CLAP, a Novel Caspase Recruitment Domain-containing Protein in the Tumor Necrosis Factor Receptor Pathway, Regulates NF-κB Activation and Apoptosis*

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Molecules that regulate NF-κB activation play critical roles in apoptosis and inflammation. We describe the cloning of the cellular homolog of the equine herpesvirus-2 protein E10 and show that both proteins regulate apoptosis and NF-κB activation. These proteins were found to contain N-terminal caspase-recruitment domains (CARDs) and novel C-terminal domains (CTDs) and were therefore named CLAPs (CARD-like apoptotic proteins). The cellular and viral CLAPs induce apoptosis downstream of caspase-8 by activating the Apaf-1-caspase-9 pathway and activate NF-κB by acting upstream of the NF-κB-inducing kinase, NIK, and the IkB kinase, IKKα. Deletion of either the CARD or the CTD domain inhibits both activities. The CARD domain was found to be important for homo- and heterodimerization of CLAPs. Substitution of the CARD domain with an inducible FKBP12 oligomerization domain produced a molecule that can induce NF-κB activation, suggesting that the CARD domain functions as an oligomerization domain, whereas the CTD domain functions as the effector domain in the NF-κB activation pathway. Expression of the CARD domain of human CLAP abrogates tumor necrosis factor-α-induced NF-κB activation, suggesting that cellular CLAP plays an essential role in this pathway of NF-κB activation.

The transcription factor NF-κB is a critical regulator of cellular response to infectious agents, stress, injury, and inflammation (reviewed in Refs. 1 and 2). NF-κB has also been implicated in the regulation of apoptosis (3–7). The transcriptionally active NF-κB is a homo- or a heterodimer of two subunits, which belong to a family of transcriptional regulatory proteins known as the NF-κB/Rel family (reviewed in Refs. 8 and 9). In mammalian cells five family members are known to date that include RelA/p65, c-Rel, RelB, p50, and p52. Among these, the p50 and p52 are made as inactive precursor molecules (p105 and p100, respectively), which are proteolytically processed to the smaller transcriptionally active forms. All family members contain a conserved N-terminal domain called the Rel homology domain that contains the necessary residues for dimerization, nuclear translocation, and DNA binding (8, 9).

The best characterized active NF-κB complex is the heterodimer p50-p65 (10–13). In unstimulated cells, the NF-κB heterodimer is sequestered in an inactive form in the cytosol through non-covalent interactions with the family of the IkB inhibitory proteins which includes IκBa, IκBβ, and IκBe (1, 2, 14). These interactions mask the nuclear localization signals of the NF-κB heterodimer, thereby preventing it from translocating to the nucleus. In response to a variety of signaling events, IκBa is phosphorylated, which targets the protein to degradation via a ubiquitination-dependent pathway. This unmask the NF-κB nuclear localization signals allowing it to translocate to the nucleus and induce the expression of a number of target genes (9). Some of the target gene products, such as cIAP-1, cIAP-2, TRAF1, and TRAF2, have been shown to protect cells from cell death induced by TNF-α1.

The proinflammatory cytokines TNF-α and interleukin-1 induce NF-κB activation by binding to their cell-surface receptors, TNF-R1, TNF-R2, or IL-1R, respectively (8, 15–17). Although these receptors do not share sequence homology, they all activate the NF-κB-inducing kinase NIK and NF-κB through distinct signaling pathways (9, 17). For example, the TNF-R1, like several other members of the TNF-R family, contains an intracellular region known as the death domain which is required for both apoptosis induction and NF-κB activation (reviewed in Refs. 18 and 19). Binding of TNF-α to the extracellular ligand binding domain of TNF-R1 induces aggregation of its death domain and assembly of signaling complex containing TRADD (TNF-R1-associated death domain protein), TRAP2 (TNF-associated factor 2), and RIP (receptor interacting protein) (20–24). Similarly, aggregation of the IL-1R by its ligand results in the assembly of an intracellular signaling complex containing the IL-1R accessory protein Acp, the adaptors MyD88, the IL-1-activated kinases, IRAK1 and IRAK2, and the TRAF protein, TRAF6 (25–29). These two distinct complexes somehow trigger activation of NIK which in turn phosphorylates the IκBa kinases α and β (IKKα and IKKβ) that then phosphorylate IκBa at Ser-32 and Ser-36 (reviewed in Refs. 9 and 30). The phosphorylation of IκBa targets it for ubiquitination and proteosomal degradation, allowing the activation of IκBα kinase, which degrades IκBα releasing NF-κB subunits which translocate to the nucleus.

