The Inhibitor of Apoptosis Protein Fusion c-IAP2-MALT1 Stimulates NF-κB Activation Independently of TRAF1 AND TRAF2*

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The inhibitors of apoptosis (IAPs) are a family of cell death inhibitors found in viruses and metazoans. All members of the IAP family have at least one baculovirus IAP repeat (BIR) motif that is essential for their anti-apoptotic activity. The t(11, 18)(q21;q21) translocation fuses the BIR domains of c-IAP2 with the paracaspase/MALT1 (mucosa-associated lymphoid tissue) protein, a critical mediator of T cell receptor-stimulated activation of NF-κB. The c-IAP2-MALT1 fusion protein constitutively activates the NF-κB pathway, and this is considered critical to malignant B cell transformation and lymphoma progression. The BIR domains of c-IAP1 and c-IAP2 interact with tumor necrosis factor receptor-associated factors 1 and 2 (TRAF1 and TRAF2). Here we investigated the importance of TRAF1 and TRAF2 for c-IAP2-MALT1-stimulated NF-κB activation. We identified a novel epitope within the BIR1 domains of c-IAP1 and c-IAP2 that is crucial for their physical interaction with TRAF1 and TRAF2. The c-IAP2-MALT1 fusion protein associates with TRAF1 and TRAF2 using the same binding site. We explored the functional relevance of this interaction and established that binding to TRAF1 and TRAF2 is not required for c-IAP2-MALT1-stimulated NF-κB activation. Furthermore, gene ablation of TRAF2 or combined down-regulation of TRAF1 and TRAF2 did not affect c-IAP2-MALT1-stimulated signaling. However, TRAF1/2-binding mutants of c-IAP2-MALT1 still oligomerize and activate NF-κB, suggesting that oligomerization might be important for signaling of the fusion protein. Therefore, the t(11, 18)(q21;q21) translocation creating the c-IAP2-MALT1 fusion protein activates NF-κB and contributes to human malignancy in the absence of signaling adaptors that might otherwise regulate its activity.

A family of anti-apoptotic regulators known as inhibitor of apoptosis (IAP) proteins was initially identified and functionally described in baculoviruses, and IAP protein homologues are now known in both invertebrates and vertebrates (1). IAP proteins can interact directly with a variety of inducers and effectors of apoptosis and block apoptosis induced by diverse stimuli (2). This places IAP proteins in a central position as inhibitors of death signals that proceed through a number of different pathways (3). Members of the IAP family are characterized by the presence of at least one and, most commonly in humans, three tandem baculovirus IAP repeat (BIR) domains (3). In addition, most IAP proteins possess a carboxyl-terminal RING finger motif involved in ubiquitination (4), and some, like c-IAP1 and c-IAP2, also possess a caspase-associated recruitment domain (CARD) (5). c-IAP1 and c-IAP2 were originally identified through their ability to physically interact with tumor necrosis factor receptor-associated factors (TRAFs) (6), namely TRAF2 (7). Through TRAF2 interactions, c-IAP1 and c-IAP2 are recruited to TNF receptor I- and II-associated complexes where they can regulate receptor-mediated apoptosis (8, 9). c-IAPs and TRAF2 associate through their BIR and TRAF-N domains (7), and recent studies indicate that the BIR1 domain of c-IAP1 and c-IAP2 is critical for that interaction (10, 11). c-IAP1 and c-IAP2 are also RING finger-containing ubiquitin ligases capable of promoting ubiquitination and proteasomal degradation of TRAF1, TRAF2, and several of their binding partners (4, 12, 13).

Interestingly, the t(11, 18)(q21;q21) genetic translocation fuses the BIR domains of c-IAP2 with the central and carboxyl-terminal portions of paracaspase/MALT1 (14–16). This translocation is the most frequent chromosomal aberration associated with MALT lymphoma (17). MALT lymphoma is the most common extranodal non-Hodgkin lymphoma, arising from a background of chronic inflammation and autoimmune disease (18). The c-IAP2-MALT1 fusion protein constitutively activates the NF-κB pathway, which is potentially seminal for development of inflammation-associated tumors (19–21). However, c-IAP2 and MALT1 independently do not possess the same activation capacity (20, 21). MALT1, also known as paracaspase, contains a death domain, two immunoglobulin-like domains (Ig-like), a region homologous to caspases, and a globular domain. Despite having a caspase-like region, MALT1 does not have any protease activity (22). Instead, it mediates antigen receptor-stimulated NF-κB activation by ubiquitinating NF-κB essential modulator (NEMO) (23, 24). The c-IAP2-MALT1 protein appears to stimulate NF-κB sig-
naling through self-oligomerization that deregulates the ubiquitin ligase activity of MALT1 (24).

