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Splice Variants of mIAP1 Have an Enhanced Ability to Inhibit Apoptosis

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Abstract

c-IAP1 is a member of the Inhibitor of Apoptosis protein family. Functions ascribed to c-IAP1 include inhibition of apoptosis and activation of NF- κ B. Herein, we show that murine c-IAP1 (mIAP1) undergoes alternative splicing, generating two truncated proteins; one that lacks the CARD and RING domains (mIAP1- Δ CARD Δ RING) and the other that lacks only the CARD domain (mIAP1- Δ CARD). mIAP1- Δ CARD Δ RING mRNA is expressed at 2–3% of the levels of full-length mIAP1 (FL-mIAP1) in mouse tissues, yet it encodes a protein that accumulates at 50-fold higher levels than the FL-mIAP1 in cultured cells. This protein has an enhanced ability to inhibit Bax-induced apoptosis, but does not activate an NF- κ B reporter. In contrast to mIAP1- Δ CARD Δ RING, the mIAP1- Δ CARD mRNA displays distinct tissue variation, ranging from 5 to 15% of the FL-mIAP1 mRNA levels and it levels increase in the mammary gland during involution. This isoform also has enhanced anti-apoptotic activity, but diminished NF- κ B activation. In summary, mIAP1 is alternatively spliced, generating protein isoforms with distinct functional characteristics.

Keywords

c-IAP1; mIAP1; mIAP2; alternative splicing; apoptosis; CARD/RING domains

INTRODUCTION

The IAP (Inhibitor of Apoptosis) proteins constitute a family of proteins characterized by the presence of one or more Baculoviral Inhibitory Repeat (BIR) domains (see [1] for review). BIR containing proteins were originally characterized in baculoviruses, which employ these caspase-binding motifs to block apoptosis in infected host cells. c-IAP1 was initially identified in mammalian cells as a component of the Tumor Necrosis Factor Receptor 2 (TNFR2) complex, a cytokine receptor which modulates life and death decisions in numerous cell types [2]. Two broad functions have been ascribed to c-IAP1: direct binding with one or more proteins and E3 ligase activity. This latter function enables cIAP-1 to promote ubiquitination of numerous substrates, including itself. Among the specific biological functions assigned to c-IAP1 are regulation of apoptosis, activation of NF- κ B signaling, post-translational regulation of protein abundance and modulation of TNF- α signaling. The contributions of four of c-IAP1's five named domains to these functions have been well-delineated. The function of the fifth domain, the CARD domain, however, has yet to be fully elucidated.

c-IAP1 is a multi-domain protein comprised of three BIR domains located at the amino-terminus, a CARD domain and a RING domain located at the carboxy-terminus. The BIR

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domains bind to a variety of proteins including TRAFs (TNF receptor associated factor) [3], NEMO (IKK- λ) [4], the IAP inhibitor SMAC/DIABLO [5] and caspases, including caspase-9 and the executioner caspase, caspase-3 [6]. Ectopic expression of c-IAP1 fragments containing only the BIR domains is sufficient to block apoptosis in cultured cells [7]. While it is possible that this inhibition is due to direct caspase inhibition [6], caspase binding is relatively weak and is insufficient to inhibit the proteolytic activity of caspases *in vitro* [8]. An alternative anti-apoptotic mechanism is BIR-mediated sequestration of the IAP inhibitor SMAC/DIABLO, which may enhance the activity of other, more potent, endogenous apoptosis inhibitors such as XIAP [9].

The RING domain of c-IAP1 functions as an E3 ubiquitin ligase and, in conjunction with an E2 ubiquitin conjugating enzyme, mediates the transfer of one or more ubiquitin moieties onto target proteins. Much of the functionality of c-IAP1 has been attributed to the ligase activity of this domain [10]. Disruption of the RING domain (via either mutagenesis or deletion of the domain) can substantially alter the characteristics of c-IAP1. For example, c-IAP1 undergoes RING-dependent auto-ubiquitination, making it a short-lived protein [11]. In contrast, ectopic expression of RING-inactive forms of c-IAP1 results in significant protein accumulation as compared to wild type c-IAP1 [7]. The RING domain is also necessary for several of the signaling functions attributed to c-IAP1. It ubiquitinates and causes the degradation of TRAF2 in response to TNFR2 activation [12] and is required for activation of the NF- κ B pathway following ectopic overexpression of c-IAP1 [4]. It has been proposed that the RING domain also contributes to the anti-apoptotic activity of c-IAP1 by either ubiquitinating [13] or hindering [6] caspases or by ubiquitinating SMAC/DIABLO [14].

In contrast to the BIR and RING domains, little is known about the function of the CARD domain of c-IAP1. As a class, CARD domains are alpha-helical bundles that promote formation of protein complexes through CARD-CARD interactions. This domain was first described in caspases, and is found in a number of apoptotic mediators as well as proteins that activate the NF- κ B pathway [15]. While c-IAP1 can both inhibit apoptosis and activate NF- κ B signaling [4], neither of these functions has been attributed to the CARD domain. Clem et. al. have shown that removal of the CARD domain stabilizes c-IAP1 and its cleavage fragments [7]. The CARD domain has also been reported to contain a nuclear export signal, thereby regulating cellular localization of c-IAP1 [16]. Whether either of these properties involves CARD-mediated binding to other proteins is not known.