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* The abbreviations used are: TNF, tumor necrosis factor; CARD, caspase-recruitment domains; IL-1R, interleukin-1 receptor; DISC, death-inducing signaling complex; CTD, C-terminal domains; PFR, polypeptide chain reaction; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; DED, death-effector domain; EHV, equine herpesvirus; HA, hemagglutinin.
tive NF-κB heterodimer to translocate to the nucleus (9, 30).

Signals are relayed from the non-enzymatic components of the TNF-R1 or IL-1R complexes to the enzymatic effector components via homotypic and heterotypic interactions between domains present in these components. The death domain for example, which is present in TNF-R1, TRADD, and RIP, mediates interactions necessary for the assembly of the NF-κB-activating complex and the death-inducing signaling complex (DISC) (9, 17, 19). This domain is also present in proteins of the IL-1R complex, such as MyD88, IRAK1, and IRAK2 (25–29).

Two other structurally related domains, known as the death-effector domain (DED) and the caspase-recruitment domain (CARD), mediate interactions between the initiator caspases and death-inducing signaling complexes (31–33). The DISC of TNF-R1 utilizes the adaptor molecule FADD which contains both death and DED domains to relay its death signal (19). After stimulation of TNF-R1 by its ligand, TNF-R1 recruits TRADD and FADD through homologous death domain interactions. The DED domain of FADD then interacts with the homologous DED domains of procaspase-8 and -10, causing their oligomerization and activation (19). The DISC of TNF-R1 also recruits another adaptor molecule with a death domain called CRADD or RAIDD to transmit its death signal (34, 35). Similar to FADD, CRADD uses its CARD domain to interact with the homologous CARD domain of procaspase-2 to induce its oligomerization and activation (34, 35). The CARD is also found in the promdomain of other apical caspases such as procaspase-1, -4, -5, and -9 and the apoptosis regulatory molecule Apaf-1 (31, 36). In the Apaf-1 DISC, this domain is involved in binding and subsequent oligomerization of procaspase-9 by Apaf-1 (37, 38).

Because of the importance of CARD-containing proteins as regulators of apoptosis, we decided to search the public data base for novel sequences that contain homologous CARD domains. Here we report the identification of a CARD-containing viral protein and its mammalian homolog, and we provide evidence that these proteins, named CLAPs, are involved in regulation of NF-κB activation and apoptosis.

**MATERIALS AND METHODS**

**cDNA Cloning**—The full-length open reading frame of E10 (vCLAP) within the genome of the equine herpesvirus-2 (EHV-2) (GenBank™ accession number U20824) was cloned by PCR using the EHV-2 genomic DNA template and given as a gift from Dr. A. J. Davison, University of Glasgow, UK) and the following PCR primer adaptors: E10-start, GAA-GATCTTATGGCGGAAAAATCCCTC; E10-stop, CGGCTGAGTCTCTTTGAGTTGGC. The resulting PCR product was digested with BglII and XhoI and ligated to a BamHI/XhoI cut T7-pcDNA3 vector. By using the TBLASTN program, several human and mouse partial EST sequences with homology to the CARD domain of vCLAP were identified. The corresponding EST clones (human clones 703916 and 812172; mouse clone 902716) were obtained from the IMAGE consortium, and their entire nucleotide sequences were determined by automated sequencing. These clones were found to encode identical full-length open reading frames as evident from the presence of stop codons upstream of the initiator methionine.

**Northern Blot Analysis**—Tissue distribution analysis of human CLAP mRNA was performed by Northern blot analysis on normal and tumor MTN Blots (purchased from CLONTECH). Each lane contains 2 μg of poly(A)+ RNA from specific tissues or cell lines. The blots were probed with a radiolabeled riboprobe prepared from a full-length human CLAP cDNA template and then subjected to autoradiography.

