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c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor α (TNFα)-induced NF-κB Activation*[S]

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The inhibitor of apoptosis (IAP) proteins are a family of anti-apoptotic regulators found in viruses and metazoans. c-IAP1 and c-IAP2 are recruited to tumor necrosis factor receptor 1 (TNFR1)-associated complexes where they can regulate receptor-mediated signaling. Both c-IAP1 and c-IAP2 have been implicated in TNFα-stimulated NF-κB activation. However, individual *c-IAP1* and *c-IAP2* gene knock-outs in mice did not reveal changes in TNF signaling pathways, and the phenotype of a combined deficiency of c-IAPs has yet to be reported. Here we investigate the role of c-IAP1 and c-IAP2 in TNFα-stimulated activation of NF-κB. We demonstrate that TNFα-induced NF-κB activation is severely diminished in the absence of both c-IAP proteins. In addition, combined absence of c-IAP1 and c-IAP2 rendered cells sensitive to TNFα-induced cell death. Using cells with genetic ablation of c-IAP1 or cells where the c-IAP proteins were eliminated using IAP antagonists, we show that TNFα-induced RIP1 ubiquitination is abrogated in the absence of c-IAPs. Furthermore, we reconstitute the ubiquitination process with purified components *in vitro* and demonstrate that c-IAP1, in collaboration with the ubiquitin conjugating enzyme (E2) enzyme UbcH5a, mediates polymerization of Lys-63-linked chains on RIP1. Therefore, c-IAP1 and c-IAP2 are required for TNFα-stimulated RIP1 ubiquitination and NF-κB activation.

Cellular IAP1[2] and IAP2 (c-IAP1 and c-IAP2) were identified in a search for proteins associated with TNF receptors (TNFRs) (1). Through binding to TNFR-associated factor 2 (TRAF2), c-IAP1 and c-IAP2 are recruited to TNFR signaling complexes, where they regulate the activation of caspase-8 (1, 2). c-IAP1 and c-IAP2 were also proposed to modulate activation of the canonical NF-κB pathway, although most of these studies relied on overexpression (3, 4). In contrast, however, targeted deletion of *c-IAP1* or *c-IAP2* genes in mice did not reveal any abnormalities in TNFα-induced NF-κB (nuclear factor κB) activation (5, 6). The absence of any appreciable phenotype in single c-IAP knock-out mice has been attributed to the putative redundancy of c-IAP1 and c-IAP2 due to their high level of sequence and functional similarities (7). Thus, combined deficiency of cellular IAPs might expose their role in this signaling pathway. In support of this possibility, a null mutation in the sole cellular IAP in zebrafish results in severe defects in NF-κB activation (8). c-IAP1 and c-IAP2 are also RING domain-containing ubiquitin ligases capable of promoting ubiquitination of several of their binding partners, including TRAF2 and SMAC (second mitochondrial activator of caspases) (4, 5, 9–12).

TNFR1 mediates activation of several signaling pathways, among them the canonical NF-κB pathway (13). Binding of TNFα to TNFR1 induces recruitment of the adaptor protein TNFR-associated death domain (TRADD) to the death domain of the receptor (14). Through its death domain and amino-terminal region, TRADD recruits RIP1 (receptor-interacting protein), TRAF2, and through its interaction with TRAF2, c-IAP1 and c-IAP2 (13). Following binding to TRADD, TRAF2 was thought to mediate non-degradative Lys-63-linked polyubiquitination of RIP1 via its RING E3 ligase domain (15, 16). This RIP1 modification induces assembly of two RIP1-associated kinase complexes, TAK1-TABs (transforming growth factor β-activated kinase 1-TAK1-binding proteins) and IκB kinase (IKK) (17–19). Binding of these two complexes to Lys-63-linked polyubiquitin chains on RIP1 leads to phosphorylation of IKKβ and subsequent phosphorylation and proteasomal degradation of IκB (20). Loss of IκB allows translocation of p50/RelA dimer to the nucleus and induction of gene expression (20).