Alternative mRNA splicing is a common regulatory mechanism among proteins that modulate apoptosis. Often, alternative splicing can generate protein isoforms that have fundamentally different properties. For example, the Bcl-xl splice form of Bcl-X has anti-apoptotic properties, while the Bcl-xs splice variant promotes apoptosis [17]. In the present paper, we describe two novel splice variants of murine c-IAP1 (mIAP1) that arise due to the use of alternative splice acceptor sites for the intron 6–7 donor site. One splice variant generates a frame-shift mutation which gives rise to a truncated protein that lacks the CARD and RING domains. mRNA levels for this variant are approximately 2–3% of the levels for the full-length c-IAP1 in all tissues except the spleen, where they are significantly higher. When expressed ectopically, the protein product from this variant is 50 fold more stable than the full-length mIAP1. The second splice variant arises due to complete excision of exon 7, giving rise to a protein product that lacks the CARD domain but has an intact RING domain. A similar CARDless splice variant can be identified for mIAP2. Expression levels of this variant fluctuates among tissues and increases in an environment of elevated apoptosis. Both variants have enhanced anti-apoptotic activity and diminished abilities to activate an NF- κ B reporter construct.

EXPERIMENTAL PROCEDURES

Reagents—Murine c-IAP1 (Flag-mIAP1), Flag-mIAP1- Δ CARD and Flag-mIAP1- Δ CARD Δ RING constructs were generated using cDNAs cloned from tissues collected from FVB/N mice using standard RT-PCR procedures. All inserts were cloned into the pcDNA3 vector (Invitrogen). cDNA sequences were confirmed against NCBI sequences. The Myc-hBax vector was a generous gift of Shigemi Matsuyama [18]. Additional commercial vectors used include pEGFPC2 (Clontech), pHRG-TK (Promega) and pNF- κ B-Luc (Stratagene). Primary antibodies used were anti-flag M5 monoclonal antibody (Sigma, F 4042) and anti- β -actin (Sigma, A-1978). Primary antibodies were detected by horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology).

Cell culture—293T cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% pen/strep. Transient transfections were performed in 6 well plates, with cells plated at 60–80% confluency, using Superfect reagent (Qiagen) according to the manufacturer's recommendations.

Animal Tissues—Mouse tissues were collected from adult male and female FVB/N mice (Jackson Laboratories). Mice were housed in microisolator cages with a 12-hour light/dark cycle, and were given food and water ad libitum. Tissues were immediately frozen on dry ice after removal and stored at -80°C . All animal procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Immunoblotting—Following transient transfections, cells were washed in ice cold PBS, and then collected in RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA) containing Protease Inhibitor Cocktail (Sigma) and 1 mM Na_3VO_4 . Homogenates were clarified by centrifugation (12 000 g) for 15 min at 4°C . Proteins were quantified by Bradford Protein Assay (Biorad, Hercules, CA, USA). 150 μg of protein from whole cell lysates was denatured in Laemmli Sample Buffer (BioRad), resolved by SDS-PAGE, and then transferred to PVDF membranes (BioRad). Membranes were blocked with 5% (w/v) skim milk in PBS with 0.05% (v/v) Tween-20 (PBS-T) (1 hr, RT). They were then incubated with primary antibody in 5% (w/v) bovine serum albumin (2 hr RT) in PBS-T plus sodium azide (0.02% (v/v) followed by incubation with appropriate secondary antibody conjugated to horseradish peroxidase in 5% skim milk in PBS-T (1 hr, RT). Bound antibodies were detected by chemiluminescence (Amersham).

RNA Extraction—Total RNA was obtained from mouse tissues or cultured cells using Trizol (Invitrogen) reagent. Mouse tissues (0.1 g) were briefly homogenized in 1.5 ml of Trizol reagent. Cultured cells were collected in 1 ml of Trizol reagent. RNA was resuspended in DEPC treated water and stored at -80°C .

RT-PCR—A total of 3 μg of DNase I (Invitrogen) treated, total RNA was reverse transcribed using Superscript III reagent (Invitrogen) and Oligo(dT) primer. To measure mIAP1 splice variant expression, 10% of the RT reaction was PCR-amplified for 25 cycles (94C, 1 min; 60C, 30 sec; 72C 40 sec) in the presence of 0.5 μCi α - ^{32}P -dCTP using Taq Polymerase (Invitrogen) with the following primers: Fwd: 5'-AAAGCAGCCTTGGAAATGGGC-3', Rev: 5'-CACAGGGAATGAACACGATG-3'. PCR products were resolved on a 5% PAGE gel. Bands were quantified using a Packard PhosphorImager and the OptiQuant software supplied by the manufacturer. The relative level of each splice variant is expressed as a percentage of the amount of the variant compared to the full-length c-IAP1. To adjust for differences in radioactive cytosine content between the PCR products, values were scaled by a factor of 1.2 and 1.9 for the mIAP1- Δ CARD Δ RING and mIAP1- Δ CARD variants, respectively. RT-PCR

for the mIAP2- CARD splice variant was performed similarly as for mIAP1, using the following primers: Fwd: 5'-TGGAAATGGGCTTCAGTAGG-3', Rev: 5'-ACCACACGGAATGAACACAA-3'. mIAP2- CARD values were adjusted by a factor of 2.2 to adjust for radioactive cytosine content, when compared to full-length mIAP2 values.

