Receptor interacting protein is ubiquitinated by cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) in vitro

Sun-Mi Park[a,b], Jong-Bok Yoon[b], Tae H. Lee[a,b,*]

[a] *Department of Biology, Yonsei University, 134 Shinchon-dong, Seodaemoon-gu, Seoul 120-749, Republic of Korea*
[b] *Protein Network Research Center, Yonsei University, 134 Shinchon-dong, Seodaemoon-gu, Seoul 120-749, Republic of Korea*

Received 11 March 2004; accepted 14 April 2004

Available online 22 April 2004

Edited by Horst Feldmann

Abstract Receptor interacting protein (RIP) is recruited to tumor necrosis factor-α receptor 1 (TNFR1) complex upon stimulation and plays a crucial role in the receptor-mediated NF-κB activation. Among the components of the TNFR1 complex are proteins that possess ubiquitin-protein isopeptide ligase (E3) activities, such as TNFR1-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins (c-IAPs) namely, c-IAP1 and c-IAP2. Here, we showed that ectopically expressed RIP is ubiquitinated, and either the intermediate or death domain of RIP is required for this modification. Expression of c-IAP1 and c-IAP2 decreased the steady-state level of RIP, which was blocked by inhibition of the 26S proteasome. RIP degradation requires intact c-IAP2 containing the RING domain. Our in vitro ubiquitination assay revealed that while TRAF2 had no effect, both c-IAP1 and c-IAP2-mediated RIP ubiquitination with similar efficiency, indicating that c-IAPs can function as E3 toward RIP.

Keywords: Receptor interacting protein; Ubiquitination; Cellular inhibitor of apoptosis protein; Tumor necrosis factor-α receptor 1-associated factor 2

1. Introduction

Tumor necrosis factor-α receptor 1 (TNFR1) mediates a variety of signals, including activation of transcription factor NF-κB, c-Jun N-terminal kinase (JNK) as well as induction of apoptosis [1,2]. Triggering of TNFR1 with trimeric TNFα leads to the recruitment of TNFR1-associated death domain (TRADD) protein to the receptor cytoplasmic death domain (DD) [3]. TRADD subsequently serves as a platform for recruiting a group of signaling proteins to the receptor complex. These include Fas-associated death domain (FADD) protein, TNFR1-associated factor 2 (TRAF2) and receptor interacting protein (RIP) [4,5]. While the association of FADD with TRADD initiates the apoptosis program by recruiting procaspase-8, the recruitment of RIP and TRAF2 elicits activation of NF-κB and JNK, respectively [4,6,7]. The crucial role of RIP in the activation of NF-κB has been demonstrated in RIP knock-out mice, as cells derived from RIP-deficient mice are unable to activate NF-κB following TNFα treatment [8].

RIP consists of three distinct domains: the N-terminal serine/threonine protein kinase domain (KD), an intermediate domain (ID), and a C-terminal DD. The RIP KD is dispensable for NF-κB activation, whereas the ID mediates the activation upon RIP recruitment to the TNFR1 complex through the DD-mediated interaction with the DD of TRADD or with the DD of TNFR1. Overexpression of the ID alone suffices to activate NF-κB, but expression of DD alone blocks NF-κB activation and induces apoptosis [5,6].

The proteins that bind to RIP include TRAF2, which in turn interacts with cellular inhibitor of apoptosis proteins (c-IAPs), c-IAP1 (HIAP2/MIHB) and c-IAP2 (HIAP1/MIHC) [9,10]. Structurally, the c-IAPs contain three baculovirus IAP repeats (BIRs) and a C-terminal RING domain. These c-IAPs can directly inhibit caspase-3 and -7 through their BIR domains and intermediary linker region [11,12]. The RING domains of c-IAPs have been shown to possess ubiquitin-protein isopeptide ligase (E3) activities [13–17]. Protein polyubiquitination requires the cooperation of three enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a E3, which transfers the activated ubiquitin from E2 to lysine residues of the bound substrate. Substrate specificity is determined mainly by the E3 enzymes. Once the multiubiquitin chain is assembled on a protein substrate, the target protein is recognized and degraded by the 26S proteasome [18,19]. Substrates that are targeted by E3 enzymatic activities of c-IAPs include themselves [13,14] and proteins implicated in the regulation of apoptosis and NF-κB activation [15–17]. For example, c-IAP1 mediates the ubiquitination of TRAF2 [15] and NEMO (IKK-γ) [16]. A recent report indicates that both c-IAP1 and c-IAP2 are E3 for mitochondrial cell death inducer Smac/DIABLO [17]. In addition, it was also reported that c-IAP2 mediates in vitro mono-ubiquitination of caspase-3 and -7 [14]. In light of the complexity of proteins recruited to the TNFR1 in conjunction with the c-IAPs, it is reasonable to assume that a protein(s) constituting the receptor complex other than TRAF2 can be ubiquitinated by c-IAPs. In fact, it has been shown that upon recruitment to the TNFR1 complex, some species of RIP undergo covalent modification and exhibit

* Corresponding author. Fax: +82-2-312-2242.
E-mail address: thlee@yonsei.ac.kr (T.H. Lee).