In the present study, we investigate the role of c-IAP1 and c-IAP2 in TNFα-induced NF-κB activation. We discover that c-IAP proteins are important mediators of canonical NF-κB signaling and demonstrate that the absence of c-IAPs severely attenuates TNFα-induced NF-κB activation. Finally, we show that c-IAPs are ubiquitin ligases capable of promoting polymerization of Lys-63-linked polyubiquitin chains on the critical adapter in the canonical NF-κB signaling pathway, RIP1.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, Western Blot Analyses, and Immunoprecipitations—HT1080 human fibrosarcoma cells were obtained from ATCC. c-IAP1-, c-IAP2-, and XIAP-deficient and matched wild-type mouse embryonic fibroblasts (MEFs) were kindly provided by Drs. John Silke, David Vaux, and Vince James (21, 22). All cell lines were grown in 50:50 Dulbecco's modified Eagle's/FK12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. MEFs were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen). Human and mouse recombinant soluble TNFα

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[2] The abbreviations used are: IAP, inhibitor of apoptosis; c-IAP, cellular IAP; XIAP, X-linked inhibitor of apoptosis; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; NF-κB, nuclear factor κB; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; IKK, IκB kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; WT, wild type; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; DMSO, dimethyl sulfoxide.

was from Genentech, Inc. The primary antibodies against mouse c-IAP1 were kindly provided by Drs. John Silke and David Vaux; anti-human c-IAP1 antibodies were purchased from R&D (affinity-purified goat antibody) or Protein Tech Group Inc.; pan c-IAP1/2 human/murine antibody was from R&D; and anti-c-IAP2 antibodies were purchased from AbCam. Anti-XIAP, anti-TRADD, anti-ubiquitin, anti-phospho-specific IκB, and anti-IκB antibodies were from Cell Signaling Technology; anti-TRAF2 antibodies were from Santa Cruz Biotechnology, Inc.; anti-TNFR1 antibodies were from R&D; anti-RIP1 antibody was from BD Biosciences; anti-FLAG M2 antibody was from Sigma; anti-Myc antibody was from Roche Applied Science; and anti-β-tubulin antibody was from ICN Biomedicals. MG132 was purchased from Calbiochem, and IAP antagonist BV6 was from Genentech (23). Western blot analyses and immunoprecipitations were performed as described previously (23–25).

Ubiquitination Assays and Protein Purification—Ubiquitination assays were performed as described previously (23, 26). Briefly, reconstituted autoubiquitination assays were performed in a 25-μl reaction volume with 0.5 μg of ubiquitin (wild type (WT), Lys-48 only, or Lys-63 only), 0.1 μg of E1, 0.5 μg of E2 UbcH5a or UbcH13 (always used in complex with Uev1) (all from Boston Biochem), 0.25 μg of recombinant c-IAP1, and 0.5 μg of recombinant RIP1 or RIP1 immunoprecipitated from c-IAP1-deficient MEFs in a buffer containing 30 mM HEPES, 2 mM dithiothreitol, 10 μM $ZnCl_2$, and 5 mM $MgCl_2$-ATP. Reactions were incubated at 30 °C for 45 min, stopped by adding 4× SDS loading buffer, boiled at 95 °C for 10 min, and immunoblotted with anti-RIP1 antibody. The full-length c-IAP1 (residues 1–618) wild type or RING mutant (H588A) and RIP1 proteins were produced and purified as described previously (23, 26, 27).

Gene Silencing and Viability Experiments—Sequences of siRNA oligonucleotides were designed by using the Dharmacon siDESIGN Center (Dharmacon Research Inc., Lafayette, CO) software and synthesized at Genentech, Inc. The following siRNA pairs were used for gene knockdown experiments: *c-IAP1*, 5′-GAGTCAAGGTGGTGATATA-3′ and 5′-GCAAGTGCTGGATTCTATT-3′; *c-IAP2*, 5′-GGACACAGCGTGCGAAAGT-3′ and 5′-GCACAAGTCCCTACCACTT-3′. Viability experiments were performed as described previously (23).