**Mammalian Expression Vectors**—The entire open reading frames of vCLAP and hCLAP were amplified by PCR using complementary PCR adaptor primers spanning the initiation and stop codons of these genes. CLAP deletion mutants were also generated by PCR using modified complementary PCR adaptor primers. The conserved L41 in the CARD domain of hCLAP was mutated by site-directed mutagenesis using overlapping PCR as described before (39). FLAG and T7 epitope tagging was done by cloning the PCR-generated cDNAs of the respective genes in-frame into pFLAG CMV-2 and pcDNA-3-T7 vectors, respectively.

GFP fusion constructs were generated by cloning the PCR-generated cDNAs of the respective genes in frame into the mammalian expression vector pEGFP (CLONTECH). FKBP12 fusion of CLAP-L14R and CLAP-CTD was constructed in pPkp3-HA vector (generous gift of Dr. X. Yang, University of Pennsylvania) which contain three tandem repeats of FKBP12 with an N-terminally fused c-Src myristylation signal and a C-terminally fused HA tag (40). Constructs encoding CRADD, caspase-9ΔN, Apaf-1, DR5, X-IPAP, Bcl-xL, p35, and kinase-inactive mutants of NIH or IKKα have been described before (34, 37, 41–44). The 5xκB-luciferase reporter plasmid was from Stratagene.

**Transfection, Immunoprecipitation, and Immunoblot Analysis**—293 or CHO cells were transfected with 5 × 10⁶ cells/well in 6-cm dishes were then transiently transfected with the expression plasmids (5 μg/plasmid) using the LipofectAMINE™ (Life Technologies, Inc.) method. Cells were lysed in a lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40) and incubated with anti-FLAG-M5 monoclonal antibody (Eastman Kodak Co.). The immune complexes were precipitated with protein G-Sepharose, washed extensively, and then eluted by boiling in SDS sample buffer. The eluted proteins were resolved by SDS-PAGE and detected by Western analysis with a horseradish peroxidase-conjugated TT-antibody (Novagen). The total lysates were also resolved by SDS-PAGE and detected by Western analysis using the anti-FLAG-M5 and T7 antibody.

**Phosphatase Assays**—The phosphatase treatments of hCLAP were described as described in (44).

**Apoptosis Assays**—MCF-Fas cells (0.5 × 10⁶ cells/well) in 12-well plates were transfected with 0.1 μg of LacZ reporter plasmid, 0.25 μg of various expression plasmids, and 0.75 μg of various apoptosis inhibitor/empty vector plasmids using the LipofectAMINE™ method. Cells were stained for β-galactosidase activity 30–36 h after transfection. In some experiments, the cells were treated with cycloheximide (1 μg/ml) with or without IFN-α (10 ng/ml) or Fas antibody (50 ng/ml) or recombinant soluble TRAIL (200 ng/ml) for 6 h before staining. Normal and apoptotic blue cells were counted. The percentage of apoptotic cells in each experiment was expressed as the mean percentage of stained apoptotic cells as a fraction of the total number of blue cells. Data represent the average of at least three individual experiments (n = 3 and S.D. ±1–4%).

**Assay of NF-κB Activation**—NF-κB activation was done using a luciferase reporter gene. 2 × 10⁶ cells/well in a 12-well plate were transfected with 5xκB-luciferase reporter plasmid and various expression plasmids using the LipofectAMINE™ method as per the manufacturer’s instructions. For Jurkat cells, transfections were done by electroporation using Bio-Rad Gene Pulser II. 24 h after transfection, cells were harvested and subjected to luciferase assay as described in Lin et al. (44). In certain experiments, cells were treated with hTNF-α (20 ng/ml) for 5 h prior to harvesting. To normalize for transfection efficiency, all transfections included a LacZ-expressing plasmid, and the lysates were assayed for β-galactosidase activity. Data represent the average of at least three individual experiments (n = 3 and S.D. ±0.1–0.4%).

