FADD, a Novel Death Domain–Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis

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Summary

Using the cytoplasmic domain of Fas in the yeast two-hybrid system, we have identified a novel interacting protein, FADD, which binds Fas and Fas-FD5, a mutant of Fas possessing enhanced killing activity, but not the functionally inactive mutants Fas-LPR and Fas-FD8. FADD contains a death domain homologous to the death domains of Fas and TNFR-1. A point mutation in FADD, analogous to the *lpr* mutation of Fas, abolishes its ability to bind Fas, suggesting a death domain to death domain interaction. Overexpression of FADD in MCF7 and BJAB cells induces apoptosis, which, like Fas-induced apoptosis, is blocked by CrmA, a specific inhibitor of the interleukin-1β-converting enzyme. These findings suggest that FADD may play an important role in the proximal signal transduction of Fas.

Introduction

Programmed cell death (PCD) is a physiological process essential to the normal development and homeostatic maintenance of multicellular organisms (reviewed by Vaux et al., 1994; Ellis et al., 1991). Apoptosis, often equated with PCD, refers to the morphological alterations exhibited by "actively" dying cells that include cell shrinkage, membrane blebbing, and chromatin condensation (Cohen, 1993). In contrast, necrosis, sometimes referred to as accidental cell death, is defined by the swelling and lysis of cells that are exposed to toxic stimuli.

Though the morphological features of cell death are well described, the molecular mechanisms behind apoptosis remain undefined. Recent work on PCD in the nematode Caenorhabditis elegans showed that the *ced-3* gene is required for apoptosis (Yuan et al., 1993). Sequence analysis revealed that CED-3 is similar to the mammalian interleukin-1β (IL-1β)-converting enzyme (ICE) (Yuan et al., 1993), which is a cysteine proteinase involved in the processing and activation of pro-IL-1β to the active cytokine (Cerretti et al., 1992; Thornberry et al., 1992). Overexpression of ICE in mammalian cells induced apoptosis, suggesting that ICE, or a related protease, may be an essential component of the cell death pathway (Miura et al., 1993).

Although a CED-3-like protease is suspected to be a distal effector of the mammalian cell death pathway, the proximal components that lead to its activation remain to be identified. Two cell surface cytokine receptors, Fas/APO-1 antigen and the receptor for tumor necrosis factor (TNF), have been shown to trigger apoptosis by natural ligands or specific agonist antibodies (Baglioni, 1992; Yonehara et al., 1989; Itoh et al., 1991; Trauth et al., 1989). Mice carrying a point mutation in the cytoplasmic domain of Fas exhibit a lupus-like lymphoproliferative autoimmune disorder (*lpr*), (Watanabe-Fukunaga et al., 1992), and the Fas-mediated cell death pathway has been recently implicated in the activation-induced death of T cells (Dhein et al., 1995; Brunner et al., 1995; Ju et al., 1995). While the main activity of Fas is to trigger cell death, the TNF receptor (TNFR) can signal an array of diverse activities such as fibroblast proliferation, resistance to chlamidiae, and synthesis of prostaglandin E2 (Tartaglia and Goeddel, 1992). Recent work in our laboratory suggests that the stimulation of Fas or the TNFR triggers the activation of a common component of the cell death pathway. CrmA, a poxvirus gene product, was shown to potently block both Fas- and TNF-induced cell death (Tewari and Dixit, 1995). Interestingly, the only reported target for CrmA is the CED-3 homolog ICE (Ray et al., 1992), suggesting that ICE, or an ICE-like protease, is the common effector of cytokine receptor–mediated cell death.

The activation of Fas and TNFR is caused by receptor aggregation mediated by the respective ligands or agonist antibodies. The signal is thought to be transduced by clustering of the intracellular domain (Boldin et al., 1995; Song et al., 1994), which encompasses a region that is significantly conserved in the Fas antigen as well as in TNFR-1 (Tartaglia et al., 1993; Itoh and Nagata, 1993). This shared "death domain" suggests that both receptors interact with a related set of signal transduction molecules that, thus far, remain unidentified.

Here, we report the molecular cloning and characterization of FADD, a Fas-associating protein with a novel death domain. The specific interaction of Fas and FADD is due to the association of their respective homologous death domains. Remarkably similar to Fas-induced killing, overexpression of FADD induces apoptosis that is inhibitable by CrmA. Taken together, our results suggest that FADD is a component of the Fas-mediated cell death pathway.

