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The TNFR2-TRAF Signaling Complex Contains Two Novel Proteins Related to Baculoviral Inhibitor of Apoptosis Proteins

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Summary

The 75 kDa tumor necrosis factor receptor (TNFR2) transduces extracellular signals via receptor-associated cytoplasmic proteins. Two of these signal transducers, TRAF1 and TRAF2, were isolated and characterized previously. We report here the biochemical purification and subsequent molecular cloning of two novel TNFR2-associated proteins, designated c-IAP1 and c-IAP2, that are closely related mammalian members of the inhibitor of apoptosis protein (IAP) family orginally identified in baculoviruses. The viral and cellular IAPs contain N-terminal baculovirus IAP repeat (BIR) motifs and a C-terminal RING finger. The c-IAPs do not directly contact TNFR2, but rather associate with TRAF1 and TRAF2 through their N-terminal BIR motif-comprising domain. The recruitment of c-IAP1 or c-IAP2 to the TNFR2 signaling complex requires a TRAF2-TRAF1 heterocomplex.

Introduction

Tumor necrosis factor (TNF) is a cytokine that mediates pleiotropic inflammatory and immunoregulatory responses via two distinct cell surface receptors of approximately 55 kDa (TNFR1) and 75 kDa (TNFR2) (reviewed by Tartaglia and Goeddel, 1992; Rothe et al., 1992; Vandenabeele et al., 1995). Both TNFRs are members of the larger TNFR superfamily, which includes the Fas antigen, the lymphotoxin-β receptor, CD40, and CD30, among others (reviewed by Smith et al., 1994). Signaling within this receptor family is triggered by receptor clustering upon binding of the respective trimeric ligands to the conserved extracellular domains (reviewed by Tartaglia and Goeddel, 1992; Smith et al., 1994; Vandenabeele et al., 1995).

Similar to the related Fas antigen, TNFR1 has been widely characterized to mediate TNF-induced apoptosis, also known as programmed cell death (reviewed by Smith et al., 1994). This naturally occurring process of cell suicide plays a crucial role in the development and maintenance of multicellular organisms by eliminating superfluous or unwanted cells (reviewed by Raff, 1992). Although TNFR2 is capable of transducing apoptotic signals in certain cell lines (Grell et al., 1993), ligation of TNFR2 by TNF does usually not trigger cell death. Rather, TNFR2 has been implicated in cell proliferation or survival, e.g., in thymocytes, where TNF-induced proliferation is specifically mediated by TNFR2 (Tartaglia et al., 1991, 1993a).

The cytoplasmic domains of members of the TNFR family do not possess sequences indicative of catalytic activity, nor do they show resemblance to components of other known signal transduction pathways (reviewed by Tartaglia and Goeddel, 1992; Smith et al., 1994). Elucidation of the intracellular events involved in signaling by TNF and related cytokines has begun to emerge from studies leading to the identification of several proteins that associate with the cytoplasmic domains of the cognate receptors (Rothe et al., 1994; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995a, 1995b; Song and Donner, 1995; Hsu et al., 1995; Boldin et al., 1995; Chinnaiyan et al., 1995; Stanger et al., 1995). TNFR-associated factor 1 (TRAF1) and 2 (TRAF2) were found to interact with a region within the cytoplasmic domain of TNFR2 that is required for signal transduction (Rothe et al., 1994). In the yeast two-hybrid system, TNFR2 associates directly with TRAF2, whereas its interaction with TRAF1 is dependent upon TRAF2 heterocomplex formation. TRAF2 and a third TRAF family member, TRAF3, also bind to the cytoplasmic domain of CD40 and, presumably, other members of the TNFR superfamily (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995a; Rothe et al., 1995). TRAF1 and TRAF2 share a conserved C-terminal TRAF domain of approximately 230 amino acids that is involved in oligomerization and receptor association (Rothe et al., 1994). Inspection of this region in TRAF3 identified two subdomains, the TRAF-C domain, comprising the C-terminal 150 amino acids, and the TRAF-N domain, which consists of a putative coiled coil (Cheng et al., 1995). Whereas the TRAF-C domain is highly conserved among the three known TRAFs, the TRAF-N domain is diverged in TRAF3. In additon, TRAF2 and TRAF3 contain an N-terminal RING finger and five zinc finger structures of weak sequence similarity (Rothe et al., 1994; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995a; Song and Donner, 1995).

Both TNFR2 and CD40 can signal activation of the transcription factor NF-κB (Rothe et al., 1994; Lægreid et al., 1994; Berberich et al., 1994; Sarma et al., 1995). Functional analysis demonstrated that TRAF2 is a common signal transducer for TNFR2 and CD40 that mediates activation of NF-κB by these two receptors (Rothe et al., 1995). This effector function of TRAF2 requires its N-terminal RING finger domain. The role of TRAF3 in signal transduction is less well defined, but it has been implicated in CD40-mediated induction of CD23 (Cheng et al., 1995).

Here we report the isolation and characterization of two novel components of the TNFR2 signaling complex, designated c-IAP1 and c-IAP2. Sequence analysis suggests that c-IAP1 and c-IAP2 are cellular members of the baculoviral inhibitor of apoptosis protein (IAP) family. Biochemi-

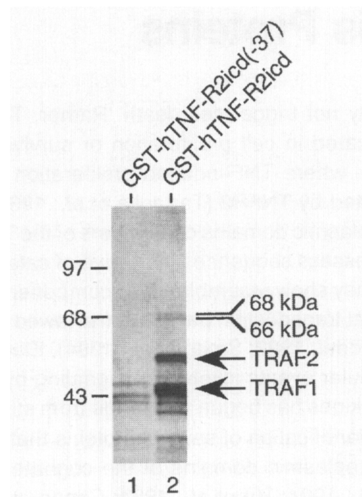

Figure 1. Purification of TNFR2-Associated Proteins

Large-scale purification of TNFR2-associated proteins from CT6 cells by GST–hTNF–R2icd fusion protein affinity chromatography was performed as described in Experimental Procedures. An aliquot of the purified material was analyzed by SDS-PAGE and silver staining. The protein bands of 68 kDa and 66 kDa that were eluted specifically from the wild-type GST–hTNF–R2icd fusion protein affinity column compared with the control GST–hTNF–R2icd(−37) fusion protein column are marked. Arrows indicate the 45 kDa TRAF1 band and the 56 kDa TRAF2 band. Positions of molecular mass standards (in kilodaltons) are shown on the left.

cal studies indicate that both c-IAPs are recruited to TNFR2 via their interaction with a TRAF2–TRAF1 heterocomplex.