**RESULTS AND DISCUSSION**

**Identification and Cloning of Viral and Cellular CLAPs**—To identify novel molecules that contain CARD domains, we searched public data bases for sequences that are homologous to the CARD sequence of Apaf-1 (residues 1–100). By using this strategy, we found that the 210-amino acid deduced open reading frame of the EHV-2 E10 (ORF-E10) contains an N-terminal CARD domain (residues 21–107) that is ~27% identical to the CARD of Apaf-1 (Fig. 1, A and C). Interestingly, the E10 CARD domain exhibits higher identity with the N-terminal prodomains of caspase-9 (~31%), caspase-2 (~28%), and CED-3 (~29%) and exhibits significant homology with the CARD domain of the adaptor molecule CRADD/RAIDD (23% identity).

To clone the ORF-E10, we generated PCR primers complementary to the start and stop codons of E10 based on the GenBank™ genomic sequence of EHV-2-E10 (accession number U20824). The PCR primers were then used on an EHV-2 genomic template to amplify the ORF-E10 by PCR. Interestingly, after sequencing of the cloned PCR products, we found an extra base (G) at position 428 relative to the ATG start site. This extra base shifts the reading frame of E10, resulting in a longer open reading frame which encodes a 311-amino acid-
long protein (Fig. 1A). The corrected ORF-E10, which represents the actual ORF of the EHV-2-E10 gene, differs from the GenBank™ ORF-E10 after amino acid 142.

To identify cellular homologs of EHV-2-E10, we searched the GenBank™ expressed sequence tags (EST) database with the ORF-E10 sequence. Several human and mouse EST clones were identified, and their 3' and 5' sequences were compiled.

Sequence analysis of the human and mouse cDNAs revealed that they encode proteins of 233 amino acids with an overall 34% identity to the viral E10 (Fig. 1B). The two proteins share a high sequence identity (91%), suggesting that they are the human and mouse orthologs. Interestingly, the highest sequence identity between the cellular and viral proteins lies within the N-terminal CARD homology domain (cellular pro-

**Fig. 1. Sequence and domain structure of viral and cellular CLAPs.** Predicted amino acid sequences of viral CLAP (A) and cellular CLAP (human and mouse) (B) and their domain structures. The sequences of the CARD homology domains in A and B are highlighted. The domain structures of vCLAP and cCLAP are represented by bar diagrams below the sequences. The CARD, Ser/Thr-rich, and Gly-rich domains are labeled inside the bar diagrams. The numbers indicate the boundaries of these domains. C, co-linear alignment of the CARD homology domain of CLAPs with that of several regulators of apoptosis. Identical residues in at least four of seven sequences are highlighted. D, hydrophilicity plot analysis of hCLAP and vCLAP. The hydrophilicities of the entire amino acid sequence of hCLAP (upper panel) and vCLAP (lower panel) were calculated and plotted using the MacVector 3.5 program.
The expression of hCLAP mRNA in normal tissues and tumor cell lines. The expression of hCLAP mRNA in normal tissues (upper panel) and tumor cell lines (lower panel) was determined by Northern blot analysis using premade multi-tissue Northern blots from CLONTECH. The cell lines are as follows: HL-60, promyelocytic leukemia; HeLa cell line S3, K-562, chronic myelogenous leukemia; Raji, Burkitt’s lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma. Numbers on the right indicate kilobases. PBL, peripheral blood leukocyte.

FIG. 2 Expression of hCLAP in normal tissues and tumor cell lines. The expression of hCLAP mRNA in normal tissues (upper panel) and tumor cell lines (lower panel) was determined by Northern blot analysis using premade multi-tissue Northern blots from CLONTECH. The cell lines are as follows: HL-60, promyelocytic leukemia; HeLa cell line S3, K-562, chronic myelogenous leukemia; Raji, Burkitt’s lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma. Numbers on the right indicate kilobases. PBL, peripheral blood leukocyte.

protein, residues 13–99; viral protein, residues 21–107). In this region the two proteins share ~47% identity. In this region also, the cellular protein has 20, 21, 26, and 29% identity to the CARD domain of Apaf-1, caspase-9, caspase-2, and CRADD, respectively (Fig. 1C). The selective conservation of the CARD homology domain within the mammalian and viral proteins suggests that they might play a role in regulating apoptosis. Based on their homology to the CARD domain and their apoptotic activity (see below), we call these proteins CLAPs (for CARD-like apoptotic proteins).