A report by Zhou et al. (24) demonstrated that the BIR1 domain of c-IAP2 is critical for c-IAP2-MALT1-induced NF-κB activation. In the present study, we investigated the importance of TRAF1 and TRAF2 for c-IAP2-MALT1-stimulated NF-κB activation. We identified a novel epitope within the BIR1 domain, comprising residues in the first α-helix of c-IAP1 and c-IAP2, that is critical for the physical interaction with TRAF1 and TRAF2. The importance of these residues is highlighted in binding studies with endogenous TRAF proteins. Finally, we established that binding to TRAF1 and TRAF2 is not required for c-IAP2-MALT1-stimulated NF-κB activation. These results suggest that, despite binding TRAF1 and TRAF2 through the BIR1 domain of c-IAP2, the c-IAP2-MALT1 fusion activates NF-κB through oligomerization.

EXPERIMENTAL PROCEDURES

Expression Constructs—Plasmids expressing FLAG-ML-IAP, Myc-SMAC, TRAF1-FLAG, TRAF2-Myc, HA-c-IAP2-MALT1 (case2), FLAG-case2, and HA-case2 deletions have been described previously (21, 24–26). The breakpoint for c-IAP2 is nucleotide 2048, and for paracaspase/MALT1, it is nucleotide 814 in case2 (G6071) genetic fusion (14–16). Full-length XIAP, c-IAP1, and c-IAP2, as well as deletions and point mutations of c-IAP1 and c-IAP2, were generated by PCR and subcloned into the p3xFLAG-CMV14 vector (Sigma). Site-specific mutants in c-IAP1, c-IAP2, and case2 were generated using a QuikChange site-directed mutagenesis kit (Stratagene).

Cell Culture, Antibodies, and Immunoprecipitations—Human 293T embryonic kidney cells, HT1080 human fibrosarcoma cells, and mouse embryonic fibroblasts (MEF) were cultured using standard procedures (27). TRAF2-deficient and matched wild-type MEFs were kindly provided by Dr. David Goeddel. 293T, HT1080, and MEF cells were transfected using GenePorter reagent (Gene Therapy Systems), Lipofectamine 2000 (Invitrogen), and FuGENE 6 (Sigma), respectively. The primary antibodies used were anti-FLAG M2 (Sigma), anti-Myc (Covance), anti-HA (Covance), anti-TRAF1 (BioCarta), and anti-TRAF2 (Santa Cruz Biotechnology). Monoclonal antibodies specific to BIR1 of c-IAP1 were raised in Genentech using the BIR1 domain as an antigen. Immunoprecipitations were performed as described previously (25, 28).

Sequence Analysis and Three-dimensional Modeling—Amino acid sequence alignments were performed using ClustalW. An homology model of the c-IAP1 BIR1 domain was constructed using the HOMOLOGY module of InsightII 2000.1 (Accelrys Software, Inc.) and using the structures of XIAP-BIR3 (Protein Data Bank ID 1NW9) (29) and ML-IAP-BIR (Protein Data Bank ID 1OXQ) (30) as templates. The final model was validated using the program Profiles-3D, as implemented in InsightII.

NF-κB Reporter Assay—293T, HT1080, wild-type, and TRAF2-null MEFs were transfected with the indicated constructs. NF-κB luciferase activity was measured at the indicated periods of time using the Promega Dual-Luciferase reporter assay system as described previously (23, 24).

Gene Silencing Experiments—Sequences of small interfering RNA oligonucleotides were designed by using the Dharmacon siDESIGN Center software (Dharmacon Research Inc., Lafayette, CO) and synthesized at aGenentech, Inc. The following small interfering RNA pairs were used for gene knockdown experiments: TRAF1, 5′-UGUUGGAAGACCAAAUGU-3′ and 5′-ACAUUGGUAUCUUCACA-3′; TRAF2, 5′-UGUCAGUGCUCCCUUCCAGA-3′ and 5′-UCUGCAAGGGACUCGACAC-3′, 5′-GGUCUUGGAGGAGGCACAC-3′ and 5′-UGCCUCUCAUCUCAAAGACC-3′. Cells were transfected as described previously (27).

