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Casper Is a FADD- and Caspase-Related Inducer of Apoptosis

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Summary

Caspases are cysteine proteases that play a central role in apoptosis. Caspase-8 may be the first enzyme of the proteolytic cascade activated by the Fas ligand and tumor necrosis factor (TNF). Caspase-8 is recruited to Fas and TNF receptor-1 (TNF-R1) through interaction of its prodomain with the death effector domain (DED) of the receptor-associating FADD. Here we describe a novel 55 kDa protein, Casper, that has sequence similarity to caspase-8 throughout its length. However, Casper is not a caspase since it lacks several conserved amino acids found in all caspases. Casper interacts with FADD, caspase-8, caspase-3, TRAF1, and TRAF2 through distinct domains. When overexpressed in mammalian cells, Casper potently induces apoptosis. A C-terminal deletion mutant of Casper inhibits TNF- and Fas-induced cell death, suggesting that Casper is involved in these apoptotic pathways.

Introduction

Apoptosis is a cell suicide process of sequential biochemical events triggered by a variety of physiological and stress stimuli. Several lines of evidence indicate that a family of cysteine proteases, or caspases (Alnemri et al., 1996), plays a crucial role in the execution of apoptosis. First, genetic experiments in Caenorhabditis elegans demonstrated that the *ced-3* gene is required for cell death during hermaphrodite development of the nematode (Ellis and Horvitz, 1986). Subsequently, it was found that the mammalian interleukin-1β–converting enzyme (ICE or caspase-1) shares significant homology with CED-3 and can cause apoptosis when overexpressed in mammalian cells (Yuan et al., 1993).

The second line of evidence for caspase involvement in mammalian apoptosis comes from studies showing that caspase inhibitors block apoptosis induced by a wide range of stimuli. One of the best-known caspase inhibitors is the protein encoded by the cowpox virus *crmA* gene (Ray et al., 1992). CrmA inhibits apoptotic pathways induced by divergent stimuli, including tumor necrosis factor (TNF) and FasL, among others (Miura et al., 1993; Enari et al., 1995; Hsu et al., 1995; Tewari and Dixit, 1995; Hsu et al., 1996a, 1996b; Henkart, 1996).

Several members of the caspase family have been identified (reviewed by Henkart, 1996; nomenclature proposed by Alnemri et al., 1996), and they share certain characteristic features. For example, all known caspases contain a conserved motif QAC(R/Q)G, in which the cysteine residue is the structural hallmark of a caspase. This cysteine residue, together with two highly conserved residues, corresponding to H237 and G238 in caspase-1, form the active site of a caspase (Wilson et al., 1994; Walker et al., 1994). In addition, many members of the family are capable of inducing apoptosis when overexpressed in mammalian cells (Henkart, 1996).

The apoptosis-inducing caspases are usually synthesized as inactive proenzymes that are activated by specific proteolytic cleavage. When activated, caspases specifically cleave substrates at sites following an aspartate residue (Henkart, 1996). It has been presumed that the caspases exist within hierarchies of auto- and trans-cleavage (Fraser and Evan, 1996). Activation and amplification of the caspase cascades result in proteolytic degradation of specific targets, such as poly(ADP-ribose) polymerase, nuclear lamin, and actin (Martin and Green, 1995). Proteolytic degradation of these targets may eventually result in cell death.

Many types of stimuli can activate the caspase cascades, leading to apoptosis. In recent years, apoptosis induced by TNF and FasL has received extensive attention. TNF elicits a broad range of biological effects (Goeddel et al., 1986; Beutler and Cerami, 1988; Fiers, 1991) through two distinct membrane receptors, TNF-R1 (~55 kDa) and TNF-R2 (~75 kDa), which are expressed at low levels on most cell types (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Fiers, 1991; Tartaglia and Goeddel, 1992). Apoptosis induced by TNF is mediated primarily through TNF-R1. The intracellular domain of TNF-R1 contains a "death domain" of ~80 amino acids (aa) that is responsible for signaling cell death (Tartaglia et al., 1993). A homologous death domain is also found in the cytoplasmic region of Fas (Itoh and Nagata, 1993) and DR3/Wsl/Apo-3 (Chinnaiyan et al., 1996b; Kitson et al., 1996; Marsters et al., 1996), two other members of the TNF receptor family that can potently induce apoptosis.

Efforts to understand the early events of TNF- and Fas-induced apoptosis have been aided by the discoveries of several proteins that interact with the intracellular domains of TNF-R1 and Fas. TRADD, a 34 kDa cytoplasmic protein containing a C-terminal death domain, interacts with the death domain of TNF-R1 in a ligand-dependent process (Hsu et al., 1995; Hsu et al., 1996a). As observed for TNF-R1, overexpression of TRADD causes both apoptosis and activation of NF-κB (Hsu et al., 1995). The death domain of TRADD also interacts with the death domain of another cytoplasmic protein, FADD (Hsu et al., 1996a). Since a dominant negative mutant of FADD blocks TNF-R1– and TRADD-induced cell death (Hsu et al., 1996a), the TRADD–FADD interaction may be part of the TNF-R1 pathway that signals apoptosis.

Fas and FADD also interact directly through their respective death domains (Boldin et al., 1995; Chinnaiyan et al., 1995). Although the death domains of TNF-R1, Fas, and TRADD induce apoptosis following overexpression in mammalian cells (Tartaglia et al., 1993; Hsu

A

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Casper      MS-AEVIHQVEEALDTDEKEMLLFLCRDVAIDVVPPNVRD---LLDILRE   46
caspase-8   MDFSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDALMLFQRLQE   50

Casper      RGKLSVGDLA---ELLYRVRRFDLLKRILKMDRKAVETHL-LRNPHLVSD   92
caspase-8   KRMLEESNLSFLKELLFRINRLDLLITYLNTRKEEMERELQTPGRAQISA  100

Casper      YRVLMAEIGEDLDKSDVSSLIFLMKDYMGRGKISKEKSFLDLVVELEKLN  142
caspase-8   YRVMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNLLDIFIEMEKRV  150

Casper      LVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSVQGAGTSYRNVLQAAIQK  192
caspase-8   ILGEGKLDILKRVCAQINKSLLKI-INDYEEFSKERSSSLEGS-PDEFSN  198

Casper      SLKDPSNNFRLHNGRS-KEQRLKEQLGAQQEPVKKSIQESEAFLPQSIPE  241
caspase-8   G-EELCGVMTISD--SPREQDSESQTL-DKVYQMKSKPRGYCLIINNHNF  244

Casper      E--RYKMKSKPLGICLIIDCIGNETELLRDTFTSLGYEVQKFLHLSMHGI  289
caspase-8   AKAREKV-PK-LHSIRDRNGTHLDAGALTTTFEELHFEIKPHDDCTVEQI  292

Casper      SQILGQFACMPEHRDYDSFVCVLVSRGGSQSVYGVDQTHSGLPLHHIRRM  339
caspase-8   YEILKIYQLMD-HSNMDCFICCILSHGDKGIIYGTDGQEP--PIYELTSQ  339
                                     ••
Casper      FMGDSCPYLAGKPKMFFIQNYVVSEGQLEDSSLLEVDGPAMKNVEFKAQK  389
caspase-8   FTGLKCPSLAGKPKVFFIQACQGDNYQKG--IPVETDSEEQPYLEMDLSS  387
                               •
Casper      RGLCTVHREADFFWSLCTADMSLLEQSHSSPSLYLQCLSQKLRQERKRPL  439
caspase-8   PQTRYIPDEADFLLGMATVNNCVSYRNPAEGTWYIQSLCQSLRERCPRGD  437

Casper      LDLHIELN-GYMYDWNSRVSAKEKYYVWLQHTLRKKLILSYT          480
caspase-8   DILTILTEVNYEVSNKDDKKNMGKQMPQPTFTLRKKLVFPSD          479
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Figure 1. Casper Amino Acid Sequence and Sequence Comparisons

(A) The predicted amino acid sequence of Casper is aligned with the sequence of caspase-8. Identical amino acids are shaded. Caspase-8 amino acids involved in catalysis are indicated by dots, and the conserved caspase pentapeptide is underlined.

