

CRADD, a Novel Human Apoptotic Adaptor Molecule for Caspase-2, and FasL/Tumor Necrosis Factor Receptor-interacting Protein RIP¹

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Abstract

FADD/MORT1 is a death domain (DD)-containing adaptor/signaling molecule that interacts with the intracellular DD of FAS/APO-1 (CD95) and tumor necrosis factor receptor 1 and the prodomain of caspase-8 (Mch5/MACH/FLICE). FADD engagement of caspase-8 presumably activates this caspase and leads to apoptosis. Another DD-containing adaptor/signaling molecule, CRADD, was identified and was shown to induce apoptosis. CRADD has a dual-domain structure similar to that of FADD. It has an NH₂-terminal caspase homology domain that interacts with caspase-2 and a COOH-terminal DD that interacts with RIP. CRADD is constitutively expressed in many tissues and thus could play a role in regulating apoptosis in mammalian cells.

Introduction

The cell surface receptor FAS/APO-1 (CD95) belongs to a family of transmembrane cytokine receptors that includes TNFR-1,³ TNFR-2, and the nerve growth factor receptor (for reviews, see Refs. 1 and 2). FAS/APO-1 and TNFR-1 trigger apoptosis in mammalian cells after binding to their respective natural ligands or specific agonist antibodies (3-5). These two receptors share significant homology, especially in their COOH-terminal cytoplasmic domain, which is also called the DD (6, 7). Recent evidence suggests that receptor-ligand interaction causes receptor aggregation and oligomerization of the DD (8-10). The apoptotic signal is thought to be transduced through interaction of the oligomerized receptor DD with a set of cytoplasmic adaptor/signaling molecules (9, 11-16). These adaptors couple the receptors to the upstream signal-transducing caspases (17-20). Several candidate adaptor/signaling molecules that interact with the DD of FAS and TNFR-1 have been identified, including FADD/MORT1, TRADD, and RIP (11-13, 15). Significantly, all of these proteins have a dual-domain structure (an NH₂-terminal domain and a COOH-terminal DD analogous to that of FAS and TNFR-1; Ref. 21). Interestingly, the FADD NH₂-terminal domain, which is also called the DED, is related to the NH₂-terminal prodomain of the signal-transducing caspases-8 and -10 (Mch5/MACH/FLICE and Mch4; Refs. 17-20). Association of FADD DED with procaspase-8 is thought to be responsible for triggering the activation of this protease by an as yet unknown mechanism (17-19).

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³ The abbreviations used are: TNFR, tumor necrosis factor receptor; DED, death effector domain; CD, caspase domain; DD, death domain; GST, glutathione-S-transferase; EST, expressed sequence tag.

Once caspase-8 is activated, it can potentially activate the remaining downstream executioner caspases (CPP32, Mch2, and Mch3) through a protease amplification cascade (19, 22).

In this paper, we report the identification and molecular cloning of an adaptor/signaling molecule structurally related to FADD. Its COOH-terminal domain is significantly related to the COOH-terminal DD of FADD, TNFR-1, and RIP. However, its NH₂-terminal domain is significantly related to the prodomain of human caspase-2 (ICH-1; Ref. 23) and caspase-9 (mch6/ICE-LAP6; Refs. 24 and 25) and to the prodomain of the nematode caspase CED-3 (26). This protein can interact with caspase-2 and RIP and is thus named CRADD⁴ (caspase and RIP adaptor with death domain). CRADD could play a role in the FAS/TNFR apoptotic pathway by recruiting caspase-2 to this pathway.

Materials and Methods

cDNA Cloning. CRADD was cloned from a human Jurkat Uni-ZAP XR cDNA library (27) through a PCR approach. Briefly, the primary amplification was done using vector-specific (T3) primer and a primer (GCCACAGT-TCAAGTCTC) derived from the 3' sequence of human EST clone 24032 (GenBank accession number r37937). The primary PCR products were then subjected to a secondary amplification with primers (ATGGAGCCAGAGACAAAC and ACCTGCCTGTAATGTGATG) derived from the 5' sequence of human EST clone 24032 (GenBank accession number t78285). The PCR product was cloned into the *Sma*I site of pBluescript II KS+ vector and then sequenced. The open reading frame of CRADD was subcloned in-frame into *Bam*HI/*Xho*I-cut pET21(b), and a T7-tagged CRADD was generated from the pET-CRADD plasmid by PCR and subcloned into the mammalian expression vector pcDNA-3 at the *Eco*RV site.