Results

Purification and Molecular Cloning of the 68 kDa TNFR2-Associated Protein

Previous biochemical analysis had identified three cytoplasmic proteins of approximately 45 kDa, 56 kDa, and 68 kDa that associate with a 78 amino acid C-terminal region of human TNFR2 (hTNFR2) involved in signaling (Rothe et al., 1994). Subsequent large-scale purification, followed by partial amino acid sequencing and molecular cloning, led to the isolation of the cDNAs for TRAF1 and TRAF2, corresponding to the 45 kDa and 56 kDa proteins, respectively (Rothe et al., 1994). In contrast, the amount of 68 kDa TNFR2-associated protein initially purified was insufficient for generating partial amino acid sequence.

The 68 kDa TNFR2-associated protein was purified on a larger scale from 120 liters of murine CT6 cells by affinity chromatography using a fusion protein between glutathione S-transferase (GST) and the intracellular domain of hTNFR2 (GST–hTNF–R2icd) as described in Experimental Procedures. The purification yielded two protein bands of approximately 68 kDa and 66 kDa (Figure 1; see also

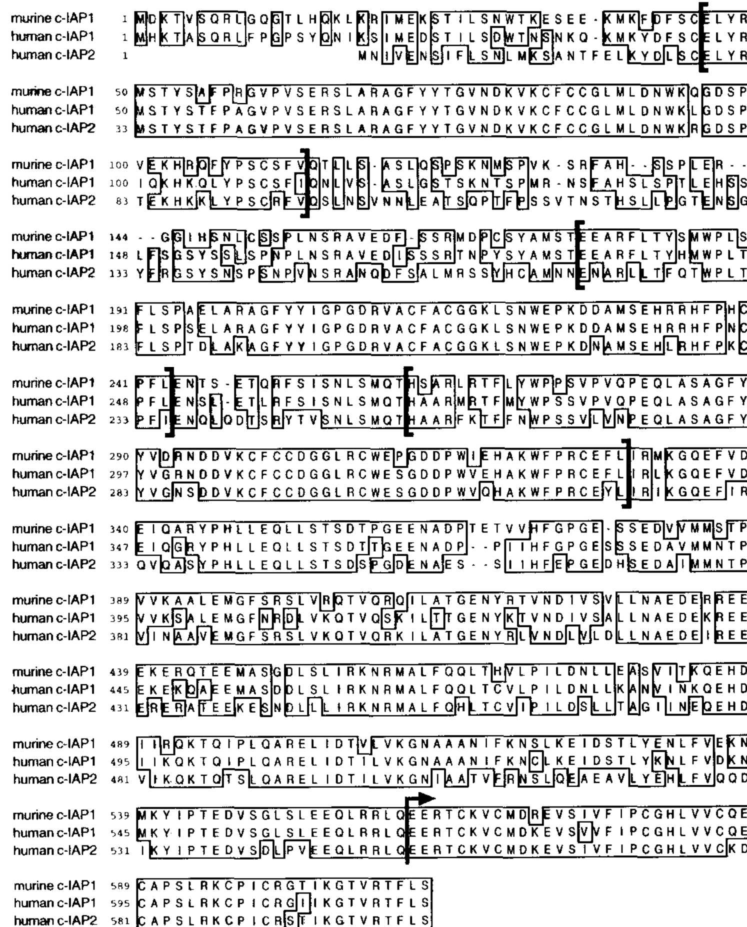

Figure 2. Homology between c-IAP1 and c-IAP2

An optimized alignment of the protein sequences of murine and human c-IAP1 and human c-IAP2 is shown. Identical amino acids are boxed. Brackets indicate the boundaries of the three BIR motifs. The arrow delineates the boundary of the C-terminal RING finger domain.

Figure 3. Sequence Similarity of c-IAP1 and c-IAP2 with other Proteins

(A) Diagrams of the putative domain structure of IAP family members. Shown are the positions of the conserved BIR motifs and RING finger domains of human c-IAP1, OpIAP, and NAIP.

(B) Optimized alignment of BIR motifs of IAP family members. The sequences are compared both among and within different members of the IAP family. Residues corresponding to the consensus sequence are stippled. Asterisks in the consensus sequence represent hydrophobic residues. See text for details.

(C) Comparison of amino acid sequences containing RING finger motifs. The RING finger domains of c-IAP1 and c-IAP2 are aligned with the respective zinc-binding motifs of v-IAPs and TRAFs (see text), the products of the *neuralized* gene from D. melanogaster and the human proto-oncogene *c-cbl*, the human 52 kDa ribonculeoprotein SS-A/Ro, and human RING1 and MDM2 (see Freemont, 1993; Schwabe and Klug, 1994). Residues corresponding to the consensus sequence are stippled.

