRAF2 serves both to 'reset' the signaling pathway and to facilitate crosstalk with other classes of receptors that also utilize TRAF2. Since c-IAP1 is a known TRAF2 binding protein that has recently been described as a regulator of NF-κB, we sought to determine whether c-IAP1 was also depleted following receptor activation, along with TRAF2, or whether the loss of TRAF2 displaced the c-IAP1 protein.

To compare the stability of c-IAP1 before and after receptor activation, we employed an experimental approach in which $CD30^+$ anaplastic large cell lymphoma (ALCL) cells are activated by the ligand for CD30 (CD30L), using an adherent cell line in which CD30L has been stably expressed (Wright et al., 2007). ALCL cells were layered onto adherent CD30L-expressing cells, or control cells, and c-IAP1 levels in the ALCL cells were subsequently examined between 0 to 5 hours by immunoblotting.

Interestingly, activation by CD30 resulted in a rapid loss of c-IAP1 (Figure 3.1A), concomitant with the loss of TRAF2, which we described previously using this system (Wright et al., 2007). Importantly, CD30 activation also induced the non-canonical NF-κB pathway, as monitored by analysis of the processing of the p100 NF-κB subunit to its mature, p52 form (Figure 3.1A, upper panel). In light of recently proposed models in which c-IAP1 functions to suppress the levels and activities of factors involved in the non-canonical NF-κB pathway, notably NIK and RIP1, through mechanisms thought to involve the E3 ubiquitin ligase activity of c-IAP1 (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007), these findings suggest a mechanism by which CD30 signaling activates the non-canonical pathway by triggering c-IAP proteins for degradation.



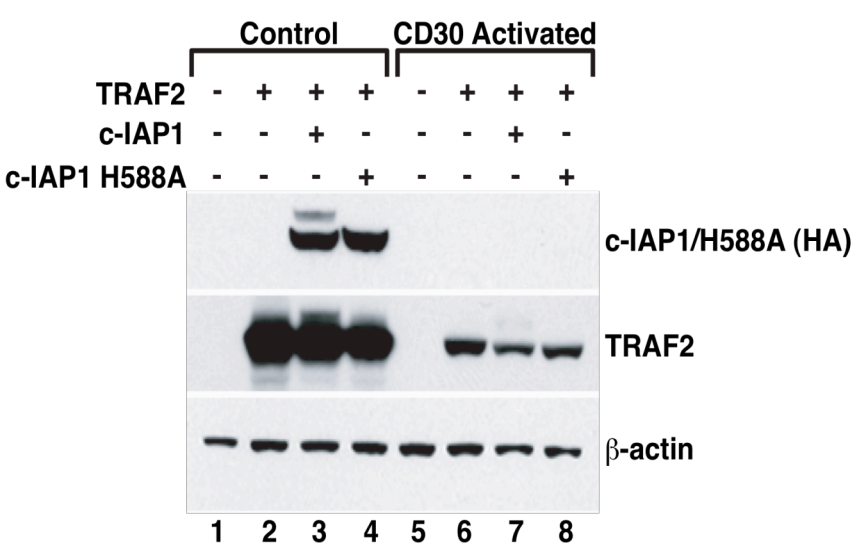


Figure 3.1: CD30 activation induces the degradation of both c-IAP1 and TRAF2. (A) Karpas 299 cells were layered onto Chinese hamster ovary (CHO) cells, or CHO cells expressing CD30 ligand, for the indicated periods of time. Cells were recovered, lysed in RIPA buffer, and immunoblotted, for p100/p52, c-IAP1, TRAF2, or β-actin. (B) Site-directed mutagenesis was performed to convert the histidine residue 588 to alanine (H588A) located within the RING domain of c-IAP1 to generate a mutant c-IAP1 deficient in E3 ubiquitin ligase activity. HEK293 cells were transfected with plasmids encoding TRAF2, HA c-IAP1 or HA c-IAP1 H588A as indicated, together with a control plasmid or a constitutively active form of CD30. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for HA c-IAP1/H588A and TRAF2. Equivalent protein loading was verified by immunoblotting for β-actin.

Receptor-induced degradation of the TRAF2:c-IAP1 complex does not require the E3 ubiquitin ligase activity of c-IAP1

Previous studies found that receptor-induced degradation of TRAF2 required an intact amino terminal RING domain (Duckett and Thompson, 1997), consistent with subsequent reports that the TRAF2 RING functions as an E3 ubiquitin ligase (Li et al., 2002). Since c-IAP1 also functions as an E3 ubiquitin ligase, mediated by a carboxy-terminal RING domain, we examined the involvement of the RING in the degradative process of c-IAP1, as mediated by receptor activation. The E3 ubiquitin ligase activity of

c-IAP1 was rendered inert by changing a critical histidine residue at position 588 within the RING domain to alanine (H588A) (Conze et al., 2005; Du et al., 2000). Human embryonic kidney (HEK) 293 cells were subsequently transfected with plasmids encoding either wildtype c-IAP1 or c-IAP1 H588A and TRAF2 in combination with a control plasmid or a constitutively signaling form of CD30 (Figure 3.1B). Coexpression of c-IAP1 with TRAF2 results in a stabilized form of c-IAP1 (Figure 3.3A and R.A.C & C.S.D. manuscript in preparation), while the E3 ubiquitin ligase deficient c-IAP1 H588A can be readily detected, and is not further stabilized by the inclusion of TRAF2 (Figure 3.3A, lane 3). Upon coexpression with the constitutively active CD30 and subsequent analysis of total detergent-soluble proteins by immunoblotting, cellular TRAF2 levels were observed to be markedly reduced relative to cells in which the control vector was included (Figure 3.1B compare lanes 2-4 versus lanes 6-8), consistent with previous reports (Duckett and Thompson, 1997; Wright et al., 2007). Similarly, c-IAP1 levels were reduced in the presence of CD30 signaling (Figure 3.1B, lane 3 versus lane 7), supporting the data with endogenous c-IAP1 described in Figure 3.1A. Interestingly, the c-IAP1 H588A RING mutant was also degraded (Figure 3.1B, compare lanes 4 versus 8), indicating that the E3 ubiquitin ligase activity of c-IAP1 is not required for the degradation of c-IAP1 or TRAF2, as induced by CD30 signaling. This finding that the E3 ubiquitin ligase activity of c-IAP1 is not required for degradation following CD30 activation contrasts the requirement of the RING of TRAF2 for CD30-mediated degradation.

Cytosolic Smac selectively induces autoubiquination and proteasome-mediate degradation of c-IAP1, but not TRAF2

The cellular IAP antagonist Smac has been shown to regulate the levels and activity of several IAP proteins following the release of Smac from mitochondria into the cytosol (Yang and Du, 2004). Interestingly, despite its mitochondrial targeting, cytosolic Smac was readily detectable in ALCL and HL cells (Figure 3.2). To determine whether the mechanism by which Smac targets c-IAP1 for degradation was related to CD30-induced degradation of c-IAP1 we used a mature cytosolic version of Smac that bypasses the mitochondria (Hunter et al., 2003). Expression of mature Smac resulted in a decrease in unmodified c-IAP1 protein and a ladder-like appearance of higher c-IAP1 moieties (Figure 3.3A). In contrast, the expression of mature Smac had no significant impact on TRAF2 expression levels. These findings suggest that the mechanism by which Smac regulates c-IAP1 expression is distinct from the mechanism by which c-IAP1 and TRAF2 are targeted following CD30 activation since only c-IAP1 protein levels are altered in the presence of Smac.

The laddering pattern of c-IAP1 that was observed in the presence of Smac is highly suggestive of polyubiquitination. To determine if Smac could function to induce ubiquitination of c-IAP1, HEK293 cells were transfected with a plasmid encoding histidine-tagged ubiquitin and combinations of c-IAP1, TRAF2 and Smac. Cellular lysates were prepared and ubiquitinated proteins were precipitated with agarose beads coupled to Nickel (Ni-agarose). Smac expression induced the accumulation of high molecular weight, ubiquitinated c-IAP1 (Figure 3.3B). Interestingly, the ubiquitination pattern of TRAF2 was unaffected by Smac (Figure 3.3B, center panel). Since Smac

could induce the polyubiquitination of c-IAP1, but not TRAF2, these data support a model in which Smac functions to specifically target c-IAP1 for degradation.