The DD of RIP was also cloned from the human Jurkat Uni-ZAP XR cDNA library using a PCR-based approach. The primary amplification was done using vector-specific (T3) primer and a primer (CTGAATGCTCTGAG-GCAG) derived from the 3' nontranslated sequence of human RIP (GenBank accession number u25994). The final PCR product was obtained using nested primers (GATAATACCACTAGTCTG and TTAGTTCTGGCTGACGTA-AATC) derived from the open reading frame of human RIP sequence. The PCR product was cloned into the *Sma*I site of pBluescript II KS+ vector and the blunted *Bam*HI site of pET28 (c).

Northern Blot Analysis. Tissue distribution analysis of CRADD mRNA was performed on Northern blots prepared by Clontech containing 2 μg/lane polyadenylated RNA. A radioactive CRADD cDNA probe was prepared using the random-primed DNA-labeling method in the presence of [α -³²P]dCTP. The blots were hybridized, washed, and then visualized by autoradiography.

GST Fusion Proteins and *In Vitro* Binding Assay. The open reading frame of CRADD was subcloned into the bacterial expression vector pGEX-2T in-frame with an NH₂-terminal GST tag. To produce GST-CRADD DD and GST-CRADD CD, CRADD cDNA was digested with *Bam*HI, which cleaves between the CD and DD coding regions, and the two fragments were subcloned in-frame into pGEX-2T vector. The GST fusion proteins were expressed in DH5 α bacteria and then immobilized on glutathione-Sepharose.

⁴ The sequence reported in this paper has been deposited in the GenBank database (accession number U84388).