Ribonuclease Protection Assay—The ribonuclease protection assay (RPA) probe template was PCR-amplified from the Flag-mIAP1 vector using the following primers: Fwd: 5'-TGACGTGTGTGACACCAATG-3' and Rev: 5'-TGCTGCAGTGTTCCTTTTG-3'. The template fragment was then cloned into the pcDNA3 vector (Invitrogen) and confirmed by sequencing. α -[³²P]-GTP cRNA riboprobes were generated using the SP6 RNA polymerase (Sigma). Ribonuclease protection assays were performed using the RPAIII kit (Ambion) according to the manufacturer's recommended procedures. 20 μ g of murine total RNA was used for each reaction. Protected fragments were separated on a 9% acrylamide/8M Urea gel. Autoradiograms were quantified by densitometry.

hBax-induced apoptosis— 1×10^5 293T cells were plated on 60 mm plates. After 24 hours, cells were cotransfected with 1 μ g pEGFPC2, 1 μ g Myc-hBax and 2 μ g test plasmid. Apoptotic cells were identified by staining cells with Hoechst 33258 (Sigma) and analyzing the nuclear morphology of GFP expressing cells by microscopy. A minimum of 100 GFP-positive cells was counted from each plate 24 hours after transfection.

NF- κ B reporter assays—293T cells were plated in 6 well dishes. After 24 hours, cells were transfected with 60 ng pHRG-TK (renilla), 300 ng pNF- κ B-Luc and 100–1000 ng test plasmid. Luciferase activity was measured after 24 or 48 hours using the Dual Luciferase Reporter Assay System (Promega). Relative luciferase expression values represent the ratio of relative light units of pNF- κ B-Luc to renilla. Assays were run in either duplicates or triplicates. All experiments were repeated at least twice.

Data analysis—Statistical tests were performed using Microsoft EXCEL and WINSTAT (Microsoft). For normally distributed data, T-Tests were used to compare groups. The Mann-Whitney U-Test was used in all other cases. A p-value of less than 0.05 was considered significant.

RESULTS

Alternative mRNA splice acceptor sites for intron 6–7 generate two novel murine c-IAP1 splice variants

Murine c-IAP1 (mIAP1) is a 9 exon gene that encodes a multi-domain protein (figure 1A). While cloning mIAP1 from mouse tissues, two smaller RT-PCR products in addition to a product of the expected size were repeatedly observed (Figure 1B). The PCR products were cloned and sequenced and were determined to be alternative splice variants of mIAP1. Sequence analysis revealed that both splice variants arose through the use of alternative splice acceptor sites for the intron 6–7 donor site (figure 1A). The longer mRNA variant arises through the use of a splice acceptor site located 85 base pairs within exon 7. This acceptor site induces a frameshift, beginning at amino acid 484, that generates a truncated protein containing 12 novel amino acids at the carboxy-terminus, but lacks both the CARD and RING domains (mIAP1- Δ CARD Δ RING) (figure 1C). The smallest RT-PCR product is caused by complete excision of exon 7. Exon 7 is a cassette exon that encodes the majority of the CARD domain of c-IAP1. This splicing event removes 84 amino acids (amino acids 484 to 568), but preserves the reading frame, thereby generating a protein that lacks the CARD domain (mIAP1- Δ CARD), but retains an intact RING domain (Figure 1C). A comparison of the genomic sequences flanking the alternative splice acceptor sites shows that both variants have identical

nucleotide sequences immediately surrounding the splice site, although the mIAP1- Δ CARD Δ RING site has a notably shorter polypyrimidine tract (figure 1D).