Abbreviations: TNFR1, tumor necrosis factor-α receptor 1; JNK, c-Jun N-terminal kinase; TRADD, TNFR1-associated death domain; FADD, Fas-associated death domain protein; TRAF2, TNFR1-associated factor 2; RIP, receptor interacting protein; DD, death domain; c-IAP, cellular inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.021

ladder-like appearance, a characteristic of proteins that are ubiquitinated [20–24]. However, how RIP is ubiquitinated has not been studied. Here, we present evidence that RIP is a target of the E3 activities of c-IAPs in vitro. Our data provide clues to understanding the mechanism of how RIP is ubiquitinated and degraded upon recruitment to the TNFR1 complex.

2. Materials and methods

2.1. Cell culture and reagents

Culture media were purchased from Life Technologies Inc. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. *N*-Acetylleucylleucylnorleucinal (ALLN) and MG132 that were used at the respective concentration of 10 μg/ml and 10 μM were purchased from Calbiochem. Antibodies were sourced as follows: monoclonal anti-RIP Ab was from BD Pharmingen, anti-Flag M2 monoclonal Ab from Sigma, anti-ubiquitin Ab from Zymed, anti-HA Ab from Santa Cruz, anti-c-IAP2 Ab from R&D systems and anti-GST Ab from Amersham.

2.2. Plasmid constructs

Expression plasmids for HA-tagged murine RIP and its deletion mutants were provided by Dr. E.-J. Choi (Korea University, Korea) [25]. The cDNA for Flag-tagged RIP and GST-fused RIP is of human origin. The cDNAs for c-IAP1, c-IAP2 and its deletions were PCR-amplified using the cDNA templates originated from pcDNA-myc-c-IAP1 and -c-IAP2 [12, a kind gift of J.C. Reed] and cloned downstream of the sequence encoding Flag epitope of pRK5 plasmid. The amino acid residues present in the c-IAP2 and its deletion constructs are as follows: c-IAP2, which is designated as C2 in Fig. 4, 1–604; C2ΔR, 1–552; C2C, 324–604; C2CΔR, 324–552; R, 553–604. To generate GST-fusion constructs, the cDNAs encoding full-length RIP, c-IAP1, and c-IAP2 were cloned downstream of GST-coding sequence in pGEX-5X (Clontech).

2.3. Transfection, immunoprecipitation and Western blot analysis

HEK293 cells were seeded in 6-cm dishes and the following day, transfection was carried out by the $CaPO_4$-DNA precipitation method using *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid buffer. After 16 h, cells were lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 1.0% Triton X-100, protease inhibitor cocktail (Sigma) and 10 μM MG132. Cell lysates were incubated with the appropriate Ab for 2 h at 4 °C and further incubated with protein-G agarose beads (Sigma) for 2 h at 4 °C. The immune complexes were washed extensively with the lysis buffer for several times. Two times sample loading buffer was added to immune complexes and boiled, and the eluted proteins were separated by SDS–PAGE. Then, proteins were transferred to nitrocellulose membrane (Bio-Rad) and visualized by Western blotting with the enhanced chemiluminescence reagents (Amersham Pharmacia).

2.4. Expression and purification of recombinant proteins

Six-His-tagged yeast Uba1 (E1) and human UbcH5A (E2) [26, a kind gift from J.W. Conaway] were expressed in *Escherichia coli* strain BL21 (DE3) and purified by Ni^{2+}–agarose beads (Qiagen). Ubiquitin containing a six-His tag and a protein kinase C recognition site (a kind gift from Z.Q. Pan) was purified as described [27]. Integrity of the prepared recombinant proteins has been confirmed and described [28]. GST-fused proteins were expressed in *E. coli* BL21 by adding 0.1 mM IPTG for 2 h and purified using glutathione–Sepharose (Pharmacia Biotech) according to the manufacturer's instructions. For in vitro ubiquitination assay, Flag-RIP was translated in vitro in rabbit reticulocyte lysate with a SP6 Quick Coupled Transcription/Translation System (Promega). Five in vitro translation reactions were performed and pooled for immunoprecipitation. Lysis buffer was added to these reactions and incubated with anti-Flag M2 Ab-coupled agarose beads (Sigma) at 4 °C for 4 h to immunoprecipitate Flag-RIP. After extensive washing of the beads with lysis buffer, bound RIP protein was eluted with the lysis buffer without detergent containing 0.3 mg/ml Flag peptide. The eluted Flag-RIP was used as substrate for in vitro ubiquitination reaction.

2.5. In vitro ubiquitination assay

GST-RIP expressed in bacteria or Flag-RIP synthesized by in vitro translation was purified and used as substrate for in vitro ubiquitination assay. In some experiments, Flag-c-IAP2 or Flag-TRAF2 were immunoprecipitated by Flag Ab agarose from lysates of transfected 293 cells (10^7 cells) and used as E3 enzymes in this bead bound form. These collected beads were washed twice with ubiquitination reaction buffer prior to the reaction. The ubiquitination assay was carried out in ubiquitination buffer (50 mM Tris, pH 7.5, 2 mM ATP, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.05% NP-40) containing yeast E1 (50 ng), E2 (200 ng) and His_6-ubiquitin (0.8 μg) in a total volume of 30 μl. The mixture was incubated at 30 °C for 1 h where in experiments using bead bound substrates, gentle agitation was given to keep beads in suspension. The reactions were terminated with sample loading buffer and run on SDS–PAGE gels. Higher molecular ladder of ubiquitinated proteins was visualized by performing immunoblot analysis with anti-RIP Ab or anti-c-IAP2 Ab.