Results

Isolation of FADD

The yeast two-hybrid system was used to screen for proteins that interact with the cytoplasmic domain of Fas. An expression vector was constructed by fusing the GAL4 DNA-binding domain to the cytoplasmic tail of the human Fas antigen (GAL4bd–Fas). This bait plasmid was cotransformed in yeast with a prey plasmid containing a human B cell cDNA expression library fused to the GAL4 activation domain. Seventeen positive clones were obtained from 2×10^6 transformants screened. To determine the specificity of interaction, plasmids containing the activation domain fusion proteins were recovered from the putative positive clones and cotransformed with GAL4bd–Fas and control heterologous baits. Two clones (8 and 15) were
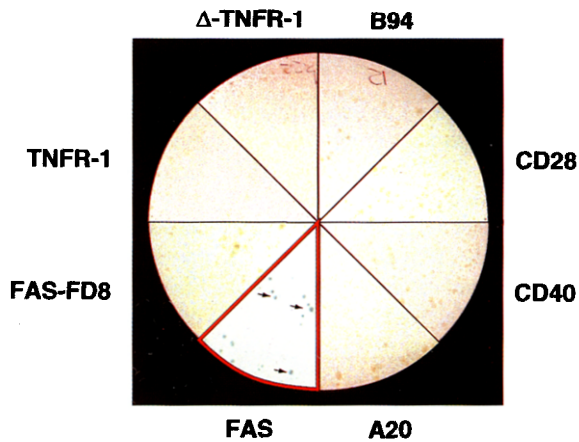
*The first two authors contributed equally to this work.

Figure 1. FADD Specifically Interacts with the Cytoplasmic Domain of Fas in Yeast

β-Galactosidase filter assays performed on Y190 yeast expressing the GAL4 activation domain–FADD fusion protein and indicated heterologous GAL4 DNA-binding domain fusion proteins.
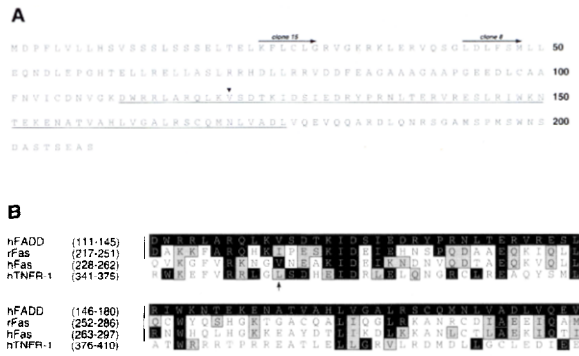

Figure 2. Sequence Analysis of FADD and Its Novel Death Domain

(A) The deduced amino acid sequence of the FADD protein product. The N-termini of clones 8 and 15 isolated in the yeast two-hybrid screen are indicated with arrows. The death domain is underlined, and the valine residue altered to an asparagine in FADDmt is indicated by the closed triangle.

(B) The death domain of FADD and its amino acid sequence homology to other death domains. Solid black shading refers to identical residues, and gray shading indicates conservative amino acid substitutions relative to the sequence of FADD. The arrow indicates the amino acid residue, which when substituted by an asparagine, disrupts binding, signaling, or both in the respective proteins.

found to interact with the GAL4 DNA-binding domain fusion protein containing the cytoplasmic domain of wild-type Fas and not the functionally inactive deletion mutant Fas-FD8 (Itoh and Nagata, 1993) or the indicated heterologous baits (Figure 1).

FADD is a Novel Protein with a Death Domain

Clones 8 and 15 isolated by the yeast two-hybrid screen were found to contain overlapping sequence fused to the GAL4 activation domain in the same reading frame. To obtain a full-length coding sequence, a human umbilical vein endothelial cell (HUVEC) library was screened with a cDNA insert obtained from clone 15. Two independent clones yielded a 1.6 kb cDNA containing an open reading frame that begins with an initiator methionine conforming to Kozak's consensus (Kozak, 1989) and that ends 625 nt later at an Opal codon. Given the presence of an in-frame stop codon 130 bp upstream of the initiator methionine and the size of the transcript (1.6 kb; Figure 3), it is likely that Figure 2A represents the full-length coding sequence. This gene encodes a previously undescribed protein of 208 amino acids with a predicted molecular mass of 23.3 kDa, designated FADD.

A BLAST search revealed that residues 111–170 of FADD matched residues 233–292 of rat Fas antigen (rFas, p = 0.0012) and shared 27% identity (51% of the amino acids were conserved). This region in the cytoplasmic domain of rFas corresponds to the death domain, a region of homology shared by both Fas and TNFR-1 that signals cell death (Tartaglia et al., 1993; Itoh and Nagata, 1993).

Dependent upon the alignment and boundaries selected, the death domains of FADD, Fas, and TNFR-1 share 25%–30% identity (Figure 2B). When conservative amino acid substitutions are included, the homologies approach 50%. These values are consistent with those previously reported for the death domain homology between TNFR-1 and Fas (Tartaglia et al., 1993; Itoh and Nagata, 1993). Interestingly, V121 of FADD is aligned and con-

served with V238 of Fas, which when altered to an asparagine, abolishes the cell-killing activity of Fas and in mice is responsible for the lymphoproliferation (lpr) phenotype (Watanabe-Fukunaga et al., 1992). A corresponding inactivating mutation also exists in TNFR-1, L351→N351 (Tartaglia et al., 1993).

Tissue Distribution of FADD

Northern blot analysis revealed that FADD is constitutively expressed in many fetal and adult human tissues (Figure 3). The mRNA transcript is approximately 1.6 kb, consistent with the size of the cDNA clones isolated from the HUVEC library.