RESULTS AND DISCUSSION

The Amino-terminal Portion of the BIR1 of c-IAP1 and c-IAP2 Is Responsible for Binding TRAF2—TRAF2 has been shown to associate with c-IAP1 and c-IAP2 but not with other IAPs (7, 31). To verify the specificity of this interaction, we co-expressed TRAF2 with c-IAP1, c-IAP2, XIAP, and ML-IAP (Fig. 1A). c-IAP1 and c-IAP2 immunoprecipitated TRAF2, whereas no interaction was observed between either XIAP or ML-IAP and TRAF2 (Fig. 1A). To identify the portion of c-IAP1 and c-IAP2 responsible for TRAF2 binding, we expressed individual BIR domains and CARD-RING finger motifs of c-IAP1 and c-IAP2 (Fig. 1B). For both c-IAP1 and c-IAP2, the BIR1 domain associated with TRAF2, whereas the BIR2, BIR3, and CARD-RING finger motifs did not (Fig. 1, C and D). Functional properties of XIAP, ML-IAP, and the other BIR domains of c-IAP1 and c-IAP2 were confirmed by binding to SMAC (data not shown).

To define the region within the BIR1 domain of c-IAP1 and c-IAP2 responsible for interaction with TRAF2, we undertook an unbiased approach and replaced portions of the BIR1 domain with corresponding portions of the BIR2 domain of c-IAP1. BIR domains of IAP proteins generally contain two α-helices followed by three β-strands and an additional three α-helices at the carboxyl-terminal portion of the domain (Fig. 2A). We made constructs that exchanged either the last three α-helices or the first two α-helices and three β-strands or just the first two α-helices between BIR1 and BIR2 of c-IAP1 (Fig. 2B). Substitution of the last three α-helices resulted in a construct that still co-immunoprecipitated with TRAF2, whereas constructs involving substitutions of the first two α-helices did not (Fig. 2C). This result suggested that the amino-terminal portion of the BIR1 domain might be critical for the interaction with TRAF2. To further verify this hypothesis, we substituted the first α-helix of the BIR1 domain with the corresponding region of the BIR2 domain from c-IAP1 (Fig. 2B). This mutant protein failed to bind TRAF2, either in the context of full-length c-IAP1 protein or as an individual BIR1 domain (Fig. 2D and data not shown).

Closer examination of the first α-helix of the BIR1 domain and sequence comparisons between IAP proteins revealed a signature motif containing several amino acid residues specific to c-IAP1 and c-IAP2 that are not present in XIAP or ML-IAP (Fig. 2E). We substituted these residues with alanine and found that substitution prevented binding to TRAF2 (Fig. 2F). A recent report by Samuel et al. (11) suggested that another pair of residues (Glu-64 and Arg-65) at the amino-terminal end of the second α-helix of the BIR1 domain is required for TRAF2 binding. To compare our mutants with those reported previ-
ously, we generated constructs of c-IAP1 and c-IAP2 with the following substitutions: substitutions of the four α-helix-1-encompassing residues (Cys-45, Leu-47, Tyr-48, Ser-51) with Ala, substitutions of the two residues in α-helix-2 (Glu-64 and Arg-65) with Ala, or a combination of all six substitutions (Fig. 2A).

