

# Identification of a Variant Form of Cellular Inhibitor of Apoptosis Protein (c-IAP2) That Contains a Disrupted Ring Domain

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## ABSTRACT

Among the members of the inhibitor of apoptosis (IAP) protein family, only Livin and survivin have been reported to have variant forms. We have found a variant form of c-IAP2 through the interaction with the X protein of HBV using the yeast two-hybrid system. In contrast to the wild-type c-IAP2, the variant form has two stretches of sequence in the RING domain that are repeated in the C-terminus that would disrupt the RING domain. We demonstrate that the variant form has an inhibitory effect on TNF-mediated NF- $\kappa$ B activation unlike the wild-type c-IAP2, which increases TNF-mediated NF- $\kappa$ B activation. These results suggest that this variant form has different activities from the wild-type and the RING domain may be involved in the regulation of TNF-induced NF- $\kappa$ B activation. (*Immune Network* 2002;2(3):137-141)

**Key Words:** c-IAP2, TNF, NF- $\kappa$ B, TRAF2, TRAF6

## Introduction

Members of the inhibitor of apoptosis (IAP) protein family have been reported to be involved in signaling that prevent cell death induced by TNFR (1), Fas signaling (2) and etoposide (3). To date, seven human IAP proteins, NAIP (4,5), c-IAP1 (6), c-IAP2 (6), XIAP (7,8), Livin (9), Bruce (10) and survivin (11) have been identified. c-IAP1, c-IAP2 and XIAP all contain two or three copies of the BIR (baculovirus IAP repeat) motif at their N termini and a RING finger at their C termini (12,13). In the case of c-IAP1 and c-IAP2, these molecules contain a CARD domain (14) and interact with TRAF2 (3,15).

Recent reports have shown that NF- $\kappa$ B is required for TNF-mediated induction of human c-IAP2 (16, 17). When c-IAP2 is overexpressed in mammalian cells, c-IAP2 activates NF- $\kappa$ B and suppresses TNF cytotoxicity (1). These c-IAP2 activities are blocked in vivo when a dominant negative form of I $\kappa$ B $\alpha$  that is resistant to TNF-induced degradation is co-transfected (1). A mutant lacking the C-terminal RING domain was shown to inhibit NF- $\kappa$ B activation by TNF and enhances TNF-mediated apoptosis (1). These reports implicate that c-IAP2 is critically

involved in TNF signaling and exerts positive feedback control on NF- $\kappa$ B via an I $\kappa$ B $\alpha$  targeting mechanism.

There have been reports that have shown variant forms of murine survivin and Livin exist and have different functions (18,19). Three murine survivin cDNA variants are formed through alternative splicing and these variants have distinct antiapoptotic functions (18). There has been no previous report that has shown the existence of a variant form of c-IAP2. We have found this variant form of c-IAP2 through the interaction with HBV X protein using yeast two-hybrid system. We have tried to search this variant form through library and genomic screening, however, we failed to find it. In contrast to the wild-type c-IAP2, the variant form has repeated sequences in the C terminus that would disrupt the RING domain. We demonstrate that c-IAP2V inhibits TNF-mediated NF- $\kappa$ B, a similar observation that has been reported in a mutant lacking the C-terminal RING domain. However, the variant form increases TRAF2-mediated NF- $\kappa$ B activation whereas the wild-type has no effect. Therefore, these results demonstrate that the variant form has different properties from the wild-type and suggest that it may have distinct functions from the wild-type in vivo.

## Materials and Methods

*Cell culture and reagents.* HEK293, human embryonic kidney cells were grown in Dulbecco's modified

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Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum in 5% CO<sub>2</sub> at 37°C.

**Transfection.** HEK293 cells were seeded into 12-well dishes the day before transfection and grown to 70% confluency. Transfection was carried out by the Ca<sub>2</sub> PO<sub>4</sub>-DNA precipitation method using N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer as described elsewhere (17) with a total of 1µg of DNA containing the indicated constructs. At 16h post-transfection, transfectants were left untreated or treated with TNF at the final concentration of 20 ng/ml for 4 h.

**Luciferase assay.** Transfectants were lysed in 0.1ml of lysis buffer (Promega) and centrifuged at 10,000 X g for 5 min to remove cell debris. The resulting clear lysates were assayed for luciferase activity according to the manufacturer's instructions and the luciferase activity was measured using the Luciferase Kit (Promega).