In Vitro Association of FADD and Fas

To confirm the interaction observed in yeast, radiolabeled in vitro translated FADD was precipitated with various glutathione S-transferase (GST) fusion proteins immobilized on glutathione–Sepharose beads (Figures 4A and 4B). As predicted, FADD specifically associated with GST–Fas but not GST, GST–Fas-FD8, or GST–Fas-LPR, which contains the cytoplasmic domain of the functionally inactive point mutant of Fas (Itoh and Nagata, 1993). A very weak interaction was observed between FADD and TNFR-1. Interestingly, relative to its association with GST–Fas, FADD strongly interacted with GST–Fas-FD5, which is a 15 amino acid C-terminal deletion mutant of Fas possessing enhanced killing activity (Itoh and Nagata, 1993). Similar results were obtained when detergent lysates of 293T cells expressing FADD were precipitated with the various GST fusion proteins (Figure 4C).

Coimmunoprecipitation of FADD and Fas

To demonstrate the interaction of FADD and Fas in vivo, 293T cells were transiently transfected with HA epitope–
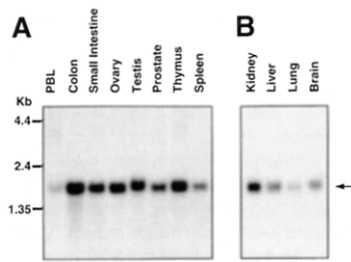
Figure 3. FADD Is Expressed in a Variety of Tissues and Developmental Stages

(A) A human adult tissue Northern blot (Clontech) was probed with FADD cDNA. PBL, peripheral blood leukocyte.
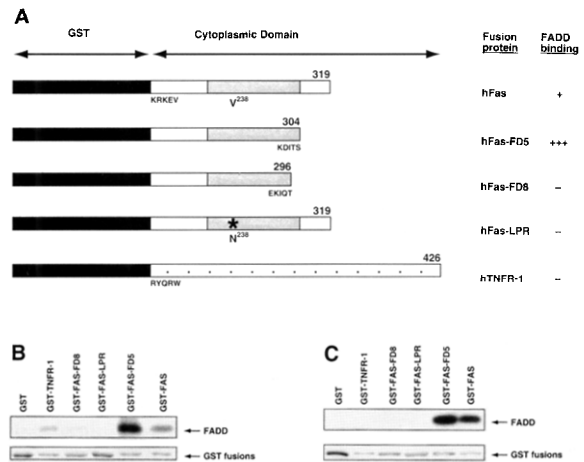(B) A human fetal Northern blot (Clontech) was probed as in (A).

Figure 4. Specific Interaction of GST–Fas and GST–Fas-FD5 with In Vitro Translated FADD and FADD Expressed in Transfected 293T Cells

(A) Schematic representation of the GST fusion proteins containing the cytoplasmic domains of Fas, Fas mutants, and TNFR-1. Amino acid residues are given for selected junctions, and numbering is based on the mature form of the receptor. The lpr mutant (V238→N238) of Fas is represented by an asterisk. Gray shading represents the death domain of Fas. Binding of FADD to the various GST fusion proteins is depicted at the right and is based on data from (B).
(B) Interaction of in vitro translated, ^{35}S-labeled FADD with various GST fusion proteins immobilized on glutathione–Sepharose beads. After the beads were washed, retained FADD protein was analyzed by SDS–PAGE and autoradiography (upper panel). The gel was Coomassie stained, and the bands representing the various GST fusion proteins were aligned to show equivalency of loading (lower panel).
(C) 293T cells were transfected with HA epitope–tagged FADD (HA-FADD) and metabolically labeled with [^{35}S]methionine and cysteine. Detergent lysates were prepared and incubated with the various GST fusion proteins immobilized on glutathione–Sepharose beads. After washing, the complexed beads were dissociated and immunoprecipitated with an anti-HA (α-HA) antibody that should recognize HA-FADD. The samples were then analyzed by SDS–PAGE and autoradiography (upper panel). The respective GST fusion proteins are shown as in (B) (lower panel).

tagged FADD (HA-FADD) and FLAG epitope–tagged Fas (FLAG-Fas) and mutants (Figure 5). Expression of the FLAG-tagged constructs was shown by immunoprecipitation with an anti-FLAG (α-FLAG) antibody (Figure 5B). Likewise, immunoprecipitation with anti-HA (α-HA) antibody showed expression of HA-FADD, and as expected, FLAG-Fas and FLAG-Fas-FD5 individually coprecipitated, while the functionally inactive mutants FLAG-Fas-FD8 and FLAG-Fas-LPR did not (Figure 5C). The α-HA immunoprecipitates were dissociated and subjected to a second round of immunoprecipitation with α-FLAG antibody. Consistent with results of the primary immunoprecipitation (with α-HA), a double immunoprecipitation with α-HA followed by α-FLAG confirmed the presence of FLAG-Fas and FLAG-Fas-FD5 in the original immune complexes (Figure 5D).