We tested the ability of these constructs to bind endogenous TRAF2 protein from 293T cells and found that both sets of mutations, as well as their combination, failed to immunoprecipitate TRAF2 (Fig. 2F). Structural integrity of TRAF2-binding mutants was confirmed by recognition of these proteins by c-IAP1 BIR1-specific antibodies (data not shown). Homology modeling of the BIR1 domain of c-IAP1 revealed that the conserved residues are clustered together on the surface of the BIR domain, either proximal to (Cys-45) or within (Leu-47, Tyr-48, and Ser-51) α-helix-1 (Fig. 2G). Amino acids Glu-64 and Arg-65 are located at the amino-terminal end of α-helix-2,
FIGURE 2. c-IAP1 and c-IAP2 bind TRAF2 through a conserved region at the amino-terminal end of the BIR1 domain. A, sequence alignment of the BIR1 and BIR2 domains of c-IAP1 and c-IAP2. Predicted secondary structures, α-helices (cylinders) and β strands (arrows), are indicated above the alignment. Mutated residues in the first α-helix are boxed in red, and in the second helix, they are boxed in purple. B, schematic representation of c-IAP1 BIR1-BIR2 chimeric expression constructs. C and D, α-helix 1 in the BIR1 domain of c-IAP1 is critical for the physical interaction with TRAF2. The indicated FLAG-tagged c-IAP1 BIR1-BIR2 constructs (C) or FLAG-tagged c-IAP1 constructs (D) and Myc-tagged TRAF2 were expressed in 293T cells. Lysis, immunoprecipitations (IP), and immunoblotting (WB) were carried out as described in the legend for Fig. 1 (panel A). E, sequence alignment of the amino acid residues within and surrounding the first α-helix of amino-terminal BIR domains. Small letters preceding the protein name indicate species: h, human; m, mouse; r, rat; d, dog; ch, chicken. Arrows point to the residues unique to c-IAP1 and c-IAP2 that were mutated. F, residues in the first two α-helices of BIR1 are crucial for TRAF2 binding. 293T cells were transfected with the indicated c-IAP1 and c-IAP2 wild-type and mutant constructs (4mut, substitutions of Cys-45, Leu-47, Tyr-48, and Ser-51 to alanine in the first α-helix; ER, substitutions of Glu-64 and Arg-65 to alanine in the second α-helix; comb, combination of both). 40 h after transfection, cells were lysed in Nonidet P-40 lysis buffer, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-TRAF2 and anti-FLAG antibodies. G, ribbon representation of the homology model of cIAP1-BIR1 showing the predicted positions of mutated residues that are critical for the cIAP1-TRAF2 interaction (Cys-45, Leu-47, Tyr-48, Ser-51, Glu-64, and Arg-65). The figure was produced using the program PyMOL (38).
FIGURE 3. TRAF2 is not critical for case2-stimulated NF-κB activation. A, schematic representation of c-IAP2-MALT1 (case2) protein domains and expression constructs. B, 293T cells were transfected with the indicated HA-tagged case2 constructs and TRAF2-Myc. After 40 h, cells were lysed in Noniied P-40 lysis buffer. Lysates were immunoprecipitated (IP) with the indicated anti-epitope tag antibodies and immunoblotted (WB) with anti-HA and anti-Myc antibodies. C, the indicated case2 constructs were expressed in 293T cells. 40 h after transfection, cells were lysed in Noniied P-40 lysis buffer, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-TRAF2 and anti-HA antibodies. WT, wild type. D, the indicated case2 wild-type and mutant constructs (designations as in the legend for Fig. 2F) were expressed in 293T cells. Lysis, immunoprecipitations, and immunoblotting were carried as in panel C. E–G, 293T cells (E), wild-type MEFs (F), and TRAF2-deficient MEFs (G) were transfected with the indicated expression constructs and NF-κB reporter plasmid. Fold change in NF-κB activation was determined by the NF-κB activation assay relative to the vector control. Data indicate mean ± standard deviation of triplicate transfections and are representative of five independent experiments.
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immediately adjacent to the conserved residues identified in the current study (Fig. 2G). Therefore, we have identified a novel binding site for TRAF2 on the BIR1 domain of c-IAPs that represents a contiguous surface and that includes the previously described binding site.

c-IAP2–MALT1-stimulated NF-κB Activation Is Not TRAF2-dependent—The BIR1 domain of c-IAP2 has been implicated as critical for c-IAP2–MALT1-induced NF-κB activation (24). Since we had identified the BIR1 domain as a crucial region for binding TRAF2, we investigated the importance of TRAF2 for c-IAP2–MALT1-induced NF-κB activation. Additionally, Samuel et al. (11) suggested in their report that TRAF2, by virtue of its binding to the BIR1 domain of c-IAP2, might be required for NF-κB activation by c-IAP2–MALT1. To that end, we tested BIR deletion constructs of the c-IAP2 protein (hereafter referred to as case2) for binding to TRAF2 (Fig. 3A). Binding assays demonstrated that the BIR1 domain of case2 is required for binding of either overexpressed or endogenous TRAF2 (Fig. 3, B and C). To verify the importance of the identified conserved residues in the first and second α-helices of the BIR1 domain, we introduced the aforementioned substitutions into case2 expression constructs and analyzed for TRAF2 binding. Neither set of mutations, alone or in combination, immunoprecipitated endogenous TRAF2, confirming the importance of those residues for TRAF2 binding by case2 as well as by the c-IAPs (Fig. 3D).

