CD95 (Fas/APO-1) and tumor necrosis factor receptor-1 (TNFR-1) are related molecules that signal apoptosis. Recently, a number of novel binding proteins have been proposed to mediate the signaling of these death receptors. Here we report that an N-terminal truncation of one of these candidate signal transducers, FADD/MORT1, abrogates CD95-induced apoptosis, ceramide generation, and activation of the cell death protease Yama/CPP32. In addition, this dominant-negative derivative, and activation of the cell death protease MORT1, abrogates CD95-induced apoptosis, ceramide of one of these candidate signal transducers, FADD/MORT1, abrogates CD95-induced apoptosis, ceramide generation, and activation of the cell death protease Yama/CPP32. In addition, this dominant-negative derivative of FADD (FADD-DN) blocked TNF-induced apoptosis while not affecting NF-kB activation. FADD-DN bound both receptors, and in the case of CD95, it disrupted the assembly of a signaling complex. Taken together, our results functionally establish FADD as the apoptotic trigger of CD95 and TNFR-1.

Recently, major advances have been made in understanding the signal transduction of the tumor necrosis factor (TNF)/nerve growth factor receptor family. Activation of these receptors is caused by aggregation mediated by the respective ligands or agonist antibodies (1–4). There are no identifiable catalytic motifs (e.g. kinase or phosphatase) in the cytoplasmic domains of these cell surface proteins. Instead, it is becoming apparent that signal transduction is accomplished via association with an emerging class of novel and diverse signaling molecules. For example, a dominant-negative mutant of TRAF2 was shown to block TNFR-2-mediated NF-kB activation (5), while an analogous mutant of CD40bp (also known as TRAF3, C, or LAP1 (6–8)) was shown to inhibit CD40-mediated up-regulation of CD23 (7). Numerous candidate signal transducers have been identified for the two death receptors, TNFR-1 and CD95, including FADD/MORT1, TRADD, RIP, F-1, FAF1, and TRAP1 (9–13). Both death receptors share a region of homology of about 80 amino acids in their cytoplasmic domain required to signal apoptosis (14, 15). This shared “death domain” suggests that both receptors engage a common component of the apoptotic machinery. Here, we investigated the role of FADD in the proximal signal transduction of CD95 and TNFR-1.

MATERIALS AND METHODS

Antibodies and Reagents—The anti-CD95 monoclonal antibodies used in this study include anti-Fas IgM (Upstate Biotechnology, Inc.), anti-APO-1 (lgG3, (2)), and phosphatidylethanolamine-conjugated anti-Fas (MBL Inc.). Anti-AU1 murine ascites was obtained from Babco, Inc. Anti-PARP antibody was clone C-2-10, which is described previously (16), and recognizes an epitope near the N terminus of PARP located between amino acids 216 and 375 (generous gift of Dr. G. Poirier). Antibodies were raised against recombinant GST-FADD fusion protein as described previously (17). Rabbit anti-peptide antibodies (Lampire) were raised against NKKNFHKSSTGMSRSGTD of the p17 subunit of Yama and STAPGYSSWRNSKDGS of the p12 subunit. C-terminal (CD8-erythro) and C2-erythro (CD4-erythro) were purchased from Matreya, Inc. and dissolved in ethanol.

Cell Lines and Culture—The B lymphoma cell line BJ AB and the breast carcinoma cell line MCF7 were grown in RPMI 1640 complete medium (10% heat-inactivated fetal bovine serum (Hyclone), l-glutamine, penicillin/streptomycin, and non-essential amino acids). MCF7-Fas cells, as described previously (18), were grown in RPMI 1640 complete media supplemented with 0.5 mg/ml G418 (Life Technologies, Inc.). BJ AB and MCF7 stable cell lines were grown in complete media supplemented with 0.3 and 0.5 mg/ml G418, respectively.

Stable and Transient Transfections—To generate the pooled stable cell lines, BJ AB-FADD-DN and MCF7-FADD-DN, cells were transfected with pDNA3-FADD-DN using a protocol described previously (19). From the pooled populations, individual clones were obtained and plated in duplicate on 48-well Costar plates. One set of cells was treated with anti-Fas IgM (100 ng/ml), and clones resistant to Fas-induced cell death were identified. The untreated, resistant clones were then pooled to obtain BJ AB-sFADD-DN (which represents a pool of seven resistant clones) and MCF7-sFADD-DN (which represents a pool of nine resistant clones). All stable lines generated were assessed for expression of FADD-DN by immunoblotting. MCF7 cells were transiently transfected with lipofectamine as described previously (9).