(B) Schematic representation of FADD, Casper, caspase-8, and caspase-10. Casper contains two DED modules and a protease-like domain. The conserved motifs HG and QAC(R/Q)G of caspases are lacking in Casper.

(C) Phylogenetic analysis of the DED modules of FADD, Casper, caspase-8 and caspase-10. The numbers in parentheses refer to the N-terminal (1) and C-terminal (2) DED modules.

(D) Sequence alignments of DED modules. The DED modules of FADD, Casper, caspase-8 and caspase-10 are classified into two groups according to their similarity, determined by phylogenetic analysis. Sequences are aligned within each groups. Identical amino acids in each groups are shaded. Consensus amino acids between the two groups are also indicated.

(E) Sequence alignment of aa 13–176 of Casper with the protein encoded by the *MC159L* gene of molluscum contagiosum virus sub-type 1, the hypothetical protein E8 of equine herpesvirus 2, and the open reading frame K13 protein of Kaposi's sarcoma–associated herpesvirus. Amino acids of the virus proteins identical to that of Casper are shaded.

et al., 1995; Itoh and Nagata, 1993; Hsu et al., 1996b), overexpression of the C-terminal death domain of FADD inhibits TNF- and Fas-induced cell death (Chinnaiyan et al., 1996a; Hsu et al., 1996a). The N-terminal domain of FADD, termed death effector domain (DED), induces apoptosis after overexpression, suggesting that the DED of FADD may activate a downstream cell death signaling component (Chinnaiyan et al., 1996a; Hsu et al., 1996a).

Recently, three groups identified a cysteine protease, which they named Mch5, MACH, and FLICE, respectively (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996). The unique structure of this protein, which is now called caspase-8, suggests that it may represent the missing link between FADD and the basic cell death machinery. The N-terminal domain of caspase-8 contains two DED-like modules through which it interacts with FADD (Boldin et al., 1996; Muzio et al., 1996). The C-terminal domain of caspase-8 is homologous to members of the caspase family and has protease activity toward most known caspases and poly (ADP-ribose) polymerase (Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Srinivasula et al., 1996). These studies suggest that caspase-8 may function at the apex of the caspase cascade activated by Fas. Currently, it is not clear how caspase-8 itself is activated following Fas activation.

In this study we have identified a cDNA encoding a protein we term Casper (caspase-eight–related protein), which is structurally related to caspase-8. Casper contains two DED-like modules and a protease-like domain. Despite its high sequence similarity to caspase-8, Casper does not contain the conserved motifs that are hallmarks of caspases. Casper interacts with FADD, caspase-8, caspase-3, TRAF1, and TRAF2 through distinct domains. Overexpression of Casper or its C-terminal protease-like domain potently induces apoptosis, whereas a deletion mutant lacking 45 C-terminal aa inhibits TNF- and Fas-induced apoptosis, suggesting that Casper may play a role in cell death pathways activated by TNF and Fas.

Results

Identification of Casper

TRADD and FADD interact when overexpressed in yeast and mammalian systems (Hsu et al., 1996a), and a dominant negative mutant of FADD blocks TNF-induced apoptosis (Hsu et al., 1996a; Chinnaiyan et al., 1996a). However, we have been unable to detect FADD in the endogenous TNF-R1 signaling complex (Shu et al., 1996; unpublished data). This observation implies the possibility that a FADD-like molecule, rather than FADD itself, may be a physiological component of the cell death pathway activated by TNF. To identify potential FADD-related genes, we searched a public database of human expressed sequence tags (ESTs). Two ESTs encoding two distinct peptides were identified, each of which has ∼30% sequence identity with the DED of FADD. Subsequent cDNA cloning and sequence analysis demonstrated that the two ESTs represent different fragments of the same gene. The longest cDNA clones obtained had an ∼2.1 kb insert and an open reading frame capable of encoding a 480 aa protein (Figure 1). We have designed this protein Casper (for caspase-eight–related protein).

Casper has significant sequence similarity to the recently cloned caspase-8 (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996) throughout its length (Figure 1A). It contains two DED-like modules at its N-terminus, each of which shares ∼25% sequence identity with the DEDs of FADD, caspase-8 and caspase-10 (Fernandes-Alnemri et al., 1996) (Figures 1B–

(*Figure 1 continued from previous page*)

1D). The C-terminal 270 aa are ~25% identical to the C-terminal protease domain of the 479 aa caspase-8. Importantly, the conserved motifs, QACR(Q)G and HG, which are involved in catalysis and are present in all identified caspases, are not found in Casper (Figures 1A and 1B), suggesting that Casper is not a cysteine protease. We also obtained alternatively spliced cDNAs that encode truncated forms of Casper and have stop codons after aa 202 and aa 435.

Interestingly, a blast search indicated that the DED modules of Casper are most homologous to three hypothetical viral proteins: the protein product (241 aa) encoded by the *MC159L* gene of molluscum contagiosum virus subtype 1 (Senkevich et al., 1996), the hypothetical protein E8 (171 aa) of equine herpesvirus 2 (Telford et al., 1995), and the open reading frame K13 protein (139 aa) of Kaposi's sarcoma-associated herpesvirus (Russo et al., 1996). These virus proteins have ~35% sequence identity with the DED modules of Casper (Figure 1E), but less than 20% sequence identity with corresponding domains of caspase-8, caspase-10, and FADD.

Northern blot analysis suggests that the human *Casper* gene is expressed as several different transcripts of approximately 1.4, 2.4, 3.8, 6.0, and 9.5 kb. Its expression is relatively high in skeletal muscle, pancreas, and heart, but was undetectable in the brain (Figure 2A). To determine whether Casper protein is present under physiologic conditions, U937 cell lysates were immunoprecipitated with either a preimmune serum or a polyclonal antibody directed against a Casper-derived peptide (aa 160–192). The immunoprecipitating proteins were then detected by Western blot analysis using an antibody derived against a different Casper peptide (aa 215–249). This antibody detected specific bands at ~55 and ~28 kDa (Figure 2B). The size of the ~55 kDa protein is consistent with the predicted molecular weight of full-length Casper. The ~28 kDa protein probably corresponds to a proteolytic fragment or an alternatively spliced variant of Casper.

Induction of Apoptosis by Casper and Its Protease-like Domain

Since Casper is structurally related to both FADD and caspase-8, we examined whether Casper is involved in signaling pathways leading to apoptosis. Using an established assay (Hsu et al., 1995; 1996a; 1996b), we

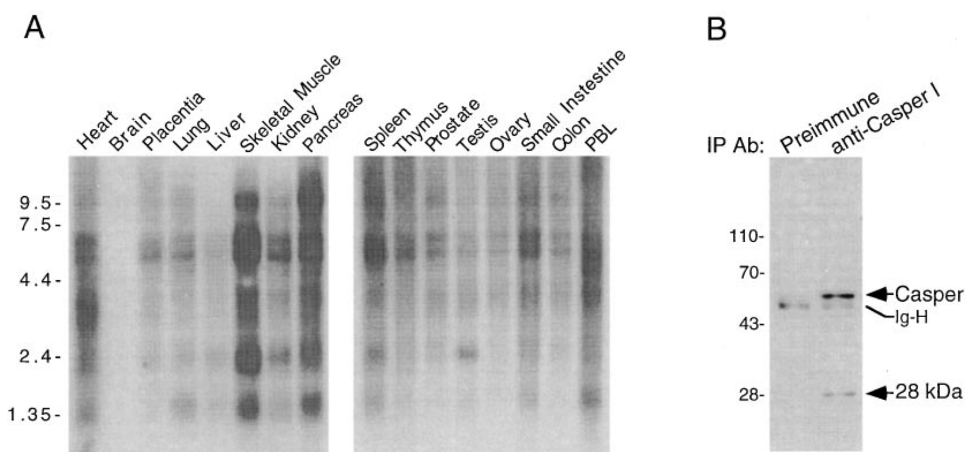

Figure 2. Expression of Casper

(A) Northern blot analysis of Casper mRNA in human tissues.