To investigate the contribution of TRAF2 binding in case2-induced NF-κB activation, we transfected the TRAF2-binding mutants of case2 into 293T cells and tested their ability to activate NF-κB-dependent reporter gene expression. Case2 constructs with TRAF2-binding mutations stimulated NF-κB activity at comparable levels with the wild-type case2 (Fig. 3E). As reported previously (21, 24), a BIR1 deletion construct of case2, or c-IAP2 or MALT1 alone, did not appreciably activate NF-κB (Fig. 3E). This result suggests that a BIR1 domain-mediated physical interaction with TRAF2 is not required for case2-stimulated NF-κB activation. To confirm that TRAF2 is not required in case2-induced NF-κB activation, we tested case2 mutants for their ability to activate NF-κB in wild-type or TRAF2-deficient MEFs. TRAF2-binding mutants of case2 and wild-type case2 activated NF-κB in both wild-type and TRAF2-deficient MEFs (Fig. 3, F and G). These data indicate that TRAF2 is not required for case2-stimulated NF-κB activation.

c-IAP1 and c-IAP2 Physically Interact with TRAF1 in a Similar Manner to TRAF2—In addition to TRAF2, c-IAP1 and c-IAP2 also interact with TRAF1 (7, 31). To examine whether TRAF2-binding mutations in the BIR1 domain of case2 affect association with TRAF1, we expressed TRAF1 along with BIR domain deletions and mutations of case2 and tested for binding. As with TRAF2, case2 associated with TRAF1 through the BIR1 domain of its c-IAP2 component, and mutations in the first and in the second α-helix of the BIR1 domain abrogated that interaction (Fig. 4A). We performed similar experiments with endogenous TRAF1 and confirmed the importance of the conserved amino acid residues at the amino-terminal portion of the BIR1 domain (Fig. 4B). Finally, we examined the same mutations in the context of c-IAP1 and demonstrated that they eliminate binding to endogenous TRAF1 (Fig. 4C). We used XIAP and ML-IAP, IAP proteins that do not interact with TRAFs, as additional controls to emphasize the specificity of this association (Fig. 4C). Thus, TRAF1 and TRAF2 associate with c-IAP1 and c-IAP2 in a similar manner by binding to a surface comprising residues within the first two α-helices of the BIR1 domain.

Knockdown of TRAF1 and TRAF2 Does Not Inhibit Case2-stimulated NF-κB Activation—To investigate the joint contribution of TRAF1 and TRAF2 in case2-induced NF-κB activation, we used small interfering RNA technology to down-
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regulate these two TRAFs individually or in combination. Knockdown of TRAF1 or TRAF2 or simultaneous knockdown of both did not prevent NF-κB activation by case2 (Fig. 5A). As we have shown in Fig. 3, the case2 construct containing all six TRAF1-/TRAF2-binding mutations (case2-comb) failed to immunoprecipitate these two TRAFs but still activated NF-κB. Thus, we have shown in Fig. 3, the case2 construct containing all mutations (comb) indicates a combination of all mutations) failed to six TRAF1-/TRAF2-binding mutations (case2-comb (where we have shown in Fig. 3, the case2 construct containing all mutations). HA-tagged case2 of case2 since the BIR1 domain of c-IAP2 has been implicated of interaction of these proteins.