Figure 2D in Rothe et al., 1994). Both proteins were individually digested with proteases, and the amino acid sequence of internal peptides was determined. Peptides with common sequences were found in the two proteins, suggesting that the 66 kDa protein may represent a degradation product or modification of the 68 kDa protein (data not shown). Fully degenerate oligonucleotides corresponding to two isolated peptides from the 68 kDa protein, YYIGPGD and EEMASGD, were used to amplify a 0.75 kb DNA fragment from mouse CT6 RNA by reverse transcription–polymerase chain reaction (RT–PCR). This DNA fragment was used to isolate a 2.85 kb cDNA clone from a mouse CT6 cDNA library. DNA sequence analysis of this cDNA clone revealed an open reading frame encoding a 612 amino acid protein with a predicted molecular mass of 69 kDa (Figure 2). Three additional peptides obtained from protein sequencing, QIPLQAR, KEIDSTLYENLFVEK, and TVRTFLS, were found in the translated mouse protein. These results confirm that the 2.85 kb cDNA clone encodes the purified murine 68 kDa TNFR2-associated protein.

c-IAP1 Is a Novel Member of the IAP Family of RING Finger Proteins

A database search did not reveal any proteins identical in primary amino acid sequence to the murine 68 kDa TNFR2-associated protein. However, substantial sequence similarity was observed with members of the IAP family previously found in baculoviruses (reviewed by Clem and Miller, 1994). The 68 kDa protein was therefore designated cellular IAP1 (c-IAP1), and we henceforth refer to its viral counterparts as v-IAPs.

Comparison of the primary structure of murine c-IAP1 and v-IAPs indicates that they are composed of at least two distinct domains (Figures 2 and 3). The N-terminal region of members of the IAP family contains characteristic tandem repeat units spanning approximately 70 amino acids that have been termed baculovirus IAP repeat (BIR) motifs (Birnbaum et al., 1994; Figures 3A and 3B). Among v-IAPs, the BIR motifs of the IAP from Orgyia pseudotsugata nuclear polyhedrosis virus (OpIAP; Birnbaum et al., 1994) are 68% and 30% identical with the equivalent domains of the IAPs from Cydia pomonella granulosis virus

(CpIAP; Crook et al., 1993) and from Autographa californica nuclear polyhedrosis virus (AcIAP; Braunagel et al., 1992), respectively (Figure 3B). The repeat motifs within the BIR domain of murine c-IAP1 (amino acids 1–329) share 50% amino acid identity with OpIAP, 42% identity with CpIAP, and 30% identity with AcIAP (Figure 3B). In addition, c-IAP1 displays 43% amino acid identity in its BIR motifs with the recently identified neuronal apoptosis inhibitory protein (NAIP) (Figure 3B), a mammalian IAP-related protein that has been implicated in spinal muscular atrophy disorders in humans (Roy et al., 1995). The BIR motifs of NAIP show 33% identity with v-IAPs (Roy et al., 1995; Figure 3B). This comparative analysis demonstrates that the BIR domains of mammalian c-IAP1 and NAIP are roughly as closely related to the corresponding domains of v-IAPs as those are to one another, suggesting an evolutionary conserved function for this structural motif. However, whereas the BIR domains of known v-IAPs are comprised of two repeat motifs, mammalian c-IAP1 and NAIP possess three tandem repeats (Figures 3A and 3B). The three BIR motifs of c-IAP1 are 33% identical (Figure 3B).

The C-terminal domain of v-IAPs contains a RING finger motif (reviewed by Clem and Miller, 1994; Figures 3A and 3C). This zinc-binding domain is also present in c-IAP1 but missing in NAIP (Figures 3A and 3C). The RING finger domain of c-IAP1 (amino acids 561–612) shares 42% and 36% identity over 52 amino acids with OpIAP (or CpIAP) and AcIAP, respectively (Figure 3C). In comparison, the RING finger domains of OpIAP and CpIAP are 54% identical, whereas they exhibit only 25% identity with the RING finger domain of AcIAP. RING finger structures have also been observed at the N-terminus of TRAF2 and TRAF3, where they mediate effector functions (Rothe et al., 1994, 1995; Cheng et al., 1995). Whereas the IAP RING finger motifs display a distribution of hydrophobic and charged residues that resembles several other RING finger family members (see Freemont, 1993), they do not share significant similarity with the TRAF RING finger domains beyond the conserved cysteine and histidine residues (Figure 3B).

c-IAP1 and c-IAP2 Are Closely Related Members of the IAP Family

To obtain the human homolog of murine c-IAP1, we screened a human HeLa cDNA library with a murine c-*IAP1* DNA probe under reduced hybridization stringency. Two distinct human cDNA clones of approximately 2.6 kb were isolated. One cDNA clone encoded a 70 kDa protein of 618 amino acids that is 84% identical to mouse c-IAP1, suggesting that it represents the human homolog of murine c-IAP1. The other human cDNA clone predicted a 68 kDa protein of 604 amino acids that is a second cellular member of the IAP family, termed c-IAP2. c-IAP2 is closely related to both murine and human c-IAP1, sharing 72% and 73% amino acid identity, respectively.

Like murine c-IAP1, human c-IAP1 and c-IAP2 contain three N-terminal BIR motifs and a C-terminal RING finger domain (Figures 2 and 3). In v-IAPs, these two domains are separated by a short linker region of approximately 30–50 amino acids (Clem and Miller, 1994). In contrast, this region extends over roughly 230 amino acids in both murine and human c-IAP1 (amino acids 330–560 and 337–566, respectively) as well as human c-IAP2 (amino acids 223–552) (see Figure 2). This protein domain displays strong sequence conservation among c-IAPs (87% identity between mouse and human c-IAP1 and 70% identity between human c-IAP1 and c-IAP2; see Figure 2), but does not bear any resemblance to v-IAPs nor to other known proteins.

The discovery of two closely related human IAPs along with their characterization as TNFR2-associated proteins (see below) suggests that the purified 66 kDa protein (see above) is probably the murine homolog of human c-IAP2 rather than a degradation product or modification of the purified 68 kDa protein.