NF-κB Reporter Assay and Expression Analysis by Real-time PCR— c-IAP2-deficient MEFs were transfected with control or mouse c-IAP1-specific siRNA oligonucleotides. NF-κB luciferase activity was measured using Promega Dual-Luciferase reporter assay according to the manufacturer's instructions. For expression analysis by real-time PCR, c-IAP2-deficient MEFs were transfected with control or mouse c-IAP1-specific siRNA oligonucleotides, treated as indicated, and the RNA was prepared with the RNeasy MiniKit (Qiagen) following standard protocols. Fold change in MCP1 mRNA was analyzed as described previously (23). The following sequences were used: for detection of MCP-1, 5′-TCAGCCAGATGCAGTTAACGC (forward), 5′-TGATCCTCTTGTAGCTCTCCAGC (reverse), 5′-FAM-CCACTCACCTGCTGCTACTCATTCACCA-TAMRA (probe), and for GAPDH, 5′-GGCATTGCTCTCAATGACAA (forward), 5′-CTGTTGCTGTAGCCGTATTCA (reverse), 5′-FAM-TGTCATACCAGGAAATGAGCTTGACAAAG-TAMRA (probe).

RESULTS AND DISCUSSION

Activation of the Canonical NF-κB Pathway by TNFα Requires c-IAP1 and c-IAP2—To examine the role of IAP proteins in TNFα-induced NF-κB activation, we treated wild-type MEFs or MEFs with genetic ablation of c-IAP1, c-IAP2, or XIAP with TNFα for various periods of time and monitored IκB degradation as a readout for the activation of canonical NF-κB. XIAP or c-IAP2 deficiencies did not significantly affect degradation of IκB, whereas the absence of c-IAP1 reduced TNFα-stimulated IκB degradation (Fig. 1*A*). We also combined genetic ablation of c-IAP2 with siRNA-mediated down-regulation of c-IAP1 to explore the effect of the joint absence of c-IAP1 and c-IAP2 on canonical NF-κB signaling (Fig. 1*B*). These experiments demonstrated that the combined absence of both c-IAPs prevented TNFα-induced IκB degradation (Fig. 1*B*). In addition, NF-κB-regulated gene expression was also severely reduced as shown by lower induction of *MCP-1* mRNA expression and decreased luciferase activation from a NF-κB-responsive reporter (supplemental Figs. 1 and 2).

Administration of IAP antagonists leads to rapid induction of autoubiquitination and proteasomal degradation of cellular IAPs (22, 23). Using the IAP antagonist BV6 to eliminate c-IAP proteins generates a cellular milieu where signaling events can be examined in their absence. Thus, we treated HT1080 cells with BV6 for 5 h and then examined NF-κB activation by TNFα. Notably, BV6 administration does not induce cell death or activation of canonical NF-κB signaling in these cells (Ref. 23 and data not shown). In vehicle-treated cells, c-IAP1 was present (expression of c-IAP2 is below detection levels in these cells), and TNFα triggered degradation of IκB (Fig. 1*C*). However, in cells pretreated with BV6, c-IAP1 protein was not detected, and the addition of TNFα did not lead to IκB degradation (Fig. 1*C*). Similarly, elimination of c-IAPs in MEFs by pretreatment with BV6 also prevented TNFα-stimulated NF-κB activation as measured by IκB degradation (data not shown). Therefore, c-IAP1 and c-IAP2 are key mediators of TNFα-induced canonical NF-κB signaling.

Activation of NF-κB ensures survival of cells in response to TNFα through expression of several anti-apoptotic proteins (28). c-IAP1 and c-IAP2 inhibit TNFα-stimulated apoptosis, most likely by regulating activation of caspase-8 (1, 2). Thus, the absence of both c-IAP1 and c-IAP2 might sensitize cells to TNFα-induced cell death. To test this hypothesis, c-IAP1- and c-IAP2-deficient MEFs were transfected with siRNA oligonucleotides targeting mouse c-IAP2 or c-IAP1, respectively, and treated with TNFα for 20 h (Fig. 1*D*). TNFα addition caused little to no cell death in c-IAP1 or cIAP2 knock-out MEFs (Fig. 1*D*). However, cells lacking both c-IAP1 and c-IAP2 were sensitive to TNFα, as demonstrated by significant decline in cellular viability (Fig. 1*D*). These results suggest that c-IAP1 and c-IAP2 have a critical role in protection against TNFα-induced cell death.