The Death Domain of FADD Interacts with the Death Domain of Fas

Previous studies have reported that the death domains of TNFR-1 and Fas self-associate (Boldin et al., 1995). The two clones (8 and 15) isolated in our two-hybrid screen (using the cytoplasmic domain of Fas as bait) did not contain various portions of the N-terminus of wild-type FADD (Figure 2A). The shortest of the two, clone 8, is missing the N-terminal 40 amino acids, suggesting that the C-terminal half of FADD, which contains the death domain, interacts with the cytoplasmic tail of Fas. More specifically, our results show that FADD interacts with the death domain of Fas, since it fails to associate with Fas-LPR and Fas-FD8, a point mutant and deletion mutant, respectively, of the Fas death domain.

Thus, it is reasonable to propose that the death domain of FADD is interacting with its homologous counterpart in Fas. To test this hypothesis, we engineered a point mutant of FADD (FADDmt) in which V121 is altered to an asparagine. This mutation corresponds to the inactivating lpr mutation (V238→N238) of Fas and the L351→N351 mutation of TNFR-1. 293T cells were transiently transfected with expression constructs containing AU1 epitope–tagged FADD (AU1-FADD) and AU1-FADDmt. Detergent lysates were prepared and subsequently precipitated with GST, GST–Fas, and GST–Fas-LPR immobilized on glutathione–Sepharose beads (Figure 6). As predicted, AU1-

FADD bound GST–Fas and not GST or GST–Fas-LPR, while, in contrast, AU1-FADDmt failed to bind any of the GST fusions. Taken together, our results suggest that a death domain to death domain interaction is responsible for the association of FADD and Fas.

Overexpression of FADD Initiates Apoptosis That Is Suppressed by CrmA

To study the functional role of FADD, we chose MCF7 cells, a breast carcinoma cell line that expresses Fas and is sensitive to Fas-induced killing in the presence of the protein synthesis inhibitor cycloheximide (Tewari and Dixit, 1995). Two stably transfected and previously characterized clonal cell lines of MCF7 were used in our study: one expresses CrmA, a viral serpin inhibitor that has been shown to potently block Fas-mediated cell death (Tewari and Dixit, 1995), and a corresponding control cell line expresses an inactive CrmA mutant (Tewari et al., submitted). The mutant CrmA possesses a single amino acid substitution within its reactive site loop, abolishing its ability to inhibit Fas- and TNF-mediated apoptosis. The re-
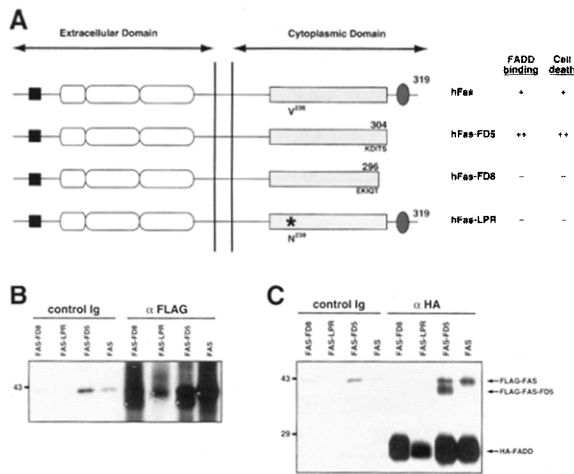
A

B

C

D

Figure 5. In Vivo Association of FADD with Fas and Fas-FD5

(A) Schematic representation of Fas and Fas mutants transfected into 293T cells. The closed square represents the FLAG epitope tag engineered five amino acids downstream of the putative signal sequence of Fas. The open rounded rectangles represent the three cysteine-rich subdomains of the extracellular domain of Fas, while the cytoplasmic residues contain the death domain (gray rectangle) and a putative negative regulatory domain (shaded oval). Residue numbering is based on the mature form of the receptor and the amino acid sequence is given for selected junctions. The lpr mutant (V238→N238) of Fas is represented by an asterisk. In vivo FADD binding is described to the right of the schematic (based on [D]) along with relative cell death caused by Fas and its mutants as described by Itoh and Nagata (1993).

(B) 293T cells were cotransfected with HA-FADD and FLAG epitope–tagged Fas and Fas mutants (as depicted in [A]) and metabolically labeled with [^{35}S]methionine and cysteine. Detergent lysates were then immunoprecipitated with anti-FLAG (α-FLAG) monoclonal antibody and isotype-matched control antibody and analyzed by SDS–PAGE and autoradiography to show expression of FLAG-tagged Fas and Fas mutants. White asterisks indicate relative position of Fas and its mutants.

(C) 293T lysates (as in [B]) were also immunoprecipitated with α-HA antibody to show HA-FADD expression.

(D) Coimmunoprecipitation of FADD with Fas and mutants. A fraction of the α-HA immunoprecipitates (used in [C]) were dissociated and reimmunoprecipitated with an α-FLAG antibody.

spective MCF7 cell lines were transiently transfected with a FADD expression vector in the presence of an equimolar amount of the pCMV β-galactosidase reporter and examined by phase contrast microscopy 24 hr later. The MCF7/ mutant CrmA cell lines expressing FADD (β-galactosidase-positive blue cells) displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Figure 7A, left). The nuclei of the rounded MCF7 cells exhibited apoptotic morphology indistinguishable from MCF7 cells treated with anti-Fas antibody in the presence of cycloheximide as assessed by propidium iodide staining (data not shown). In contrast, MCF7/CrmA cells transfected with FADD were significantly protected from cell death and the accompanying morphological alterations of apoptosis (Figure 7A, right). A quantitative representation of this data is shown in Table 1.