FIGURE 5. Case2 activates NF-κB and oligomerizes independently of TRAF1 and TRAF2. A, NF-κB activation by case2 in TRAF1- and TRAF2-depleted HT180 cells. HT180 cells were transfected with control scramble (Control), TRAF1 (T1), TRAF2 (T2), or a combination of TRAF1 and TRAF2 (T1&T2) small interfering RNA duplexes. 24 h later, cells were transfected with the indicated case2 constructs and NF-κB reporter expression constructs. NF-κB activation was determined by luciferase reporter assay 24 h later (left panel). Data indicate mean ± standard deviation of triplicate transfections and are representative of three independent experiments. Expression levels of TRAF1 and TRAF2 were determined by immunoblotting with the indicated antibodies (right panels). Actin blots show equal protein loading (right panels). WT, wild type. B, the indicated case2 constructs were expressed in HT180 cells. After 48 h, cells were lysed in Nonidet P-40 lysis buffer, immunoprecipitated (IP) with anti-HA antibody, and immunoblotted (WB) with anti-FLAG, anti-HA, and anti-HA antibodies. C, analysis of oligomerization of wild-type and TRAF-binding mutants of case2. FLAG-tagged case2 and the indicated HA-tagged case2 constructs were expressed in 293T cells. 40 h after transfection, cells were lysed in Nonidet P-40 lysis buffer, immunoprecipitated with the indicated anti-tag antibodies, and immunoblotted with anti-FLAG and anti-HA antibodies.

The most common chromosomal translocation associated with MALT lymphoma, t(11;18)(q21;q21), generates a fusion protein (case2) containing the BIR domains of c-IAP2 and the caspase-like and IgG-like regions of paracaspase/MALT1. The BIR1 domain of case2 seems to be critical for case2-stimulated NF-κB activation and is also essential for the interaction with TRAF2. Therefore, we and others (11) have speculated that TRAF2 might be crucial for case2-induced NF-κB activation, either by serving as an adaptor for NF-κB activation or as an oligomerization facilitator for case2. In support of this hypothesis, a TRAF2 dominant negative RING-less construct blocks case2-stimulated NF-κB activation in overexpression studies (21). Additionally, another TRAF that has been implicated in

own since they associate with TRAF1 and TRAF2. We have investigated the importance of the individual BIR domains of c-IAPs for TRAF1 and -2 binding and have found the BIR1 domain to be both necessary and sufficient for this interaction. Most of the well-studied TRAF2 interactions (TRADD, TNFR2) involve the TRAF-C domain and have led to the identification of a TRAF-binding motif (32, 33). However, TRAF2 associates with c-IAPs via the TRAF-N domain that encompasses the coiled-coil region (7). The TRAF-N domain has not been extensively studied, and there are no known structures that would allow modeling of binding sites. To gain further insight into the interaction between c-IAP1/2 and TRAF1/2, we undertook an unbiased approach and examined different portions of the BIR1 domain for TRAF1/2 binding. We found that residues proximal to and within the first α-helix are critical for the c-IAP-TRAF1/2 interaction. These residues constitute a novel interaction site for TRAF1 and -2 on the BIR1 domains of the c-IAPs that is evolutionarily conserved and highly specific for c-IAPs. Interestingly, these residues, in combination with two previously identified residues at the amino-proximal end of α-helix-2, form a contiguous area on the surface of the BIR1 domain. This highlights the difference between the potentially larger binding interface for c-IAP-BIR1-TRAF1/2 association and the BIR3-SMAC-binding interface where a surface groove on the BIR3 domain creates a binding pocket for the amino-terminal four amino acids of mature processed SMAC (30, 34–36). Structural studies of the interaction between the c-IAP BIR1 domain and the TRAF-N domain should further elucidate the mechanism of interaction of these proteins.

IAP proteins interact with a variety of inducers and effectors of apoptosis, and the IAP signature motifs, the BIR domains, mediate most of those interactions. However, with the possible exception of SMAC, most IAP proteins and their BIR domains have distinct binding partners. XIAP, for example, is a potent binder and inhibitor of caspases 3, 7, and -9, whereas other IAPs are not. c-IAP1 and c-IAP2 make an IAP subgroup of their
case2-induced NF-kB activation, TRAF6 (37), does not appear to be important for this case2-mediated signaling (24). However, our mutagenesis studies, experiments in TRAF2-deficient cells, and down-regulation of TRAF2 all demonstrate that TRAF2 does not play a role in case2-stimulated NF-kB activation. We have also investigated the possibility that another c-IAP-binding TRAF, TRAF1, mediates case2-induced NF-

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