Structural Organization of Viral and Cellular CLAPs—Sequence analysis of the cellular and viral CLAPs revealed that they have a dual domain structure similar to several adaptor molecules that regulate apoptosis, such as FADD and CRADD. As mentioned above, the vCLAP and cCLAP have N-terminal CARD homology domains that might be necessary for interaction with proximal signal transducing molecules. However, their C-terminal domains (CTD, cCLAP residues 100–233; vCLAP residues 108–311) are unique in that they share no significant sequence homology with each other or any other known proteins. Interestingly, the CTD domain of cCLAP is rich in Ser/Thr residues, whereas that of vCLAP is rich in Gly residues. An abundance of the Ser/Thr residues in cCLAP suggests that it might be regulated by phosphorylation.

Hydropathy plots revealed that the CARDs of CLAPs contain characteristic hydrophobic stretches similar to the ones present in the CARDs of several CARD-containing proteins such as caspase-9, Apaf-1, or CRADD (Fig. 1D). Surprisingly, the hydropathy plots revealed that their CTD domains are also structurally related and contain similar profiles of hydrophilic and hydrophobic stretches (Fig. 1D). Therefore, the cellular and viral CLAPs may be overall structurally related and may function as adaptor molecules for an as yet unidentified signaling complex(es).

Tissue Distribution of cCLAP—To determine the distribution of cCLAP, various normal human tissues and tumor cell line mRNA samples were subjected to Northern blot analysis. As shown in Fig. 2A, cCLAP mRNA (~2.5 kilobase pairs) is constitutively expressed in all the normal tissues examined with particularly high expression in the pancreas and in lymphoid organs. Smaller transcripts (~2.0, ~1.6, and ~1.3 kilobase pairs), which could represent alternatively spliced cCLAP mRNAs, were detected in skeletal muscle, placenta, spleen, and peripheral blood leukocyte. The major cCLAP mRNA transcript and the smaller isoforms were also detected in all the tumor cell mRNA samples examined (Fig. 2, lower panel).

Viral and Cellular CLAPs Induce Apoptosis in Human Cells—Many regulators of apoptosis such as the death receptors, the adaptor molecules FADD, CRADD, and RIP, the pro-apoptotic Bcl-2 family members and others, induce apoptosis when overexpressed in the absence of additional apoptotic signals (34, 41, 42, 46, 47). It is possible that overexpression of these molecules mimics or provides the necessary signal to initiate the apoptotic cascade. We therefore examined whether the viral and cellular CLAPs are able to induce apoptosis when overexpressed in human cells. To this end we transiently transfected MCF-7 cells with constructs encoding full-length CLAP proteins or the CARD or CTD domains of cCLAP. The transfected cells were then examined for morphological features of apoptosis. Both the full-length viral and cellular CLAP proteins induced apoptosis in the absence of additional apoptotic signals (Fig. 3A). However, the CARD or the CTD of cCLAP had marginal apoptotic activity, suggesting that the intact protein is required for induction of apoptosis. Interestingly, both intact vCLAP and cCLAPs exhibited higher apoptotic activity in the presence of cycloheximide, suggesting that these molecules could also regulate genes that inhibit apoptosis (see below).

CLAPs Activate Apoptosis Downstream of the CrmA Block—To gain a better understanding of how vCLAP and cCLAP might engage the death pathway, we investigated their activities with respect to various inhibitors of apoptosis. The viral serpin CrmA inhibits the initiator caspase-8 but has relatively weak inhibitory activity toward the initiator caspase-9 or the effector caspases (caspase-3, -6, and -7) (48–50). As a result, CrmA inhibits death receptor-induced apoptosis but not Apaf-1/caspase-9-mediated apoptosis (41, 42, 51). On the other hand the caspase inhibitor Z-VAD-fmk and the baculovirus p35 protein have a broad inhibitory spectrum (50) and can inhibit multiple apoptotic pathways including the death receptor and Apaf-1/caspase-9 pathways. When MCF-7 cells were co-transfected with constructs encoding CrmA and DR5, CrmA was able to block DR5-induced apoptosis (Fig. 3B). However, when MCF-7 cells were co-transfected with constructs encoding CrmA and vCLAP or cCLAP, CrmA failed to block CLAP-induced apoptosis. As expected, Z-VAD-fmk and p35 were able to block both DR5- and CLAP-induced apoptosis (Fig. 3B). These data suggest that CLAPs initiate apoptosis by activating caspases downstream of caspase-8.