FADD-induced apoptosis is not peculiar to MCF7 cells, since a similar phenotype was observed in the B cell lymphoma cell line, BJAB, which is exquisitely sensitive to anti-Fas antibody–induced apoptosis (Tewari and Dixit, 1995). CrmA-expressing and vector control BJAB cell lines (Tewari and Dixit, 1995) were transfected with the pCMV β-galactosidase reporter in the presence or absence of an equimolar amount of an expression construct encoding FADD. As expected, expression of β-galactosidase alone in both the CrmA and vector clones did not induce apoptotic cell death as assessed by propidium iodide staining of nuclei of β-galactosidase-positive cells (data not shown). In contrast, however, the vector control cell line cotransfected with pCMV β-galactosidase and pcDNA3 AU1-FADD exhibited prominent apoptotic morphology including chromatin condensation and cellular shrinkage. More importantly, FADD-induced apoptosis, like Fas-induced apoptosis, was inhibited in the CrmA-expressing line. In the vector control lines, over 90% of the transfected cells
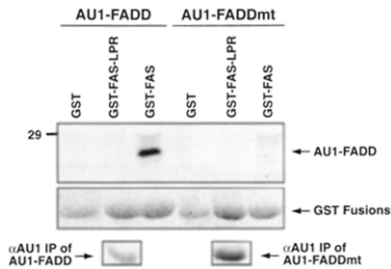

Figure 6. FADDmt Fails to Bind Fas, Suggesting a Death Domain to Death Domain Interaction

293T cells were transfected with AU1 epitope–tagged FADD (AU1-FADD) or AU1-FADDmt and metabolically labeled with [^{35}S]methionine and cysteine. Detergent lysates were prepared and incubated with various GST fusion proteins immobilized on glutathione–Sepharose beads. The samples were analyzed by SDS–PAGE and autoradiography (upper panel). The respective GST fusion proteins are shown as in Figure 4B (middle panel). To show that equivalent amounts of AU1-FADD and AU1-FADDmt were expressed and subsequently incubated with the beads, an aliquot of the respective lysates was immunoprecipitated with α-AU1 antibody and visualized by SDS–PAGE and autoradiography (bottom panels).

expressing FADD were apoptotic, while less then 10% exhibited similar morphology in the corresponding CrmA-expressing lines (Table 1). As a control, expression of AU1-TRAF1 (Rothe et al., 1994) and HA-CD40bp (Hu et al., 1994), which associate with the cytoplasmic domains of TNFR-2 and CD40, respectively, revealed less than 10% apoptotic morphology in either the CrmA or vector cell lines.

Delineation of the Death Effector Domain of FADD

Deletion mutants of FADD were constructed to determine the region responsible for initiating the cell death program (Figure 7B). As described above, MCF7 cells expressing
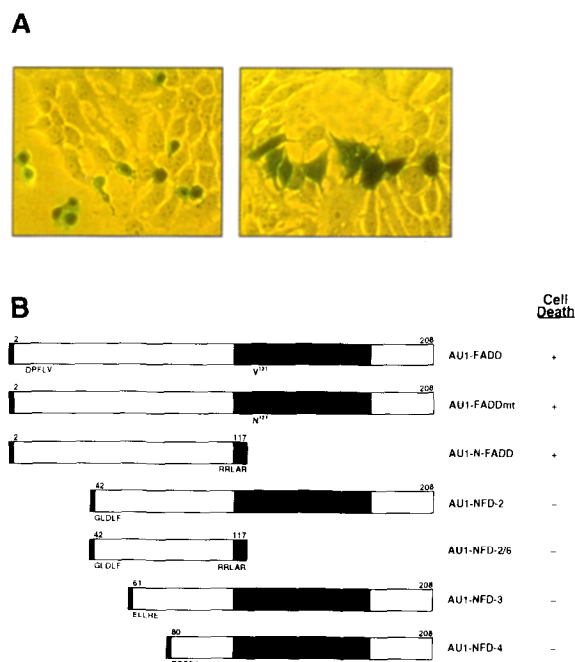
A

B

Figure 7. Functional Effects of Expression of FADD and mutants in MCF7 Cells

(A, left) Overexpression of FADD and β-galactosidase in MCF7/mutant CrmA cells. (Right) Overexpression of FADD and β-galactosidase in MCF7/CrmA cells. Cells were stained with X-Gal and examined by phase contrast microscopy. See Table 1 for details.

(B) Schematic representation of FADD and mutants. Amino acid residues are given for selected junctions. The point mutation of FADD (V121→N121) is represented by an asterisk. The gray and black rectangles represent the death domain of FADD and an AU1 epitope tag, respectively. Ability of the various mutants to induce cell death in MCF7 cells is described to the right of the schematic and is based on data from Table 1.