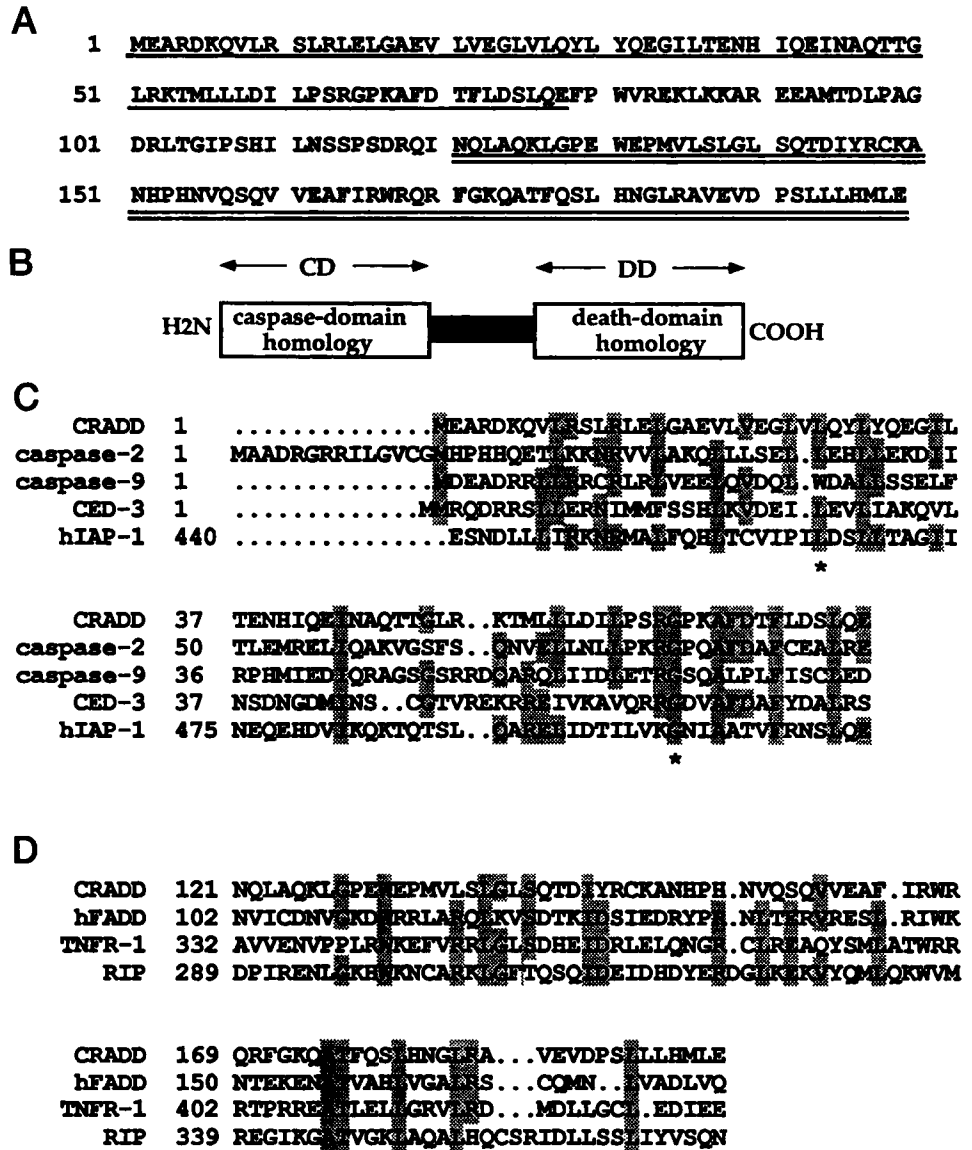


Fig. 1. Amino acid sequence of human CRADD and its homology to other proteins. *A*, predicted amino acid sequence of human CRADD. The NH₂-terminal CD homology region and the COOH-terminal DD homology region are *single-* and *double-underlined*, respectively. *B*, a schematic illustrating the dual-domain structure of CRADD. The CD domain of CRADD is equivalent to the DED domain of FADD. *C*, colinear alignment of the CRADD CD region with the prodomains of caspase-2, caspase-9, and CED-3 and the COOH terminus of human inhibitor of apoptosis IAP-1. Asterisks, the critical L27 and G65 of CED-3 that are conserved in CRADD and the other proteins. *D*, colinear alignment of the DD of CRADD with the DDs of FADD, TNFR-1, and RIP. Amino acids that are identical in at least three of the sequences are shaded. The amino acid residues are numbered to the left.

Labeled interacting proteins were prepared by *in vitro* transcription and translation in the presence of [³⁵S]methionine using Promega's T7-coupled transcription/translation TNT kit according to the manufacturer's recommendations. After translation, equal amounts of the translation reactions were diluted to 100 μ l in GST binding buffer [50 mM Tris-Cl (pH 7.6), 120 mM NaCl, and 0.5% Brij supplemented with protease inhibitors] and allowed to incubate with the various GST fusion proteins on ice for 2–3 h. The beads were washed 3–4 times with the same buffer and boiled in SDS sample buffer. The proteins were then resolved on a 10% SDS gel and visualized by autoradiography.

Transfections and Histochemical Detection. Human embryonic kidney 293 cells and MCF-7 cells were transiently transfected using the LipofectAMINE method. Approximately 2×10^5 cells were transfected with 0.25 μ g of the pcDNA3.1/His/lacZ vector (Invitrogen) and 1 μ g of CRADD cDNA cloned in pcDNA3, using LipofectAMINE (Life Technologies) according to the manufacturer's recommendations. After 36–40 h, expression of the lacZ gene product in cells was detected using a β -galactosidase staining kit (Invitrogen). Cells were visualized under the microscope, and blue cells (healthy and apoptotic) were counted. The percentage of dead cells was expressed as the mean percentage of blue apoptotic cells as a fraction of the total number of blue cells counted.

Cleavage Assays. Baculovirus p35 and human caspases were ³⁵S-labeled *in vitro* using Promega's TNT kit as described above. Two μ l of the translation reactions were incubated with purified recombinant caspase-2 (100 ng) in ICE buffer [25 mM HEPES, 1 mM EDTA, 5 mM DTT, and 0.1% 3-[(3-cholamido-

propyl)dimethylamino]-1-propanesulfonate (pH 7.5)] in a final volume of 10 μ l. The reaction was incubated at 37°C for 1 h and then analyzed by Tricine-SDS-PAGE and autoradiography.