(B) Western blot analysis of Casper protein in U937 cells. Lysates of U937 cells (~1 × 10^8) were immunoprecipitated with either a preimmune serum control or the Casper peptide I polyclonal antibody against Casper. Immunoprecipitating proteins were detected with the Casper peptide II antibody. Ig-H, immunoglobulin heavy chain.

found that Casper overexpression in HeLa cells induced apoptosis (Table 1). Analysis of deletion mutants indicated that the protease-like domain of Casper (aa 192–480) was necessary and sufficient to induce apoptosis and was relatively more potent than full-length Casper. In contrast, caspase-8 is more potent than its protease domain (aa 217–479) in inducing apoptosis (Table 1). The Casper deletion mutants encoding aa 1–96, 192–435, 370–480, and the naturally spliced form aa 1–435 did not cause significant cell death in these assays. Interestingly, when overexpressed, the naturally spliced variant of Casper, encoding aa 1–202, had weak apoptosis-inducing acitivity, similar to that observed for aa 1–198 of caspase-8 (Table 1).

Table 1. Cell Death Induced by Casper Overexpression

	Number of Blue Cells per Well	
Expression Vector	Control	+CrmA
pRK5	384 ± 5	395 ± 8
Casper	71 ± 1	340 ± 19
Casper(1–435)	338 ± 4	356 ± 10
Casper(1–202)	175 ± 7	357 ± 13
Casper(1–96)	291 ± 10	328 ± 3
Casper(78–480)	87 ± 9	346 ± 6
Casper(192–480)	34 ± 4	339 ± 26
Casper(192–480Y/F)	140 ± 18	340 ± 20
Casper(370–480)	362 ± 12	352 ± 11
Casper(192–435)	333 ± 8	331 ± 14
caspase-8	1 ± 1	311 ± 7
caspase-8(1–198)	164 ± 12	362 ± 22
caspase-8(1–436)	310 ± 6	345 ± 5
caspase-8(217–479)	22 ± 2	325 ± 18
TRADD	33 ± 5	320 ± 8
FADD	81 ± 4	342 ± 13

HeLa cells were transfected with 2 μg of the indicated expression vector, 0.25 μg of pCMV-β-gal, and 2 μg of pRK control vector or 2 μg of crmA vector. Transfected cells were identified by staining with X-gal. Data (± SD) are shown as the number of blue cells per each of two wells. One of at least three representative experiments is shown.

The cowpox virus protein CrmA is a specific caspase inhibitor that can block apoptosis induced by TRADD, FADD, and caspase-8 (Tewari and Dixit, 1995; Hsu et al., 1995, 1996a). CrmA also protects against Casper-induced apoptosis (Table 1), suggesting that Casper activates a caspase cascade leading to apoptosis.

Although Casper is related to caspase-8 throughout its length, it does not contain the conserved QAC (R/Q)G motif present in all known caspases. The position corresponding to the C360 in caspase-8 is a tyrosine residue in Casper. To determine whether the Y360 is involved in cell killing by Casper, we mutated this residue to phenylalanine in Casper(192–480), the deletion mutant that is a potent activator of apoptosis when overexpressed. We found that Casper(192–480Y/F) had significantly weaker apoptotic activity compared to its wild-type counterpart (Table 1), suggesting that Y360 contributes to Casper's apoptosis-inducing activity.

A Deletion Mutant of Casper Blocks TNF- and Fas-Induced Apoptosis

Full-length Casper can induce apoptosis in mammalian cells, whereas Casper(1–435) does not. To determine whether Casper might be involved in Fas or TNF signaling, we investigated the effect of Casper(1–435) on TNF- and Fas-mediated apoptosis. When Casper(1–435) was expressed in HeLa cells, it behaved as a dominant negative mutant by inhibiting both TNF- and anti-Fas–induced apoptosis (Figure 3A).

Since TRADD and FADD are downstream death signaling proteins of TNF-R1 and Fas, we determined whether Casper(1–435) could inhibit apoptosis induced by these proteins. Expression vectors for TRADD or FADD was cotransfected with an expression vector for Casper(1–435). Consistent with its ability to inhibit anti-Fas– and TNF-induced apoptosis, Casper(1–435) potently inhibited FADD- and TRADD-induced apoptosis (Figure 3B). In parallel experiments, a similar deletion mutant (aa 1–436) of caspase-8 also functioned as an

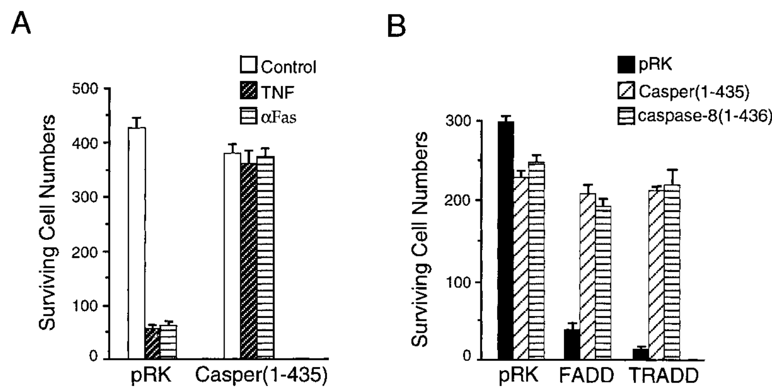

Figure 3. Functional Analysis of Casper in Mammalian Cells

(A) A dominant negative mutant of Casper (aa 1–435) blocks anti-Fas– and TNF-induced cell death. HtTA cells were transfected with 2 μg of pRK5 control vector or Casper(1–435) expression vector, together with 0.25 μg of CMV-β-gal vector. Twenty-four hours after transfection, cells were left untreated or were treated with 100 ng/ml TNF or 250 ng/ml anti-Fas antibody (Panvera) for 12 hr (in the presence of 10 μg/ml cycloheximide). Cells were then fixed and stained with X-gal, and surviving β-gal–positive cells were counted under a microscope. Numbers shown are the average of two wells of a representative experiment.

(B) Inhibition of TRADD- and FADD-induced cell death by Casper(1–435). HtTA cells were transfected with 2 μg of expression vectors for FADD, TRADD, or control vector pRK5, together with 2 μg of pRK5 control or expression vectors for aa 1–435 of Casper or aa 1–436 of caspase-8, as indicated. pCMV-β-gal vector, 0.25 μg, was added to each transfection. Cells were fixed and stained with X-gal 24 hr after transfection. The survived β-gal–positive cells were counted under a microscope. Numbers shown are the average of two wells of a representative experiment.

inhibitor of FADD- and TRADD-induced apoptosis (Figure 3B). These data suggest that Casper may be a component of the TNF- and Fas-induced cell death pathways that functions downstream of the death domain–containing proteins.

Casper Interacts with Distinct Signaling Proteins

To elucidate the signaling pathway(s) involving Casper, we searched for Casper-interacting proteins using the yeast two-hybrid system. Several proteins were identified that specifically interacted with Casper, including FADD, caspase-8, caspase-10, TRAF1, and TRAF2 (Table 2). Each of these interactions was confirmed in mammalian cells (see below).

Casper Interacts with FADD and Is Recruited to Fas

Since Casper and FADD interact in yeast two-hybrid assays, we tested whether they also interact in mammalian cells. Casper or various Casper deletion mutants were coexpressed with FADD in human 293 cells. Coimmunoprecipitation analysis indicated that the interaction between Casper and FADD is comparable to that between caspase-8 and FADD (Figure 4A). The two DED-like modules of Casper are required and sufficient for its interaction with FADD, presumably through the DED of FADD, as indicated by yeast two-hybrid assays (data not shown). The protease-like domain of Casper did not interact with FADD (Figure 4A). Deletion mutants Casper(1–96) (data not shown) and Casper(78–480) (Figure 4A), which contain either the first or second DED, interacted with FADD weakly. Casper did not interact with the death domain proteins TRADD or RIP (data not shown).