CrmA and mutant CrmA were transiently transfected with the various FADD mutant expression vectors. Interestingly, overexpression of FADDmt, a point mutant that fails to bind Fas, still induced cell death that was CrmA inhibitable and, in fact, was slightly more potent than wild-type FADD (81% versus 72% apoptotic cells). A FADD mutant (N-FADD) containing only 117 N-terminal amino acids was able to trigger cell death (Table 1; Figure 7B). Since a large portion of the death domain is absent from N-FADD, it is not surprising that it, like FADDmt, fails to associate with the cytoplasmic domain of Fas (data not shown). N-terminal deletions of FADD (NFD-2, NFD-3, and NFD-4) attenuated its ability to induce cell death (Table 1; Figure 7B). This analysis suggests that, whereas the Fas-interacting domain is in the C-terminal half of FADD, the death effector domain lies in its N-terminal portion.

Discussion

FADD was identified as a novel protein that associates specifically with the cytoplasmic domain of Fas in the yeast two-hybrid screen (Figure 1). A BLAST search using the amino acid sequence of FADD revealed a stretch of 80 amino acids that were significantly homologous to the death domain of Fas (Figure 2B). When this region of FADD was masked, the remaining sequences did not match any proteins in the data base. Interestingly, BLAST searches using the death domains of FADD, Fas, and TNFR-1 revealed a significant homology to the family of ankyrins (p < 0.001 for all three death domains) that mediate interaction between integral membrane components and cytoskeletal elements. More specifically, the respective death domains aligned with approximately 80 amino acids of the negative regulatory domain of ankyrin. A previous study reported that this region of ankyrin is homolo-

Table 1. The Effects of Overexpressing FADD and FADD Mutants in MCF7 and BJAB Cells

	Cell Lines			
	MCF7/CrmA Mutant	MCF7/CrmA	BJAB/Vector	BJAB/CrmA
β-galactosidase alone	12.9 ± 3.8	7.7 ± 4.0	6.0 ± 4.3	7.4 ± 4.3
AU1-FADD	72.1 ± 1.2	15.6 ± 4.8	91.6 ± 2.0	6.2 ± 3.6
AU1-FADDmt	81.2 ± 2.9	13.8 ± 8.4	ND	ND
AU1-N-FADD	69.9 ± 1.7	10.4 ± 6.1	ND	ND
AU1-NFD2	24.0 ± 8.8	10.9 ± 8.0	ND	ND
AU1-NFD2/6	13.6 ± 1.4	12.1 ± 7.5	ND	ND
AU1-NFD3	29.6 ± 4.2	13.0 ± 8.8	ND	ND
AU1-NFD4	21.2 ± 1.7	11.2 ± 6.2	ND	ND
AU1-TRAF1	ND	ND	5.5 ± 0.9	6.8 ± 2.6
AU1-CD40bp	ND	ND	10.8 ± 1.2	9.7 ± 1.4

Two previously characterized MCF7 and BJAB cell lines expressing CrmA and either a corresponding vector control line (Tewari and Dixit, 1995) or mutant CrmA line (Tewari et al., submitted) were transiently transfected with pCMV-β-galactosidase in the presence or absence of an approximately equimolar quantity of pcDNA3 (Invitrogen) expression constructs encoding AU1-FADD, FADD mutants (as designated in Figure 7B), AU1-TRAF1 (Rothe et al., 1994), or HA-CD40bp (Hu et al., 1994). After transfection (24 hr), MCF7 cells were fixed with 0.5% glutaraldehyde and stained with X-Gal for 4 hr. The data (mean ± SEM) shown are the percentage of round blue cells among total number of blue cells counted. Round cells were confirmed to be apoptotic by propidium iodide staining of nuclei (data not shown). After transfection (12 hr), BJAB cells were cytocentrifuged, methanol fixed, and stained for β-galactosidase and with propidium iodide. The data shown are the percentage of apoptotic cells among the total number of β-galactosidase-positive cells counted. The data were collected from at least three independent experiments. ND, not determined.

gous to the cytoplasmic domain of TNFR-1 (Peters and Lux, 1993), corroborating our observation. Why ankyrin contains a "death domain" remains unclear, but presumably this region is acting as a protein interaction domain.

In vitro and in vivo binding studies show that FADD specifically associates with the death domain of Fas, confirming the results of the yeast interaction assay. FADD failed to interact with Fas-LPR and Fas-FD8, a nonsignaling point mutant and deletion mutant, respectively, of the Fas death domain. Interestingly, upon deletion of the negative regulatory domain of Fas, an enhanced interaction with FADD was observed. Hence, a correlation exists between the cell-killing activity of the various Fas mutants and their association with FADD (Figure 5A). A weak association between FADD and TNFR-1 was observed in vitro (Figure 4B). In addition, β-galactosidase filter assays of yeast cotransformed with GAL4bd–Fas and GAL4ad–FADD turned blue within 1 hr, while those cotransformed with GAL4bd–TNFR-1 and GAL4ad–FADD turned blue overnight (the other cotransformed heterologous baits remained unchanged). If the weak interaction between FADD and TNFR-1 observed in yeast and in vitro proves to be significant, this would correlate with the relative potencies of Fas-dependent cell death and TNF-dependent cytotoxicity (Clement and Stamenkovic, 1994) and, furthermore, would suggest that TNFR-1 could interact with and utilize FADD or a FADD-like molecule as a proximal transducer of the death signal.