TNFα-stimulated RIP1 Polyubiquitination Is Dependent on c-IAPs—Because c-IAPs are E3 ligases and RIP1 ubiquitination is critical for propagation of TNFα-induced NF-κB activation, we

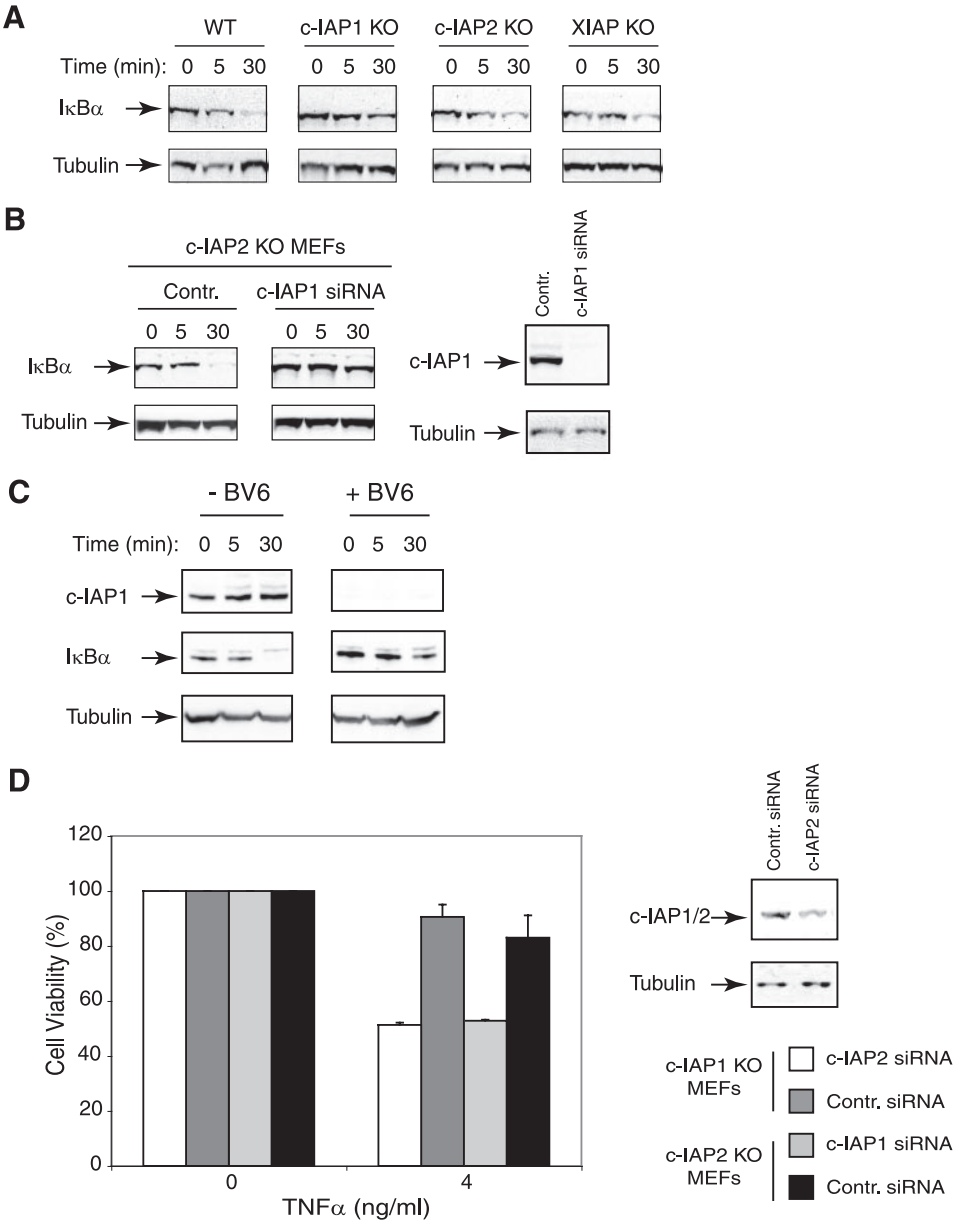

FIGURE 1. **TNFα-stimulated NF-κB activation depends on c-IAP1 and c-IAP2.** *A*, analysis of IκB protein levels in WT and in c-IAP1-, c-IAP2-, and XIAP-deficient MEFs. Cells were treated for the indicated periods of time with 20 ng/ml recombinant murine TNFα. The protein levels of IκB were analyzed by Western blotting. *KO*, knockout. *B*, c-IAP2-null MEFs were transfected with scrambled (*Contr.*) or c-IAP1-specific siRNA duplexes. 48 h later, cells were treated with recombinant murine TNFα (20 ng/ml), and protein levels were analyzed as in *A*. *C*, HT1080 cells were pretreated with vehicle (DMSO) or BV6 (5 μM) for 5 h followed by treatment with human recombinant TNFα (20 ng/ml) for the indicated time periods. The protein levels of IκB and c-IAP1 were analyzed by Western blotting. *D*, down-regulation of c-IAP1 and c-IAP2 expression reduces viability of TNFα-treated cells. Cells were transfected with scrambled, c-IAP1-specific, or c-IAP2-specific siRNA duplexes. 48 h later, cells were treated with TNFα as indicated. Cell viability was determined as described under "Experimental Procedures," and protein levels were determined as described as in *A*. The *error bars* represent standard deviation from three independent experiments. The *inset* shows down-regulation of c-IAP2 in c-IAP1-deficient MEFs; down-regulation of c-IAP1 in c-IAP2-deficient MEFs is presented in *panel B*. Western blotting was performed with indicated antibodies.