To test whether Casper can be recruited to the Fas signaling complex, we transfected 293 cells with expression vectors for Fas, FADD, and Casper. Cell extracts were immunoprecipitated with an antibody against the extracellular domain of Fas, and coimmunoprecipitating proteins were detected by immunoblotting analysis (Figure 4B). Fas did not directly interact with Casper in this assay. However, Casper was recruited to Fas when FADD was coexpressed (Figure 4B). A parallel experiment confirmed earlier observations (Muzio et al., 1996; Boldin et al., 1996) that caspase-8 is recruited to Fas through FADD (Figure 4C).

Casper Interacts with Caspase-8 through Distinct Domains

To test whether Casper interacts with caspase-8, 293 cells were transfected with expression vectors for Flag-tagged Casper and Myc-tagged caspase-8. Cell lysates were immunoprecipitated with an anti-Flag antibody and coimmunoprecipitating proteins were analyzed by immunoblotting with an anti-Myc antibody. This analysis showed that Casper interacts with caspase-8 (Figure

Table 2. Yeast Two-Hybrid Screening of Casper-Interacting Proteins

Bait	cDNA Library	Clones Screened	Specific cDNAs Obtained (Number)
Casper(1–215)	B cell	2.6×10^6	FADD (4)
			caspase-10 (2)
	Peripheral lymph node	2.5×10^6	caspase-8 (4)
Casper(192–480)	B cell	6.0×10^6	TRAF1 (3)
			TRAF2 (3)
	Fetal liver stromal	6×10^5	TRAF2 (3)

Yeast two-hybrid screening was performed using the indicated baits and cDNA libraries following established procedures (Rothe et al., 1994; Hsu et al., 1995).
His^+ and β-gal^+ clones were sequenced, and their identities were determined by blast searches of the Genbank database.

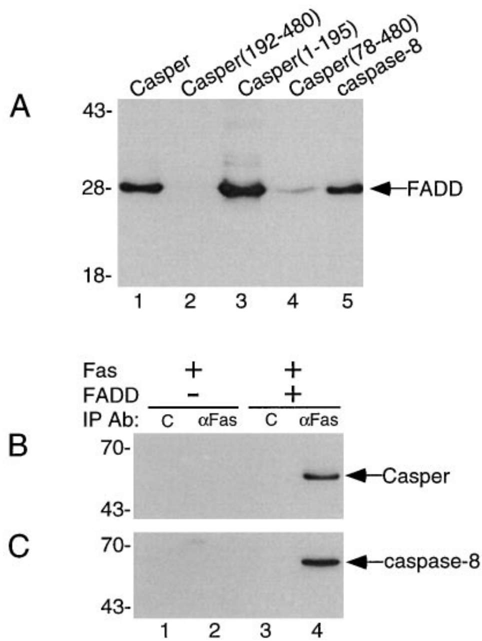

Figure 4. Casper Interacts with FADD and is Recruited to Fas

(A) Casper interacts with FADD. 293 cells were cotransfected with expression vectors for FADD and Flag-tagged Casper, various Casper deletion mutants, or caspase-8. *crmA* vector was expressed to inhibit cell death. Cell lysates were immunoprecipitated with an anti-Flag antibody, and coimmunoprecipitating proteins were analyzed by Western blotting using an anti-FADD antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(B and C) FADD recruits Casper and caspase-8 to Fas. 293 cells were transfected with expression vectors for Flag-tagged Casper (B) or caspase-8 (C), together with expression vectors for Fas and AU1-tagged FADD. CrmA vector was expressed to inhibit cell death. Cell lysates were immunoprecipitated (IP) with a rabbit polyclonal anti-Fas antibody. Immunoprecipitates were analysed by Western blotting with an anti-Flag antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

5A). The N-terminal DED modules and the C-terminal protease-like domain of Casper can independently interact with caspase-8 (Figure 5A). Interestingly, the C-terminal 91 aa of Casper is sufficient to immunoprecipitate caspase-8 (Figure 5A). Caspase-8 can also interact with itself and with FADD. Under the same conditions, Caspase-8 does not interact with TRADD (Figure 5A). These data indicate that Casper and caspase-8 can interact through distinct domains.

Since Casper can form a complex with caspase-8, we asked whether these two proteins can act collaboratively in inducing apoptosis. To test this, we examined the apoptosis-inducing activity of Casper(192–480) and caspase-8(217–479) separately and together in HeLa cells. At all doses tested, equivalent amounts of the two expression plasmids together induced apoptosis more potently than either of them alone (Figure 5B).

CrmA Interacts with Caspase-8 but Not Casper or Caspase-3

CrmA can block cell death induced by TNF, Fas, and their signaling proteins TRADD and FADD (Tewari et al., 1995; Enari et al., 1995; Hsu et al., 1995; 1996a; 1996b; Chinnaiyan et al., 1996a). In vitro interactions between CrmA and activated caspase-1 (Komiyama et al., 1994) and caspase-3 (Tewari et al., 1995) have also been reported. Since CrmA also inhibits apoptosis induced by both Casper and caspase-8, we examined whether CrmA interacts with either protein in mammalian cells. In coimmunoprecipitation experiments, neither Casper nor any of its various deletion mutants interacted with CrmA. Caspase-3 also did not interact with CrmA in this assay. However, caspase-8 coprecipitated with CrmA (Figure 6). The protease domain of caspase-8 (aa 217–479) interacts with CrmA much more weakly than does the full-length caspase-8 (data not shown). These data suggest that the most upstream target of CrmA in the Fas- and TNF-induced apoptosis pathways might be

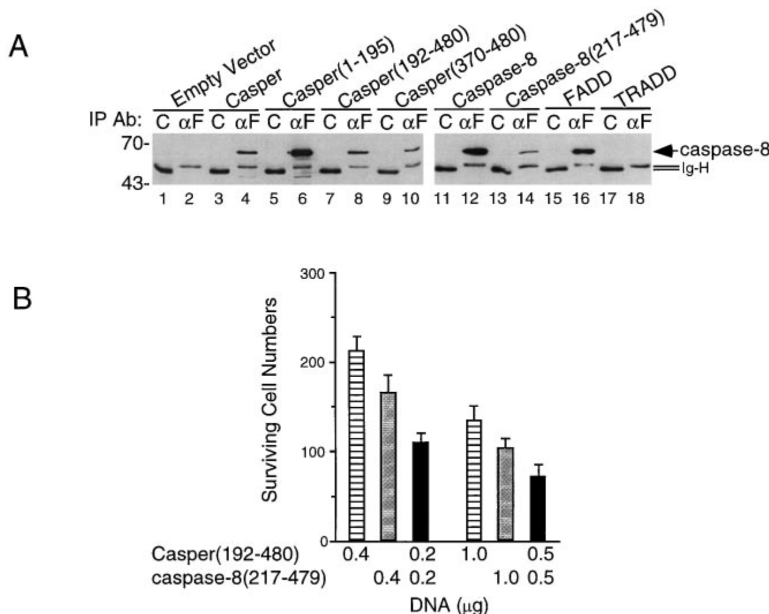

Figure 5. Physical and Functional Interaction Between Casper and Caspase-8

(A) Casper interacts with caspase-8 through distinct domains. 293 cells were transfected with an expression vector for Myc-tagged caspase-8, together with various Flag-tagged expression vectors for the indicated proteins. CrmA was coexpressed to inhibit cell death. Cell lysates were immunoprecipitated (IP) with an anti-Flag antibody or mouse immunoglobulin G control. Immunoprecipitates were anayzed by Western blotting with an anti-Myc antibody. Positions of the molecular weight standards (in kilodaltons) are shown. Ig-H, immunoglobulin heavy chain.