Having shown that FADD specifically binds the death domain of Fas, we wished to identify the corresponding interaction domain in FADD. Previous studies have shown that death domains have a propensity to self-associate (Boldin et al., 1995). It was thus reasonable to propose that the death domain of FADD was interacting with its homologous counterpart in Fas. As predicted, a point mutation in the death domain of FADD abrogated its association with Fas (Figure 6). In addition, a deletion encompassing the death domain of FADD (N-FADD) also abolished binding to Fas (data not shown). Our results support a model in which a death domain to death domain interaction is responsible for the binding of FADD to Fas.

Once the in vitro and in vivo association of FADD and Fas was established, we proceeded to determine a functional role for this novel Fas-binding protein. BJAB cells treated with α-Fas antibody undergo apoptosis within hours (M. T. and V. M. D., unpublished data), a time frame similar to FADD-induced killing. Likewise, CrmA-expressing BJAB cells are resistant to both Fas- and FADD-induced killing. To ensure that the induction of cell death by FADD was not a peculiarity of one cell type, we also examined the effects of FADD on another Fas-sensitive cell line, MCF7. As was seen in BJAB cells, overexpression of FADD in MCF7 cells induced apoptosis that was CrmA inhibitable and indistinguishable from Fas-induced killing.

Deletional analysis of FADD localized the death effector domain of FADD to its N-terminal portion (Figure 7B). In fact, the N-terminal amino acids (1–117) are sufficient to cause death and deletions of the N-terminus attenuate the cell-killing ability of FADD. In addition, the data refutes the

possibility that FADD causes cell death by simply aggregating the Fas antigen intracellularly, since both N-FADD and FADDmt trigger apoptosis without binding Fas. We are currently investigating the intriguing possibility that the C-terminus of FADD can act as a dominant negative regulator of Fas-induced signaling.

Overexpression of FADD induces apoptosis in a Fas ligand–independent fashion. A model consistent with our data is that endogenous FADD is associated with the death domain of the Fas receptor in a "resting" cell. However, upon ligand-mediated oligomerization of the Fas antigen, intracellular aggregation of the Fas death domains might displace FADD, triggering a death response, presumably mediated via the N-terminus of FADD interacting with a downstream component of the cell death pathway.

Experimental Procedures

Yeast Two-Hybrid System

The cytoplasmic domains of Fas, Fas-FD8, TNFR-1, Δ-TNFR-1, CD40, and CD28 were obtained by PCR and cloned in-frame, as confirmed by sequencing, into the GAL4 DNA-binding domain (GAL4bd) vector pAS1CYH2. Full-length A20 and B94 were similarly cloned into the bait vector. GAL4bd–Fas was cotransformed with a prey plasmid containing a human B cell cDNA expression library fused to the GAL4 activation domain (GAL4ad) in the pACT plasmid. A more detailed account of the plasmids used and the procedure for the yeast two-hybrid system can be found in our previous work (Hu et al., 1994).

DNA Sequencing and Data Base Searching

Double-stranded plasmid template was sequenced on both strands by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation). Manual sequencing was confirmed by subsequent automated sequencing. Network BLAST searches were conducted using the NCBI on-line service. Sequences were compared using the MegAlign (DNASTAR) software.

cDNA Cloning

A random-primed cDNA library was constructed in the pcDNA1 vector (Invitrogen) from TNF–cycloheximide-treated human umbilical vein endothelial cell poly(A)+ RNA. Colonies (5 × 10^5) were screened with a ^32P random-labeled XhoI restriction fragment of the yeast prey plasmid encoding GAL4ad–FADD (clone 15) using standard techniques (Sambrook et al., 1989)

Northern Blot Analysis

Adult and fetal human multiple tissue Northern blots (CLONTECH) were hybridized, according to the instructions of the manufacturer, using radiolabeled cDNA insert obtained from an XhoI digestion of the yeast prey plasmid encoding GAL4ad–FADD (clone 15).

Expression Vectors

FADD was cloned into a modified pcDNA3 expression vector (Invitrogen) in which an HA epitope tag (YPYDVPDYA) had previously been placed downstream of the cytomegalovirus promoter/enhancer (pcDNA3 HA-FADD). In addition, an AU1 epitope (DTYRYI)-tagged FADD and mutants were made with PCR primers encoding the epitope and using the FADD cDNA as template (pcDNA3 AU1-FADD). FLAG (DYKDDDDK)-tagged constructs of Fas and mutants were also made in pcDNA3 using full-length Fas as a template. The 5' FLAG PCR primer was engineered to encode a FLAG epitope five amino acids downstream of the the putative signal sequence site of Fas and is as follows: AAG CCT GGT ACC ATG CTG GGC ATC TGG ACC CTC CTA CCT CTG GTT CTT ACG TCT GTT GCT AGA TTA TCG TCC AAA GAC TAC AAG GAC GAC GAT GAC AAG AGT GTT AAT GCC CAA GTG. The amplified products were then cloned into the KpnI–XhoI site of pcDNA3. pcDNA3 AU1-FADDmt and pcDNA3 FLAG-Fas-LPR were made by site-directed mutagenesis using a two-step PCR proto-

col (Higuchi et al., 1988). The V121→N121 and V238→N238 mutations, respectively, were confirmed by sequence analysis.