Results and Discussion

Identification and Cloning of Human CRADD. Recent evidence suggests that the prodomain of caspases could mediate their interac-

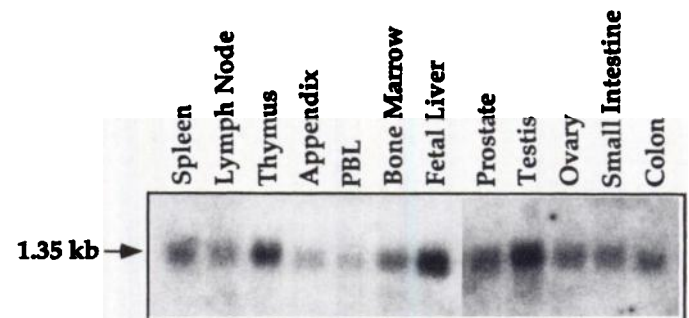


Fig. 2. CRADD expression. Human tissue Northern blots (Clontech) containing 2 μ g of polyadenylated RNA were probed with radioactive CRADD cDNA, exposed to Kodak X-ray film, and photographed. PBL, peripheral blood leukocyte.

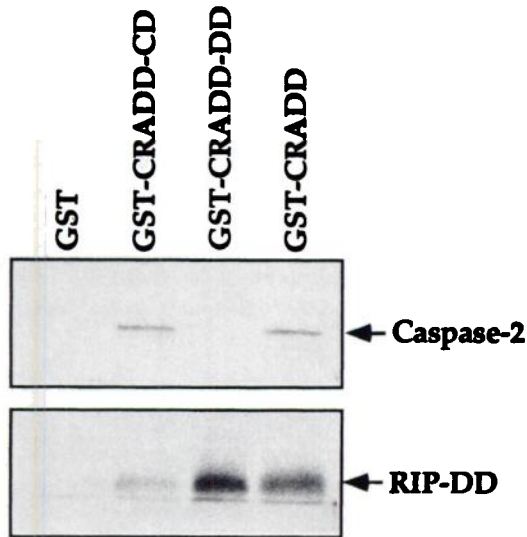


Fig. 3. Specific interactions of GST-CRADD, GST-CRADD CD, and GST-CRADD DD with *in vitro*-translated caspase-2 and the DD of RIP. [³⁵S]Methionine-labeled caspase-2 or the DD of RIP was precipitated with GST (Lane 1), GST-CRADD CD (Lane 2), GST-CRADD DD (Lane 3), or GST-CRADD (Lane 4) fusion proteins immobilized on glutathione-Sepharose beads. The bound proteins were then analyzed by SDS-PAGE and autoradiography.

tion with adaptor molecules involved in cell death-signaling pathways (17–19). The prodomains of caspase-8 and caspase-10 contain two tandem regions of homology with the NH₂-terminal DED of FADD (17–19). Caspase-8 is recruited to the FAS/tumor necrosis factor death pathway through interaction of its prodomain with the DED of FADD (17, 18). Other caspases with long prodomains such as caspase-2 or caspase-9 could also be recruited to death-signaling complexes through interaction with other novel adaptor molecules. To identify

additional adaptor molecules, we searched the GenBank EST data base for sequences with homology to the prodomain of caspase-2 or caspase-9. The sequence of an EST clone (clone 24032) from a human infant brain cDNA library had significant homology to the prodomain of caspase-2. The DNA sequence of this clone overlaps perfectly with sequences of several other clones in the EST data base. Using the 5' and 3' sequence information derived from this EST clone and others, the open reading frame was verified and then cloned by PCR. The open reading frame encodes a protein of 199 amino acids (Fig. 1A) that exhibits a dual-domain structure related to FADD (Fig. 1B).

CRADD Has CD and DD Homology Regions. A BLAST search of the GenBank/European Molecular Biology Laboratory data base revealed that the NH₂ terminus of CRADD (residues 1–78) share significant homology with the prodomains of caspase-2 (residues 15–91; 31% identity), caspase-9 (residues 1–79; 28% identity), and CED-3 (residues 2–78; 24% identity; Fig. 1C). The CD homology region of CRADD also has significant homology to the COOH terminus of human inhibitor of apoptosis IAP-1 (residues 440–516; 27% identity). Interestingly, residues L27 and G65 of CED-3, which when mutated result in loss of function (26), are aligned and conserved in CRADD and the other proteins (Fig. 1C). This suggests that these residues could also be critical for CRADD function. The BLAST search also revealed that the COOH terminus of CRADD (residues 121–199) contains a DD homology region related to the DD of FADD, TNFR-1, and RIP (Fig. 1D). The identity of the DD of CRADD with that of FADD is 23%, and the identity of the DD of CRADD with that of TNFR-1 or RIP is ~20%.