(B) Simultaneous expression of the protease-like domain of Casper and the protease domain of caspase-8 leads to enhanced apoptosis. HeLa cells were transfected with indicated amounts of vectors for the protease-like domain of Casper or the protease domain of caspse-8 or both together, and cell death assays were performed as described above.

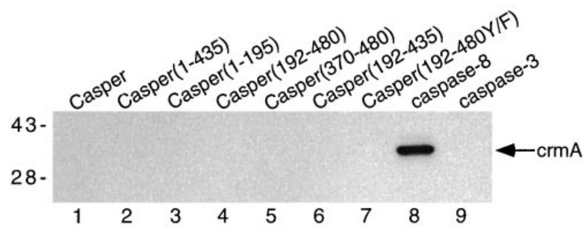

Figure 6. CrmA Interacts with Caspase-8

293 cells were transfected with the Myc-tagged *crmA* vector and various Flag-tagged expression vectors as indicated. Transfected cell lysates were immunoprecipitated with an anti-Flag antibody. Imunoprecipitating Myc-CrmA was detected by Western blotting with an anti-Myc antibody. Positions of molecular weight standards (in kilodaltons) are shown.

caspase-8, and that CrmA might inhibit Casper-induced apoptosis by blocking caspase-8 activation.

Casper Indirectly Induces Caspase-3 Activity

Since caspase-3 may be a component of Fas- and TNF-R1–induced apoptosis pathways (Fernandes-Alnemri et al., 1994; Enari et al., 1995; Tewari et al., 1995; Enari et al., 1996; Nagata, 1996), and since Casper may be an upstream component of these pathways, we examined whether Casper can activate caspase-3. We examined the protease-like domain of Casper since it induces apoptosis more potently than does full-length Casper, and since the in vitro–translated protease domain of caspase-8 but not full-length caspase-8 can enzymatically activate caspase-3 (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Srinivasula et al., 1996). Lysates from 293 cells expressing either Casper(192–480) or caspase-8(217–479) were able to process in vitro–translated ^{35}S-labeled caspase-3 precursor to its signature ~17 kDa fragment (Figure 7A). This result suggests that both the protease-like domain of Casper and the protease domain of caspase-8 can induce a signaling pathway leading to caspase-3 activation in mammalian cells.

To test for direct processing of caspase-3, we mixed in vitro–translated Casper(192–480) or caspase-8(217–479) with ^{35}S-labeled caspase-3. In vitro–translated caspase-8(217–479) was able to process caspase-3, whereas in vitro–translated Casper(192–480) could not (Figure 7B). Thus, activation of caspase-3 by Casper in mammalian cells is probably indirect, whereas caspase-8 can directly process caspase-3 to its active form.

Casper Interacts with Caspase-3

To examine how Casper overexpression leads to caspase-3 activation, we tested whether the two proteins could physically interact. Expression vectors for various forms of Casper or caspase-8 (Flag-tagged) were cotransfected with an expression vector for caspase-3. Transfected cell lysates were immunoprecipitated with an anti-Flag antibody, and coimmunoprecipitating caspase-3 was detected by immunoblotting analysis. Casper interacts strongly with caspase-3, and the protease-like domain of Casper is sufficient for this interaction (Figure 8A). Interestingly, Casper(192–480Y/F), which is less potent in inducing apoptosis than its wild-type counterpart (Figure 3A), interacts more weakly with caspase-3 (Figure 8A). In these experiments, neither caspase-8 nor its protease domain interacted with caspase-3 (Figure 8A).

Although the protease domain of caspase-8 does not interact with caspase-3, it can form a complex with caspase-3 in the presence of the protease-like domain of Casper (Figure 8B). This result suggests that the protease-like domain of Casper can recruit caspase-3 to the protease domain of caspase-8.

Casper Is Proteolytically Processed in Mammalian Cells

In our various transfection experiments, we found that Casper was proteolytically processed. When N-terminally Flag-tagged Casper was overexpressed in 293 cells and lysates were analyzed by immunoblotting with anti-Flag antibody, an N-terminal fragment of ~43 kDa was observed. Casper(78–480) and Casper(192–480) were also cleaved, and the resulting N-terminal fragments were aprroximately 34 kDa and 20 kDa in size, respectively (Figure 9A). To detect the processed C-terminal fragment, we constructed a C-terminally Flag-tagged Casper vector. However, C-terminally tagged Casper was not processed (data not shown), suggesting that a free C-terminus may be required for Casper processing.

Casper(192–480Y/F) was only weakly processed in comparison to Casper(192–480) (Figure 9A). This finding is consistent with our earlier observation that Casper(192–

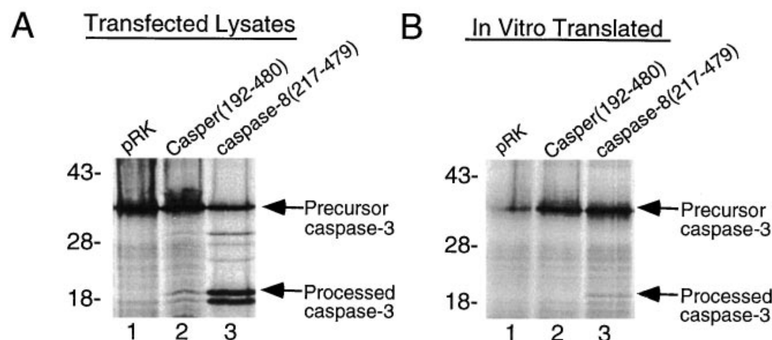

Figure 7. Casper Indirectly Activates Caspase-3

(A) Overexpression of the protease-like domain of Casper or the protease domain of caspase-8 leads to activation of caspase-3. 293 cells were transfected with the indicated vectors. Ten-microliter aliquots of transfected cell lysates were mixed with 2 μl of purified ^{35}S-labeled caspase-3 precursor, and the mixtures were incubated at 30°C for 1.5 hr. Caspase-3 processing was analyzed by SDS-PAGE and autoradiography. Positions of the molecular weight standards (in kilodaltons) are shown.

(B) Casper does not directly process caspase-3. The protease activity assay was performed as above, except that in vitro translated proteins, rather than transfected cell lysates were used to cleave caspase-3 precursor. Positions of molecular weight standards (in kilodaltons) are shown.

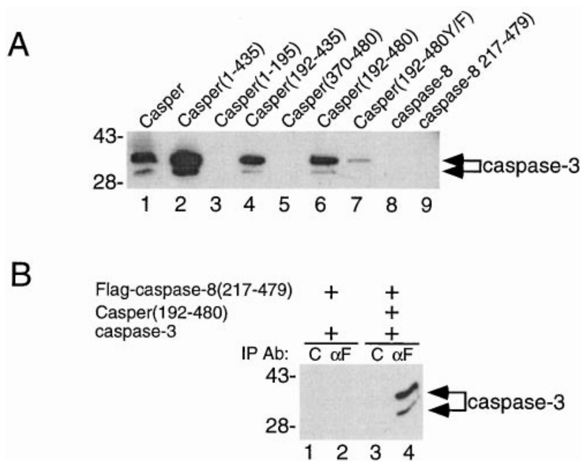

Figure 8. Interaction of Casper with Caspase-3

(A) Casper associates with caspase-3. 293 cells were transfected with Myc-tagged caspase-3 vector and various Flag-tagged expression vectors as indicated. Cell lysates were immunoprecipitated with an anti-Flag antibody. Coimmunoprecipitating caspase-3 was detected by a monoclonal anti-caspase-3 antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(B) Casper(192–480) recruits caspase-3 to activated caspase-8. 293 cells were transfected with various combinations of expression vectors as indicated. Transfected cell lysates were immunoprecipitated with mouse immunoglobulin G control or an anti-Flag antibody. Coimmunoprecipitating caspase-3 protein was detected with a monoclonal antibody against caspase-3. Positions of molecular weight standards (in kilodaltons) are shown.