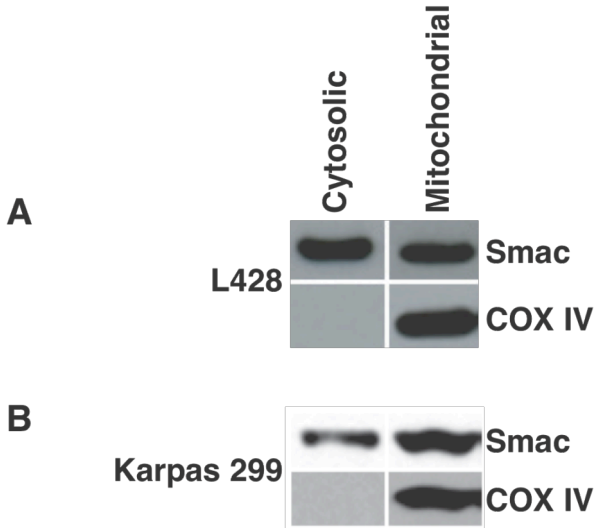
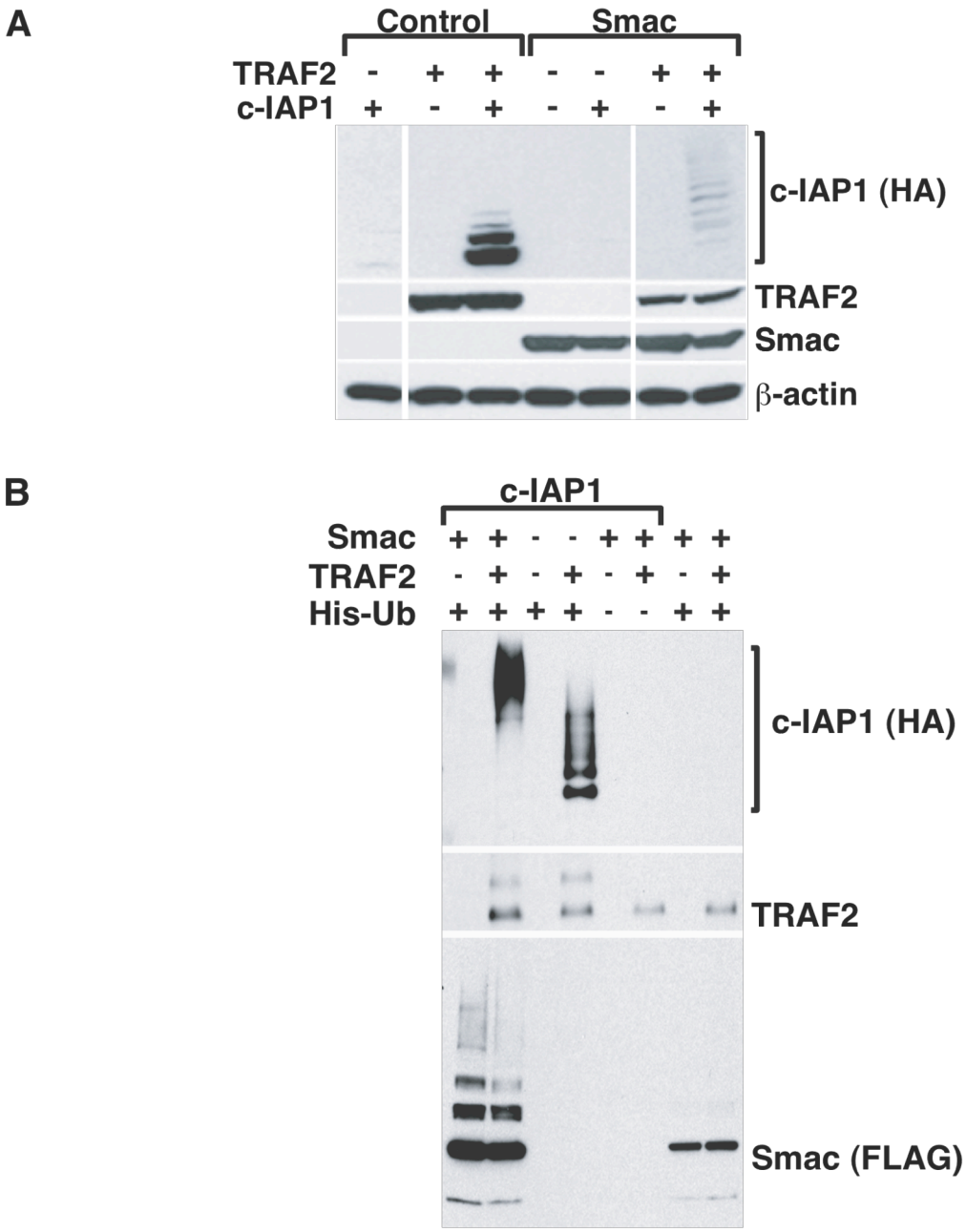


Figure 3.2: **Smac is located in cytosolic and mitochondrial fractions.** Cytosolic and mitochondrial fractions were prepared from (A) HL cells (L428) and (B) ALCL cells (Karpas 299), and the subcellular distribution of Smac was subsequently analyzed by immunoblotting. The quality and integrity of the mitochondrial and cytosolic fractions were confirmed by reprobing with an antibody directed against cytochrome oxidase subunit IV (COX IV).

Several known E3 ubiquitin ligases could be postulated to catalyze the Smac-induced ubiquitination of c-IAP1, but since c-IAP1 itself harbors E3 ubiquitin ligase activity, a likely possibility is that Smac might facilitate the autoubiquitination of c-IAP1. To test this possibility HEK293 cells were transfected with wildtype c-IAP1 or the cIAP1 H588A mutant in the absence or presence of Smac. Unlike the pronounced ubiquitination ladder observed for wildtype c-IAP1 in the presence of Smac, its expression had no effect on the levels of the E3 ubiquitin ligase deficient c-IAP1 (Figure 3.3C). These data support a model in which Smac selectively induces the ubiquitin-mediated degradation of c-IAP1 through its autoubiquitination, while leaving TRAF2 unaffected.


A
Control
Smac
TRAF2 - + + - - + +
c-IAP1 + - + - + - +
c-IAP1 (HA)
TRAF2
Smac
β-actin
B
c-IAP1
Smac + + - - + + + +
TRAF2 - + - + - + - +
His-Ub + + + + - - + +
c-IAP1 (HA)
TRAF2
Smac (FLAG)

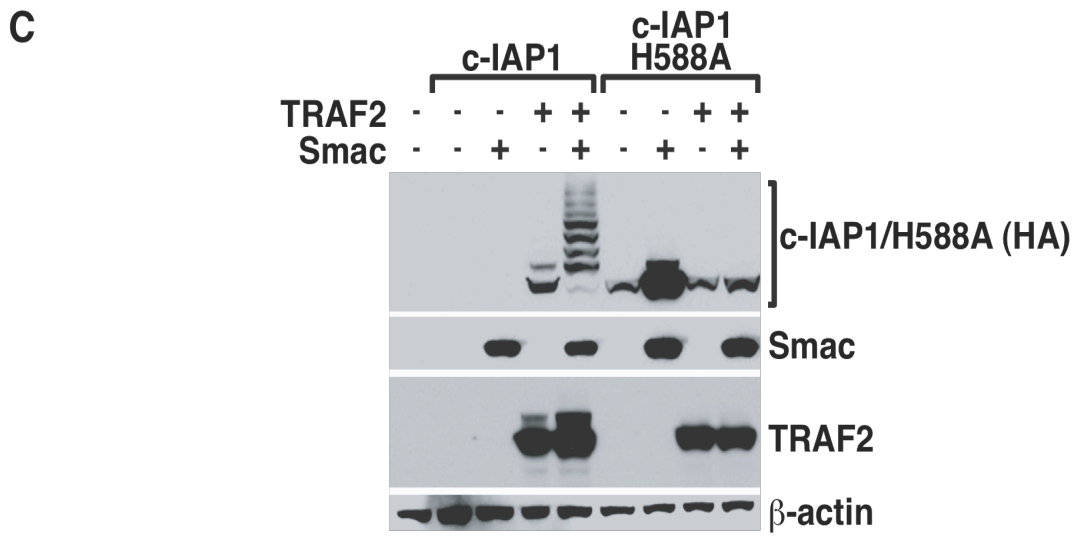


Figure 3.3: Cytosolic Smac triggers the autoubiquitination and degradation of c-IAP1, but not TRAF2. (A) HEK293 cells were transfected with plasmids encoding TRAF2 and HA c-IAP1, together with an empty control plasmid or a plasmid encoding mature Smac as indicated. Cells were harvested 24 hours following transfection, and lysates were analyzed by immunoblotting for Smac, HA c-IAP1, and TRAF2. Immunoblotting for β-actin was used to verify equivalent protein loading. (B) HEK293 cells were transfected with combinations of Smac-FLAG, HA c-IAP1, TRAF2 and His-Ub. Twenty-four hours following transfection, cell lysates were prepared in 8 M Urea buffer and precipitated using Nickel beads. The presence of HA c-IAP1, TRAF2, and FLAG Smac in the precipitate was determined by immunoblotting. (C) HEK293 cells were transiently transfected with HA c-IAP1 or the c-IAP1 H588A mutant expression plasmid along with plasmids encoding for TRAF2 and mature Smac. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for HA c-IAP1 / H588A mutant, Smac, and TRAF2. Equivalent protein loading was verified by immunoblotting for β-actin.

c-IAP1 is an inhibitor of the non-canonical NF-κB pathway, and Smac neutralizes this inhibition

Distinct targeting of c-IAP1 alone was observed in the presence of Smac, whereas CD30 activation resulted in the degradation of both c-IAP1 and TRAF2. Since the loss of c-IAP1 has been associated with the activation of the non-canonical NF-κB pathway, we evaluated the effect of Smac on non-canonical NF-κB activation, by examining the processing of the p100 protein to its transcriptionally active p52 form. Expression of

TRAF2 resulted in a significant increase in p100 processing to p52, confirming previous studies in which TRAF2 has been implicated in the non-canonical NF-κB pathway (Figure 3.4, lane 2) (Wright et al., 2007). However, coexpression of c-IAP1 with TRAF2 inhibited the ability of TRAF2 to induce p100 processing (Figure 3.4, lane 3), consistent with reports that c-IAP1 functions to suppress the non-canonical pathway by targeting NIK for degradation. Interestingly, expression of mature Smac alone induced p100 processing (Figure 3.4, compare lanes 2 & 6), and Smac counteracted the inhibitory effect of c-IAP1 on p100 processing (Figure 3.4, lane 5). In the presence of Smac, the high molecular weight ladder of polyubiquitinated c-IAP1 was again observed, while TRAF2 appeared unaffected. Therefore, Smac was found to activate the non-canonical NF-κB signaling pathway, and this activation occurred concomitantly with induced autoubiquitination of c-IAP1.