investigated whether TNFα-stimulated RIP1 polyubiquitination was dependent on c-IAP1 and c-IAP2 (29). HT1080 cells were treated with the IAP antagonist BV6 for 5 h, leading to proteasomal degradation of cellular IAP proteins (Fig. 2*A*). In agreement with our results presented in Fig. 1*C*, pretreatment of HT1080 cells with BV6 prevented TNFα-induced degradation of IκB (Fig. 2*A*). Examination of the TNFα-stimulated TNFR1 complex revealed that RIP1 was recruited and ubiquitinated in a ligand-dependent fashion in cells when c-IAPs were present (Fig. 2*A*). c-IAP deficiency did not block the recruitment of RIP1 to the TNFR1 complex, but it abrogated TNFα-induced polyubiquitination of RIP1 (Fig. 2*A*). In a similar fashion, genetic ablation of c-IAP1 greatly reduced TNFα-stimulated RIP1 ubiquitination in MEFs (Fig. 2*B*). These results suggest that c-IAPs are critical E3 ligases required for TNFα-stimulated RIP1 ubiquitination.

To further explore the properties of c-IAP mediated ubiquitination of RIP1, we reconstituted the ubiquitination process with purified components *in vitro*. To this end, RIP1 immunoprecipitated from c-IAP1-deficient MEFs was incubated with recombinant full-length c-IAP1 protein, E1 enzyme, and UbcH5a or UbcH13 E2 enzymes in an ubiquitination assay (Fig. 2*C* and supplemental Fig. S3). Incubation of RIP1 with c-IAP1 triggered significant polyubiquitination of RIP1 in the presence of E2 enzyme UbcH5a but not with UbcH13 (Fig. 2*C*). Furthermore, in addition to promoting generation of wild-type and Lys-48-linked polyubiquitin chains, c-IAP1 mediated assembly of Lys-63-linked polyubiquitin chains on RIP1, confirming its role in TNFα-stimulated RIP1 ubiquitination (Fig. 2*C*). Similarly, ubiquitination reactions with recombinant RIP1 and c-IAP1 yielded the formation of Lys-63-linked polyubiquitin chains in the presence of UbcH5a (Fig. 2*D*). As a control, c-IAP1 with a mutation in its RING domain was unable to promote RIP1 ubiquitination (Fig. 2*D* and supplemental Fig. S3). Therefore, we conclude that c-IAPs are key ubiquitin ligases capable of mediating Lys-63-linked polyubiquitination of RIP1 in the TNFR1 complex.