480Y/F) is less potent than Casper(192–480) and interacts more weakly with caspase-3. Casper(1–435), Casper(192–435), and Casper(370–480), which do not induce apoptosis, were not processed, although aa 1–435 and aa 192–435 contain the putative cleavage site (Figure 9A).

Since the size of the processed N-terminal fragment of Casper(78–480) was only slightly larger than that of nonprocessed Casper(192–480), we reasoned that the processing site should occur soon after position 366 (the size difference between aa 78–480 and aa 192–480 is 114 aa). In that region, there are two asparatate residues, at positions 369 and 376, which we mutated to alanine or asparagine, respectively. Transient transfection experiments showed that the D376N mutation abolished the processing activity of Casper, while D369A mutation had no effect (Figure 9B), suggesting Casper is processed after D376. To determine whether the processing of Casper is required for its apoptotic activtiy, we expressed these point mutants in HeLa cells. Both mutants potently induced cell death (Figure 9C), demonstrating that the processing of Casper is not required for its cell killing activity.

Casper Interacts with TRAF1 and TRAF2

In yeast two-hybrid screening experiments using aa 192–480 (the protease-like domain) of Casper as bait, we identified both TRAF1 and TRAF2 (see above), signaling proteins previously shown to be associated with signaling complexes for both TNF-R1 and TNF-R2 (Rothe et al., 1994; Hsu et al., 1996a; Shu et al., 1996). To test whether Casper interacts with TRAFs in mammalian cells, 293 cells were transfected with expression vectors for Casper, together with expression vectors for TRAF1, TRAF2, TRAF3, and various TRAF2 deletion mutants. Coimmunoprecipitation experiments showed that Casper interacts specifically with TRAF1 and TRAF2, but not with TRAF3 (Figure 10A). Furthermore, the TRAF-N domain of TRAF2 was required to bind to Casper (Figure 10A). In similar experiments, we found that TRAF1 and TRAF2 interact with the protease-like domain but not the N-terminal region of Casper (Figure 10B).

TRAF2 interacts with several signaling proteins, including members of the TRAF family, c-IAP1, c-IAP2, TRADD, TNF-R2, I-TRAF, and A20 (Rothe et al., 1994, 1995; Hsu et al., 1996a; Song et al., 1996). Among these TRAF2-interacting proteins, only c-IAP1 and c-IAP2 interact with the TRAF-N domain of TRAF2, whereas the other proteins interact with the TRAF-C domain. Since Casper interacts with the TRAF-N domain of TRAF2, we examined whether Casper can compete with c-IAP1 for binding to TRAF2. 293 cells were transfected with expression vectors for hemagglutinin (HA)–tagged TRAF2 and Myc-tagged c-IAP1, together with increasing amounts of expression vector for Casper. Coimmunoprecipitation analysis showed that binding of c-IAP1 to TRAF2 was diminished by increasing Casper expression levels (Figure 10C). Consistent with this observation, Casper and cIAP-1 do not simultaneously bind to TRAF2 when overexpressed in 293 cells (data not shown).

Since Casper and TRADD interact with different domains of TRAF2, we examined whether TRADD, TRAF2, and Casper can form a complex. Cotransfection and coimmunoprecipitation experiments indicate TRADD and Casper cannot simultaneously bind to TRAF2 (data not shown). Furthermore, Casper was not recruited to the TNF-R1 complex by the addition of TRADD, FADD, and TRAF2, either individually or combined (data not shown).

Discussion

In this report we describe the identification and characterization of Casper, a novel protein with substantial structural similarity to caspase-8. Like caspase-8, Casper contains two DED-like modules at its N-terminus and extensive homology to caspases at its C-terminus. Casper is not a protease and lacks the conserved QAC(R/Q)G motif, which is a hallmark of caspases. However, Casper induces cell death when overexpressed in mammalian cells, and its apoptotic activity maps to the C-terminal protease-like domain. The N-terminal DED-containing region of Casper is a weak inducer of cell death when overexpressed, similar to the corresponding region of caspase-8. It is possible that the interaction of overexpressed DED-like modules partially mimics the mechanism by which FADD is able to activate the cell death pathway.

A Casper deletion mutant lacking 45 C-terminal aa blocks both the TNF and Fas cell death pathways. Casper(1–435) also inhibits TRADD- and FADD-induced apoptosis pathways. Therefore, Casper may be a component of Fas- and TNF-induced cell death pathways that functions downstream of the death domain proteins. Alternatively, this mutant may bind to FADD and prevent recruitment of caspase-8 to the receptor complexes. Since Casper(1–435) occurs as a natural splice

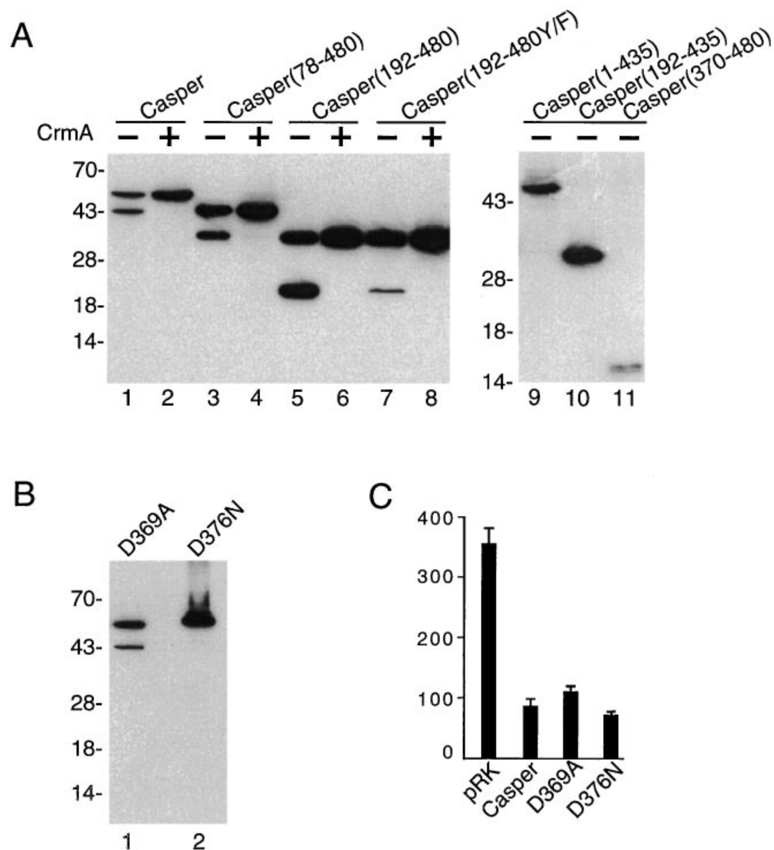

Figure 9. Casper Processing

(A) Proteolytical processing of Casper in mammalian cells. 293 cells were transfected with expression vectors for Flag-tagged Casper and various mutants as indicated, with (+) or without (-) CrmA expression. Sixteen hours after transfection, cells were lysed, and the lysates were analyzed by Western blotting with an anti-Flag antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(B) Mutation analysis of potential processing site(s) of Casper. Expression vectors pRK7-Flag-Casper (D369A, with a D-to-A mutation at position 369) and pRK7-Flag-Casper (D376N, with a D-to-N mutation at position 376) were transfected into 293 cells. Sixteen hours after transfection, cells were lysed, and the lysates were analyzed by Western blotting with anti-Flag antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(C) Induction of apoptosis by Casper and its point mutants. Cell death assays were performed as described above using the indicted vectors.

variant of Casper, alternative splicing of Casper may provide a mechanism to regulate apoptosis triggered by Fas, TNF-R1, and other cell death pathways.