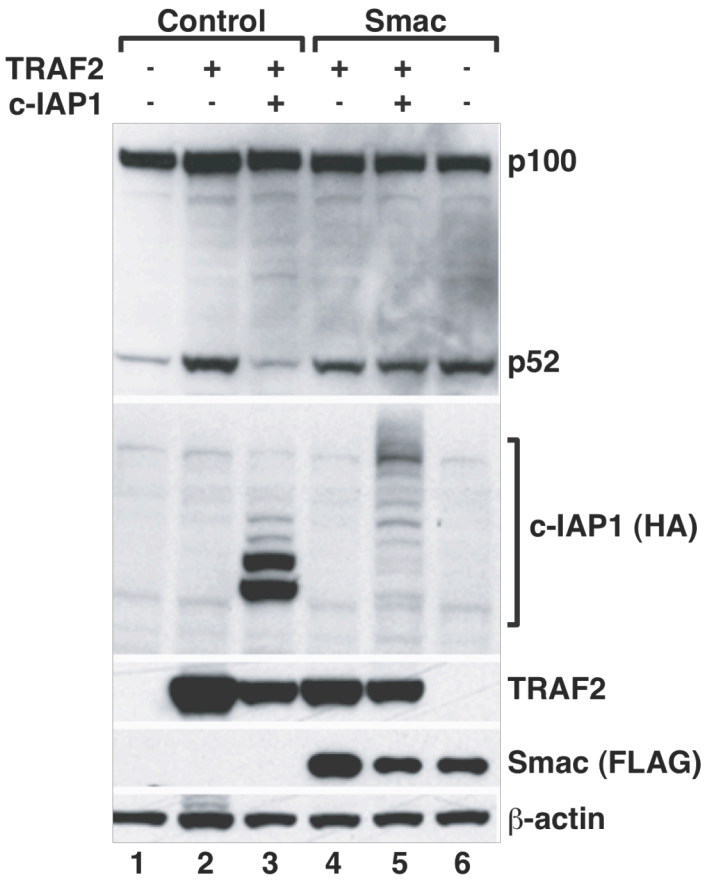


Figure 3.4: c-IAP1 is an inhibitor of the non-canonical NF-κB pathway, and Smac neutralizes c-IAP1 mediated NF-κB inhibition. HEK293 cells were transfected with mature Smac-FLAG, HA c-IAP1 and TRAF2. Twenty-four hours following transfection, cell lysates were prepared and immunoblot analysis was performed for p100/p52, HA c-IAP1, TRAF2, and Smac-FLAG. Equivalent protein loading was confirmed by immunoblotting for β-actin.

A synthetic IAP antagonist selectively targets c-IAP1 for autoubiquitination

The data presented in Figures 3.3 and 3.4 indicate that Smac induces a selective autoubiquitination of c-IAP1, while leaving TRAF2 unaffected, and that this process leads to the activation of the non-canonical NF-κB pathway by inducing the processing of the p100 NF-κB subunit. Since a number of synthetic, cell-permeable IAP antagonists have been developed based on the Smac:IAP interface, we examined the possibility that an IAP antagonist, AEG40730, would function similarly to mature Smac, by activating

the autoubiquitination of c-IAP1, releasing TRAF2, and triggering p100 processing. Interestingly, AEG40730 was capable of degrading ectopically expressed c-IAP1 in a similar fashion as mature Smac, as increasing concentrations of AEG40730 resulted in decreasing levels of c-IAP1 (Figure 3.5A). Importantly, no differences in the levels of TRAF2 were observed.

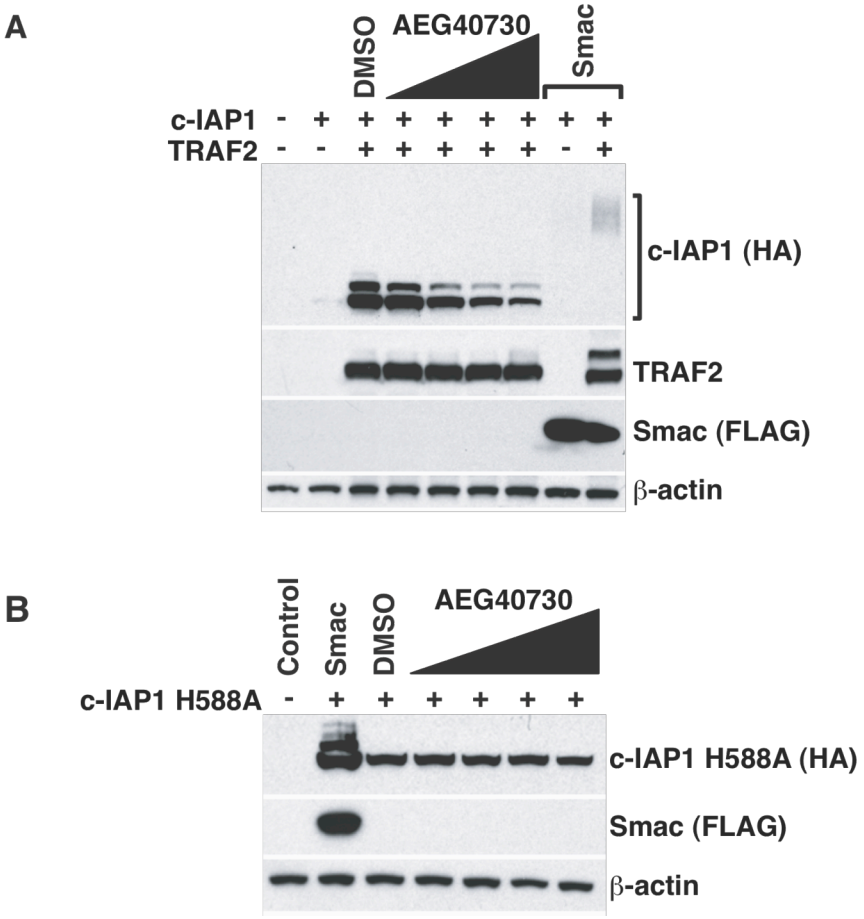


Figure 3.5: The IAP antagonist AEG40730 induces the degradation of c-IAP1, but not TRAF2. (A) HEK293 cells were transfected with HA c-IAP1 and TRAF2. Twenty-four hours following transfection, cells were treatment with 0, 1, 5, 10 or 25 nM AEG40730 or DMSO. Twenty-four hours following treatment with AEG40730, cell lysates were prepared and immunoblot analysis was performed for HA c-IAP1 and TRAF2. Equivalent protein loading was confirmed by immunoblotting for β-actin. As a control, c-IAP1 and TRAF2 were also transfected with mature Smac-FLAG. (B) HEK293 cells were transfected with HA c-IAP1 H588A. Twenty-four hours following transfection, cells were treatment with 0, 1, 5, 10, or 25 nM AEG40730 or DMSO. Twenty-four hours following treatment with AEG40730, cell lysates were prepared and immunoblotted for HA c-IAP1 H588A. As a control, c-IAP1 H588A was transfected with mature Smac-FLAG. Equivalent protein loading was verified by immunoblotting for β-actin.

Given the apparent similarities between the effects of mature Smac and the synthetic IAP antagonist, we sought to test whether AEG40730 could induce c-IAP1 autoubiquitination by analysis of the c-IAP1 E3 ubiquitin ligase mutant H588A in the presence of increasing doses of AEG40730. Similar to the results observed for c-IAP1 H588A in the presence of mature Smac, the RING mutant of c-IAP1 was insensitive to AEG40730 treatment at any tested dosage (Figure 3.5B). These findings strongly support the notion that AEG40730 functions analogously to mature Smac, in the ability to stimulate autoubiquitination of c-IAP1.

AEG40730 triggers processing of p100 NF-κB to its p52 form

Since the IAP antagonist, AEG40730, appeared to trigger c-IAP1 autoubiquitination in a similar fashion to mature Smac, we next investigated the ability of the IAP antagonist to activate the non-canonical NF-κB pathway through c-IAP1 degradation. To determine whether AEG40730 was capable of activating the alternative NF-κB pathway, we examined p100 processing to p52. HEK293 (Figure 3.6A) and Karpas 299 (Figure 3.6B) cells were treated for twenty-four hours with increasing doses of AEG40730. As before, treatment with AEG40730 induced the loss of endogenous c-IAP1, whereas endogenous TRAF2 levels were unaffected (Figure 3.6). Importantly, treatment with AEG40740 also resulted in increased processing of p100 to p52, already evident by six hours (Figure 3.6C). Therefore, AEG40730 was capable of selectively targeting the degradation of c-IAP1, but not TRAF2, and a concomitant processing of p100 similar to the results observed with mature Smac.