Tissue Distribution of c-*IAP* mRNA

Northern blot analysis indicated that murine c-IAP1 is the translation product of a 4.1 kb mRNA expressed constitutively in mouse tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis; Figure 4A). Similarly, low levels of human c-*IAP1* and c-*IAP2* transcripts of approximately 4.3 kb and 6 kb, respectively, were detected in all human tissues examined (spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes; Figures 4B and 4C). Human c-*IAP1* mRNA was most abundant in thymus, testis, and ovary (Figure 4B), whereas human c-*IAP2* expression was highest in spleen and thymus (Figure 4C).

The BIR Domains of c-IAP1 and c-IAP2 Interact with the TRAF-N Domains of TRAF1 and TRAF2

Although the subsequent functional analysis of mammalian c-IAPs is described for c-IAP1 only, virtually identical results were obtained for c-IAP2 (data not shown). Thus, functional distinction between these closely related members of the IAP family was not apparent.

The functional properties of c-IAP1 as a TNFR2-associated protein were investigated in mammalian cells. Human c-IAP1 containing an N-terminal Myc epitope was transiently expressed in human embryonic kidney 293 cells. Cell lysates were incubated with various GST–hTNF-R2icd fusion proteins (Rothe et al., 1994), and specific binding of c-IAP1 was detected using an anti-Myc antibody. This analysis demonstrated that c-IAP1 coprecipitates with GST fusion proteins containing the wild-type cytoplasmic domain of TNFR2 and the cytoplasmic domains of the functional receptor mutants hTNFR2(−16) and hTNFR2(Δ304–345) (Figure 5A). However, c-IAP1 did not coprecipitate with GST fusion proteins comprising the cytoplasmic domains of the inactive mutants hTNFR2(−37) and hTNFR2(−59) (Figure 5A). These findings confirmed that the cloned c-IAP1 corresponds to the endogenous 68 kDa TNFR2-associated protein and suggest that c-IAP1 is involved in TNFR2 signaling.

Next we used the yeast two-hybrid system to determine whether the observed coprecipitation of c-IAP1 and TNFR2 in cell extracts is mediated by direct association or via other TNFR2-associated proteins. Two-hybrid analysis

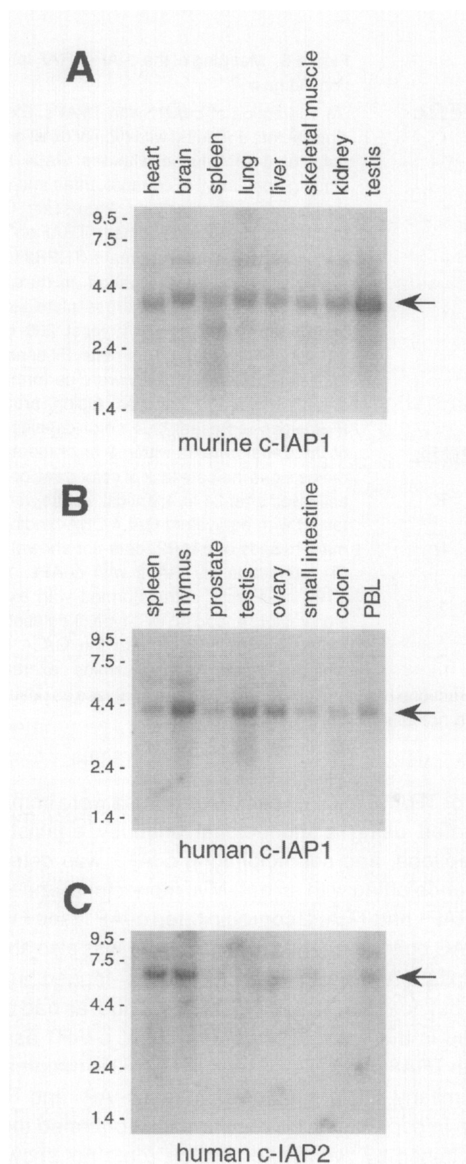

Figure 4. Northern Blot Analysis of c-*IAP1* and c-*IAP2* mRNA in Mouse and Human Tissues

Mouse (A) and human (B and C) multiple tissue Northern blots were hybridized with radiolabeled murine (A) and human c-*IAP1* (B) and human c-*IAP2* (C) probes. Arrows mark the postitions of specific transcripts of the respective c-*IAPs*. Molecular mass standards (in kilobases) are indicated on the left in each panel.

failed to reveal a direct interaction of c-IAP1 with the cytoplasmic domain of TNFR2 (Figure 6A). However, direct association could be detected between c-IAP1 and both TRAF1 and TRAF2 (Figure 6A). The conserved TRAF domain of TRAF2 (amino acids 264–501) was sufficient to mediate this interaction, and a mutant TRAF2 protein devoid of the entire TRAF domain (TRAF2[1–250]) did not associate with c-IAP1 (Figure 6B). Further two-hybrid analysis demonstrated that a mutant TRAF2 protein lacking the TRAF-C domain (TRAF2[1–358]) still associated with c-IAP1 (Figure 6B) but had lost its ability to bind to the cytoplasmic domain of TNFR2 (Figure 6B). In addition, no interaction could be detected between c-IAP1 and TRAF3