Casper is similar to caspase-8 in that it interacts with FADD and can be recruited to Fas via FADD. Several lines of evidence suggest that Casper may function as a coactivator of caspase-8-induced cell death. First, two distinct domains of Casper can interact with caspase-8. Second, CrmA, which can inhibit cell death induced by both Casper and caspase-8, physically interacts only with caspase-8. This is consistent with the hypothesis that Casper-induced apoptosis may be mediated by caspase-8. Third, caspase-3 and caspase-8, which do not directly interact with each other, can simultaneously associate with Casper. Therefore, it is possible that Casper may recruit the caspase-3 precusor to the vicinity of caspase-8, where it can be cleaved to its active form. Consistent with this hypothesis, the protease-like domain of Casper interacts with caspase-3 much more avidly than does its Y360F counterpart, which has weaker cell killing activity.

Although Casper(1–435) can interact with both caspase-3 and caspase-8, it does not induce apoptosis. Instead, it functions as a dominant negative mutant for Fas- and TNF-induced apoptosis. This shows that the simultaneous interaction of Casper with caspase-8 and caspase-3 is not sufficient to induce apoptosis. It is possible that the C-terminal fragment of Casper, which can interact with caspase-8, may regulate the enzymatic activity of caspase-8 and therefore be required for Casper-induced apoptosis. This model is attractive because it postulates that the first enzyme of the caspase cascade is activated by a nonenzymatic regulator, which may provide for a more easily controlled cell death apparatus.

Interestingly, the DED modules of Casper have ~35% sequence identity with viral proteins encoded by genomes of molluscum contagiosum virus subtype 1, equine herpesvirus 2, and Kaposi's sarcoma–associated herpesvirus. In contrast, the DED modules of caspase-8, caspase-10, and FADD are much less homologous to these viral proteins, suggesting that the DED modules of Casper may be the cellular counterpart of these viral proteins. Since the DED modules of Casper can inhibit apoptosis induced by Fas and TNF, the viral proteins may antagonize apoptosis by mimicking this function. This may provide a mechanism for enhancing survival and replication of these viruses in mammalian cells.

CrmA interacts strongly with full-length caspase-8 and prevents its processing, yet interacts only weakly with the protease domain of caspase-8. This is in contrast to caspase-3, which does not interact with CrmA under our experimental conditions (Figure 6). Previously, it was reported that CrmA interacts with processed caspase-3 subunits (Tewari et al., 1995). Thus it is possible that CrmA may inhibit different caspases through different mechanisms.

When overexpressed, Casper is proteolytically processed into two fragments. Casper deletion mutants that can induce cell death are processed, whereas the deletion mutants that cannot induce cell death are not processed. The processing site has been mapped to

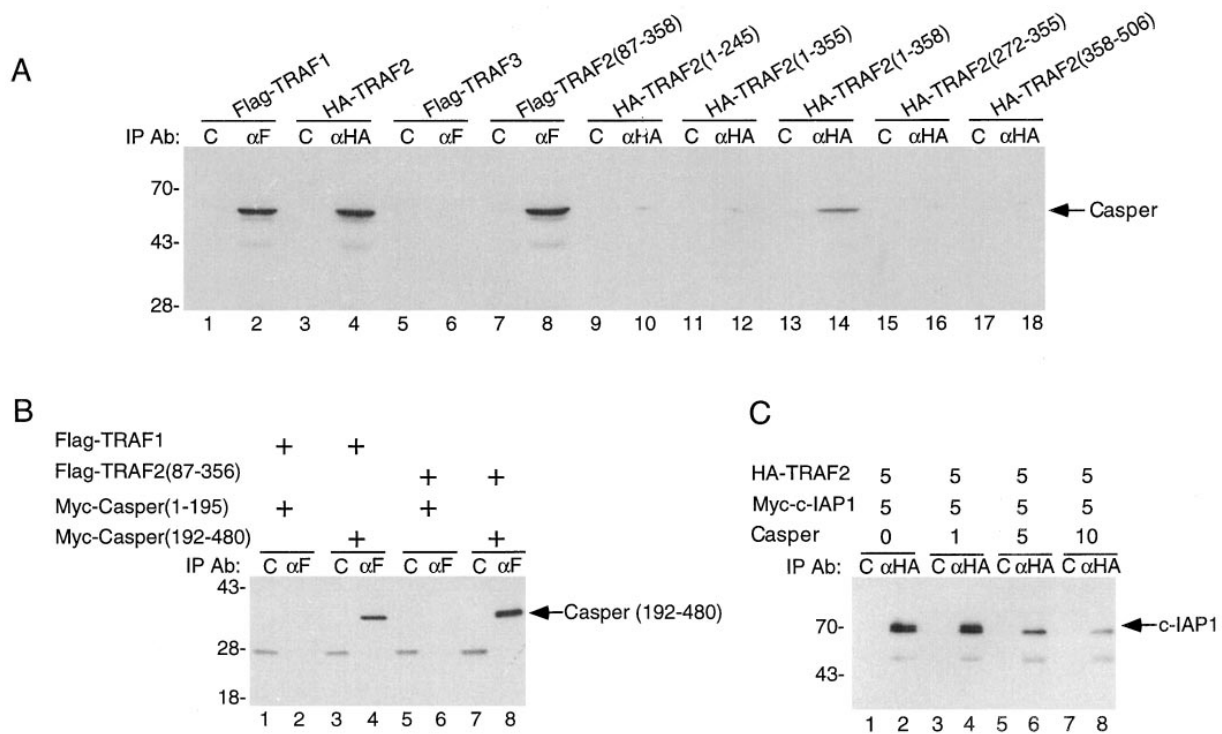

Figure 10. Characterization of Casper Interaction with TRAF1 and TRAF2

(A) Casper interacts with TRAF1 and TRAF2, but not TRAF3. 293 cells were transfected with the Casper expression vector, together with the indicated TRAF expression vectors. Transfected cell lysates were analyzed by immunoprecipitation (IP) with indicated antibodies and Western blotting with a rabbit anti-Casper antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(B) The protease-like domain of Casper is sufficient to bind to TRAF1 and TRAF2. 293 cells were transfected with indicated vectors. Transfected cell lysates were immunoprecipitated with an anti-HA antibody. Immunoprecipitating proteins were detected by Western blotting analysis with an anti-Flag antibody. Positions of the molecular weight standards (in kilodaltons) are shown.

(C) Casper competes for the binding of c-IAP1 to TRAF2. 293 cells were transfected with 5 μg of HA-tagged TRAF2 and 5 μg of Myc-tagged c-IAP1 vectors and an increased amount of expression vector for Casper as indicated. Transfected cell lysates were immunoprecipitated with an anti-HA antibody. The immunoprecipitating proteins were detected by Western blotting with an anti-Myc antibody for c-IAP1. Positions of the molecular weight standards (in kilodaltons) are shown.

A CrmA expression vector was added to the above transfection to inhibit cell death.

the D376–G377 dipeptide. Mutation of D376 to N abolishes the processing activity but not the apoptotic activity of Casper. Since Casper processing is inhibited by CrmA, it is possible that Casper is processed by activated caspase-8 or caspases activated by caspase-8. These findings suggest that Casper activates a caspase cascade, which in turn converts Casper into an inactive form.

A yeast two-hybrid screen for Casper-interacting proteins yielded TRAF1 and TRAF2. Mapping experiments demonstrated that the protease-like domain of Casper interacts with the TRAF-N domain of TRAF1 and TRAF2. The TRAF-N domain of TRAF1 and TRAF2 is known to interact with c-IAP1 and c-IAP2 and recruit these proteins to the TNF-R1 and TNF-R2 complexes (Rothe et al., 1995; Shu et al., 1996). Since the c-IAPs are thought to inhibit apoptosis, the ability of c-IAPs to compete with Casper for TRAF1 and TRAF2 binding suggests that the binding of TRAF1 and TRAF2 to different partners may regulate components associated with the TNF-R1 and/or TNF-R2 complex, and therefore regulate cell death or survival.