3. Results and discussion

3.1. Detection of ubiquitinated species of RIP

293 cells were transiently transfected with plasmid encoding HA epitope-tagged RIP (HA-RIP). To block the apoptotic process caused by RIP overexpression, caspase inhibitor CrmA expression plasmid was cotransfected. Immunoblot analysis using an anti-HA Ab revealed that cells transfected with both HA-RIP and CrmA expression plasmids produced higher amount of HA-RIP and a number of slow migrating immunoreactive bands, compared to cells transfected with the RIP expression vector alone (Fig. 1A). This ladder-like appearance of RIP is characteristic of ubiquitinated proteins. To confirm that RIP is ubiquitinated, immunoprecipitated RIP was analyzed by Western blot using the anti-ubiquitin Ab. To facilitate the detection of the ubiquitinated RIP, we also analyzed the immunoprecipitated RIP prepared from 293 cells coexpressing Flag-ubiquitin in the presence of CrmA. As shown in Fig. 1B, the precipitated RIP contained proteins reactive to ubiquitin Ab, which increased when Flag-ubiquitin was coexpressed. The same blot reprobed with anti-Flag Ab exhibited strong Flag Ab reactive products with smeared appearance above the band corresponding to RIP in the sample coexpressing Flag-ubiquitin (Fig. 1B, *right panel*). Fig. 1C shows that anti-RIP Ab is able to detect ubiquitinated RIP even without the coexpression of CrmA, and its steady-state expression level is enhanced by the treatment of 26S proteasome inhibitors (ALLN and MG132), indicating that the ubiquitinated RIP undergoes proteasome-mediated degradation.

3.2. RIP is ubiquitinated in the ID and DD region

To determine the regions required for ubiquitination, we expressed several deletion mutant proteins of RIP (HA-tagged) in 293 cells along with Flag-ubiquitin and CrmA (Fig. 2A). The expression of each deletion protein was confirmed with Western blot analysis using the anti-HA Ab (Fig. 2B, *upper panel*). After immunoprecipitating the expressed RIP proteins with HA Ab, the ubiquitinated products were analyzed by immunoblotting with Flag Ab (Fig. 2B, *lower panel*). While the KD did not produce ubiquitinated products, the deletion protein containing the ID and DD exhibited abundant amount of ubiquitinated products like the wild-type (*lanes 3* and *4* Fig. 2B). The ubiquitinated species were not clearly detectable in the immunoprecipitated samples of the ID and DD. However, the ID was consistently expressed at a low level, pre-

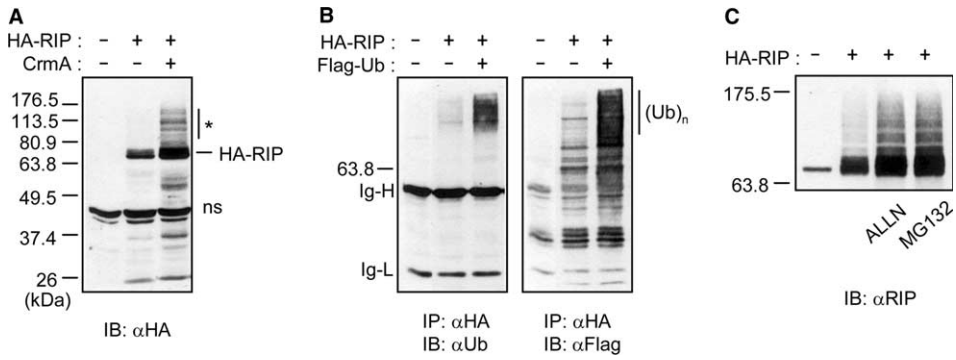

Fig. 1. RIP undergoes ubiquitination. (A) 293 cells were transfected with expression plasmids encoding HA-RIP alone or with CrmA. 16 h later, cell lysates were prepared and analyzed by immunoblotting with anti-HA Ab. Higher molecular weight products above RIP are indicated by asterisk (*ns*, non-specific). (B) 293 cells were transfected with HA-RIP plasmid alone or together with Flag-ubiquitin plasmid. Lysates were immunoprecipitated with anti-HA Ab and immunoblotted with either anti-ubiquitin Ab or anti-Flag Ab. Ubiquitin moieties attached to RIP are indicated by (*Ub*)*n*. (*Ig-H*, immunoglobulin heavy chain; *Ig-L*, light chain). (C) Cells transfected with HA-RIP plasmid were treated with proteasome inhibitors, ALLN and MG132, 4 h prior to cell lysate preparation. Cell lysates were immunoblotted with anti-RIP Ab.

sumably due to the intrinsic instability (*lane 5*, Fig. 2B; see also *lane 7* in Fig. 3B). In addition, higher molecular weight products of the DD proteins were also faintly detected in the cell lysates as well as in the immunoprecipitated preparations (*lane 6*). When ID and DD were linked to the KD, the resulting KD-ID and KD-DD were ubiquitinated as efficiently as the full-length RIP (*lanes 8 and 9*). Since the KD alone was not ubiquitinated, cellular ubiquitination machinery may target the ID or DD region of RIP for ubiquitination.