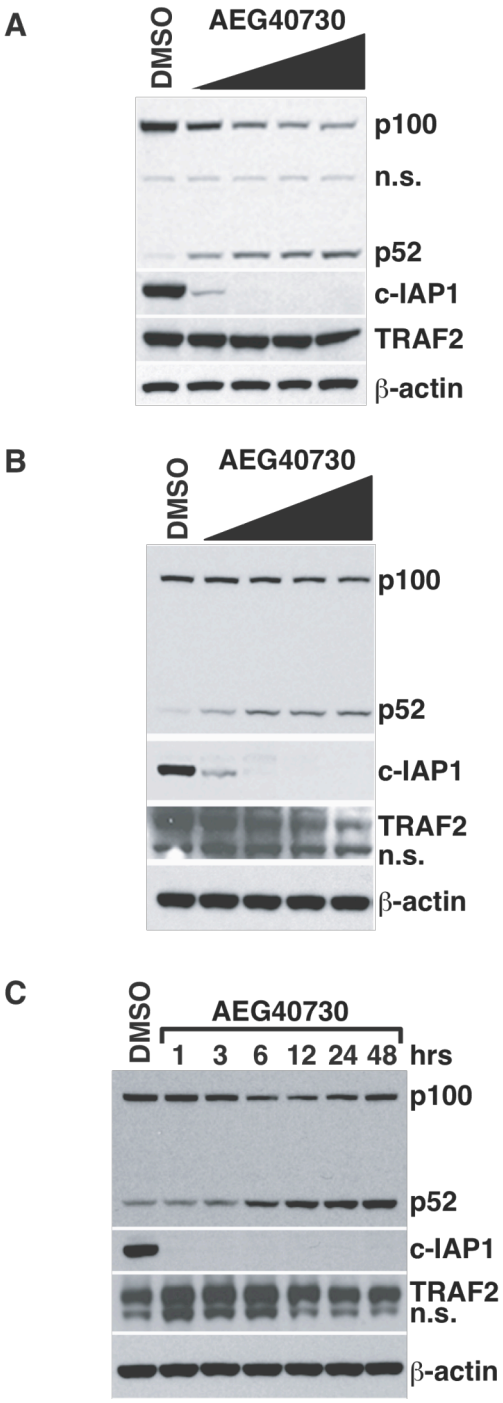


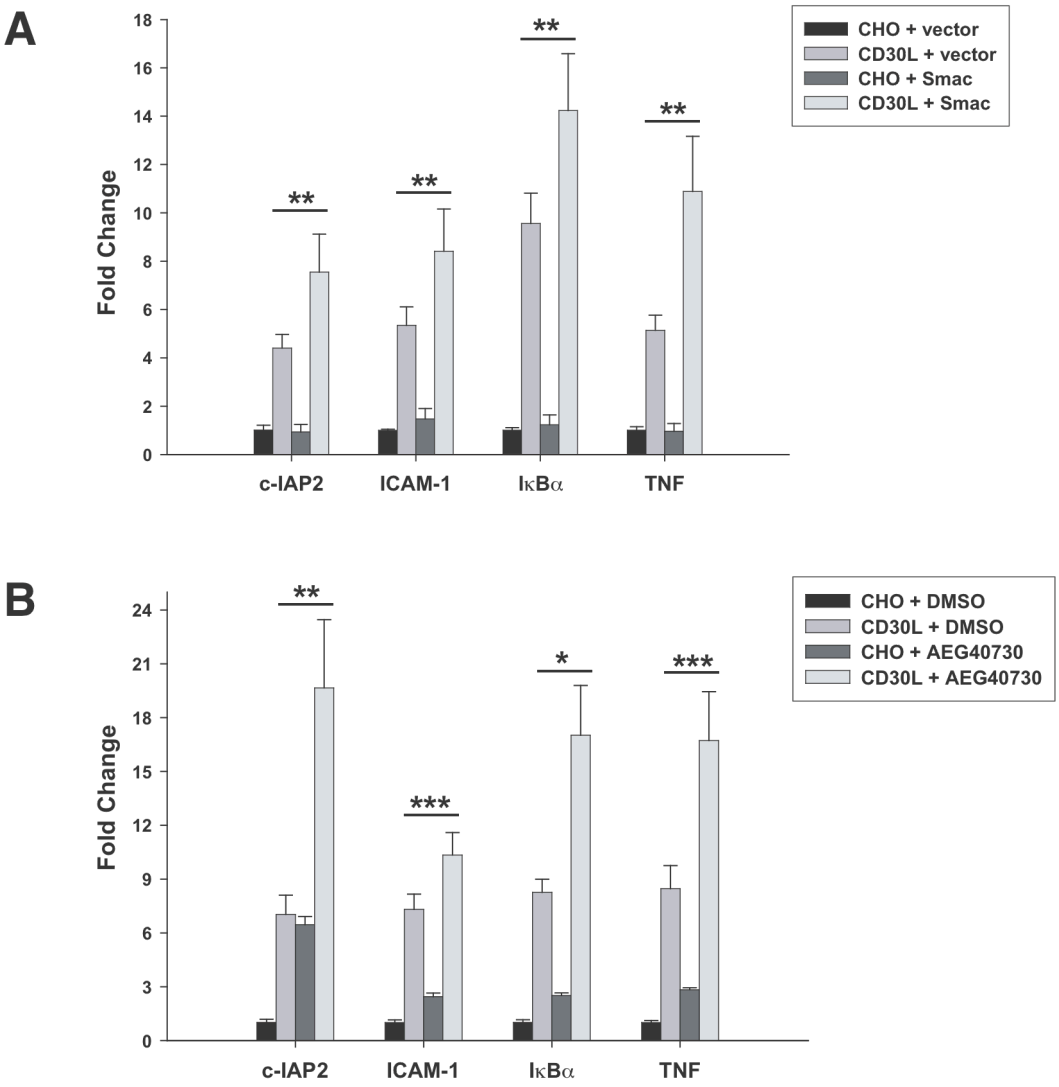
Figure 3.6: Rapid degradation of c-IAP1, but not TRAF2, precedes activation of the non-canonical NF-κB pathway. The IAP antagonist AEG40730 triggers processing of p100 to p52. (A) HEK293 cells or (B) Karpas 299 cells were treated with a vehicle control (DMSO) or 0, 1, 5, 10, 25 nM AEG40730 and incubated for 24 hours. (C) Karpas 299 cells were treated for 0 to 48 hours with 25 nM AEG40730 or DMSO. Following treatment with AEG40730, cell lysates were prepared and immunoblot analysis was performed for c-IAP1, TRAF2, and p52/p100. Equivalent protein loading was confirmed by immunoblotting for β-actin.

Both Smac and the IAP antagonist AEG40730 potentiate transcription of endogenous NF-κB-dependent genes

The finding that both Smac and the synthetic IAP antagonist can induce the processing of p100 suggested that these agents might also modulate NF-κB-dependent transcription, as induced by a defined signal from a TNF receptor superfamily member such as CD30. To test this possibility, Karpas 299 cells were transfected with a plasmid encoding mature Smac or with an empty control plasmid, and subsequently stimulated with control CHO or $CD30L^+$ CHO cells. Following stimulation, RNA was isolated and quantitative real time-PCR (qRT-PCR) analysis was performed on a panel of previously described NF-κB-responsive genes. Interestingly, the induction of NF-κB-dependent genes by CD30 was augmented in cells expressing mature Smac (Figure 3.7A). Similarly, pretreatment of Karpas 299 cells with AEG40730 prior to CD30 activation resulted in an almost identical augmentation of κB-dependent transcription (Figure 3.7B) to that observed following ectopic expression of mature Smac. Therefore, both Smac and the IAP antagonist, AEG40730, were found to function indistinguishably in their ability to potentiate κB-dependent transcription, in response to CD30 activation.

While the findings shown in Figures 3.7A and 3.7B demonstrate a potentiation of NF-κB-dependent transcription in response to the IAP antagonists or overexpression of mature Smac, it remained unclear whether endogenous Smac could play a role in NF-κB activation. To address this issue, endogenous Smac expression was experimentally suppressed and expression of defined NF-κB target genes was subsequently analyzed by qRT-PCR in response to CD30 activation (data not shown). Interestingly, Karpas 299 cells with reduced Smac levels demonstrated a significant decrease in NF-κB-responsive

genes following CD30 activation, as compared to cells transfected with control oligonucleotides and stimulated in parallel through CD30 (Figure 3.7C). Altogether, these data indicate that Smac plays a role in the expression of NF-κB target genes, consistent with its role in controlling c-IAP1 levels.