To determine the relative frequency of alternating splicing at the intron 6–7 donor site, a ribonuclease protection assay (RPA) was performed that employed a probe flanking the boundary of exons 6 and 7 of the full-length mIAP1 (figure 1E). The probe was designed so that, after RNase digestion, two products are observed. The longer product represents probe protected by the full-length mIAP1 while the shorter product represents both alternative splice forms arising from the intron 6–7 donor site. To verify that the probe worked as predicted, the RPA was used on total RNA collected from 293T cells transfected with plasmids encoding each of the three mIAP1 isoforms. Each test digestion produced a specific band of the expected size (data not shown). When the RPA was performed on total RNA extracted from selected mouse tissues, we found that 5–33% of mIAP1 mRNAs were alternatively spliced at this boundary.

mIAP1- Δ CARD Δ RING mRNA is constitutively expressed while mIAP1- Δ CARD mRNA levels vary across tissues

The results from the RPA suggested that alternative splicing at the intron 6–7 boundary was relatively frequent and displayed tissue variability. To ascertain the relative abundance of each splice variant, competitive RT-PCR was used to quantify polyadenylated mRNA levels encoding each alternative splice variant of mIAP1 in mouse tissues. Both alternative splice variants were observed in all tissues surveyed (Figure 2A). Relative amounts of the mRNAs for the mIAP1- Δ CARD Δ RING variant were comparable across tissues except for the spleen. The average level of expression was 2.7% ($\pm 1.1\%$) of the levels for the full-length mIAP1 (Figure 2B). This value is comparable to those measured by RPA using a probe that could distinguish between mIAP1- Δ CARD Δ RING and full-length mIAP1, where the average expression level was 2.5% ($\pm 3.5\%$) of full-length mIAP1 levels (data not shown). Large variation in expression was observed in the spleen, where levels of mIAP1- Δ CARD Δ RING ranged from 2.4% to 6.6% and may suggest that expression of this variant is regulated in the spleen.

In contrast to mIAP1- Δ CARD Δ RING, mIAP1- Δ CARD was variably expressed across tissues. Among the tissues examined, the highest levels of expression were observed in the lung and heart (where expression was 15% of full-length mIAP1) and the lowest levels were in the testes and thymus (relative levels of 5%).

mIAP2 (murine c-IAP2) is another member of the mammalian IAP gene family. Like mIAP1, it is also encoded by a nine exon gene that gives rise to a protein with an identical domain structure as mIAP1. RT-PCR was used to ascertain whether alternative splicing might occur in the proximity of exon 7 of mIAP2. Similar to mIAP1, mIAP2 generates a CARDless alternative splice variant that lacks exon 7 (figure 3A). When the relative abundance of this splice variant was quantified, alternative splicing occurred at approximately the same frequency as mIAP1- Δ CARD, but with a somewhat altered tissue distribution, most notably within the thymus and the heart where the abundance of alternative splicing was reversed compared to mIAP1 (figure 3B). Alternative splicing of mIAP2 occurred at the highest levels in the spleen, thymus, mammary gland and lung and less frequently in the heart and testes.

mIAP1- Δ CARD Δ RING protein accumulates at higher levels than full-length mIAP1 protein in transiently transfected 293T cells

Experimentally produced inactivating mutations in the RING domain of c-IAP1 have been shown to impact several of its properties, including inhibition of apoptosis and activation of NF- κ B. In particular, it has previously been shown that loss or inactivation of the RING domain

promotes substantially augmented accumulation of c-IAP1 in transiently transfected cells [7]. This suggests that, despite the relatively low abundance of mRNAs encoding the mIAP1- Δ CARD Δ RING variant observed in mouse tissues, there could be substantial protein accumulation of this variant due to a relatively prolonged half-life.

Transient transfection of plasmids encoding the mIAP1- Δ CARD Δ RING variant into 293T cells resulted in substantial protein accumulation over that observed with transient transfection of either the full-length mIAP1 or the mIAP1- Δ CARD variant (figure 4A). To more accurately quantify the relative magnitude of differences in protein accumulation that occur between the full-length protein and the mIAP1- Δ CARD Δ RING variant when expressed at their endogenous mRNA ratios, 293T cells were transiently cotransfected with full-length mIAP1 and mIAP1- Δ CARD Δ RING expression vectors at varying ratios and protein levels were measured by immunoblotting. Significantly greater protein accumulation was observed in cells transfected with the mIAP1- Δ CARD Δ RING vector than with the full-length mIAP1 vector, even when amount of mIAP1- Δ CARD Δ RING vector used was less than 10% of the amount of full-length vector (figure 4B). Indeed, comparable amounts of protein for each splice variant were observed when mIAP1- Δ CARD Δ RING plasmid levels were only 2% of the levels of the full-length mIAP1, the approximate levels of expression that are observed for the endogenous mRNAs. Prior to conducting this experiment, we verified that the levels of proteins produced when the full-length mIAP1 and mIAP1- Δ CARD Δ RING expression vectors were cotransfected were comparable to the levels observed when they were transfected individually, ruling out cross-regulation when both plasmids are coexpressed (data not shown). Thus, from this data, we conclude that protein accumulation for the mIAP1- Δ CARD Δ RING variant is approximately 50-fold greater than full-length mIAP1 when the mRNAs are equivalently expressed. To control for the possibility that the differences in protein levels were due to variations in expression levels of the plasmids or mRNA stability, relative mRNA levels were measured by RT-PCR in 293T cells transiently cotransfected with plasmids encoding the mIAP1- Δ CARD Δ RING and full-length mIAP1 cDNAs at varying ratios. The relative levels of mRNA extracted from cells closely approximated the relative levels of plasmid transfected (figure 4C). In summary, these data suggest that the mIAP1- Δ CARD Δ RING protein encoded by the splice variant can accumulate to substantial quantities in cells, despite relatively low levels of message.