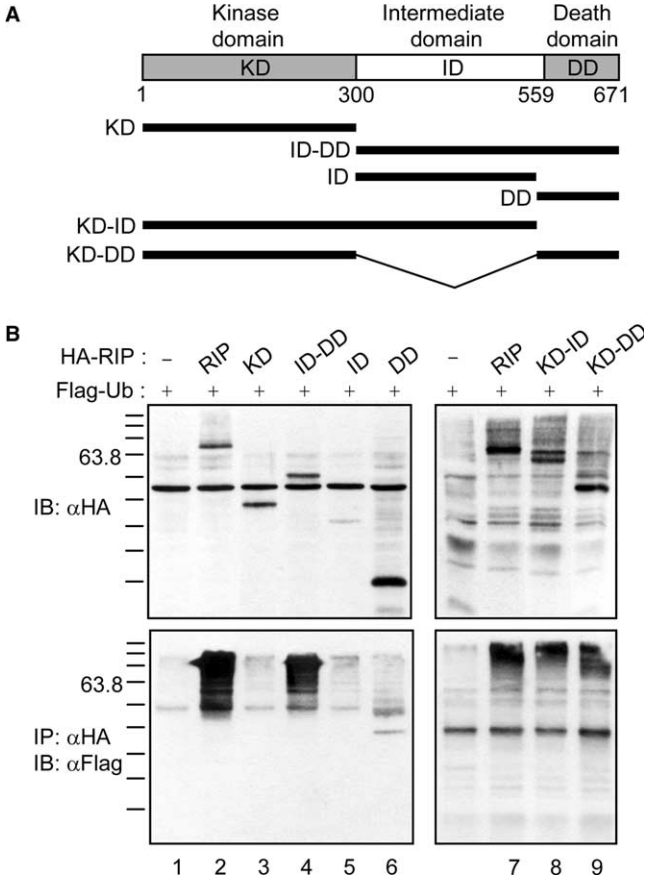

Fig. 2. ID and DD are required for RIP ubiquitination. (A) Schematic representation of RIP and its deletion constructs. KD, ID and DD are indicated. (B) Western blot analysis of the expressed HA-RIP and its deletion mutants. 293 cells were transfected with the indicated plasmids and cell lysates were prepared and processed for immunoblotting with anti-HA Ab (*upper*). The same lysates were immunoprecipitated with anti-HA Ab and immunoblotted with anti-Flag Ab to detect Flag-ubiquitin bound to HA fusion proteins (*lower*). Reference molecular weight markers are the same as indicated in Fig. 1A.

3.3. Expression of c-IAP2 decreased the steady-state level of RIP, which correlates with ubiquitination

During TNFR1 signaling, RIP is recruited to the receptor complex along with TRAF2, c-IAP1 and c-IAP2, those which possess E3 activities [13–17,29,30]. Previous reports have mentioned that within the receptor complex, RIP undergoes rapid and transient covalent modification, which has been shown to be ubiquitination [23,24]. Having hypothesized that RIP is a target of an E3 enzyme recruited to the receptor complex, we tested whether c-IAP2 has any effect on RIP expression. RIP was cotransfected with different amounts of c-IAP2 expression plasmids in 293 cells and cell lysates from these transfectants were examined for the level of RIP by immunoblotting with anti-RIP Ab. As shown in Fig. 3A, the expression of c-IAP2 dose-dependently reduced the level of RIP and the degradation was blocked by the treatment of cells with ALLN and MG132, showing that 26S proteasome is involved in the degradation of RIP induced by c-IAP2. The breakdown of RIP by c-IAP2 was also confirmed in HeLa cells (data not shown). The functional consequence of dose-dependent RIP degradation by c-IAP2 was confirmed by concomitant decrease of NF-κB activation induced by RIP expression (Fig. 3A, *right panel*). In addition, we observed that while KD was not degraded, the ID-DD, ID and DD proteins that can be ubiquitinated were efficiently degraded by coexpression of c-IAP2, demonstrating that breakdown of RIP and its deletion derivatives by c-IAP2 correlates with their ubiquitination (Fig. 3B).