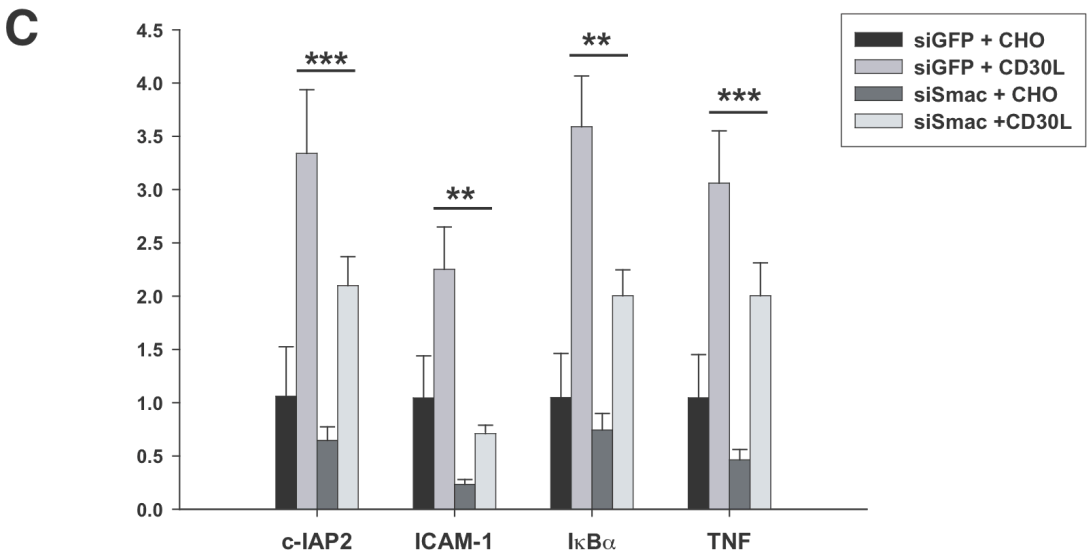


Figure 3.7: Smac is a modulator of CD30-mediated signaling and activates transcription of endogenous NF-κB-dependent genes. (A) Karpas 299 cells were electroporated with an expression plasmid encoding mature Smac-FLAG, or an empty control plasmid, as indicated. Twenty-four hours following transfection, cells were layered onto $CD30L^+$ CHO cells or control CHO cells for 2 hours. Total RNA was extracted and reverse transcribed, cDNA was analyzed by quantitative real time PCR (qRT-PCR) using Taqman probes for the indicated NF-κB-dependent genes. (B) Karpas 299 cells were treated with 25 nM AEG40730 or DMSO. Twenty-four hours following treatment, cells were layered onto $CD30L^+$ CHO cells or control CHO cells, as indicated, for 2 hours. Total RNA was extracted and reverse transcribed, and cDNA was analyzed by quantitative real time PCR (qRT-PCR) using Taqman probes for the indicated NF-κB-dependent genes. (C) Karpas 299 cells were electroporated with double-stranded siRNA oligonucleotides targeting Smac or an irrelevant sequence, as indicated. Forty-eight hours following transfection, cells were layered onto $CD30L^+$ CHO cells or control CHO cells, as indicated, for 2 hours. Total RNA was extracted and reverse transcribed, cDNA was analyzed by qRT-PCR using Taqman probes for the indicated NF-κB-responsive genes. The data shown in A, B, and C are the averages S.D. for multiple independent samples. T-test analysis was performed to calculate significance, p-values of less than 0.01 are indicated with an asterisk (*), p-values of less than 0.05 are indicated with two asterisks (**), and p-values of less than 0.10 are indicated with three asterisks (***).

Discussion

In this study, we examined the cellular regulatory mechanisms for c-IAP1, which has recently been shown to function as an NF-κB inhibitory factor. We found that c-IAP1 can be regulated by two distinct mechanisms (Figure 3.8). During CD30 signaling

c-IAP1 is targeted for degradation through its association with TRAF2 (Figure 3.1). On the other hand, cytosolic Smac selectively induces the autoubiquitination and proteasomal degradation of c-IAP1, while TRAF2 is unaffected by Smac (Figure 3.3 and 3.4). These constitute two endogenous cellular mechanism by which c-IAP1 is controlled. Interestingly, a number of earlier reports describe the autoubiquitination and degradation of c-IAP proteins after treatment with IAP antagonists (Varfolomeev et al., 2007; Vince et al., 2007; Yang and Du, 2004). Our studies indicate that endogenous Smac plays a similar role and places this factor in the regulation of NF-kB signaling through its effects on c-IAP1. In addition, our studies provide functional evidence in support of the original biochemical screen where c-IAP proteins were found to associate with TNFR2 (Rothe et al., 1995).

The enhanced expression of CD30 in several forms of leukemia and lymphoma suggest that CD30 signaling is critical to pathogenesis of these tumors (Horie et al., 2004; Horie et al., 2002). Previous studies have described CD30 as an activator of NF-κB signaling and showed that CD30 induces the degradation of TRAF2 (Duckett and Thompson, 1997; Wright et al., 2007). However, the significance of TRAF2 depletion on NF-κB activation remained unclear. Here, we show that CD30 activation not only results in the degradation of TRAF2, but also c-IAP1 (Figure 3.1). Interestingly, the degradation of TRAF2 was previously shown to require the RING of TRAF2, whereas the findings shown in Figure 3.1B indicate that the E3 ubiquitin ligase activity of the RING in c-IAP1 is not required for CD30-mediated degradation. Although exact downstream targets of c-IAP1 remain unclear, c-IAP1 has been reported to function as an E3 ubiquitin ligase for NIK, RIP, and NEMO, and to function as an inhibitor of NF-κB activation (Gaither et al.,

2007; Petersen et al., 2007; Tang et al., 2003; Varfolomeev et al., 2007; Vince et al., 2007). In this context, receptor mediated degradation of c-IAP1 is expected to facilitate NF-κB activation. Our data also provide support for the rationale of targeting c-IAP proteins to promote cell death through the activation of an autocrine TNF dependent pathway. Since several immunotherapeutic approaches are being tested for $CD30^{+}$ malignancies, the targeting of IAP proteins in combination with CD30 might be especially effective.

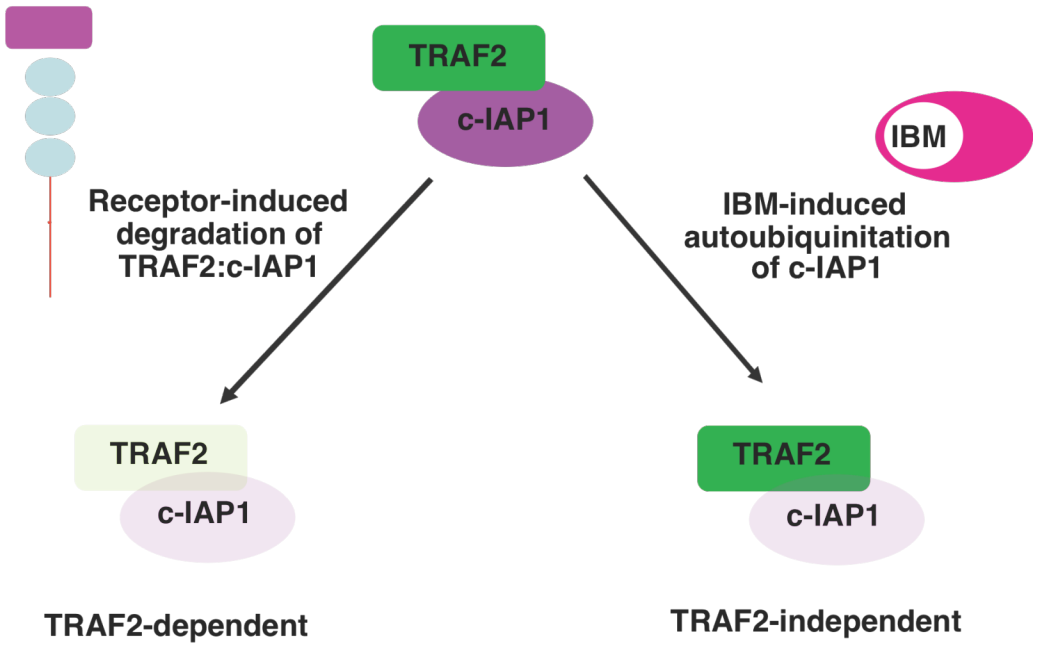


Figure 3.8: Model of the two distinct mechanisms by which the NF-κB regulatory factor c-IAP1 can be regulated. Smac and the IAP antagonist induce the selective autoubiquitination and degradation of c-IAP1. In the presence of mature Smac, c-IAP1 but not TRAF2 is degraded. Smac promotes processing of p100 to p52 and augments non-canonical NF-κB signaling. CD30 activation promotes the degradation of c-IAP1 and TRAF2. Degradation of c-IAP1 and TRAF2 requires the RING in TRAF2 but does not require the E3 ubiquitin ligase activity of c-IAP1. CD30 signaling results in induction of NF-κB signaling through processing of p100 to the active p52 subunit.

The classical role described for IAP proteins was to protect against cell death. Although XIAP has been shown to bind to and inhibit caspases, the c-IAP proteins do not

inhibit caspase activity. Similarly, prior work supported Smac as a c-IAP protein antagonist, but simple overexpression of Smac does not induce cell death. These findings suggest a non-apoptotic role for the c-IAP1:Smac interaction. Indeed, earlier reports have described a cytosolic localization of Smac in non-apoptotic cells. Notably, Deng and coworkers (Deng et al., 2003) reported the selective release of Smac from the mitochondria in colon carcinoma cells following TNF treatment, and others reported the existence of an exclusively cytoplasmic variant of Smac, which is thought to be generated by alternative splicing of the Smac mRNA transcript (Roberts et al., 2001). Consistent with these reports, we observed the presence of Smac in the cytosol of healthy ALCL and HL cells (Figure 3.2), and examined the regulation of c-IAP1 by mature Smac or an IAP antagonist. We found that c-IAP1 underwent autoubiquitination in the presence of mature Smac or the IAP antagonist (Figures 3.3, 3.5 and 3.6), supporting prior studies. Interestingly, Smac not only induced autoubiquitination of c-IAP1, but also activated the non-canonical NF-κB pathway (Figures 3.4, 3.6 and 3.7), as demonstrated by the ability of mature Smac to promote processing of p100 to p52 (Figures 3.4, 3.6 and 3.7).