mIAP1- Δ CARD Δ RING is a more effective inhibitor of Bax-induced apoptosis than full-length mIAP1, but is minimally able to activate NF- κ B

Murine c-IAP1 is a relatively low-abundance, short half-lived protein and functional characterization of the endogenous protein has been limited, in part, by a lack of sufficiently sensitive antibodies that can detect this protein *in vivo*. Consequently, the majority of studies characterizing the functions of this protein have relied on overexpression systems in transfected cells [6,19,20]. Two functions commonly attributed to c-IAP1 are inhibition of apoptosis and activation of NF- κ B signaling. Both of these functions are significantly altered by experimental inactivation of the RING domain. In general, c-IAP1 is considered a relatively weak inhibitor of apoptosis, as compared to other apoptotic regulators such as XIAP. However, previous studies have shown that truncated c-IAP1 mutants that contain only the BIR domains are significantly more effective at inhibiting apoptosis than the full-length c-IAP1 [7]. Since mIAP1- Δ CARD Δ RING has an almost identical domain structure to the truncated mutants (with the exception of the 12 novel amino acids on the carboxy terminus), we hypothesized that mIAP1- Δ CARD Δ RING would also have an enhanced ability to inhibit apoptosis, as compared to the full-length mIAP1. To test this, 293T cells were challenged with hBax, an activator of the intrinsic apoptosis pathway. The full-length mIAP1 vector was no more effective at inhibiting Bax-induced apoptosis than the empty vector at 24 hours post-transfection (Figure 5A). In contrast, the mIAP1- Δ CARD Δ RING variant had significantly

improved anti-apoptotic ability, generating a 25% increase in cell survival over the empty vector control (figure 5A).

Another function of c-IAP1 is the activation of NF- κ B via RING-mediated ubiquitination of NEMO (IKK- λ) [4]. This can be indirectly measured using a luciferase reporter vector that contains multimerized NF- κ B binding sites upstream of a minimal promoter. We measured NF- κ B activation by mIAP1- Δ CARD Δ RING in transiently transfected 293T cells. Ectopically expressed full-length mIAP1 demonstrated robust, dose-dependent activation of an NF- κ B luciferase reporter construct. In contrast, mIAP1- Δ CARD Δ RING demonstrated less than a two-fold activation of the reporter (Figure 5B), suggesting that this variant does not retain the ability to activate NF- κ B. In summary, these data show that the mIAP1- Δ CARD Δ RING has an enhanced ability to block Bax-induced apoptosis, as compared to the full-length mIAP1. However, inhibition of apoptosis is not due to activation of NF- κ B, a potent pro-survival transcriptional modulator.

Relative levels of mIAP1- Δ CARD mRNA increase during early mammary gland involution

In contrast to mIAP1- Δ CARD Δ RING, mIAP1- Δ CARD mRNA levels varied across tissues (figure 2B). This observation suggests that mRNA splicing for this variant may be regulated. To determine whether splice levels for this variant could be dynamically regulated within a tissue, relative expression of mIAP1- Δ CARD levels were measured in mammary glands at various stages of development. The mammary gland was examined because it provides an excellent *in vivo* model of splicing modulation in the presence of alternating cycles of cellular proliferation and apoptosis. During pregnancy, mammary epithelia undergo massive proliferation in preparation for lactation. Upon removal of pups, mammary glands undergo involution, which is marked by extensive apoptosis [21]. Levels of apoptosis reach a maximum at day 2 of involution, and are considerably reduced by day 5. There is substantial alteration in the general splicing environment within mammary epithelial cells throughout this process, and modulation between splicing isoforms has been observed for a number of apoptotic regulators [22]. For example, the levels of the pro-apoptotic splice forms of apoptotic regulators such as BCL-X are elevated during early involution and are lowest during pregnancy and late involution [23]. To examine whether mIAP1 alternative splicing is also regulated during mammary gland development, mRNA from mammary glands was collected from virgin mice, pregnant mice and mice after 2 and 5 days of forced involution and the relative levels of mIAP1- Δ CARD mRNA were quantified. Levels were similar between virgin and pregnant glands (figure 6). However, the relative levels of mIAP1- Δ CARD mRNA increased by approximately 40 percent on day 2 of involution, before returning to baseline by day 5 of involution. Thus mIAP1- Δ CARD mRNA expression appears to be regulated in the mammary gland and is highest when levels of apoptosis are maximal and pro-apoptotic splice forms of apoptotic regulators are favored.

mIAP1- Δ CARD is a more effective inhibitor of Bax-induced apoptosis than full-length mIAP1, but has an attenuated ability to activate NF- κ B

The pattern of mIAP1- Δ CARD regulation observed in the mammary gland suggests that mIAP1- Δ CARD may be a less effective inhibitor of apoptosis than full-length mIAP1. To assess this directly, the ability of mIAP1- Δ CARD to delay Bax-induced apoptosis was compared to the full-length mIAP1. Contrary to expectations, mIAP1- Δ CARD was significantly more effective at blocking Bax-induced apoptosis than the full-length isoform (figure 7A). Thus, loss of the CARD domain enhances the anti-apoptotic activity mIAP1.