Tissue Distribution of CRADD. To determine the tissue distribution of CRADD mRNA, various tissue mRNA samples were subjected to Northern blot analysis. Expression of the CRADD mRNA was detected in most adult tissues and in the fetal liver (Fig. 2). The highest expression was observed in the thymus, testes, and fetal liver. The size of the CRADD mRNA is approximately 1.35 kb, which is

Fig. 4. Induction of apoptosis in cells transfected with CRADD and β -galactosidase expression plasmids. Human embryonic kidney 293 cells were transfected, fixed, and then stained to visualize β -galactosidase activity. A and B, two representative fields of cells transfected with β -galactosidase plasmid alone. C and D, two representative fields of cells transfected with CRADD and β -galactosidase plasmids.

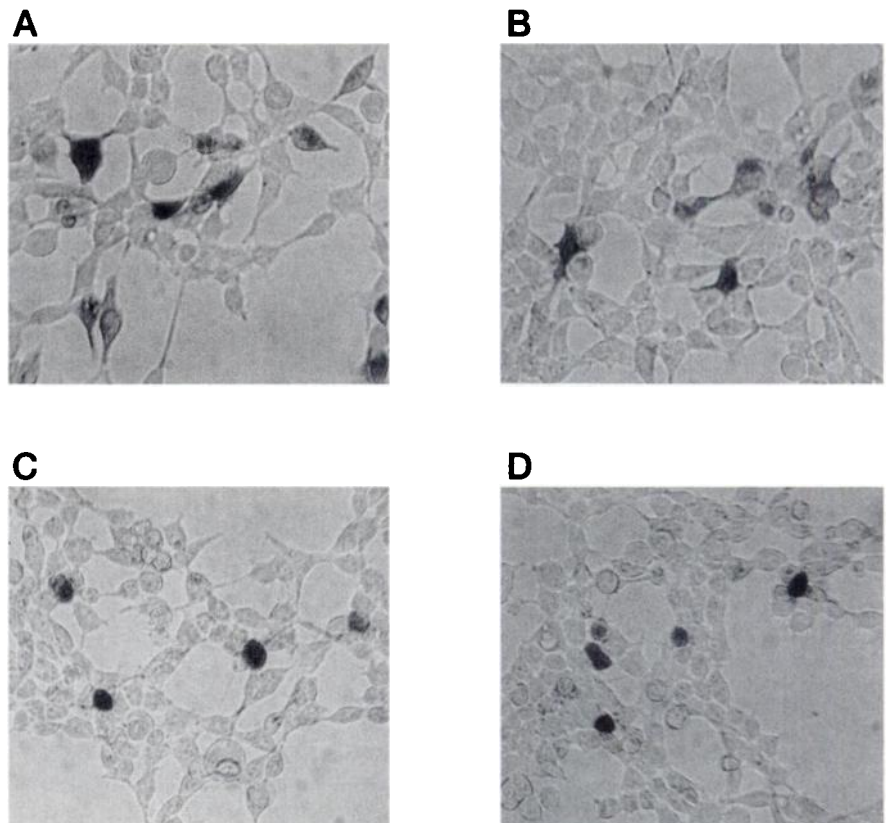


Table 1 Quantitative analysis of the apoptotic activity of CRADD

Human embryonic kidney 293 cells were cotransfected with CRADD or mutant CRADD and β -galactosidase cDNAs and then fixed, stained, and counted as described in "Materials and Methods." The data represent the percentage of apoptotic cells \pm SD.

cDNA	% \pm SD (No. of cells counted)
β -galactosidase	17 \pm 2.8 (771)
CRADD	76 \pm 1.9 (894)
CRADD (27L-F)	27 \pm 3.5 (730)
CRADD (65G-R)	26 \pm 2.8 (450)

consistent with the size of the cDNA clone obtained by PCR from the human Jurkat T-lymphocyte library.