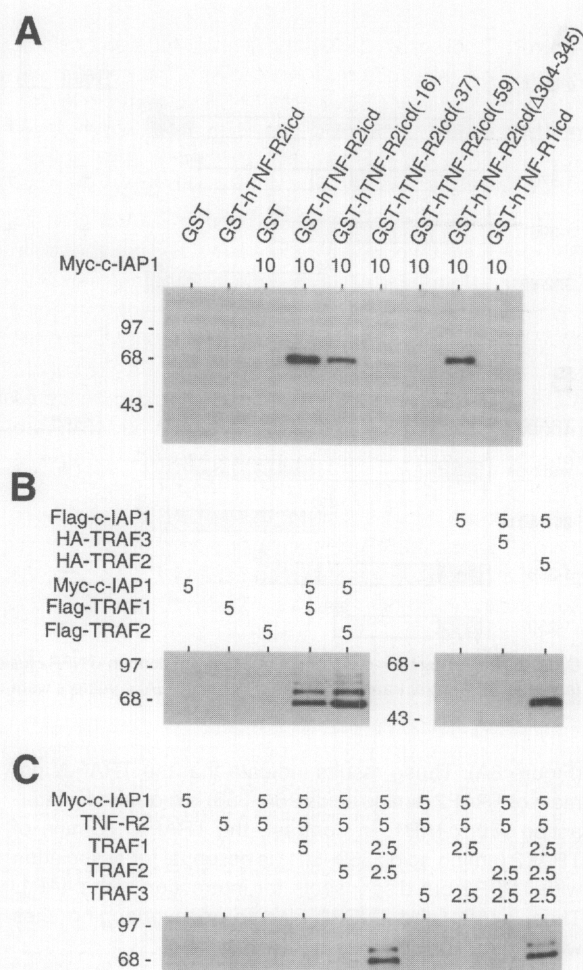

Figure 5. Interaction of c-IAP1 with TNFR2 and TRAFs

(A) Interaction of c-IAP1 with the intracellular regions of TNFR2 mutants expressed as GST fusion proteins. 293 cells (5×10^6) were transiently transfected with the indicated amounts (in micrograms) of an expression vector encoding Myc epitope–tagged c-IAP1. After 36 hr, lysates were prepared and incubated with the indicated GST fusion proteins. Coprecipitating Myc-tagged c-IAP1 was detected by immunoblot analysis using the anti-Myc monoclonal antibody. Note that the association of c-IAP1 with TNFR2 is indirect and can only be observed when the coprecipitation is performed in larger scale cell lysates, which provide sufficient amounts of endogenous TRAF1 and TRAF2 (see below).

(B) Interaction of c-IAP1 with TRAFs. 293 cells were transiently transfected with the indicated amounts (in micrograms) of epitope-tagged c-IAP1 and TRAF expression vectors. After 24 hr, aliquots of cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody. Coprecipitating Myc-tagged c-IAP1 and HA epitope–tagged TRAFs were detected by immunoblot analysis using the anti-Myc monoclonal antibody (right) and the anti-HA monoclonal antibody (left).

(C) TRAF2–TRAF1-mediated interaction of c-IAP1 with TNFR2. 293 cells (2×10^6) were transiently transfected with the indicated amounts (in micrograms) of *TNFR2*, *TRAF*, and Myc epitope-tagged c-*IAP1* expression vectors for 24 hr. Aliquots of cell lysates (one fith) were immunoprecipitated with polyclonal antibody to TNFR2. Coprecipitating Myc-tagged c-IAP1 was detected by immunoblot analysis using the anti-Myc monoclonal antibody.

The positions of molecular mass markers (in kilodaltons) are shown on the left in all panels.

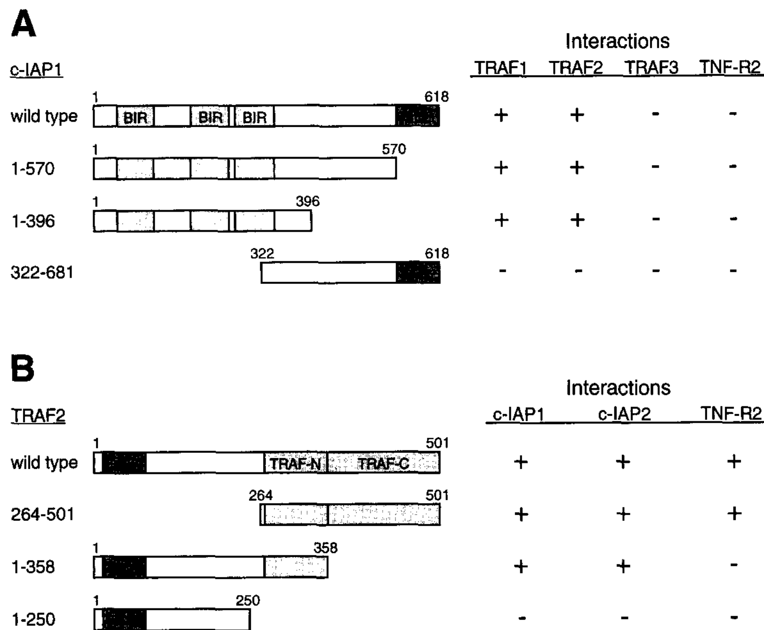

Figure 6. Mapping of the c-IAP–TRAF Interaction Domain

(A) Interaction of c-IAP1 with TRAFs. Expression vectors encoding wild-type or deletion mutants of c-IAP1 fused with the GAL4 DNA-binding domain were cotransformed into yeast Y190 cells with plasmids expressing GAL4 transcription activation domain–TRAF or GAL4 transcription activation domain–TNFR2 fusion proteins. Each transformation mixture was plated on a synthetic dextrose plate lacking tryptophan and leucine. At least 200 transformants were obtained per plate. Filter assays for β-galactosidose activity were performed to detect interaction between fusion proteins. Plus signs represent blue color development of the transformants within 2 hr of the assay; minus signs indicate lack of color development assessed after 24 hr. Identical results were obtained with equivalent GAL4 DNA-binding domain hybrids of c-IAP2 (data not shown).

(B) Interaction of TRAF2 with c-IAPs. Yeast Y190 cells were cotransformed with expression vectors encoding wild-type or deletion mutants of TRAF2 fused with the GAL4 DNA-binding domain and plasmids expressing GAL4 activation domain–c-IAP or GAL4 activation domain–TNFR2 fusion proteins. Interactions between fusion proteins were analyzed as described (see above). Control transformations with empty GAL4 vectors were negative and are not listed.

(Figure 6A). These results indicate that the TRAF-N domain of TRAF2 (amino acids 264–358) is required for interaction with c-IAP1. In contrast, the TRAF-C domain of TRAF2 (amino acids 359–501) is essential for association with TNFR2 but dispensable for interaction with c-IAP1. Thus, c-IAP1 and TNFR2 bind to nonoverlapping sites within the TRAF domain of TRAF2.