3.4. RIP breakdown requires intact c-IAP2 protein

To delineate the structural requirement responsible for the c-IAP2-mediated RIP destabilization, we constructed various c-IAP2 deletion mutants (Fig. 4A) and tested whether they mediate RIP destabilization. Fig. 4B shows that RING domain-deleted c-IAP2 mutant proteins (C2ΔR and C2CΔR) failed to degrade RIP. It has been reported that the RING

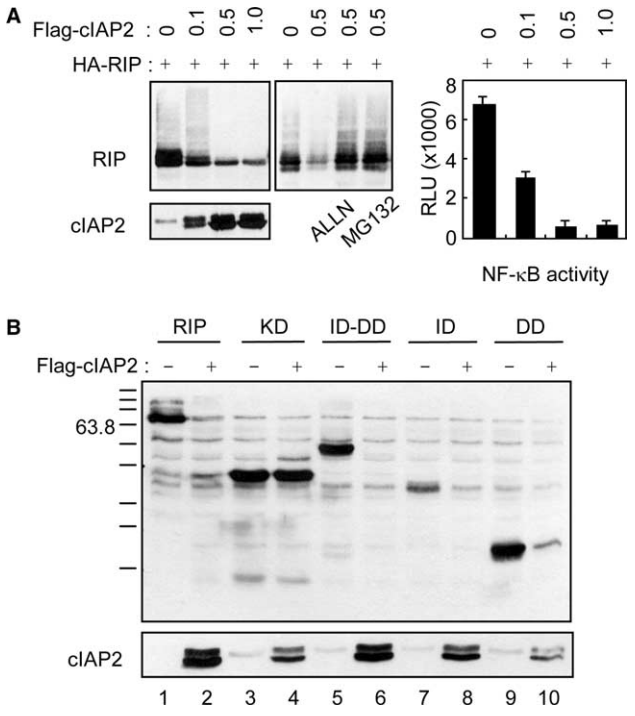

Fig. 3. Expression of c-IAP2 dose-dependently induces the degradation of RIP. (A) Subconfluent 293 cells on 6-well-plates were transfected with HA-RIP and the indicated amounts (μg) of Flag-c-IAP2 plasmids. After transfection (16 h), the cell lysates were analyzed by immunoblotting with anti-RIP Ab and anti-Flag Ab. In other set of experiments, the transfected cells were treated with 26S proteasome inhibitors, 10 μg/ml ALLN and 10 μM MG132 for 4 h. Then, cell lysates were prepared and the level of RIP was examined by immunoblotting with anti-HA Ab. 293 cells were transiently transfected with 0.5 μg of HA-RIP, the indicated amounts of Flag-c-IAP2 and 0.2 μg of κB-luciferase reporter plasmid, which contains two NF-κB binding elements derived from the promoter region of c-IAP2 gene [31]. 18 h after transfection, luciferase activities from lysates were measured. Values are representative of three independent experiments. (B) 293 cells expressing each indicated HA-RIP deletion construct together with or without Flag-c-IAP2 were processed for Western blot analysis to detect the level of expression of RIP proteins with anti-HA Ab (*upper*) and of c-IAP2 with anti-Flag Ab (*lower*).

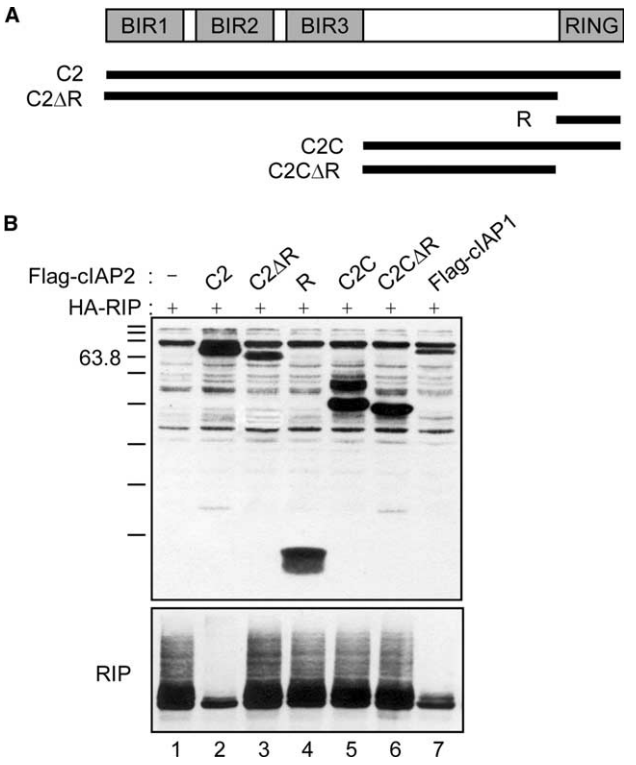

Fig. 4. RIP degradation by c-IAP2 requires intact full-length c-IAP2 in vivo. (A) Schematic representation of c-IAP2 and its deletion constructs. Indicated are domains of c-IAP2. (B) 293 cells were cotransfected with HA-RIP and various Flag-tagged c-IAP2 deletion constructs as well as Flag-c-IAP1 as indicated. Cell lysates were prepared, and protein expression levels were analyzed by immunoblotting with anti-Flag Ab (*upper*) and by anti-RIP Ab (*lower*).

domain of c-IAP2 is essential for its E3 enzymatic activity and the RING alone can promote self-ubiquitination as well as mono-ubiquitination of caspase-7 in vitro [14]. However, the RING domain (R) protein as well as the BIR-deleted protein (C2C) also failed to degrade RIP, presumably due to the lack of the region of c-IAP2 to recruit the substrate RIP and other regulatory proteins. Our observations indicate that RIP degradation by c-IAP2 is RING domain-dependent and that the BIR domain of c-IAP2 is indispensable for RIP degradation in vivo. Consistent with our results, it has been recently reported that in the context of c-IAPs-dependent ubiquitination and degradation of Smac/DIABLO, the BIR domains of c-IAPs are required for specific interaction with Smac/DIABLO [17]. Lane 7 of Fig. 4B shows that in addition to c-IAP2, c-IAP1 promoted RIP degradation.