Optimal activation of NF- κ B signaling by c-IAP1 has been previously shown to require intact BIR domains and a functional RING domain [4]. The BIR domains directly bind NEMO (IKK- λ), while the RING domain mediates the transfer of a ubiquitin moiety onto NEMO. The

contribution of the CARD domain is not known. To assess the impact losing of the CARD domain on mIAP1's activation of NF- κ B signaling, we compared the abilities of the full-length mIAP1 and the mIAP1- Δ CARD splice variant to activate an NF- κ B reporter. We found that mIAP1- Δ CARD is significantly less effective at activating NF- κ B than the full-length protein (Figure 7B). This result suggests that the CARD domain, in addition to the RING and BIR domains, is necessary for optimal activation of the NF- κ B.

DISCUSSION

In the present study, we characterized two novel splice variants of murine c-IAP1 arising from alternative splicing at intron 6–7. One variant (mIAP1- Δ CARD Δ RING) encodes a truncated protein that lacks both the RING and CARD domains. What is particularly striking about this variant is its vastly enhanced apparent stability in transiently transfected cells, as compared to the full-length mIAP1. The gain in stability is most likely due to loss of RING-mediated autoubiquitination, as has been previously described [11]. Poly-adenylated mRNA levels for this variant are approximately 2–3% of levels for the full-length isoform in most tissues. However, in light of the almost 50-fold enhancement in protein accumulation observed with this variant, it is likely that its protein levels in tissues are comparable to those for the full-length protein. In contrast to mIAP1- Δ CARD Δ RING, the mRNA for the second variant (mIAP1- Δ CARD) accumulates to a much greater degree, with a broad range of expression levels across different tissues. This suggests that splicing of mIAP1- Δ CARD is regulated in a tissue specific manner. We also found that the relative expression of this isoform changes during mammary gland development at times when alternative splicing for other apoptosis-regulating proteins is differentially regulated [22,23] suggesting that the mIAP1- Δ CARD variant has a biological function that is dynamically regulated within this tissue. Unlike mIAP1- Δ CARD Δ RING, the accumulation of this protein appears to follow similar kinetics as the full-length mIAP1. This finding contrasts with previous reports indicating that deletion of the CARD domain enhanced c-IAP1 protein accumulation in virally transfected cells [7]. A likely explanation for this alternative outcome is that viral-based approaches have much higher transfection efficiencies than liposomal-mediated transfection, and this gain in efficiency may be capable of revealing modest differences in stability. Direct *in vivo* confirmation and comparisons of protein accumulation for all three mIAP1 isoforms will require the future development of NH₃-terminally directed antibodies, which are currently unavailable.

The *in vivo* functions of c-IAP1 have not been fully elucidated. While this protein was named based on a proposed anti-apoptotic function, it is a fairly weak inhibitor of apoptosis [7,8]. This has led to speculation that inhibition of apoptosis is not the primary function of c-IAP1. A second function attributed to c-IAP1 is activation of NF- κ B [4]. This involves RING-mediated ubiquitination of NEMO, an inhibitor of NF- κ B activation. To determine if the splice variants had altered activities, we evaluated both functions of mIAP1. We found that the mIAP1- Δ CARD Δ RING variant was a more potent inhibitor of Bax-induced apoptosis than full-length mIAP1 in transiently transfected cells. This is consistent with previous reports that employed contrived deletion mutants lacking the RING and CARD domains [7]. We also found that the increased ability of mIAP1- Δ CARD Δ RING to inhibit apoptosis was not due to NF- κ B activation, because this isoform was only minimally able to activate an NF- κ B reporter due to the lack of the RING function. The modest NF- κ B inducing activity is likely due to retained protein:protein interactions mediated by the BIR domains. Indeed, Samuel et. al. showed that a synthetic c-IAP1 mutant that lacked the RING and CARD domains potentiated the NF- κ B inducing activity of TRAF2 [3].

The BIR domains of c-IAP1 directly interact with caspases, and it has been proposed that this interaction is involved in apoptosis inhibition [6]. These domains also interact with SMAC/DIABLO, a protein inhibitor of the anti-apoptotic activity of c-IAP1 [9]. Thus, the mIAP1-

Δ CARD Δ RING variant, which retains all three BIR domains, contains the necessary domain structures to bind and inhibit these apoptotic regulators. The mIAP1- Δ CARD Δ RING variant accumulates to a far greater extent than the full-length mIAP1 in transiently transfected cells. From these data, we conclude that the increased accumulation of this protein significantly contributes to its ability to inhibit Bax-mediated apoptosis. This conclusion is further supported by our studies evaluating a form of mIAP1 with an inactivating point mutation in the RING domain [24,25]. Indeed, we found that the anti-apoptotic activity of mIAP1- Δ CARD Δ RING was comparable to this RING-dead mutant (data not shown), suggesting that loss of the RING domain plays a major role in the increase in anti-apoptotic activity observed with mIAP1- Δ CARD Δ RING.