The importance of c-IAP1 and c-IAP2 for TNFα-induced NF-κB activation has never been clearly established. Overexpression studies suggested their involvement, but genetic ablation of either c-IAP1 or c-IAP2 did not provide support for their role in this important cellular signaling event. Definitive answers will be obtained once the effort of knocking out the two proximal genes is accomplished. In the meantime, here we reveal the critical involvement of c-IAPs in canonical NF-κB activation downstream of TNFα

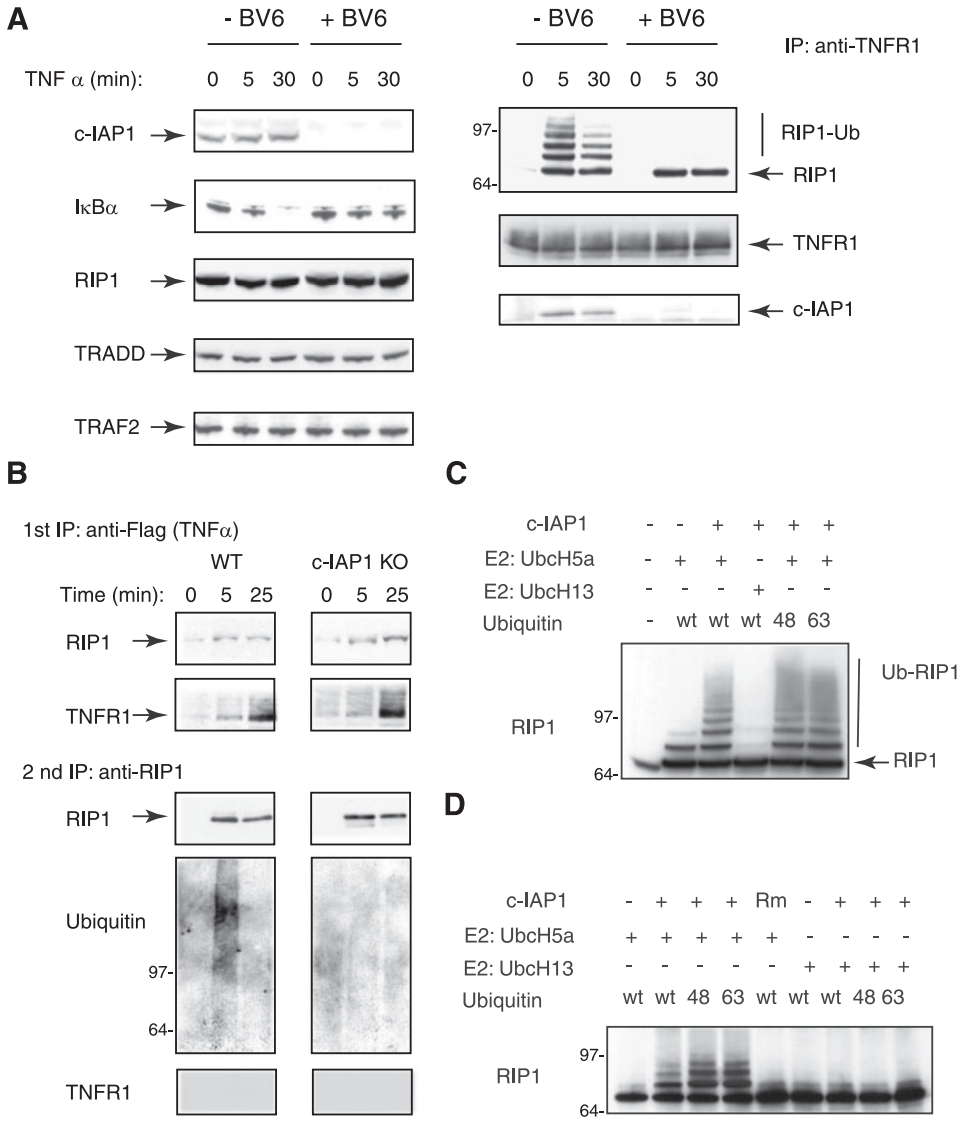

FIGURE 2. **c-IAPs are critical for TNFα-induced RIP1 polyubiquitination.** *A*, HT1080 cells were pretreated with DMSO or BV6 (5 μM) for 5 h followed by TNFα treatment as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-TNFR1 antibodies, and protein levels in cellular lysates and in the TNFR1-associated complex were determined by Western blotting with the indicated antibodies. *Ub*, ubiquitin. *B*, WT or c-IAP1-null MEF cells were treated for the indicated periods of time with FLAG-tagged TNFα (1 mg/ml) followed by immunoprecipitation and Western blot analysis with the indicated antibodies. One-half of the immunoprecipitated protein complexes described above were dissociated by a 20-min incubation in 6 M urea. Collected supernatants were diluted 25-fold in the lysate buffer followed by immunoprecipitation and Western blot analysis with the indicated antibodies. *KO*, knock-out. *C*, RIP1 was immunoprecipitated from c-IAP1-null MEFs and incubated for 45 min in ubiquitination reactions with the indicated combination of recombinant c-IAP1, E2 enzymes, and ubiquitin proteins (WT, Lys-48 only, or Lys-63 only). RIP1 modifications were determined with anti-RIP1 antibodies. *D*, recombinant RIP1 was incubated for 45 min in ubiquitination reaction in the absence or presence of recombinant c-IAP1 or c-IAP1 RING mutant (*Rm*; H588A) and the indicated combination of E2 enzymes and ubiquitin proteins (WT, Lys-48 only, or Lys-63 only). RIP1 modifications were determined with anti-RIP1 antibodies.