Whereas TRAF1 and TRAF2 can form homo- and heteromeric complexes, no homo- or heterodimerization of c-IAP1 and c-IAP2 was observed either by two-hybrid analysis or coimmunoprecipitation experiments (data not shown). Mutational analysis of human c-IAP1 was performed to investigate the structural requirements for its association with TRAFs. In the yeast two-hybrid system, the C-terminal RING finger domain of c-IAP1 was expendable for interaction with TRAFs, since a mutant c-IAP1 protein lacking this domain (c-IAP1[1–570]) still associated with TRAF1 and TRAF2 (Figure 6A). Further deletion analysis indicated that the region of c-IAP1 containing the BIR motifs mediates the interaction with TRAFs, since a mutant c-IAP1 protein comprising the N-terminal half of the molecule (c-IAP1[1–396]) associated with TRAF1 and TRAF2 whereas a mutant protein devoid of BIR motifs (c-IAP1[322–618]) lacked this ability (Figure 6A). This observation suggests that BIR motifs of IAPs may constitute a protein–protein interaction domain.

c-IAP1 and c-IAP2 Are Components of TNFR2-TRAF Signaling Complex

A transfection-based coimmunoprecipitation assay was used to further investigate the interaction of c-IAP1 with components of the TNFR2–TRAF complex in mammalian cells. An expression vector encoding the Myc epitope-tagged human c-IAP1 was transfected alone or with expression vectors encoding FLAG epitope-tagged murine TRAF1 or TRAF2 into 293 cells. Cell lysates were immunoprecipitated using a monoclonal antibody against the FLAG epitope, and coprecipitating c-IAP1 was detected by immunoblotting with an anti-Myc monoclonal antibody. Both TRAF1 and TRAF2 coprecipitated c-IAP1 (see Figure 5B). FLAG epitope–tagged murine c-IAP1 was also able to coprecipitate hemagglutinin (HA) epitope–tagged human TRAF2 but not TRAF3 (see Figure 5B). Thus, as had been observed in the yeast two-hybrid system, c-IAP1 associates with TRAF1 and TRAF2 but not TRAF3 in mammalian cells. Similarly, the interaction of mutant c-IAP1 and TRAF proteins in coprecipitation experiments confirmed the results obtained by two-hybrid analysis (data not shown).

Because the interaction between c-IAP1 and TNFR2 was found to be indirect and both proteins bind to distinct regions within TRAF2, we reasoned that TRAF2 was likely to mediate the association of c-IAP1 with TNFR2. Similarly, TRAF2 has been shown to mediate the interaction between TNFR2 and TRAF1 in the yeast two-hybrid system (Rothe et al., 1994). To test this assumption, TNFR2 and Myc epitope–tagged c-IAP1 were coexpressed in 293 cells in the absence or presence of overproduced TRAF proteins. Cell lysates were immunoprecipitated with polyclonal anti-TNFR2 antibodies, followed by immunoblotting with anti-Myc monoclonal antibody. In this assay, TNFR2 did not coprecipitate detectable levels of c-IAP1 through endogenous factors since the amount of cell extract used for immunoprecipitation was approximately 25-fold less than in the coprecipitation experiments with GST–hTNF-R2icd fusion proteins (see above) (see Figure 5C). Surprisingly, coexpression of neither TRAF1 nor TRAF2 was sufficient to mediate association of TNFR2 with c-IAP1 (see Figure 5C). However, when equivalent amounts of both TRAF1 and TRAF2 were coexpressed with TNFR2 and c-IAP1, strong coprecipitation of c-IAP1 with TNFR2 was

detected (see Figure 5C). This association of TNFR2 and c-IAP1 was specifically mediated by the TRAF1–TRAF2 heterocomplex, since it was not observed upon coexpression of either TRAF1 or TRAF2 with TRAF3 (see Figure 5C). These findings demonstrate that whereas c-IAP1 is capable of associating directly with TRAF1 and TRAF2, its interaction with TNFR2 is indirect and requires both TRAF1 and TRAF2. c-IAP2 behaved indistinguishably from c-IAP1 in this analysis (data not shown). Thus, both c-IAP1 and c-IAP2 constitute components of the TNFR2 signaling complex.

Discussion

The recent identification of two distinct families of signal transducers has provided insights into how members of the TNFR superfamily initiate downstream signaling events (reviewed by Vandenabeele et al., 1995). These signaling proteins contain either death domains or TRAF domains and are involved in signal transduction by either TNFR1 and the Fas antigen or TNFR2, CD40, and, presumably, other TNFR family members, respectively. We now report the identification and molecular characterization of a third class of putative intracellular signal transducers for this diverse group of cell surface receptors. c-IAP1 and c-IAP2 were found in association with the cytoplasmic domain of TNFR2 and are closely related mammalian members of the expanding IAP family originally identified in baculoviruses (Clem and Miller, 1994).

The common structural feature of all IAP family members are tandem repeat units, termed BIR motifs, of the general consensus sequence $X_3RX_{20-23}GX_{11}CX_2CX_{16}HX_6CX_3$ (Birnbaum et al., 1994; Figure 3B). The spacing of cysteine and histidine residues within the BIR motifs raises the possibility of metal ion coordination and nucleic acid binding, similar to known metal ion–containing protein motifs (Birnbaum et al., 1994). Our mutational analysis of c-IAP1 and c-IAP2 provides evidence that the BIR motif–containing domain of these two IAPs facilitates protein–protein interaction, allowing these proteins to associate with TRAFs. This finding suggests that other viral and cellular members of the IAP superfamily may interact with still unidentified TRAF-related proteins as part of their mechanism of action.