Cell Death and Viability Assays—To assess nuclear morphology, fluorescent DNA-staining dyes were utilized as described previously (19). DNA fragmentation (TUNEL staining) was determined using the in situ cell death detection kit (Boehringer Mannheim). BJ AB cells were air-dried onto Colorfrost/Plus microscope slides (Fisher) using 4% parafomaldehyde, and the manufacture’s protocol was followed. The TUNEL-stained cells were then counterstained with propidium iodide (10 μg/ml) and visualized by fluorescence microscopy. To assess cell viability, the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide conversion assay was done on the BJ AB cell lines as described previously (20).
**FIG. 1.** **FADD mediates CD95 signal transduction.** A, schematic representation of FADD and FADD-DN (NFD-4). Amino acid residues are given for selected junctures. B, BJAB cells expressing FADD-DN are resistant to CD95-induced apoptosis. The indicated cell lines were incubated for 16 h with various concentrations of anti-Fas IgM and cell death assessed by nuclear morphology. At least 250 cells were counted in three independent experiments (mean ± S.D.). Expression of FADD-DN is shown in the photographic insets. FADD-DN migrates as a doublet around 18 kDa due to post-translational modification(23). The TUNEL assay is shown in the graphical inset, and at least 250 cells were counted in three independent experiments (mean ± S.D.; n = 3). Significant levels of ceramide could not be detected at 5-, 10-, 30- and 60-min timepoints (inset). D, C2-ceramide (C2), but not C2-dihydroceramide (DH-C2), can bypass the dominant negative effect of the FADD derivative. The cells characterized include BJAB vector, BJAB-FADD-DN, and BJAB-sFADD-DN. As a control, cells were also exposed to the structurally related inactive analog, C2-dihydroceramide (33). Viabilities were not decreased significantly,
FADD Mediates the Death Signal of CD95 and TNFR-1

Fig. 2. FADD mediates TNF-induced NF-κB activation. A, the stable cell lines utilize include MCF7-sFADD-DN, which represents a pool of nine resistant clones and a corresponding MCF7 vector control. Expression of FADD-DN is shown in the left panels. FADD-DN migrates as a doublet around 18 kDa, possibly due to post-translational modification. The indicated cell lines were either left untreated (UnRx) or treated with anti-Fas IgM (250 ng/ml) plus cycloheximide (CHX, 10 μg/ml) or 100 ng/ml TNF for 24 h and stained with propidium iodide. Similar results were obtained using anti-APC-1 antibody plus soluble protein A in the absence of cycloheximide (data not shown). Phase contrast micrographs are shown with corresponding confocal micrographs (insets) depicting nuclear morphology. B, MCF7 vector or MCF7-sFADD-DN cells were transfected with an NF-κB-dependent E-selectin-luciferase reporter construct (5) and were either untreated or treated with TNF for 9 h. Luciferase activities were assessed as described previously (5), and values shown are mean ± S.D. of three independent experiments.

Ceramide Assays—Ceramide levels were determined by a modified diacylglycerol kinase assay (21, 22) as described previously (20).

Immunoprecipitations, Western Blotting, and Two-dimensional Gels—Immunoblotting of cell lysates for PARP was carried out as described previously (21). Yama processing was assessed using x 107 B) AB vector and B) AB-sFADD-DN cells untreated or treated with 10 ng/ml anti-Fas IgM for 18 h. Cells were then lysed in 60 μl of 0.1% Nonidet P-40, free-thawed three times, and centrifuged at 14,000 rpm for 20 min. Cytoplasmic extracts were carefully added to sample buffer and run on a 15% gel, transferred to a nitrocellulose membrane, and immunoblotted with antibodies directed against the p17 and p12 subunits of Yama. To show expression of FADD-DN in B) AB and MCF7 cells, cells were immunoprecipitated in phosphate-buffered saline-TDS (TDS, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS) with anti-AU1 antibody (1:1000) and Western blotted with anti-FADD polyclonal antisera (1:1000). Immunoprecipitation of the DISC and analysis on two-dimensional gels was done as described previously (23). Alternatively, cell lysates were immunoprecipitated with anti-AU1 antibody coupled to protein A-Sepharose beads as described previously (23).

NF-κB Assay—MCF7 vector and MCF7-sFADD-DN cells were transfected with the NF-κB-dependent E-selectin reporter construct and luciferase activity assessed as described previously (5).

Table I

| % | Untreated | Anti-Fas IgM | Tumor necrosis factor | Staurosporine | ICE |
|---|----------|-------------|----------------------|---------------|-----|
| MCF7-Vector | 85.8 ± 5.0 | 82.9 ± 5.5 | 86.4 ± 0.8 | 6.0 ± 1.8 | 35.5 ± 1.8 |
| MCF7-FADD-DN | 18.1 ± 6.0 | 51.0 ± 5.7 | 80.5 ± 3.7 | 2.8 ± 2.1 | 26.5 ± 8.7 |
| MCF7-sFADD-DN | 6.0 ± 5.5 | 82.9 ± 0.6 | 86.4 ± 0.8 | 5.5 ± 3.0 | 35.5 ± 1.8 |