In Vitro Interaction of CRADD with Caspase-2 and RIP. To identify interacting partners of CRADD, *in vitro*-translated caspase-2, caspase-9, FADD, TNFR-1, and the DD of RIP were precipitated with various GST fusion proteins immobilized on glutathione-Sepharose resin. As shown in Fig. 3, caspase-2 specifically associated with GST-CRADD (Lane 4) and GST-CRADD CD (Lane 2), which contains the CRADD CD homology region. Caspase-2 did not interact with GST or with GST-CRADD DD, which contains the DD but lacks the CD homology region. No interaction was detected between CRADD and caspase-9, FADD, or TNFR-1 (data not shown). Interestingly, RIP DD specifically associated with GST-CRADD and GST-CRADD DD but not with GST or GST-CRADD CD (Fig. 3). These observations suggest that CRADD is an adaptor molecule for caspase-2 and RIP.

Overexpression of CRADD Induces Apoptosis. Overexpression of FADD, which is an adaptor molecule for caspase-8 and FAS/TNFR-1, induces apoptosis in mammalian cells (11, 12), most likely by activating caspase-8. To study the functional role of CRADD in mammalian cells, human embryonic kidney 293 cells were transiently cotransfected with two expression plasmids encoding CRADD or β -galactosidase at a 1:4 ratio of β -galactosidase plasmid to CRADD plasmid. Histochemical examination of β -galactosidase-expressing cells (blue cells) revealed that cells transfected with β -galactosidase and CRADD expression plasmids had morphological changes typical of adherent cells undergoing apoptosis (Fig. 4C and D). Most blue cells were intensely stained, round, and shrunken, with typical membrane blebbing and loss of adherence. In contrast, cells transfected with the β -galactosidase expression plasmid alone seemed healthy, with a predominantly uniform cytoplasmic β -galactosidase staining pattern (Fig. 4A and B). Similar results were obtained with the human breast adenocarcinoma cell line MCF-7 (data not shown). Quantitative analysis of these results revealed that \sim 76 \pm 1.9% of blue cells cotransfected with CRADD and β -galactosidase exhibited morphological changes typical of apoptosis (Table 1). Approximately, 17 \pm 2.8% of blue cells transfected with the β -galactosidase expression plasmid exhibited such a phenotype. Interestingly, site-directed mutagenesis of L27 to F or G65 to R resulted in the loss of CRADD apoptotic activity (Table 1). These data clearly demonstrate that CRADD is an apoptosis-inducing protein and that, like CED-3, L27 and G65 are important for its apoptotic activity. This also suggests that the CD domain of CRADD is necessary for its activity.

Caspase-2 Does Not Activate Other Known Caspases. Recently, we demonstrated that mature caspase-8 (Mch5) or caspase-10 (Mch4) could activate all known caspases (22). Thus, clustering of caspase-8 and/or caspase-10 by association of their prodomains with FADD could trigger their activation. This could initiate a proteolytic cascade leading to activation of the remaining caspases. By analogy, clustering of procaspase-2 by association with CRADD could trigger its activation and initiation of a proteolytic cascade. To determine the activity of caspase-2 toward the other known caspases, mature caspase-2 was

incubated with *in vitro*-translated [³⁵S]methionine-labeled caspase proenzymes, and the reaction products were analyzed by SDS-PAGE and autoradiography. Mature caspase-2 was able to process its own precursor proenzyme but not other procaspases (Fig. 5). Mature caspase-2 was also able to cleave baculovirus p35 (Fig. 5C). This suggests that caspase-2 has a very narrow specificity and that it prefers a cleavage site with a DQXD \uparrow G sequence. This sequence is present in procaspase-2 and p35 but not in the other known procaspases examined. These data suggest that CRADD could recruit caspase-2, which could then execute the apoptotic program by itself without directly processing/activating other caspases. Alternatively,