In addition to BIR motifs, all known IAP family members contain RING finger domains, with the exception of NAIP. This sequence motif has been observed in a wide variety of nuclear and cytoplasmic proteins and is thought to form two zinc-binding finger structures involved in protein–DNA interactions and possibly protein–protein interactions (reviewed by Schwabe and Klug, 1994). Unlike most RING finger proteins characterized to date, the IAP RING finger domains are located C-terminally rather than N-terminally (Birnbaum et al., 1994). N-terminal RING finger domains are also present in two members of the TRAF family, TRAF2 and TRAF3 (Rothe et al., 1994; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995a; Song and Donner, 1995). The TRAF2 RING finger domain is required for TRAF2-mediated NF-κB activation (Rothe et al., 1995). By analogy, the RING finger domains of IAPs may represent effector or regulatory domains that are involved in IAP function.

Our analysis identifies c-IAP1 and c-IAP2 as candidate signal transducers based on their association with a 78 amino acid region in TNFR2 cytoplasmic domain required for signaling (Rothe et al., 1994). Both IAPs are recruited to TNFR2 by binding to a TRAF2–TRAF1 heterocomplex. This indirect mode of interaction suggests that c-IAP1 and c-IAP2 may be signaling molecules not only for TNFR2 but also for other members of the TNFR superfamily that bind the TRAF2–TRAF1 heterocomplex (Rothe et al., 1995). Also, these findings bear implications with respect to the function of TRAF1. Whereas TRAF2 and TRAF3 have each been linked to downstream signaling events, i.e., NF-κB activation (Rothe et al., 1995) and induction of CD23 expression (Cheng et al., 1995), respectively, a direct contribution of TRAF1 to receptor signaling has not yet been uncovered. Our observations now support at least a structural role for TRAF1 in the TNFR2 signaling complex by functioning as an adaptor protein that enables the recruitment of additional signaling proteins. Conceptually, TNFR2 signaling appears to proceed via a multisubunit signaling complex, in which discrete intracellular signals may emanate from its individual components. Because expression of *TRAF1* transcripts is tissue specific (Rothe et al., 1994; Mosialos et al., 1995), this model implies a mechanism for generating locally restricted responses upon ligand stimulation. In addition, TRAF1 may also directly initiate signaling cascades distinct from TRAF2 and TRAF3.

Similar to its role in the development and maintenance of multicellular organisms, apoptosis has been recognized as an efficient host antiviral defense mechanism through which virally infected cells are destroyed (reviewed by Vaux et al., 1994; Oltvai and Korsmeyer, 1994). To ensure viral propagation, many DNA-containing viruses have evolved molecular strategies to circumvent and antagonize host death signals (reviewed by Oltvai and Korsmeyer, 1994; Vaux et al., 1994). Among these anti-death mechanisms are virally acquired mimics of cellular proteins, such as BCL2, that regulate apoptosis during specific stages of host development (reviewed by Vaux et al., 1994; Oltvai and Korsmeyer, 1994). However, several viral proteins have been identified that interfere with apoptosis, but for which putative cellular counterparts have remained elusive. Among these are the p35 and IAP families from baculoviruses (reviewed by Clem and Miller, 1994). Although initially isolated by genetic analysis in Spodoptera frugiperda cells, the p35 protein was subsequently demonstrated to suppress apoptosis in Caenorhabditis elegans, Drosophila melanogaster, and mammalian cells (Sugimoto et al., 1994; Hay et al., 1994; Martinou et al., 1995; Beidler et al., 1995). In contrast, functional analysis of baculoviral IAPs has been limited to insect cells.

Our discovery of two closely related mammalian members of the IAP family raises the possibility that c-IAP1 and c-IAP2 function to regulate cellular apoptosis. In support of this notion, v-IAPs have recently been shown to antagonize apoptosis in Drosophila (Hay et al., 1995 [this issue of *Cell*]). However, overexpression of c-IAP1 and c-IAP2

in mammalian cells failed to suppress cell death induced by TNF or Fas antigen stimulation, overproduction of the TNFR1-associated signaling protein TRADD, or growth factor deprivation (M. R., T. M. A., H. Hsu, and D. V. G., unpublished data). Also, whereas v-IAPs have been shown to suppress apoptosis induced by actinomycin D in insect cells (Clem and Miller, 1994), overexpression of c-IAP1 or c-IAP2 did not antagonize actinomycin D–induced cell death in human HL60 cells (T. M. A. and M. R., unpublished data). The finding that c-IAP1 and c-IAP2 are most abundantly expressed in lymphoid tissues suggests that they may be involved in protecting against apoptosis initiated by other events in the immune system. In this respect, it appears possible that TNFR2-associated IAPs might participate in TNF-induced proliferation of thymocytes, since this TNF response is specifically mediated by TNFR2 (Tartaglia et al., 1991, 1993a).

Alternatively, c-IAP1 and c-IAP2 may not contribute to the regulation of apoptosis. Unlike the closely related OpIAP and CpIAP, the AcIAP does not sustain survival of infected insect cells (Birnbaum et al., 1994). This apparent lack of apoptosis-antagonizing acitivity of AcIAP may indicate functional diversity among viral and, by analogy, perhaps mammalian IAPs. In this respect, a potential involvment of c-IAPs in TRAF2-mediated NF-κB activation by TNFR2 and CD40 was not substantiated (M. R. and D. V. G., unpublished data). Thus, although our interaction analysis implicates an important role for c-IAP1 and c-IAP2 in signaling by members of the TNFR superfamily, their specific function in this process remains to be elucidated. Similarly, the functional properties of viral AcIAP await further studies. However, all IAP family members display highly conserved structural features, including BIR motifs and RING finger domains. This common architecture predicts equally conserved biochemical properties intrinsic to IAPs. To this end, our analysis represents a conceptual paradigm for the mechanism of action of members of the IAP family.