GST Fusion Protein Expression and In Vitro Binding Assay

The cytoplasmic domains of Fas, Fas-FD5, Fas-FD8, and TNFR-1 were amplified by PCR using appropriate templates and primers and cloned in-frame into pGSTag (Ron and Dressler, 1992). Fas-LPR was made by site-directed mutagenesis using a two-step PCR protocol (Higuchi et al., 1988) and cloned into pGSTag. The V238→N238 mutation was confirmed by sequence analysis. The pGSTag constructs were then transformed into the E. coli strain BL21(DE3)pLysS (Studier, 1991). GST and GST fusions were prepared using published procedures (Studier, 1991), and the recombinant proteins immobilized onto glutathione–agarose beads (Harper et al., 1993).

Labeled FADD was prepared by in vitro transcription/translation using TNT T7-coupled reticulocyte lysate system from Promega according to the instructions of the manufacturer, using pcDNA3 HA-FADD as template.

Following translation, equal amounts of total ^{35}S-labeled reticulocyte lysate were diluted into 150 μl of GST binding buffer (50 mM Tris [pH 7.6], 120 mM NaCl, 1% Brij) and incubated for 2 hr at 4°C with the various GST fusion proteins complexed to beads, following which beads were pelleted by pulse centrifugation, washed three times in GST buffer, boiled in SDS sample buffer, and resolved on a 10% SDS–polyacrylamide gel. Bound proteins were visualized following autoradiography at −80°C.

Lysates of FADD or FADDmt-transfected 293T cells were processed as above, except that the GST binding buffer also had 10% glycerol and a protease inhibitor cocktail. For some experiments, the complexed GST beads were dissociated by boiling in PBS plus 1% SDS, diluted 10-fold in PBS containing 1% deoxycholate, and subsequently subjected to immunoprecipitation analysis.

Transfection, Metabolic Labeling, and Immunoprecipitation Analysis

These were performed essentially as described previously (O'Rourke et al., 1992). For reimmunoprecipitation analysis, the initial immune complex was dissociated by boiling in PBS plus 1% SDS, diluted 10-fold in PBS containing 1% deoxycholate, and subjected to a second round of immunoprecipitation analysis.

Functional Assay and Immunocytochemistry

BJAB and MCF7 clonal cell lines stably transfected with either vector, CrmA, or CrmA mutant expression constructs were utilized (Tewari and Dixit, 1995; Tewari et al., submitted). For transient transfection of BJAB lines, 5 × 10⁶ cells were electroporated at 220 V, 960 μF in 0.4 cm cuvettes (Bio-Rad) using 20 μg of pCMV β-galactosidase plus or minus 30 μg of pcDNA3 AU1-FADD. After 12 hr, cells were cytocentrifuged, fixed with 1% paraformaldehyde, permeabilized with 0.1% Triton/PBS, blocked with horse serum, and incubated with rabbit anti-β-galactosidase (1:200 dilution, Cappel) for 1 hr. The cells were subsequently washed with PBS, incubated with biotinylated anti-rabbit antibody (1:200 dilution, Vector Laboratories) for 20 min, washed with PBS, and incubated with Avidin-FITC (1:100 dilution, Vector Laboratories) for 20 min. The nuclei were stained with a 10 μg/ml solution of propidium iodide (Sigma) for 10 min. Cells were visualized by fluorescence microscopy using a FITC range barrier filter cube. At least 100 β-galactosidase-positive cells were counted for each transfection (n = 3) and identified as apoptotic or nonapoptotic.

MCF7 lines were transiently transfected with 5 μl of lipofectamine (GIBCO-BRL) at 50% confluency in 6-well dishes using 0.25 μg of pCMV β-galactosidase plus or minus 1 μg of AU1-FADD or mutants. Transfections were carried out in 1 ml of OptiMEM Minimal Media (GIBCO-BRL) and incubated for 5 hr after which 1 ml of serum-containing growth media was added. After 24 hr, cells were fixed with 0.5% glutaraldehyde and stained with X-Gal for 4 hr. Cells were visualized by phase-contrast microscopy.

Acknowledgments

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GenBank Accession Number

The accession number for the human FADD cDNA sequence reported in this paper is U24231.

Note Added in Proof

In the April 7, 1995, issue of the Journal of Biological Chemistry, Boldin et al. independently utilized a Fas bait in the yeast two-hybrid system and cloned a cDNA that is nearly identical to the FADD cDNA. Consistent with our results, they report that expression in this protein is cytotoxic to Hela cells and that it interacts with Fas in vivo.