3.5. c-IAP1 and c-IAP2 ubiquitinate RIP in vitro

To examine whether c-IAP2 ubiquitinates RIP directly, in vitro ubiquitination assay was performed. Recombinant GST-RIP was incubated with E1 (His-tagged yeast Uba1), E2 (His-tagged human UbcH5A) and His-ubiquitin in the absence or presence of purified c-IAP2 proteins. Flag-tagged full-length c-IAP2 (C2) and its RING deletion mutant (C2ΔR) were expressed in 293 cells and affinity-purified using anti-Flag M2 Ab-coupled agarose beads. The reaction products were resolved on SDS–PAGE and probed with anti-RIP Ab or with anti-c-IAP2 Ab. The results showed that the intact c-IAP2 (designated as C2 in Fig. 5A) promoted the formation of higher molecular ladder of GST-RIP protein, an indication of ubiquitination, only in the presence of E1 and E2 (*lane 6*, *left panel* Fig. 5A), however no such modification was observed with the C2ΔR (*lane 5*, *left panel*), indicating that the RING domain is essential for E3 activity of c-IAP2. The addition of E1 and E2 without functional E3 enzyme produced shifted bands which appeared to be mono- and di-ubiquitinated forms of GST-RIP (*lane 2* and *5*, *left panel*). At the moment we could not evaluate what causes this modification. When blotted with anti-c-IAP2 Ab, higher molecular weight species which are indicative of self-ubiquitinated products of Flag-c-IAP2 were detected in the reaction performed with the intact c-IAP2, but not with the RING-deleted c-IAP2 (*right panel*, Fig. 5A).

To exclude the possibility that other cellular proteins co-precipitated with c-IAP2 from the transfected 293 cell lysates may influence RIP ubiquitination, we used bacterially expressed GST-fused forms of c-IAP2 as E3 for in vitro RIP ubiquitination assays. Since GST proteins are known to interact with each other, instead of GST-RIP, in vitro-synthesized Flag-RIP was used as substrate. Under this assay condition, we also tested whether c-IAP1 can mediate RIP

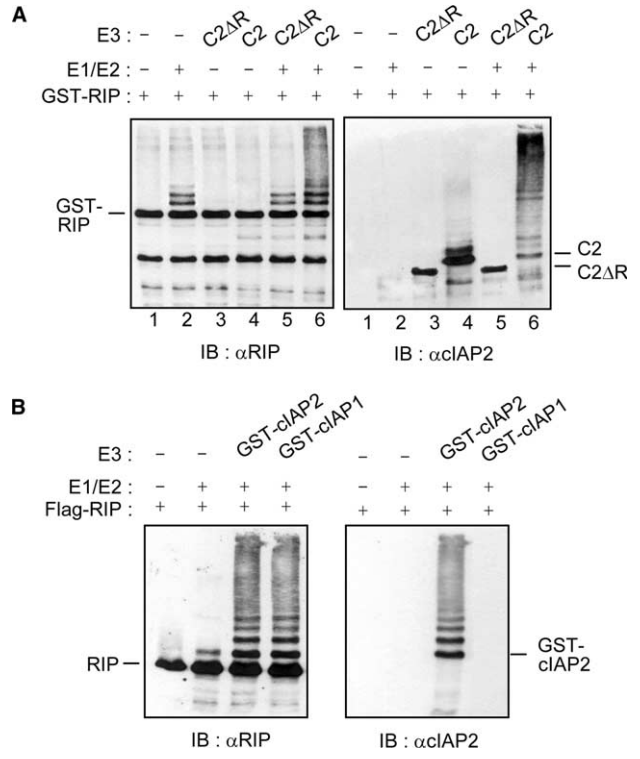

Fig. 5. c-IAP1 and c-IAP2 ubiquitinate RIP in vitro. (A) The c-IAP2 RING domain is required for RIP ubiquitination in vitro. Bacterially expressed GST-RIP was incubated with Flag-c-IAP2 (indicated as C2) or c-IAP2 without RING (C2ΔR) that were affinity-purified from transfected 293 cells, together with purified E1, E2 and His6-ubiquitin, at 30 °C for 60 min. After the reaction, the samples were resolved on 6% SDS–PAGE and analyzed by immunoblotting with anti-RIP Ab (*left*). To examine self-ubiquitinating activity of c-IAP2, the same lysates were probed with anti-c-IAP2 Ab (*right*). (B) c-IAP1 ubiquitinates RIP as efficiently as c-IAP2. In vitro translated Flag-RIP was incubated with the indicated bacterially expressed GST-fused proteins. The reaction was performed in the same condition as written in panel *A*. The reaction products were analyzed by immunoblotting with anti-RIP Ab (*left*) and c-IAP2 Ab (*right*). Self-ubiquitinated products of GST-c-IAP1 were not visualized with anti-c-IAP2 Ab.