To confirm that CLAPs initiate apoptosis downstream of caspase-8, we used the apoptotic inhibitors Bcl-xL, X-IAP, and caspase-9DN. Bcl-xL and caspase-9DN act specifically at the level of the Apaf-1/caspase-9 apoptotic complex. Bcl-xL is believed to block the cytochrome c release from the mitochondria, whereas caspase-9DN interferes with formation of a functional Apaf-1/caspase-9 complex by a dominant negative mechanism (38, 52–54). X-IAP directly blocks the activities of the effector caspase-3 and -7 and may also interfere with the Apaf-1/caspase-9 complex (55, 56). As shown in Fig. 3B, CLAPs failed to induce apoptosis in MCF-7 cells cotransfected with constructs encoding CLAPs and either Bcl-xL, X-IAP, or caspase-9DN. Taken together, these observations suggest that CLAPs initiate apoptosis some how by triggering the activation of Apaf-1/caspase-9 pathway via caspase-8-independent mechanism.

Viral and Cellular CLAPs Potentiate Death Receptor-induced Apoptosis in MCF-7 Cells—Activation of the cytochrome
NF-κB activity. Since the CTD domain (residues 97–233) migrates as a doublet or triplet ranging in size from 29 to 32 kDa (Fig. 4B), the slower migrating bands appear to be phosphorylated cCLAP. The transfected cells were stimulated with TNF-α, Fas agonist antibody, or TRAIL, and then examined for morphological features of apoptosis. As expected, we observed that both full-length vCLAP and hCLAP significantly enhanced the apoptotic activities of TNF-α, Fas agonist antibody, or TRAIL, compared with the vector controls (Fig. 3C). These findings suggest that activation of the cytochrome c/Apaf-1/caspase-9 pathway by the CLAP proteins enhances apoptosis induction by death receptors in some cells.

The CARD and CTD domains of cCLAP slightly enhanced the apoptotic activities of TNF-α, Fas agonist antibody, or TRAIL, compared with the vector controls. Cycloheximide also produced a similar effect on MCF-7 cells comparable to that observed with the CARD and CTD (Fig. 3C). The slight enhancement effect produced by the CARD and CTD could be due to their marginal proapoptotic activity (see below). It is also possible that the dominant negative effect of the CARD on NF-κB activation (see below) may contribute to its marginal apoptosis enhancement activity.

**CLAPs Induce NF-κB Activation**—The ability of cycloheximide to potentiate the apoptotic activity of CLAPs (Fig. 3A) suggests that these molecules may have an intrinsic anti-apoptotic signaling activity that is inhibited by protein synthesis inhibitors. Cycloheximide has also been shown to potentiate apoptosis induced by stimulation of the TNF-R1 (59), possibly by inhibiting the synthesis of NF-κB-regulated anti-apoptotic proteins. Since TNF-R1 can induce both apoptosis through activation of caspase-8 and proliferation through induction of NF-κB activation, we reasoned that CLAPs could also have a dual signaling role that leads either to apoptosis or to NF-κB activation. To test this possibility, we transiently transfected 293T cells with an NF-κB reporter construct and increasing amounts of expression constructs encoding these proteins. The transfected cells were lysed 16–20 h later and assayed for NF-κB activity and protein expression. As shown in Fig. 4, A and B, both human and viral CLAPs were capable of inducing NF-κB in a dose-dependent manner. At comparable expression levels, vCLAP was ∼40–50-fold more potent than cCLAP in inducing NF-κB activation. Similar results were also obtained with the human MCF-7 and Jurkat cell lines (data not shown). Furthermore, both human and viral CLAPs failed to induce AP1 or NFAT reporter activity (Fig. 4C), suggesting that the activation is NF-κB-specific.