## Results

A cDNA encoding a variant form of c-IAP2 was found through the interaction with the X protein of HBV using the yeast two-hybrid system and was named c-IAP2V. Sequencing analysis of the wild-type and variant form of c-IAP2 has revealed that the variant form is exactly the same as the wild-type except for the C-terminal portion where insertion of particular stretches of sequence found in the RING domain exist in one or two other sites in the C terminal (Fig. 1).

Pairs of two direct repeat sequence, DR1 (indicated by single arrow) and DR2 (indicated by double line arrow) and an inverted repeat, IR (indicated by thick arrow) exist in the RING domain of the wild-type c-IAP2. The first stretch of sequence that is duplicated in the c-IAP2V is the 104 bp sequence (indicated by upper arrowed line in Fig. 1A) within the pair of the DR1. The second gene duplication sequence is a 61 bp sequence (indicated by shadowed box in Fig. 1A) between the pair of the DR2 and this sequence is repeated three times whereby the last repeat has only two bp that are different, thus showing a nearly perfect repeat. The duplication of these sequences suggests that it may have resulted from somatic gene rearrangement because previous reports have shown that this rearrangement occurs in sites of genes containing direct repeat sequence or inverted repeat sequence. Furthermore, it is unlikely that this variant form is created by incorrect cDNA synthesis because these artifacts usually have a particular sequence either in the 5'- or 3'- end or have fragments of other cDNAs attached.

The duplication of these sequences causes the dis-

### A. C-terminal sequence of variant c- IAP2V

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GAAGAAAGAACATGTAAAGTGTGTATGGACAAAGAGTGCCATAGTGTATTTCATG:
E E R T C K V C M D K E V S I V F I P C
GGTCATCTAGTAGTAGTATGCAAGATTGTGCTTCTTTTAAAGAAAGAACATGTAAAGTGT:
G H L V V C K D C A P S L R K N M *
TATGGACAAAGAGTGCCATAGTGTATTTCCTTGTGGTCATCTAGTAGTAGTATGCAAG:
TTGTGCTCCTTCTTTAAGAAAGTGCCATAGTGTATTTCCTTGTGGTCATCTAGTAGT:
TGCAAGATTGTACTCCTACAAATA
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### B. C-terminal sequence of wild-type c-IAP2

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DR1 IR DR2
GAAGAAAGAACATGTAAAGTGTGTATGGACAAAGAGTGCCATAGTGTATTTCATG:
E E R T C K V C M D K E V S I V F I P C
GGTCATCTAGTAGTAGTATGCAAGATTGTGCTTCTTTTAAAGAGTGCTTATTGTAGC:
G H L V V C K D C A P S L R K C P I C R
AGTACAATCAAGGGTACAGTTCGTACATTTCCTTCATGA
S T I K G T V R T F L S *
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**Figure 1.** The C-terminal sequence of variant c-IAP2 encoding the truncated Ring finger motif. A, the DNA and amino acid sequence of the C-terminal of c-IAP2V. The possible gene duplication region is marked by the upper-lined arrow and shadowed. B, the DNA and amino acid sequence of the Ring finger motif of wild-type c-IAP2. The pairs of direct repeats DR1, DR2 and indirect repeat IR are marked.

ruption of the RING domain in c-IAP2V that may act as a negative regulator on the activities of c-IAP2. There are two to three BIR (baculovirus IAP repeat) motifs in the IAP family members and these motifs are essential for the anti-apoptotic function of these proteins. The role of the RING domain in the anti-apoptotic function of the IAPs has not been clearly defined. However, previous studies have shown that c-IAP1 and c-IAP2 without the RING domains exhibit anti-apoptotic activities suggesting that the BIR motifs are sufficient for the anti-apoptotic function (3,6). Furthermore, previous report has demonstrated that expression of the RING domain alone increases apoptosis in *Drosophila* (20). Other studies have shown that full-length c-IAP2 degrades IκBα leading to NF-κB activation, which inhibits TNF-mediated apoptosis (1). Also, truncated form of c-IAP2 without the RING domain blocks c-IAP2 mediated NF-κB activation (1). Thus, the RING finger domain is essential for the role of c-IAP2 in TNF-mediated NF-κB activation.