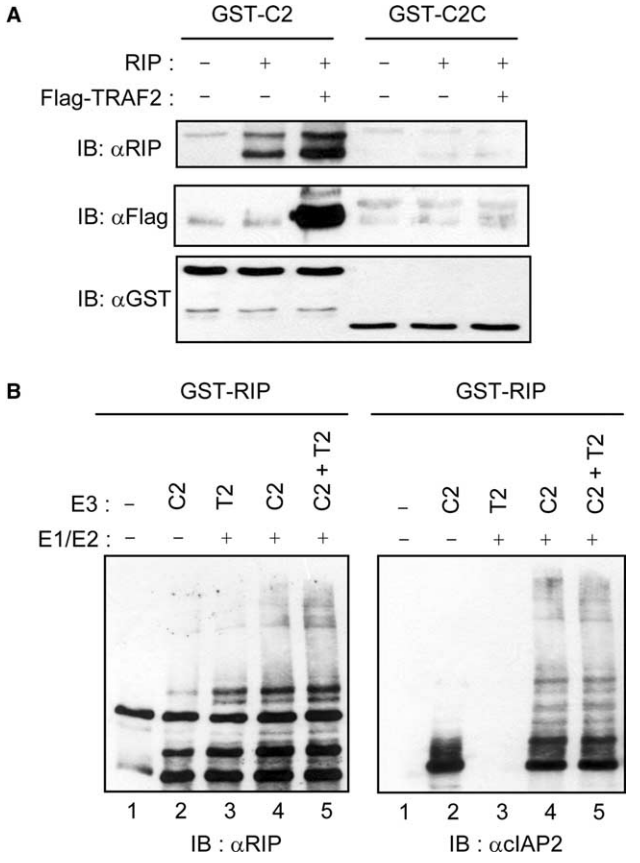

Fig. 6. (A) RIP interacts weakly with c-IAP2 and this interaction is enhanced in the presence of TRAF2. Bacterially expressed and purified GST-C2 or GST-C2C were incubated with 293 cell lysates expressing the indicated proteins, together with glutathione–Sepharose beads under continuous shaking at 4 °C for 4 h. The beads were washed extensively and the interacting proteins were analyzed by immunoblotting with anti-RIP Ab (*upper*), anti-Flag Ab (*middle*) and anti-GST Ab (*lower*). (B) TRAF2 has no effect on c-IAP2-mediated RIP ubiquitination.Flag-c-IAP2 and Flag-TRAF2, which were expressed in 293 cells, were purified in the bead-immobilized state and used for in vitro ubiquitination reaction containing the substrate GST-RIP and other components (E1, E2, His6-ubiquitin). After reaction at 30 °C for 60 min, the samples were analyzed by immunoblotting with anti-RIP Ab (*left*) and anti-c-IAP2 Ab (*right*).

ubiquitination. As shown in Fig. 5B, both GST-c-IAP1 and -c-IAP2 mediated ubiquitination of Flag-RIP with similar efficiency, consistent with the observation that RIP was degraded by c-IAP1 and c-IAP2 in vivo (*left panel*). When the same blot was probed with anti-c-IAP2 Ab, self-ubiquitinated products of GST-c-IAP2 were visualized, but those of GST-c-IAP1 were not able to be seen due to no reactivity of anti-c-IAP2 Ab to GST-c-IAP1 (*right panel*). These results suggest that c-IAP1 and c-IAP2 can promote RIP ubiquitination without the aid of other cellular proteins originated from the transfected 293 cells.

3.6. *TRAF2 promotes the interaction between c-IAP2 and RIP, but has no effect on c-IAP2-mediated RIP ubiquitination in vitro*

It has been shown that TRAF2 interacts with both c-IAP2 and RIP through its TRAF domain [5,7,9]. However, direct association between RIP and c-IAP2 has not been reported. Our observation that c-IAP2 induces the degradation of RIP in vivo and ubiquitinates RIP directly in vitro suggests that a close contact occurs between RIP and c-IAP2. Therefore, we examined whether c-IAP2 interacts with RIP directly and whether such interaction is influenced by TRAF2. Because cotransfection of RIP and c-IAP2 in 293 cells results in the degradation of RIP, study of physical association between RIP and c-IAP2 was not feasible in these transfected cells. Thus, we performed in vitro GST pull-down assay to examine the interaction between these proteins in the presence or absence of TRAF2 (Fig. 6A). Lysates of 293 cells expressing HA-RIP and Flag-TRAF2 were incubated with GST-c-IAP2 or GST-C2C together with glutathione–Sepharose beads at 4 °C for 4 h. The analysis of the complex pulled down by GST-c-IAP2 showed that RIP and TRAF2 were found in this complex. Even in the absence of TRAF2, RIP was pulled down by GST-c-IAP2 but at a lesser amount compared to the reaction containing TRAF2. Neither TRAF2 nor RIP was found in the complex pulled-down by GST-C2C, indicating that both proteins interact with c-IAP2 at the N terminal BIR domain of c-IAP2. These results demonstrate that RIP and c-IAP2 interact

weakly and this interaction is enhanced in the presence of TRAF2.