Our data indicating that loss of just the CARD domain (mIAP1- Δ CARD) also enhances the anti-apoptotic ability of mIAP1 was unexpected. While CARD domains function as protein:protein interaction motifs, the role of the CARD domain in mIAP1 has not been fully explored and its contributions to inhibiting apoptosis have not been evaluated independently of the other domains in this protein. Our characterization of this naturally occurring splice variant suggests that loss of the CARD domain impacts both apoptosis and NF- κ B activity. The precise mechanisms involved are not clear. One possibility is that loss of the CARD domain impacts the function of the RING domain of mIAP1, possibly due to alterations in the tertiary structure of the protein. This is supported by the observation that the mIAP1- Δ CARD variant is a less potent activator of NF- κ B, a previously identified RING-dependent function [4]. Altered RING function may also explain the enhancement in apoptotic activity, as RING functionality impacts c-IAP1's complex interaction with the IAP inhibitor SMAC/DIABLO [14,26,27].

In summary, we have shown that mIAP1 alternative splice variants have significantly different functional properties than the full-length mIAP1, *in vitro*. As more is learned about the *in vivo* functions of mIAP1, the physiological roles for these splice variants may become better understood. However, it is also possible that the various isoforms have biologically distinct properties. If so, comparisons of the alternative splice forms with the full-length protein may not illuminate their functions. In this case, use of sophisticated mIAP1 splice-specific knock-in and knock-out mouse models may be necessary to fully elucidate the functional significance of each of these proteins.

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Figure 1. Alternative mRNA splice acceptor sites for intron 6–7 generate two novel murine c-IAP1 splice variants

(A) c-IAP1 is a 9 exon gene. Alternative splice acceptor sites at intron 6–7 generate two splice variants, one that completely excises exon 7 (miAP1-ΔCARD) and a second which is spliced 85 bp past the 5' end of exon 7 (miAP1-ΔCARDΔRING). Arrowheads denote the positions of PCR primers used in (1B). (B) RT-PCR using forward and reverse primers located within exons 3 and 9 of miAP1 generates a band corresponding to the full-length miAP1 (FL-miAP1) in addition to two smaller bands representing the two alternative splice variants. The cDNA used for this reaction was generated from mouse heart total RNA. (C) Predicted domain structures of the full-length miAP1 and the two alternative splice variants. miAP1 contains 3 BIR (B)

domains, a CARD domain and a RING domain. The bold line at the amino-terminus of the mIAP1- Δ CARD Δ RING indicates 12 amino acids of novel protein sequence that arises due to a frame shift. These amino acids are not found in the full-length protein. (D) Alignment of the genomic sequences immediately surrounding the major splice acceptor site and the two alternative splice acceptor sites. The perpendicular line indicates the position where splicing occurs. Polypyrimidine tracts are underlined. Identical sequences surrounding the splice acceptor sites are shown in bold. (E) Ribonuclease protection assay (RPA) reveals that alternative splicing occurs in multiple tissues. The black line indicates the region of hybridization of a 268 bp radiolabeled cRNA riboprobe which spans the boundary of exon 6 and 7 of the mature mIAP1 mRNA. The full-length mIAP1 mRNA protects a 208 bp band while both alternatively spliced mRNAs protect a 120 bp band. A representative autoradiograph of the RPA performed on various murine tissues is shown.

Figure 2. mIAP1-ΔCARDΔRING mRNA is constitutively expressed while mIAP1-ΔCARD mRNA levels vary across tissues

(A) Competitive RT-PCR of murine tissue cDNA. RT-PCR of poly-adenylated cDNA using primers flanking exon 7 of mIAP1 was used to simultaneously amplify all three mIAP1 splice forms. Shown is a representative autoradiograph of radiolabeled PCR products. No RT indicates a representative sample where the reverse transcriptase enzyme was omitted from the RT reaction. (B/C) Quantification of cDNA levels of the variants relative to full-length mIAP1. Values for the alternative splice variants were adjusted to compensate for differences in [α^{32} P] dCTP incorporation between the variants and the full-length mIAP1 (as described in Materials and Methods). Values are the amount of (B) mIAP1-ΔCARDΔRING and (C) mIAP1-ΔCARD

expressed as a percentage of the amount of full-length mIAP1. Error bars represent standard deviations. At least two independent sources of tissue were used in each assay and the RT-PCR reaction was performed twice for each tissue.