A provocative earlier study revealed the ability of Smac to be recruited into the lymphotoxin-β receptor-signaling complex (Kuai et al., 2003). Since we found that Smac caused increased p100 processing and existed in the cytosol of healthy cells, we examined whether Smac would affect CD30-mediated activation of NF-κB. Indeed, ectopic expression of Smac was found to potentiate transcription of endogenous NF-κB -responsive genes (Figures 3.7A and 3.7B). Suppression of Smac also resulted in a reduction of NF-κB induction (Figure 3.7C). Therefore, by targeting c-IAP proteins for degradation, Smac can function as a highly versatile molecule with the ability to regulate

not only caspase-mediated apoptosis, but also caspase-independent signaling pathways including, but not restricted to, NF-κB activation. In this context, Smac might work differently to promote cell survival through NF-κB target genes. It will be of great importance to fully understand the intracellular cues that serve to trigger the abilities of Smac and potentially other IBM-containing proteins to function physiologically to regulate these processes.

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Chapter IV

Conclusions

The development and survival of complex multicellular organisms requires tight regulation of numerous intracellular signaling processes to balance the rates of cell death and cell growth (Jacobson et al., 1997; Kerr et al., 1972; Meier et al., 2000). Apoptosis or programmed cell death is a critical cellular mechanism to remove unnecessary or damaged cells in an organized manner, and programmed cell death is a highly regulated process that can be inhibited at several stages by various regulatory factors, including BCL-2 and IAP proteins (Salvesen and Duckett, 2002). Evasion of apoptosis is considered a hallmark of cancer, and upregulation of IAPs has been suggested to contribute to cancer cell survival through apoptotic inhibition (Hanahan and Weinberg, 2000). While IAP proteins share a conserved BIR domain that determines placement in the IAP family, it has become increasingly clear that the inhibitory mechanisms utilized by various IAP proteins are remarkably different, and that many IAP proteins, including c-IAP1, are critical regulators of additional intracellular signaling pathways (Eckelman and Salvesen, 2006; Eckelman et al., 2006; Srinivasula and Ashwell, 2008). The findings presented in this dissertation illustrate that c-IAP1 can function as an inhibitor of cell death through an indirect mechanism. Furthermore, this dissertation delineates how c-IAP1 plays a critical role in the regulation of NF-κB-mediated transcriptional activation

through TNF receptor signaling. Lastly, this dissertation illustrates how c-IAP1 protein levels are regulated by ubiquitination and proteasome-mediated degradation, and the consequences of its interaction with distinct factors, namely TRAF2 and Smac/DIABLO.

Cytoprotective Mechanism of c-IAP1

Previous studies have carefully investigated the anti-apoptotic properties of XIAP, the ubiquitously expressed prototypical mammalian IAP protein. Based on biochemical, structural and *in vivo* data, XIAP has been determined to be a potent caspase inhibitor. XIAP can bind directly to and inhibit the enzymatic activity of caspase-3, -7, and -9 (Eckelman et al., 2006). While XIAP is a potent inhibitor of cell death, the cellular IAP proteins have been considered much weaker inhibitory molecules. While c-IAP proteins were found to directly bind caspase-7 and -9, these IAP proteins were incapable of inhibiting the enzymatic activities of all tested caspases (Eckelman and Salvesen, 2006). Moreover, historically, it has been difficult to characterize the genuine inhibitory properties of the c-IAP proteins in biochemical studies since c-IAP proteins are often difficult to observe by Western blot. The carboxy-terminal RING domain present in both c-IAP proteins has intrinsic E3 ubiquitin ligase activity that can cause ubiquitination and degradation of target proteins (Joazeiro and Weissman, 2000; Dohi et al., 2004; Conze et al., 2005; Silke et al., 2005). Studies in Chapter 2 show that both c-IAP1 and c-IAP2 undergo autoubiquitination and proteasome-mediated degradation. Treatment of cells with a proteasome inhibitor resulted in a marked increase in c-IAP1 and c-IAP2 protein levels. Additionally, in the same study, mutation of a critical histidine residue in the

RING domains of c-IAP1 and c-IAP2, which has been shown to be important for E3 ubiquitin ligase activity, resulted in stable forms of c-IAP1 and c-IAP2. Therefore, the study presented in Chapter 2 provided novel evidence that c-IAP proteins are highly regulated by autoubiquitination, clarifying why c-IAP proteins are often difficult to detect by Western blot.

Given that a stable form of c-IAP1 was observed in a mutant of c-IAP1 deficient in E3 ubiquitin ligase activity, the anti-apoptotic properties of c-IAP1 were investigated. Interestingly, the RING mutant of c-IAP1 was found to inhibit cell death to a level comparable to XIAP. Although c-IAP1 cannot inhibit the enzymatic activities of caspases, c-IAP1 sequestered Smac/DIABLO, preventing Smac/DIABLO from inhibiting XIAP. Thus, when c-IAP1 sequesters and prevents Smac/DIABLO from antagonizing XIAP, this allows XIAP to prevent caspase activity and inhibit execution of cell death. Therefore, the studies presented in Chapter 2 of this dissertation suggest that c-IAP1 can function as a cell death inhibitor by a mechanism distinct from the mechanism utilized by XIAP. c-IAP1-mediated protection from cell death seems to also rely on the presence of XIAP, or other IAP proteins, which can effectively suppress caspases while c-IAP1 neutralizes Smac/DIABLO or other IAP antagonists. Figure 4.1 illustrates the distinct mechanisms utilized by XIAP and c-IAP1 to inhibit cell death.

Interestingly, while it was observed that c-IAP1 underwent RING-mediated autoubiquitination and degradation, the consequences of the interaction between c-IAP1 and TRAF2, a previously identified c-IAP1 binding partner, on cell death were largely unknown. Chapter 2 illustrates that c-IAP1 is highly stabilized by TRAF2, the most well described TRAF protein that can function as an adaptor molecule for several TNF

receptors, including TNFR2, CD30 and CD40 (Duckett et al., 1997; Chaudhuri et al., 1997; Rothe et al., 1995b). Association of TRAF2 with c-IAP1 resulted in stabilization of c-IAP1, and TRAF2-deficient MEFs or suppression of TRAF2 by RNA interference resulted in a decrease in endogenous c-IAP1 levels. Interestingly, the RING mutant of c-IAP1 could not be further stabilized by association with TRAF2, suggesting that association of TRAF2 inhibits the autoubiquitination properties of c-IAP1.

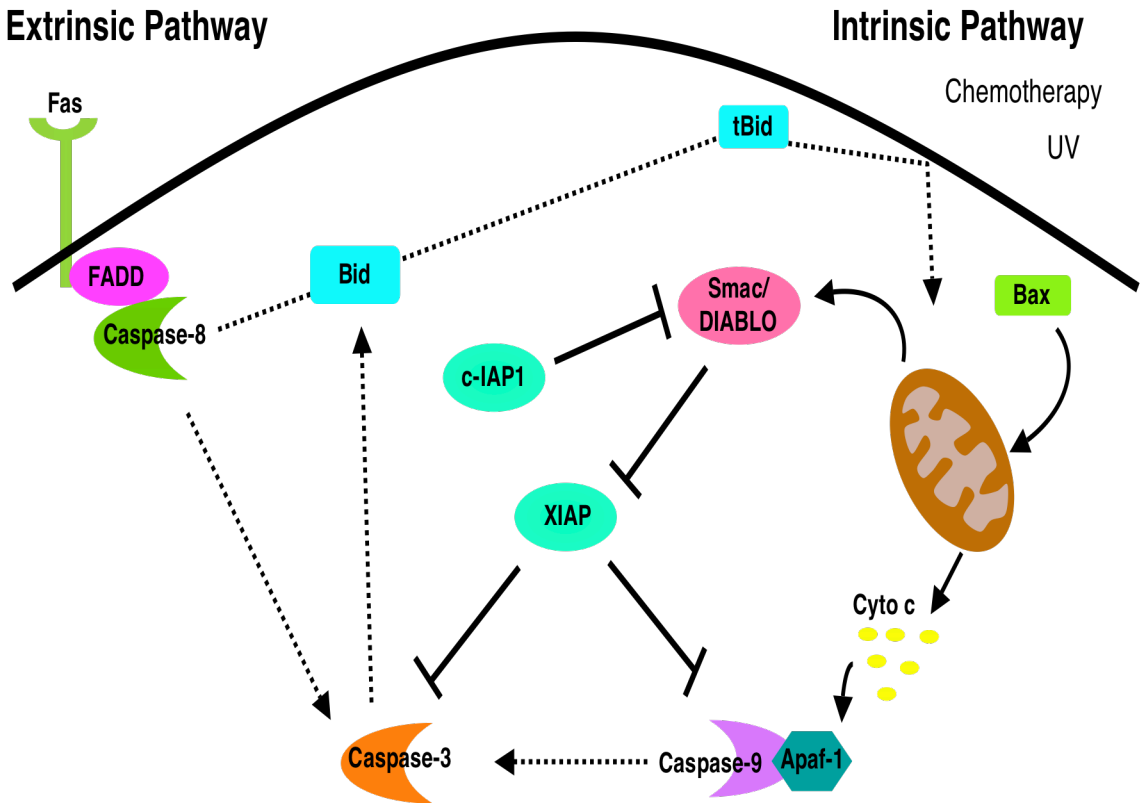

Figure 4.1: The mechanisms of cell death inhibition by XIAP and c-IAP1. Inhibitor of apoptosis (IAP) proteins can inhibit cell death by several mechanisms. XIAP can bind and inhibit the enzymatic activities of caspases, the central executioners of cell death. Therefore, XIAP is considered a potent and direct cell death inhibitor. c-IAP1 and c-IAP2 can bind to caspases, but do not inhibit the enzymatic activities of caspases. However, c-IAP1 can sequester and inhibit Smac/DIABLO, the IAP antagonist released from the mitochondria, from binding to XIAP. Therefore, c-IAP1 can function as an anti-apoptotic molecule through an indirect mechanism.