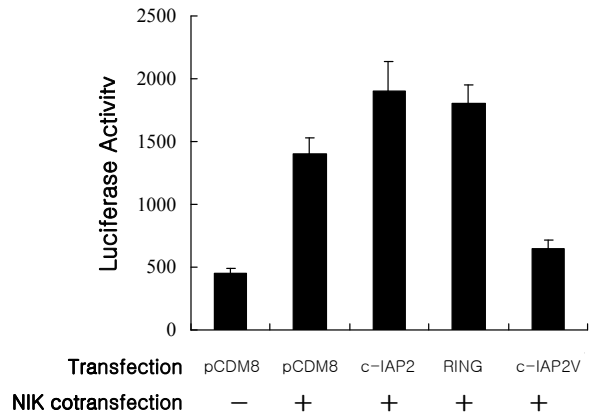
c-IAP2V with the disrupted RING domain may exhibit different functions from the wild-type. To find out if this variant form has different actions from that of the wild-type, we compared the effect of c-IAP2 and c-IAP2V in TNF-mediated NF-κB activation. We transfected these cDNAs together with the κB-luciferase reporter plasmid in human

embryonic kidney cells and treated TNF for 4hrs. The cells were then processed and the luciferase activity was measured. As shown in Fig. 2 c-IAP2 potentiates TNF-mediated NF- $\kappa$ B activation, showing the involvement of c-IAP2 in TNF-mediated NF- $\kappa$ B activation. Transfection of the RING domain alone also increased NF- $\kappa$ B activation by TNF suggesting that the RING domain in c-IAP2 is related to c-IAP2 function in potentiating TNF-mediated NF- $\kappa$ B activation. The result that shows the decrease in NF- $\kappa$ B activation by TNF in c-IAP2V is consistent with the result seen in full-length c-IAP2 and RING domain.

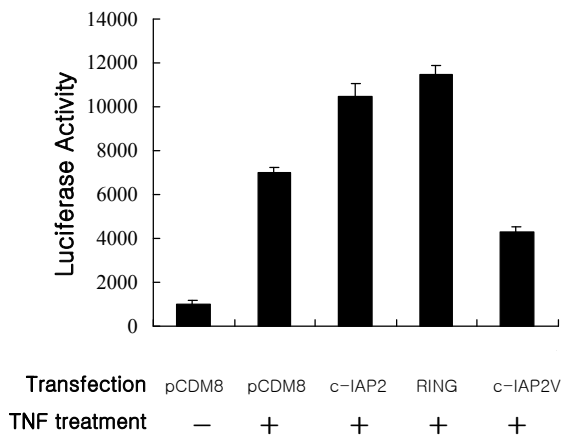
NIK is known to act upstream of the IKKs in TNF-mediated NF- $\kappa$ B activation pathway (21,22). We investigated the effect of c-IAP2 and c-IAP2V on NIK-induced NF- $\kappa$ B activation. As shown in Fig. 3 transfection of c-IAP2 or the RING domain increased NIK-induced NF- $\kappa$ B activation and c-IAP2V decreased the NF- $\kappa$ B activation. This result is similar to that seen in TNF-mediated NF- $\kappa$ B activation.

Members of the TRAF family are known to be involved in the upstream of pathways that lead to NF- $\kappa$ B activation (21,23). Therefore, similar studies were done to investigate the effects of c-IAP2 and c-IAP2V in TRAF2 and TRAF6-mediated NF- $\kappa$ B activation. As shown in Fig. 4, in TRAF2-mediated NF- $\kappa$ B activation, c-IAP2 and the RING domain have no effect, whereas the c-IAP2V shows an increase in NF- $\kappa$ B activation, suggesting that the c-IAP2 involvement in NF- $\kappa$ B activation by TRAF2 and TNF is different. In the case of TRAF6-mediated

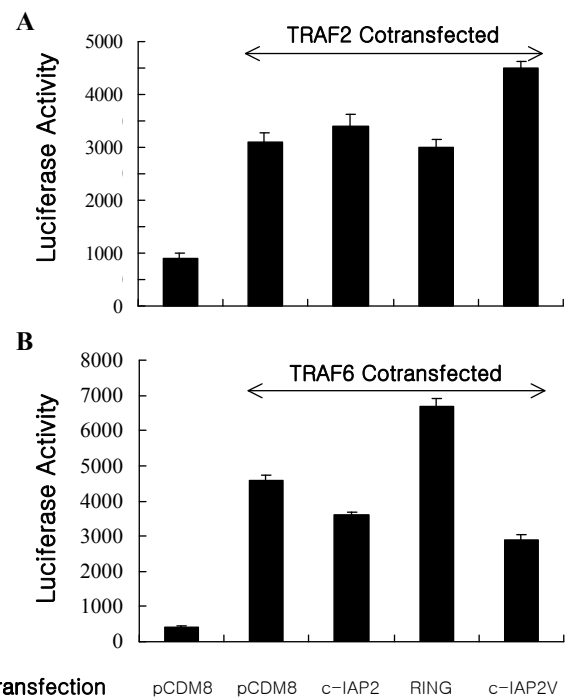
show inhibition in NF- $\kappa$ B activation. These results show that c-IAP2 acts differently on NF- $\kappa$ B activation depending on the upstream molecules that