Casper interacts with FADD and can be recruited to Fas through FADD, and the Casper(1–435) deletion mutant can protect against Fas-induced cell death. These findings suggest that Casper is physically and functionally involved in Fas signaling. Although Casper(1–435) also inhibits TNF-induced cell death, we have not been able to demonstrate physical association of Casper with the TNF-R1 signaling complex. It should also be noted that a physical association of FADD with the endogenous TNF-R1 signaling complex has yet to be demonstrated. In mammalian overexpression systems, FADD is only weakly recruited to TNF-R1 through TRADD (Hsu et al., 1996a). Although a direct interaction between TNF-R1 and FADD has been reported (Chinnaiyan et al., 1996a), we have been unable to confirm this result (H. Hsu et al., unpublished data). It is possible that a FADD-like molecule, rather than FADD itself, may be involved in the TNF-R1 signaling pathway and may recruit Casper to the TNF-R1 complex. Alternatively, it is possible that additional component(s) other than TRADD, TRAF2, and FADD are required for recruitment of Casper to TNF-R1. Finally, it is also possible that Casper is not a component of the TNF-R1 complex.

In conclusion, we have identified and characterized a

novel protein Casper that is related to both caspase-8 and FADD. Casper physically interacts with FADD, caspase-8 and caspase-3 and its overexpression induces apoptosis. A C-terminal deletion mutant of Casper potently inhibits Fas- and TNF-R1-induced apoptosis, suggesting that Casper is involved in signaling pathways leading to apoptosis.

Experimental Procedures

Reagents and Cell Lines

Recombinant human TNF was provided by Genentech. The rabbit anti-TNFR-1 and anti-Fas antisera and the monoclonal antibody against the Myc epitope tag have been described previously (Tartaglia et al., 1991; Wong and Goeddel, 1994; Hsu et al., 1995). The rabbit anti-human FADD antibody was provided by V. Dixit. The monoclonal antibodies against the Flag (Kodak International Biotechnologies), HA (BABCO, Richmond, CA), and AU1 (BABCO) epitopes; the agonistic monoclonal antibody against Fas (Panvera Corporation); and the monoclonal antibody against caspase-3 (Transduction Laboratory) were purchased from the indicated suppliers. The human 293 embryonic kidney (R. Tjian), HeLa (Y. Jiang), and its derivative HtTA-1 (H. Bujard) cell lines were obtained from the indicated sources. Rabbit polyclonal antibodies against human Casper were raised against either a 33-mer peptide (for Casper antibody I), HRIDLKTKIQKYKQSVQGAGTSYRNVLQAAIQK, or a 35-mer peptide (for Casper antibody II), EQLGAQQEPVKKSIQESEAF LPQSIPEE RYKMKSK, respectively (BABCO).

cDNA Cloning

HeLa and human umbilical vein cell (HUVEC) cDNA libraries (provided by Z. Cao) were screened with radiolabeled probes corresponding to two ESTs (GeneBank accession numbers W23795 and T30922) following standard procedures (Sambrook et al., 1989). Seven independent positive clones were obtained from HeLa cDNA library screening and three positive clones were obtained form the HUVEC library screening. The cDNA inserts of the positive phage clones were sequenced with an Applied Biosystems model 373A automated DNA sequencer.

Northern Blot Hybridization

Human multiple tissue Northern blots (Clontech) were hybridized under high-stringency conditions using radiolabeled Casper-encoding cDNA probe according to the instructions of the manufacturer.

Mammalian Expression Vectors

Mammalian expression vectors encoding TNF-R1, Flag-tagged TRADD and FADD, Myc-tagged TRADD and RIP, CrmA, pRK5 control vector, and pCMV-β-gal have been described previously (Hsu et al., 1995; Hsu et al., 1996a, 1996b). The expression vectors for Fas and AU1-tagged FADD were provided by V. Dixit. Other vectors used in this study were constructed by standard procedures.

Full-length caspase-3 cDNA was amplified from a Jurkat cDNA library and inserted into the pRK7 vector (for pRK7-caspase-3) or the pRK7-Flag vector (for pRK7-Flag-caspase-3; pRK7-Flag was provided by H. Song). pRK7-Myc-caspase-3 was constructed by replacing the Flag epitope with the Myc epitope.

DNA encoding the Myc epitope was fused in-frame to the 5′ end of CrmA cDNA in the pRK5-crmA vector (Hsu et al., 1995) to give pRK7-Myc-crmA vector.

To construct the expression vectors for the various Casper mutants, the appropriate polymerase chain reaction (PCR) fragments were inserted into the N-terminal Flag-tagged pRK7-Flag vector.

The C-terminal Flag-tagged Casper expression vector was constructed by in-frame insertion of PCR-amplified Casper cDNA (with a stop codon mutation) to pRK5-C-Flag vector (provided by Z. Cao).

All N-terminal Myc-tagged Casper and Casper deletion mutant vectors were generated by replacement of the N-terminal Flag epitope of the respective vectors with DNA encoding the Myc epitope.

Point mutation (Y to F) at position 360 of Casper on pRK7-Casper (192–480) vector was performed with Chameleon site-directed mutagenesis kit (Stratagene). The other point mutation vectors used in this study were made by PCR.

Transfections

The 293, HeLa, and HtTA-1 cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (GIBCO). For apoptosis assays, $\sim 2 \times 10^5$ cells/well were seeded on six-well (35 mm) dishes. For coimmunoprecipitations, $\sim 2 \times 10^6$ cells/well were seeded on 100 mm plates. Cells were transfected the following day by the standard calcium phosphate precipitation method (Sambrook et al., 1989).

Apoptosis Assays

β-Galactosidase cotransfection assays for determination of cell death were performed as described (Hsu et al., 1995, 1996a, 1996b). Transfected cells were stained with X-gal as previously described (Shu et al., 1995). The number of blue cells from eight viewing fields of a 35 mm dish was determined by counting. The average number from one representative experiment is shown.

Yeast Two-Hybrid Screening

DNA encoding aa 1–215 or 192–480 of Casper was inserted into the inserted in-frame into Gal4 DNA-binding domain vector pPC97 (Rothe et al., 1994) or pGBT9 (Clontech). The murine fetal liver stromal cell cDNA library (M. Rothe), human B cell cDNA library (Clontech), and human peripheral lymph node cDNA library (Clontech) were obtained from the indicated resources. The isolation of positive clones and subsequent two-hybrid interaction analyses were carried out as described (Rothe et al., 1994; Hsu et al., 1995, 1996a, 1996b).

Coimmunoprecipitation and Western Analysis

Transfected 293 cells from each 100 mm dish were lysed in 1 ml lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). For each immunoprecipitation, 0.8 ml aliquots of lysates were incubated with 1 μl polyclonal anti-Fas or anti-TNF-R1, or 0.5 μg monoclonal antibody against the epitope tag, and 25 μl of a 1:1 slurry of GammaBind G Plus Sepharose (Pharmacia) for at least 1 hr. The sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and subsequent Western blotting analyses were performed as described (Hsu et al., 1995).

Assay for Protease Activity

In vitro transcription and translation was performed with the TNT SP6 Coupled Reticulocyte Lysate System (Promega) following the manufacturer's recommendation. In vitro–translated ^{35}S-labeled Flag-tagged caspase-3 precursor was purified by immunoprecipitation with anti-Flag antibody. The purified caspase-3 precursor was aliquoted and mixed with 10 μl of nonlabeled in vitro translation product or 10 μl of transfected cell lysates from pRK7 control vector, pRK7-Casper (192–480), or pRK7-caspase-8 (217–479). The mixture was buffered with 25 mM HEPES (pH7.5), 0.1% CHAPS, 5 mM EDTA, and 10 mM dithiothreitol and incubated at 30°C for 1.5 hr. Following incubation, the samples were subjected to SDS-PAGE and autoradiography analysis.

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