using a combination of genetic ablation and siRNA-mediated knockdown. These findings were further supported by IAP antagonist-mediated elimination of c-IAP1 and c-IAP2, demonstrating that the physical presence of cellular IAP proteins in the TNFR1 complex is key for TNFα-dependent activation of NF-κB. Additionally, we have also found that cells lacking c-IAP1 and c-IAP2 are sensitive to TNFα-induced cell death. Since NF-κB activation is a crucial survival mechanism induced by TNFα, c-IAP1 and c-IAP2 appear to play a central role as regulators of TNFα signaling with direct influence on the cell fate.

TRAF2, and possibly also TRAF5, have been suggested for some time to ubiquitinate RIP1 in the context of TNFα signaling (30, 31). However, the ability of TRAF2 or TRAF5 to directly mediate polyubiquitination of RIP1 has never been demonstrated with purified components *in vitro*. Importantly, c-IAP1 and c-IAP2 are constitutively associated with TRAF2, and via TRAF2, they are recruited to TNFR1 signaling complexes (1, 32). Our studies with pharmacological elimination or genetic ablation of c-IAPs demonstrate that cellular IAPs are key E3 ligases responsible for ubiquitination of RIP1. The possibility remains that TRAF2 may possess a low level of ubiquitin ligase activity for RIP1 or that c-IAP-mediated ubiquitination of TRAF2 might modulate the E3 ligase activity of TRAF2 and consequent RIP1 ubiquitination. Nevertheless, our findings suggest that RIP1 ubiquitination in the context of TNFα signaling is largely dependent on c-IAP1 and c-IAP2.

The ubiquitin-conjugating enzyme UbcH13 promotes the formation of Lys-63-linked polyubiquitin chains, and it has been implicated as a critical E2 enzyme in polyubiquitination mediated by TRAFs (33). However, genetic ablation of UbcH13 did not affect TNFα-stimulated NF-κB activation (34). Moreover, UbcH5 was originally shown as a key activator of the IKK complex (35). Our studies provide an explanation for these observations as they identify c-IAPs and UbcH5 as critical components of the ubiquitination system responsible for Lys-63-linked polyubiquitination of RIP1. These findings also establish c-IAPs as E3 ligases with the exceptional capacity to promote both Lys-48-linked and Lys-63-linked polyubiquitination and as critical regulators of NF-κB signaling pathways.

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