Experimental Procedures

Cell Culture and Biological Reagents

The murine interleukin-2-dependent cytotoxic T cell line CT6 and human embryonic kidney 293 cells were maintained as described previously (Tartaglia et al., 1991; Pennica et al., 1992). The rabbit anti-hTNFR2 antibodies have been described previously (Pennica et al., 1992). The monoclonal antibody 9E10 against the Myc epitope was provided by R. Schreiber. The anti-FLAG epitope monoclonal antibody M2 and the anti-HA epitope monoclonal antibody 12CA5 were purchased from Eastman Kodak Company and BAbCO, respectively.

Purification and Amino Acid Sequencing of the 68 kDa TNFR2-Associated Protein

Large-scale purification of the 68 kDa TNFR2-associated protein was performed according to previously described procedures (Rothe et al., 1994). In brief, total cell extract prepared from 120 liters of CT6 cells (6×10^{10} cells) was passed through a preabsorption column of 25 ml glutathione–agarose GST–hTNF–R2icd(−37) fusion protein. The flowthrough was applied to a 0.25 ml Affigel 10/15 GST–hTNF–R2icd fusion protein affinity column. As a control, the lysate was passed through a similar Affigel 10/15 GST–hTNF–R2icd(−37) fusion protein affinity column in parallel. After extensive washing, bound proteins were eluted with ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce) containing 0.1 M DTT, precipitated with methanol/chloroform, and resuspended in SDS sample buffer containing 5% SDS. An aliquot of the material was separated by 8% SDS–PAGE under reducing conditions and visualized by silver staining. The remaining purified material was separated by SDS–PAGE and electrophoretically transfered to PVDF sequencing membrane (Millipore), and proteins were visualized by staining with Coomassie brilliant blue R-250 (Sigma). The proteins bands of 68 kDa and 66 kDa were cut out and subjected to amino acid sequencing as described previously (Rothe et al., 1994).

cDNA Cloning and Northern Blot Hybridization

Reverse transcription of poly(A)$^+$ RNA isolated from CT6 cells (Sambrook et al., 1989) was performed using the cDNA Cycle Kit (Invitrogen) according to the instructions of the manufacturer. First-strand CT6 cDNA was subjected to PCR with combinations of fully degenerate oligonucleotides corresponding to the sense and antisense sequences of obtained peptides using a Cetus GeneAmp Kit and Perkin–Elmer Thermocycler. The PCR was run for 35 cycles (45 s at 95°C; 60 s at 55°C; 150 s at 72°C) after an initial step of 6 min at 95°C and analyzed by electrophoresis on a 1.2% agarose gel. A specifically amplified 0.75 kb DNA fragment was labeled with [α-^{32}P]dCTP using the T7 Quick Prime Kit (Pharmacia). The labeled fragment was used to screen murine CT6 and human HeLa cDNA libraries (Rothe et al., 1994; Hsu et al., 1995) under standard high and reduced stringency conditions, respectively (Sambrook et al., 1989). The cDNA inserts of positive phage clones were subcloned into pBluescript KS (Stratagene) and sequenced on both strands with Sequenase (United States Biochemical).

Northern blot analysis of mouse and human multiple tissue blots (Clontech) was done under high stringency conditions according to the instructions of the manufacturer with radiolabeled *c-IAP1* and *c-IAP2* cDNA probes.

Yeast Two-Hybrid Assay

DNA fragments encoding full-length and truncated variants of human c-IAP1 and c-IAP2 were amplified from the corresponding cDNAs by PCR and cloned in-frame into the GAL4 DNA-binding domain vector pPC97 (Chevray and Nathans, 1992). Expression vectors for TRAFs and the cytoplasmic domain of TNFR2 in the yeast two-hybrid system have been reported (Rothe et al., 1994; Hu et al., 1994). Interaction analysis between bait- and prey-encoded fusion proteins in Saccharomyces cerevisiae Y190 cells was done as described by the manufacturer (Matchmaker Two-Hybrid System Protocol; Clontech).

Transfections, Coimmunoprecipitations, and Immunoblotting

For expression in mammalian cells, the cDNAs for murine and human *c-IAP1* and human *c-IAP2* were cloned into pRK5 under the transcriptional control of the cytomegalovirus immediate-early promotor–enhancer (Schall et al., 1990). Expression vectors for TNFR2 and TRAFs have been described previously (Tartaglia et al., 1993b; Hu et al., 1994; Rothe et al., 1995). Epitope tag constructs were made by N-terminal addition of DNA encoding the sequences MASMEQKLISEEDL (Myc epitope tag) or MDYKDDDDK (FLAG epitope tag).

For coprecipitation experiments with GST fusion proteins, 5×10^6 293 cells per dish were seeded into 150 mm dishes and transfected the next day by the calcium phosphate precipitation method (Ausubel et al., 1987). After incubation for 36 hr, cells were washed twice with phosphate-buffered saline and then lysed in 1 ml of 0.1% NP-40 lysis buffer containing 50 mM HEPES (pH 7.2), 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 μg/ml benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin. Cell lysates were incubated for 2 hr at 4°C with 5 μl of glutathione–agarose GST–hTNF–R2icd fusion protein beads (Rothe et al., 1994). The beads were washed extensively with lysis buffer and bound proteins fractionated by 8% SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with an anti-Myc epitope monoclonal antibody. The antibody was visualized with horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Amersham) using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System (Amersham) according to the instructions of the manufacturer.

For immunoprecipitation experiments aliquots of cell lysates (one fifth) from transfected 293 cells (2×10^6 cells per 100 mm dish) were incubated with 1 μl of anti-hTNFR2 antibody or anti-FLAG epitope

monoclonal antibody. After 3 hr at 4°C, 20 μl of protein A– or protein G–agarose beads (Oncogene Science) was added to the mixture and incubated for another hour. The precipitates were washed and analyzed by SDS-PAGE and immunoblotting (see above). In all cases, expression of transfected constructs was verified by immunoblotting of aliquots of cell lysates.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L49433, L49431, and L49432 for murine and human c-*IAP1* and human c-*IAP2*, respectively.