Interestingly, we observed that cCLAP migrates in SDS gels as a doublet or triplet ranging in size from 29 to 32 kDa (Fig. 4A). The slower migrating bands appear to be phosphorylated forms of cCLAP, since treatment of lysates with λ phosphatase reduced their intensity and increased the intensity of the faster migrating band (Fig. 4D). These observations suggest that cCLAP might be regulated by phosphorylation. It is likely that phosphorylation of hCLAP occurs in the Ser/Thr-rich CTD domain, since the CTD domain (residues 97–233) migrates as a
doublet in a high percentage of SDS gels, whereas the CARD domain (residues 1–104) migrates as a single band (data not shown).

CLAP-induced NF-κB Activation Is Inhibited by Dominant Negative NIK and IKKα—To determine how CLAP proteins activate NF-κB, we transiently transfected 293T cells with the NF-κB controlled luciferase reporter plasmid in combination with cCLAP or vCLAP expression constructs and kinase-inactive NIK or IKKα (mNIK, mIKKα) expression constructs. NIK and IKKα transduce the signals that activate NF-κB. Their kinase-inactive mutants block TNF-α-induced NF-κB activation (60, 61). As shown in Fig. 5, A and B, the two kinase mutants completely blocked NF-κB activation by CLAP proteins. These results demonstrate that the kinase activities of NIK and IKKα are essential for CLAP-induced NF-κB activation and suggest that cCLAP may contribute to the transduction of the NF-κB activation signals from the TNF receptor.

The CARD Domain of the CLAP Proteins Is Required for Homo- and Heterodimerization—To determine whether cCLAP interacts with itself and with other CARD-containing proteins, we transfected 293T cells with expression constructs encoding wild type or mutant T7-tagged cCLAP and several FLAG-tagged CARD-containing proteins including vCLAP and cCLAP. cCLAP was found to bind specifically to itself and to vCLAP and vice versa (Fig. 6, A and B, lanes 2 and 6). However, cCLAP and vCLAP did not bind other CARD-containing proteins such as CRADD, APAF-1, and caspase-9 (Fig. 6, C and D). The interactions of cCLAP with itself or vCLAP are mediated by the CARD domain, since deletion of this domain prevented these interactions (Fig. 6, A and B, lanes 4). The CARD domain of cCLAP was also able to interact with full-length cCLAP and vCLAP proteins (Fig. 6, A and B, lanes 3). A variant cCLAP with a point mutation (L41R) in the CARD domain, however, did not interact with wild type cCLAP or vCLAP (lanes 5). The Leu-41 residue is conserved in the CARD domains of several apoptotic proteins (Fig. 1C) and has been shown to be essential for heterodimerization of CED-3 and CED-4 or RAIDD and caspase-2 (35, 62). These observations suggest that the CARD domain is essential for homo- and heterodimerization of CLAPs. Mutation of a conserved residue in the CARD domain of cCLAP abrogates these interactions.

The Role of CARD-mediated Oligomerization in NF-κB Activation—To define the role of the CARD and CTD domains of the CLAP proteins in NF-κB activation, we transfected 293T cells with expression constructs encoding the CARD and CTD domains of cCLAP. In addition we transfected cells with a...
These results show that NF-κB activation is due to the oligomerization of the cCLAP CTD domain. As with the Fkp3-CTD, AP1510 restored the NF-κB inducing activity of Fkp3-CLAP-L41R (Fig. 7D). The CTD alone or the L41R mutant of cCLAP failed to activate NF-κB both in the presence or absence of the drug. Similar data were obtained with vCLAP-CTD (data not shown). These data support the hypothesis that the CARD domain functions as an oligomerization domain, whereas the CTD functions as the effector domain in the NF-κB activation pathway.