While the E3 ubiquitin ligase activity of c-IAP1 seemed to catalyze c-IAP1 autoubiquitination, it was unclear whether the RING domain within TRAF2 caused modification of c-IAP1 or was required for stabilization of c-IAP1 by TRAF2. The studies presented in Chapter 2 suggest that the RING in TRAF2 is not required for c-IAP1 stabilization by TRAF2. However, the RING of TRAF2 seemed to promote a modified form of c-IAP1, which was not observed in the presence of TRAF2 lacking its RING domain. Besides the native form of c-IAP1, a slightly higher molecular weight species of c-IAP1 was observed by Western blot upon coexpression of TRAF2 that may represent a monoubiquitinated version of c-IAP1. Future investigation of this higher molecular weight species of c-IAP1 upon coexpression of TRAF2 may reveal a novel modification of c-IAP1, which could also affect the anti-apoptotic or non-apoptotic signaling properties of c-IAP1.

Given that TRAF2 stabilized and prevented autoubiquitination of c-IAP1, the inhibitory properties of TRAF2-stabilized c-IAP1 on apoptosis were investigated. TRAF2-stabilized c-IAP1 effectively inhibited cell death to a level comparable to XIAP, and TRAF2 was not found to be responsible for any of the cytoprotective effects observed since TRAF2 alone did not protect against cell death. Therefore, although c-IAP1 does not directly inhibit the enzymatic activities of caspases, these findings suggest that c-IAP1 can function as a cytoprotective molecule when associated with TRAF2.

It remains to be investigated whether the mechanism of cell death inhibition by the c-IAP1 RING mutant and TRAF2-stabilized c-IAP1 are the same. Since c-IAP1 stabilized by TRAF2 is still sensitive to autoubiquitination because the E3 ubiquitin ligase activity is intact, the ability of TRAF2 stabilized c-IAP1 to inhibit Smac/DIABLO

might be hypothesized to be more transient because Smac/DIABLO can still promote TRAF2-stabilized c-IAP1 autoubiquitination, whereas Smac/DIABLO cannot cause autoubiquitination of the c-IAP1 RING mutant, as described in Chapter 3. Although TRAF2-stabilized c-IAP1 can undergo autoubiquitination triggered by an association with Smac/DIABLO, an intact RING in c-IAP1 might function to promote ubiquitination and degradation of Smac/DIABLO, leading to the simultaneous degradation of Smac/DIABLO and c-IAP1.

Furthermore, while the inhibitory properties of c-IAP2 were beyond the scope of this dissertation, it remains to be determined if the mechanism by which c-IAP2 can protect against cell death is similar to the mechanism described here for c-IAP1. Since c-IAP1 and c-IAP2 associate with the same signaling molecules, including TRAF2 and Smac/DIABLO, and the c-IAP proteins have highly homologous protein sequences, a plausible hypothesis is that c-IAP2 might inhibit cell death through a mechanism similar to c-IAP1. However, investigating the anti-inhibitory properties of c-IAP2 is highly challenging, since c-IAP2 has not only been shown to be a target of autoubiquitination, but has also been shown to be a target of c-IAP1-mediated ubiquitination and degradation (Conze et al., 2005). Recent data from our laboratory have suggested that c-IAP2-deficient MEFs have increased sensitivity to several apoptotic stimuli, supporting the idea that c-IAP2 has anti-apoptotic properties, although the mechanism utilized by c-IAP2 to inhibit apoptosis remains unclear.

Non-Apoptotic Function of c-IAP1 in TNF Signaling

While several IAP proteins have anti-apoptotic properties, it is now well established that IAP proteins have multiple cellular functions beyond apoptotic inhibition. As members of the IAP family, c-IAP1 and c-IAP2 were originally hypothesized to function primarily in cell death, and the findings in this dissertation support that c-IAP1 can function as a cell death inhibitor. However, involvement of c-IAP1 and c-IAP2 in TNF signaling is a property unique to the c-IAP proteins. Although the discovery that c-IAP proteins associated with TNFR2 was made in 1995, and it was subsequently found that TRAF1 and TRAF2 bridge this association, it remained unclear what roles the c-IAP proteins might play in TNF-mediated signaling until recently (Rothe et al., 1995a).

Previous studies from our laboratory and other research groups have shown that TRAF2 is an important component of signaling to NF-κB from several TNF receptor superfamily members, including CD30, which is upregulated in various forms of lymphoma and leukemia (Duckett et al., 1997; Duckett and Thompson, 1997; Boucher et al., 1997; Aizawa et al., 1997; Lee et al., 1996; Wright et al., 2007). Upon CD30 activation, it was found that TRAF2 was targeted for degradation, which corresponded to activation of both the canonical and the alternative NF-κB pathways (Wright et al., 2007). Degradation of TRAF2 following CD30 activation was found to be dependent upon the presence of the RING domain of TRAF2 (Duckett et al., 1997; Duckett and Thompson, 1997). Based on these findings, and results obtained from TRAF2-deficient B cells, TRAF2 is considered to be a critical component of NF-κB signaling that

functions to promote phosphorylation and degradation of IκBα in the canonical pathway and stabilization of NIK to promote p100 processing to the active p52 form in the alternative pathway (Grech et al., 2004).

Therefore, given that c-IAP1 and TRAF2 are established binding partners and TRAF2 stabilizes c-IAP1, the involvement of c-IAP1 in CD30 mediated NF-κB activation was hypothesized. In Chapter 3, the cellular regulatory mechanisms for c-IAP1 following CD30 activation were investigated, as well as the downstream effects on NF-κB activation. Indeed, the data presented in Chapter 3 suggest that c-IAP1 regulation is critical for NF-κB signaling following CD30 stimulation, since degradation of c-IAP1 resulted in p100 processing and activation of the non-canonical NF-κB signaling pathway. Specifically, the results in Chapter 3 suggest that like TRAF2, c-IAP1 is targeted for ubiquitination and degradation following CD30 activation. However, unlike TRAF2, it was found that the RING of c-IAP1 is not required for degradation of c-IAP1 following CD30 activation.

While the findings suggest that the RING of TRAF2 is required for TRAF2 degradation and the RING of c-IAP1 is not required for c-IAP1 degradation, it will be interesting to determine whether the RING of TRAF2 is also required for c-IAP1 degradation following CD30 stimulation. Degradation of c-IAP1, as with TRAF2, may require the RING domain within TRAF2 in order for docking of the protein(s) that catalyzes degradation of c-IAP1 and TRAF2. Alternatively, the RING of TRAF2 may be necessary to promote modifications, including ubiquitination, of c-IAP1 and TRAF2 necessary for degradation.

The findings presented in Chapter 3 support the model that c-IAP1 functions as a negative regulator of NF-κB by inducing the constitutive degradation of one of several key NF-κB signaling molecules, including NIK, RIP1, or NEMO (Tang et al., 2003; Varfolomeev et al., 2007; Vince et al., 2007). Several of these signaling molecules have been shown to trigger the non-canonical NF-κB pathway by inducing the proteolytic cleavage and maturation of p100 to the active p52 form (Senftleben et al., 2001; Xiao et al., 2001). Therefore, if c-IAP1 is targeted for degradation following CD30 activation, this allows for the accumulation of the key NF-κB regulatory factors that are normally constitutively targeted for degradation by c-IAP1. Figure 4.2 provides a schematic of the current model for the involvement of c-IAP1 in CD30-mediated NF-κB activation.