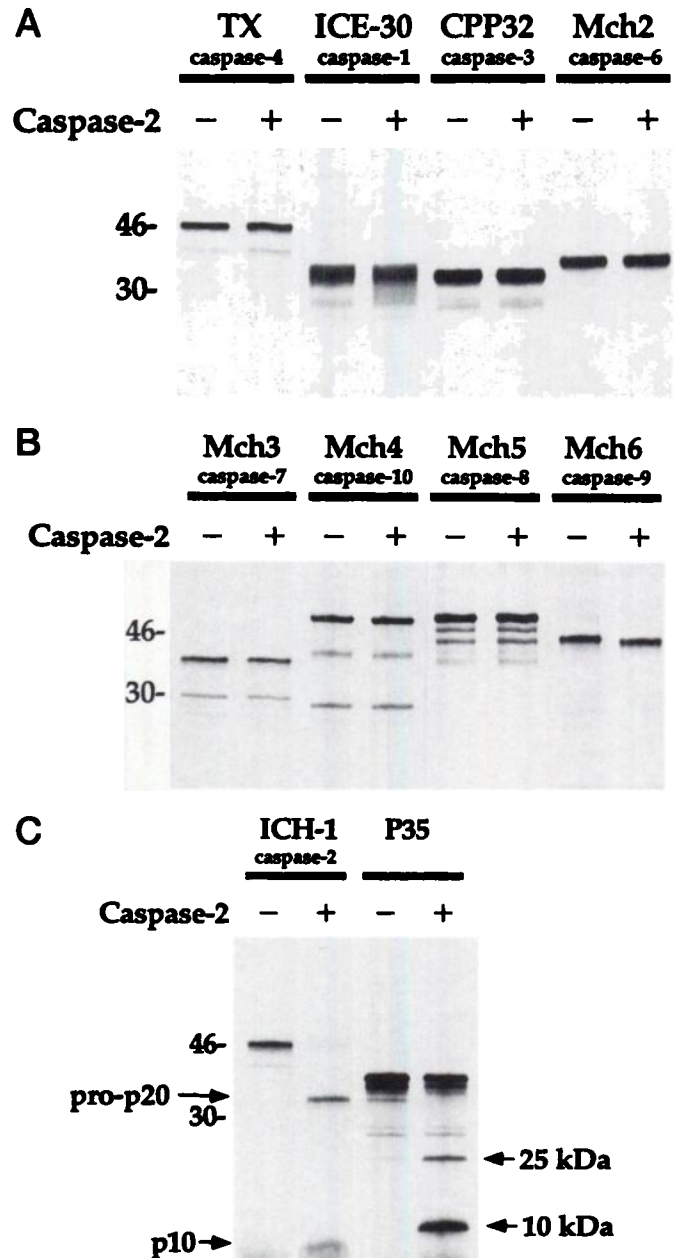


Fig. 5. Protease activity of caspase-2 toward other caspases. ³⁵S-labeled procaspases or baculovirus p35 was incubated with (+) or without (-) purified caspase-2 (100 ng/reaction) for 1 h at 37°C. The reaction products were then analyzed by Tricine-SDS-PAGE and autoradiography. A, activity of caspase-2 toward proenzymes of caspase-4 (TX), caspase-1 (ICE-30), caspase-3 (CPP32/Yama/Apopain), and caspase-6 (Mch2). B, activity of caspase-2 toward proenzymes of caspase-7 (Mch3, ICE-LAP3, CMH-1), caspase-10 (Mch4), caspase-8 (Mch5/MACH/FLICE), and caspase-9 (Mch6/ICE-LAP6). C, activity of caspase-2 toward procaspase-2 (ICH-1) and p35. The cleavage products of procaspase-2 and p35 are indicated. p35 has an NH₂-terminal T7-His6 tag, thus the 10-kDa product seems larger than 10 kDa.

there could be still another target caspase downstream of caspase-2 capable of initiating the protease cascade. It is also possible that CRADD may associate with and trigger the activation of another as yet unknown caspase.

In conclusion, we have identified and characterized a novel apoptotic adaptor molecule named CRADD. CRADD has a dual-domain structure similar to FADD and it might be a component of the FAS/TNFR-1 apoptotic signaling complex. Its ability to interact with the DD of RIP and the prodomain of caspase-2 suggests that it could play a role in the FAS/TNFR-1 apoptotic pathway by recruiting caspase-2. CRADD could also associate with the DD of other unidentified signaling/adaptor molecules or TNFR family members. This CRADD pathway might be a tissue-specific pathway complementary to the FAS/TNFR-1-FADD-caspase-8 pathway. The identification of CRADD adds another adaptor molecule to the FAS/TNFR-1 apoptotic signaling complex. The targeted disruption of the CRADD gene will allow a greater understanding of its role in the death-signaling pathways of mammalian cells.

Note Added in Proof

In the January 2, 1997 issue of the journal *Nature*, Duan and Dixit reported the identification of a death adaptor molecule that is nearly identical to CRADD. They reported the finding that this protein can interact with RIP and ICH-1, which is consistent with our results.

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