**Figure 3.** Overexpressed c-IAP2V inhibits NIK-induced NF- $\kappa$ B activation. HEK293 cells were transiently transfected with 0.1 $\mu$ g of kB-Luc reporter construct together with 0.1 $\mu$ g of NIK, 0.4 $\mu$ g of c-IAP2, RING and c-IAP2V. After 24 hr of transfection, cell lysates were prepared and assayed for luciferase activity. PCDM8 indicates cells that were transfected only with the empty vector and not with the NIK construct. The data shown represent the result of triplicate transfections in two separate experiments.



**Figure 2.** Overexpressed c-IAP2V inhibits TNF-mediated NF- $\kappa$ B activation. HEK293 cells were transiently transfected with 0.1 $\mu$ g of kB-Luc reporter construct together with 0.4 $\mu$ g of c-IAP2, RING and c-IAP2V. After 24 hr of transfection, cells were treated with TNF (20 ng/ml) for 4 hrs. Cell lysates were prepared and assayed for luciferase activity. The data shown represent the result of triplicate transfections in two separate experiments.



**Figure 4.** Overexpressed c-IAP2 and c-IAP2V show distinct effects on TRAF2 and TRAF6-induced NF- $\kappa$ B activation. Cells were transfected with 0.1 $\mu$ g of kB-Luc reporter construct together with 0.4 $\mu$ g of TRAF2, TRAF6, c-IAP2, RING or c-IAP2V. After 24 hr of transfection, cell lysates were prepared and assayed for luciferase activity. The data shown represent the result of triplicate transfections in two separate experiments.

send the NF- $\kappa$ B activation signal.

## Discussion

Previous reports have shown that variants of murine survivin and Livin, members of the IAP family, exist and have different functions. Three murine survivin cDNA variants are formed through alternative splicing and these variants have distinct antiapoptotic functions. These variants either lack the C terminal coiled-coil domain or both the coiled-coil domain and the IAP repeat (18). However, only the variant lacking both the coiled-coil domain and IAP repeat did not suppress caspase-3 activity indicating that the variants have different antiapoptotic properties (18).

The splicing variants of Livin are almost identical proteins that share the amino-terminal BIR and carboxyl-terminal RING domain (19). Livin- $\beta$ , the shorter variant lacks 18 amino acids in the BIR RING inter-linking region. Both the variant forms showed significant antiapoptotic activity in cell death triggered by TNF and anti-Fas Ab. Livin- $\alpha$  but not Livin- $\beta$  protected cells from staurosporine-induced apoptosis, however in contrast, apoptosis induced by etoposide was blocked only by the  $\beta$  variant, thus showing differences in biological activities.

This is the first report indicating that a variant form c-IAP2 exists and that it acts differently from the wild-type. Overexpression of c-IAP2 potentiates TNF-mediated and NIK-induced NF- $\kappa$ B activation whereas, c-IAP2V inhibits NF- $\kappa$ B activation in these two cases. However, the opposite effects of c-IAP2 and c-IAP2V were not seen in TRAF2 and TRAF6-induced NF- $\kappa$ B activation. This result suggests that the role of c-IAP2 in the NF- $\kappa$ B activation pathways may be different in TNF-triggered, TRAF2 and TRAF6-induced pathways. The overexpression of RING domain increased NF- $\kappa$ B activation induced by TNF, NIK and TRAF6. This result is in correlation with previous reports demonstrating the significance of the RING domain in NF- $\kappa$ B activation.

This work is unique since it suggests for the first time that variants of the IAP family protein can be formed through the result of somatic gene rearrangement other than alternative splicing and that the variant has distinct activities. These findings indicate that somatic gene rearrangement might play an important role in the fine tuning of c-IAP2 biological function. Further investigation of the mechanism that resulted in the variant form and its biological function may provide further insights into the role of IAP variants in various physiological and pathological conditions.

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