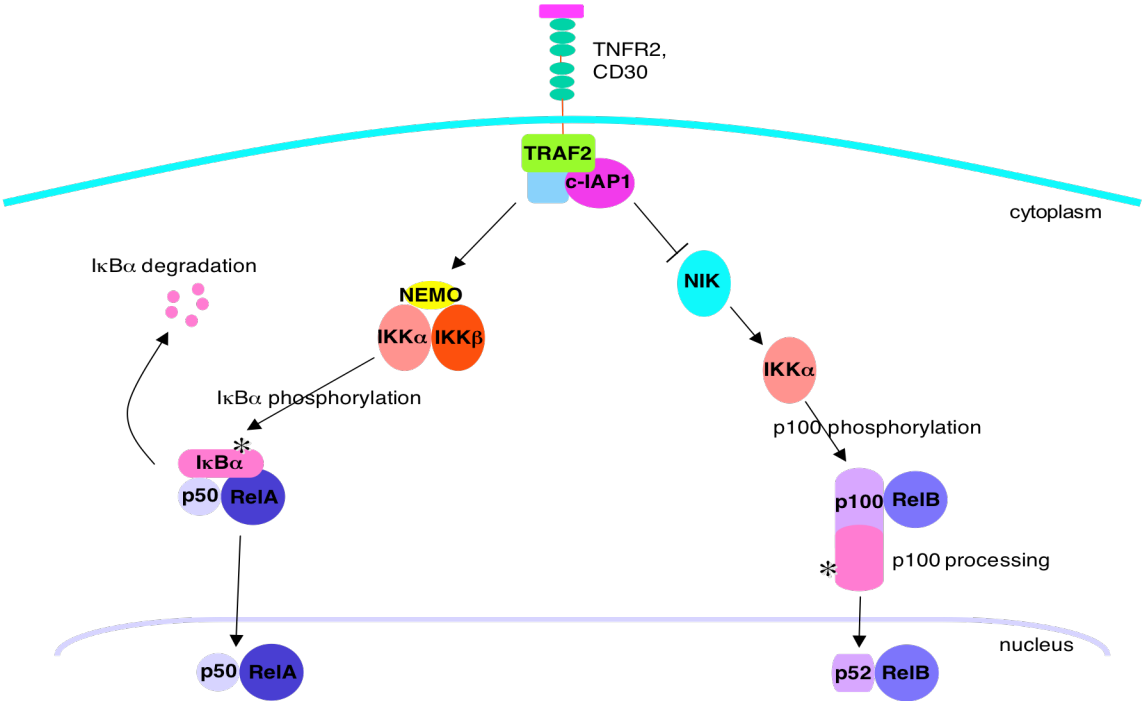

Figure 4.2: Role of c-IAP1 in the regulation of NF-κB signaling. Stimulation of TNF receptors, including CD30 and TNFR2, promotes activation of the canonical and alternative NF-κB pathways. Following TNF receptor activation, NEMO is activated through c-IAP1-mediated ubiquitination. Degradation of TRAF2 and c-IAP1 also results in accumulation of NIK, and other NF-κB regulatory factors, which promote activation of the NF-κB signaling pathways. *=phosphorylation.

Furthermore, it is clear from the results presented in Chapter 3 that there are two distinct pathways by which c-IAP1 can be targeted for degradation – Smac/DIABLO and CD30-mediated degradation. Smac/DIABLO mediated degradation of c-IAP1 is dependent upon the presence of the E3 ubiquitin ligase activity of the RING domain in c-IAP1. However, the degradation of c-IAP1 following CD30 activation does not require an intact RING domain of c-IAP1. Although the degradation of c-IAP1 by Smac/DIABLO was distinct from CD30-mediated degradation and did not result in the additional degradation of TRAF2, the surprising finding was that Smac/DIABLO-mediated c-IAP1 degradation was also capable of promoting NF-κB activation. Smac/DIABLO and AEG40730, an IAP antagonist designed around the IAP:Smac/DIABLO interface, were capable of inducing p100 processing to p52, and transcriptional activation of NF-κB targets. These findings support a model in which the effects of Smac/DIABLO are more complex that promoting cell death by targeting IAP proteins for degradation. Therefore, future evaluation of the impact of Smac/DIABLO or IAP antagonists on intracellular signaling will be highly beneficial for our understanding of how the cell integrates multiple signaling events.

Currently, while it is known that Smac/DIABLO is capable of targeting c-IAP1 for degradation and that this regulatory mechanism is dependent upon the presence of the RING within c-IAP1, there are several candidate molecules that facilitate c-IAP1 and TRAF2 degradation after CD30 stimulation. One potential molecule we are currently investigating is RIP1, a serine/threonine kinase, which has been shown to associate with both TRAF2 and c-IAP1. At the present time, preliminary data suggest that RIP1 is a good candidate, since overexpression of RIP promotes c-IAP1 and TRAF2 degradation in

a manner consistent with CD30 activation, such that the RING of c-IAP1 is not required for RIP-mediated degradation, while the RING of TRAF2 is required.

Collectively, the findings illustrated in Chapter 3 focus on the regulation of c-IAP1 by Smac/DIABLO and CD30-mediated signaling. However, it will also be interesting to determine whether the same regulation of c-IAP1 occurs by other IAP antagonists or through activation of other TNF receptors, such as CD40 and TNFR2. A recent publication by Varfolomeev *et al.* presented a similar finding for the TWEAK receptor, another TNF receptor superfamily member, where c-IAP1 is targeted for degradation, and activation of NF-κB occurs simultaneously. Therefore, it is tempting to speculate that the same mechanism of c-IAP1-mediated NF-κB regulation occurs following activation of multiple TRAF-domain containing TNF receptors.

c-IAP1 & Cancer

Currently, there is a significant amount of data suggesting that IAP proteins have altered expression and/or activity in neoplastic and lymphoproliferative diseases. Table 4.1 provides a list of several forms of cancer that have been shown to exhibit alterations in IAP expression and/or activity (Zender et al., 2006; Hunter et al., 2007; Imoto et al., 2001; Keats et al., 2007; Inagaki, 2007; Altieri, 2003; Duffy et al., 2007; Bilim et al., 2003; Yang et al., 2003). While it is now clear that many IAP proteins can promote cancer development, the exact contributions of IAP proteins to cancer are unclear and might not be as simple as cell death inhibition, since it is now evident that many IAP proteins, including c-IAP1, function in additional signaling pathways that regulate such

processes as the inflammatory response and cell proliferation. Currently, there is much investigation to uncover the functional properties of IAP proteins that contribute to tumorigenesis.

IAP	Cancer	Reference
c-IAP1	Esophageal, hepatocellular, liver, lung & ovarian carcinoma; multiple myeloma	Zender et al. (2006), Hunter et al. (2007), Imoto et al. (2007), Keats et al. (2007)
c-IAP2	MALT lymphoma, multiple myeloma	Inagaki et al. (2007), Keats et al. (2007)
Survivin	Lymphoma; Breast, colorectal, esophageal & gastric carcinoma	Alteri D.C. (2003), Duffy et al. (2007)
XIAP	Bladder, breast, colorectal, & pancreatic carcinoma	Yang et al. (2003), Bilim et al. (2003)

Table 4.1: Summary of cancers with alterations in IAP expression or activity.

Several recent studies have identified c-IAP1 as a putative oncogene. The *BIRC2* gene, which encodes c-IAP1, was present in an amplicon region associated with esophageal squamous cell carcinoma, where c-IAP1 expression correlated with resistance to chemotherapeutic agents (Imoto et al., 2001). In a murine hepatocellular carcinoma genetic screen, the *BIRC2* gene was also present in an amplified chromosomal region, and c-IAP1 was found to function synergistically with c-MYC (Zender et al., 2006). Subsequently, c-IAP1 was shown to target MAD1, the c-MYC regulatory factor, for proteasome-mediated degradation (Xu et al., 2007), supporting the idea that the E3 ubiquitin ligase activity of c-IAP1 might be the important function for the oncogenic properties of c-IAP1. Therefore, while it is now clear that c-IAP1 has oncogenic properties, it remains to be determined what functional roles of c-IAP1 are critical for it to function as an oncogene. While the apoptotic inhibitory properties of c-IAP1 could be critical to inhibit cell death of cancer cells, the newly emerging roles described here for c-

IAP1 in alternative signaling pathways such as NF-κB activation by TNF receptor might also be necessary for c-IAP1 to function as an oncogene, just as the E3 ubiquitin ligase activity of the RING domain is important for c-IAP1-mediated ubiquitination of MAD1 and might support synergy between c-IAP1 and c-MYC.

Many important recent observations have come from the characterization of IAP antagonists developed by various research labs and pharmaceutical corporations (Varfolomeev et al., 2007; Vince et al., 2007; Petersen et al., 2007). However, before c-IAP proteins can become central targets for cancer therapeutics, it will be necessary to identify all cellular functions – both apoptotic and non-apoptotic, and it will be imperative to identify which of these functions are necessary and sufficient for the oncogenic function of c-IAP1. Part of identifying the functional properties of c-IAP1 for tumorigenesis will also involve determining the mechanisms of regulating c-IAP1 expression and activities. This dissertation provides insight into the mechanisms that regulate and target c-IAP1, and in combination with future studies, will yield a thorough understanding of the signaling properties of c-IAP1 and the mechanisms critical for the regulation of c-IAP1 expression and activities.

Closing Remarks

In conclusion, this dissertation delineates the distinct mechanisms by which c-IAP1 is regulated, and provides insight into the functional roles of c-IAP1 in apoptotic and non-apoptotic pathways. These findings suggest that c-IAP1 is an important multifunctional signaling molecule, supporting recent studies suggesting that c-IAP1 is a

putative oncogene that contributes to tumorigenesis. Possibly the most surprising finding was the dynamic regulatory effects mediated by different c-IAP1 associating proteins, including TRAF2 and Smac/DIABLO, and the novel findings of the effects of these interactions on cell death and NF-κB signaling. The findings in this dissertation provide evidence that the cellular effects of c-IAP1 on NF-κB activation and cell death inhibition are largely mediated by the interactions of c-IAP1 with its cellular binding partners that inhibit or promote the E3 ubiquitin ligase activity intrinsic to the RING of c-IAP1. Therefore, while it remains to be determined which properties of c-IAP1 are critical for its oncogenic function, it will also be important to determine whether the regulatory effects mediated by difference c-IAP1 associating proteins also contribute to the oncogenic properties of c-IAP1.

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