To examine whether TRAF2 per se can ubiquitinate RIP or influence c-IAP2-mediated RIP ubiquitination, we performed in vitro ubiquitination assays with purified TRAF2. Addition of TRAF2 to the reaction neither ubiquitinated GST-RIP (*lane 3*, Fig. 6B) nor affected the c-IAP2-mediated ubiquitination of GST-RIP (compare *lane 4* to *lane 5*). Although the possibility that TRAF2 may require a different E2 enzyme instead of the UbcH5A that we tested cannot be ruled out, a recent study has suggested that TRAF2 is not the E3 responsible for the modification of RIP [23], agreeing with our in vitro result. In view of the property of TRAF2 to associate with c-IAP2 and RIP, it may function as a bridging factor that brings c-IAP2 and RIP into close proximity.

In conclusion, upon activation of TNFR1, RIP complexed with TNFR1 is ubiquitinated only after translocating to the cholesterol- and sphingolipid-enriched lipid rafts, whereas RIP in the cytoplasmic compartment appeared not to undergo such modification [23]. Lipid rafts serve as platforms for RIP modification by recruiting molecules possessing E3 activity, such as TRAF2 and c-IAPs. Our data suggest that c-IAPs can function as E3 toward RIP. Whether such event occurs in the context of TNFR1 complex in a stimulus-dependent manner remains to be determined.

Acknowledgements: We thank H.S. Park, E.Y. Ko, and E.H. Shin for supplying reagents (E1, E2, and Flag-c-IAP2). This work was supported by grants from the Korean Ministry of Science and Technology through 21C Frontier Project and from the basic research program (R02-2002-000-00043-0) of the Korea Science and Engineering Foundation. S.-M.P. is a recipient of a predoctoral fellowship provided by the Korea Research Foundation.

References

[1] Baud, V. and Karin, M. (2001) Trends Cell Biol. 11, 372–377.
[2] Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001) Cell 104, 487–501.
[3] Hsu, H., Xiong, J. and Goeddel, D.V. (1995) Cell 81, 495–504.
[4] Hsu, H., Shu, H.B., Pan, M.G. and Goeddel, D.V. (1996) Cell 84, 299–308.
[5] Hsu, H., Huang, J., Shu, H.B., Baichwal, V. and Goeddel, D.V. (1996) Immunity 4, 387–396.
[6] Ting, A.T., Pimentel-Muinos, F.X. and Seed, B. (1996) EMBO J. 15, 6189–6196.
[7] Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M. and Liu, Z. (2000) Immunity 12, 419–429.
[8] Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z. and Leder, P. (1998) Immunity 8, 297–303.
[9] Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V. (1995) Cell 83, 1243–1252.
[10] Shu, H.B., Takeuchi, M. and Goeddel, D.V. (1996) Proc. Natl. Acad. Sci. USA 93, 13973–13978.
[11] Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997) EMBO J. 16, 6914–6925.
[12] Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998) EMBO J. 17, 2215–2223.
[13] Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M. and Ashwell, J.D. (2000) Science 288, 874–877.
[14] Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Leverson, J.D. and Hunter, T. (2000) J. Biol. Chem. 275, 26661–26664.
[15] Li, X., Yang, Y. and Ashwell, J.D. (2000) Nature 416, 345–347.
[16] Tang, E.D., Wang, C.Y., Xiong, Y. and Guan, K.L. (2003) J. Biol. Chem. 278, 37297–37305.
[17] Hu, S. and Yang, X. (2003) J. Biol. Chem. 278, 10055–10060.
[18] Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439.
[19] Pickart, C.M. (2001) Annu. Rev. Biochem. 70, 503–533.
[20] Zhang, S.Q., Kovalenko, A., Cantarella, G. and Wallach, D. (2000) Immunity 12, 301–311.
[21] Chen, G., Cao, P. and Goeddel, D.V. (2002) Mol. Cell 9, 401–410.
[22] Harper, N., Hughes, M., MacFarlane, M. and Cohen, G.M. (2003) J. Biol. Chem. 278, 25534–25541.
[23] Legler, D.F., Micheau, O., Doucey, M.A., Tschopp, J. and Bron, C. (2003) Immunity 18, 655–664.
[24] Micheau, O. and Tschopp, J. (2003) Cell 114, 181–190.
[25] Kim, J.W., Joe, C.O. and Choi, E.J. (2001) J. Biol. Chem. 276, 27064–27070.
[26] Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R.C. and Conaway, J.W. (2000) Proc. Natl. Acad. Sci. USA 97, 10430–10435.
[27] Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z. and Pan, Z.Q. (1999) Mol. Cell 3, 527–533.
[28] Min, K.W., Hwang, J.W., Lee, J.S., Park, Y., Tamura, T.A. and Yoon, J.B. (2003) J. Biol. Chem. 278, 15905–15910.
[29] Brown, K.D., Hostager, B.S. and Bishop, G.A. (2002) J. Biol. Chem. 277, 19433–19438.
[30] Shi, C.S. and Kehrl, J.H. (2003) J. Biol. Chem. 278, 15429–15434.
[31] Hong, S.Y., Yoon, W.H., Park, J.H., Kang, S.G., Ahn, J.H. and Lee, T.H. (2000) J. Biol. Chem. 275, 18022–18028.