The CARD of hCLAP Inhibits TNF-α-induced NF-κB Activation—To determine the role of cCLAP in the transduction of the TNF-α signals that activate NF-κB, we transfected 293T cells with constructs encoding FLAG- or GFP-tagged wild type or mutant cCLAP proteins and then stimulated the cells with TNF-α. The ability of TNF-α to induce NF-κB activation was slightly enhanced by overexpression of FLAG-cCLAP or cCLAP-GFP (Fig. 8). Interestingly, the ability of TNF-α to induce NF-κB activation was blocked in a dose-dependent manner by overexpression of increasing amounts of the FLAG-tagged CARD domain of cCLAP but not by the FLAG-tagged CTD domain or the L41R mutant. A dose-dependent inhibition of TNF-α-induced NF-κB activation was also observed when cells were transfected with increasing amounts of FLAG-tagged CARD domain of cCLAP. These data demonstrate that the CARD domain of cCLAP is a dominant negative regulator of TNF-α-induced NF-κB activation and suggests that cCLAP might be a downstream component of the TNF-α-induced NF-κB activation pathway.

In conclusion we have identified and characterized a novel mammalian protein cCLAP and its homolog in the virus EHV-2. Both the cellular and viral CLAP proteins activate NF-κB and induce apoptosis. Although vCLAP and cCLAP induce apoptosis in transfected cells, their ability to induce NF-κB activation suggests that they may also promote cell survival. During viral infection, it is possible that inhibition of apoptosis may be temporarily advantageous for the enhancement of viral replication (64). EHV-2 has developed an anti-apoptotic strategy by acquiring genes that encode anti-apoptotic proteins such as FLIP (E8) which suppresses death receptor-induced apoptosis. Moreover, it has acquired an open reading frame (E4) that has homology to the anti-apoptotic protein Bcl-2 (64). During viral infection FLIP and the Bcl-2 homolog(s) are expected to prevent vCLAP (E10)- and death receptor-induced apoptosis. In this anti-apoptotic environment, vCLAP is likely to induce massive activation of NF-κB which may further extend the survival of the infected cells by inducing the expression of cellular anti-apoptotic proteins. NF-κB activation by vCLAP may also promote the expression of EHV-2 genes. It is possible that deregulation of the anti-apoptotic proteins (i.e. E4 and FLIP) during the lytic phase of viral replication may unmask the proapoptotic properties of vCLAP which may therefore induce apoptosis.

The exact molecular order of the TNF-R1 signaling pathway leading to NF-κB activation is not fully understood. Several components of this pathway remain to be identified as evident from the recent identification of two additional components of the IKK complex, IKAP and NEMO/IKK-γ (65–67). One of the missing links between the upstream TNF-R1-TRADD-RIP complex and the downstream NIK-IKK-IKAP complex could be CLAP. This is consistent with the ability of dominant negative NIK and IKKα to inhibit NF-κB activation by CLAP and with the ability of the CARD domain of CLAP to inhibit TNF-α-induced NF-κB activation.

The activity and the dual domain structure of CLAP suggest that it could be an adaptor molecule. Its major function is to connect the TNF-R1 complex to the downstream NIK complex. The presence of the CARD domain suggests that this may be the result of CARD domain-mediated homotypic and hetero-
homotypic protein-protein interactions, but it is essential for CLAP-induced NF-κB activation. Deletion of this domain abolishes the ability of cCLAP to induce NF-κB activation. However, artificially induced oligomerization of this domain is sufficient to induce NF-κB activation independent of the CARD domain. This indicates that the CTD domain is involved in heterotypic interactions with downstream regulators of NF-κB and that oligomerization of these regulators is necessary for induction of NF-κB activation. These regulators may include the NIK-IKK-IKAP complex and other proteins that remain to be identified.

CLAP proteins appear to have a molecular organization similar to FADD, where the CARD domain of CLAP corresponds to the structurally related death domain of FADD and the CTD domain of CLAP corresponds to the DED domain of FADD. Death receptor-mediated oligomerization of FADD via its death domain allows FADD to recruit procaspase-8 through heterotypic interactions of its DED with the DED of procaspase-8. This causes oligomerization of the C-terminal caspase domain and autoprocessing of procaspase-8. Similarly, aggregation of the TNF receptor by its ligand could induce oligomerization of cCLAP via its CARD domain leading to induction of NF-κB activation.

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