Figure 3. Murine cIAP2 is also alternatively spliced to produce a CARDless splice variant
 (A) Competitive RT-PCR of murine tissue cDNA. RT-PCR of poly-adenylated cDNA using primers flanking exon 7 of mIAP2 was used to amplify the mIAP2 splice forms. Shown is a representative autoradiograph of ^{32}P -labeled PCR products. (B) Quantification of cDNA levels of the variants relative to full-length mIAP2 (FL-mIAP2). Values for the alternative splice variants were adjusted to compensate for differences in $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ incorporation between the variants and the full-length mIAP2 (as described in Materials and Methods). Values are the amount of mIAP2- CARD expressed as a percentage of the amount of full-length mIAP2. Error bars represent standard deviations. At least two independent sources of tissue were used in each assay.

Figure 4. mIAP1-ΔCARDΔRING protein accumulates at higher levels than full-length mIAP1 protein in transiently transfected 293T cells

(A) 293T cells were transiently transfected with 5 μ g of empty vector (pcFLAG) or vectors containing Flag-tagged cDNAs for full-length mIAP1, mIAP1-ΔCARDΔRING or mIAP1-ΔCARD. Cells were harvested 18 hours after transfection. Protein from whole cell lysates was resolved by immunoblotting and probed with anti-FLAG antibody. Molecular weight markers are shown adjacent to the figure as well as arrows denoting the specific protein isoforms. NS denotes a non-specific band and demonstrates equal loading. Less intense higher molecular weight bands may represent ubiquitinated protein isoforms. (B) 293T cells were transiently cotransfected with 2 μ g of vectors containing Flag-tagged cDNAs for full-length mIAP1 and

mIAP1- Δ CARD Δ RING at varying ratios. Protein expression levels were assessed by immunoblotting. (C) mRNA expression levels correlate with the amounts of plasmids transfected. 293T cells were transiently cotransfected with 2 μ g of vectors containing Flag-tagged mIAP1 in combination with either 2 or 5% of mIAP1- Δ CARD Δ RING. Expression levels of full-length mIAP1 and mIAP1- Δ CARD Δ RING mRNAs were determined by RT-PCR as described in figure 2. Shown is a representative autoradiograph and quantification of the relative expression of mIAP1- Δ CARD Δ RING shown.

Figure 5. mIAP1-ΔCARDΔRING is a more effective inhibitor of Bax-induced apoptosis than full-length mIAP1, but is minimally able to activate NF-κB

(A) 293T cells were transiently cotransfected with a GFP plasmid (1 μ g) to identify transfected cells in conjunction with an expression plasmid encoding human Bax (1 μ g) and 2 μ g of plasmids containing empty vector (pcFLAG), or flag-tagged FL-mIAP1 or mIAP1-ΔCARDΔRING. After 24 hours, cells were stained with Hoescht dye and a minimum of 100 GFP-containing cells were ascertained for apoptosis based on nuclear morphology. Bars represent the average percent of surviving GFP-positive non-apoptotic cells measured from four independent experiments. Error bars represent standard deviations. An asterisk (*) denotes a significant difference between the means for FL-mIAP1 and mIAP1-ΔCARDΔRING

($p < 0.05$). (B) 293T cells were transiently transfected with plasmids expressing renilla (60 ng), an NF- κ B-Luciferase reporter containing 5 tandem copies of NF- κ B enhancer elements (300 ng) and 100, 400 or 800 ng of plasmids containing Flag-tagged mIAP1 or mIAP1- Δ CARD Δ RING or 800 ng or empty vector (pcFLAG). Relative luciferase values are normalized to those observed for pcFLAG. Data represent the average of two transfections. Error bars represent standard deviations.

Figure 6. Relative levels of mIAP1- Δ CARD mRNA increase during early mammary gland involution

Mammary glands were collected from virgin mice (V) and mice during mid-pregnancy (P) and days 2 (Inv 2) and 5 (Inv 5) after forced involution. RT-PCR reactions were performed and data was quantified as described in figure 2. Bars represent the average amount of mIAP1- Δ CARD expressed as a percentage of full-length mIAP1 in at least two independent tissues. Error bars represent standard deviations. An asterisk (*) denotes a significant difference in between virgin and Inv2 (Mann-Whitney U-Test, $p < 0.05$).

Figure 7. mIAP1-ΔCARD is a more effective inhibitor of Bax-induced apoptosis than full-length mIAP1, but has an attenuated ability to activate NF-κB

(A) The ability of mIAP1-ΔCARD to inhibit hBax-induced cell death was determined as described in figure 5. Bars represent means of four independent experiments. Error bars represent standard deviations. An asterisk (*) denotes a significant difference between the means for mIAP1 and mIAP1-ΔCARD ($p < 0.05$). (C) 293T cells were transiently transfected with plasmids expressing renilla (60 ng), an NF-κB-Luciferase reporter (300 ng) and 300 or 600 ng of plasmids containing Flag-tagged full-length mIAP1 or mIAP1-ΔCARD of 600 ng of empty vector (pcFLAG). Relative luciferase values are normalized to those observed for pcFLAG. Data represent the average of three replicates. Error bars represent standard deviations. An asterisk (*) indicates a significant difference between mIAP1 and mIAP1-ΔCARD relative luciferase values at comparable levels of transfected vector ($p < 0.05$).