**RESULTS AND DISCUSSION**

Overexpression of FADD causes apoptosis (9), resulting in cleavage of the death substrate poly(ADP-ribose) polymerase (PARP) to signature apoptotic fragments (data not shown). A previously reported deletion mutant of FADD, NF4D (hereon referred to as FADD-DN), was able to interact with CD95, but failed to initiate apoptosis (9) (Fig. 1A), suggesting that it may have a dominant negative effect on CD95 signaling. FADD-DN lacks 80 N-terminal amino acids, but contains the death domain responsible for association with the related death domain of CD95 (9). The B lymphoma cell line B) AB was transfected with either the expression vector pcDNA3 alone or as a FADD-DN expression construct. Stable transfectants were generated by neomycin (G418) selection and pooled populations assessed for FADD-DN expression and sensitivity to anti-Fas-induced apoptosis (B) AB-FADD-DN, Fig. 1B). Expression of FADD-DN in both a pooled population and in a mixture of selected clones (B) AB-sFADD-DN) dramatically abrogated CD95-induced cell death (Fig. 1B). The apoptotic nature of the cell death was confirmed by the TUNEL assay which detects 3’-OH DNA strand breaks. The FADD-DN expressing B) AB cells were not inherently resistant to apoptotic cell death, since the protein kinase inhibitor staurosporine and the calcium ionophore A23187 equally killed the three cell lines (data not shown). CD95 surface expression was equivalent in the vector and FADD-DN cell lines as assessed by flow cytometry (data not shown). The possibility that clonal variation in the stable lines was responsible for the observed resistance to CD95 killing was discounted by the observation that transient overexpression of the FADD derivative ablated CD95-induced cell death in B) AB and J urkat cells (data not shown).

The sphingolipid ceramide has been implicated as a signaling intermediate in the CD95 pathway (20, 24–26). To determine whether this signaling event was blocked by the FADD derivative, B) AB cells expressing FADD-DN were treated with anti-Fas IgM and, subsequently, ceramide levels assessed. Consistent with a proximal role of FADD in CD95 signaling, thus validating the specificity of the cytotoxic effect of C2-ceramide. The x axis refers to the concentration of synthetic ceramide used and the y axis refers to viability as assessed by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide conversion. Viability is expressed as percentage of vehicle-treated control ± S.E. Results are representative of three independent experiments. E, CD95-induced activation of the apoptotic protease YamaCPP32 is blocked by FADD-DN. B) AB vector and B) AB-sFADD-DN were left untreated or treated with 100 ng/ml anti-Fas IgM for 18 h. Lysates were then run on a 15% gel and immunoblotted with polyclonal antibodies directed against the p17 and p12 subunits of Yama (upper panel). Cleavage of the death substrate poly(ADP-ribose) polymerase was also assessed (lower panel).
FADD Mediates the Death Signal of CD95 and TNFR-1

FADD-DN inhibited CD95-mediated ceramide generation (Fig. 1C). Additionally, vector and FADD-DN transfected BJ AB cells were equally susceptible to cell death induced by the cell-permeable, active ceramide analogue, C2-ceramide, confirming that the block in the death pathway was prior to ceramide generation (Fig. 1D).

Mammalian homologs of the Caenorhabditis elegans cell death protease CED-3 are thought to be distal effectors of the CD95 cell death pathway. Here we show that the apoptotic protease Yama/CPP32 (17, 27, 28) is activated by CD95 engagement (Fig. 1E). Endogenous Yama is expressed as a 32-kDa pro-enzyme and upon activation is proteolytically processed into active p17 and p12 subunits (17, 27). One of the proposed substrates of Yama is the nuclear enzyme PARP (17, 27). As expected, CD95-mediated activation of Yama and resulting PARP cleavage was blocked by FADD-DN (Fig. 1E).

In the yeast two-hybrid assay, FADD had a weak but specific interaction with TNFR-1 (9). To determine whether FADD has a role in TNFR-1-induced cell death, the FADD derivative was transfected into TNF-sensitive MCF7 breast carcinoma cells and stable cell lines generated. Interestingly, FADD-DN expressing MCF7 cells were equally resistant to both CD95- and TNF-induced cell death (Fig. 2A, Table I), suggesting a proximal convergence of the cytokine-mediated cell death pathway. Additionally, 293T cells transiently overexpressing TNFR-1 (data not shown). As with the BJ AB cell lines, the FADD-DN MCF7 cell lines were not resistant to staurosporine-induced apoptosis (Table I). Overexpression of interleukin-1β converting enzyme (ICE) could “bypass” the dominant-negative effect of the FADD derivative, suggesting that the death pathway was being blocked upstream of the ICE-like proteases implicated in the apoptotic pathway.

While the main activity of CD95 is to trigger apoptosis, TNFR-1 can signal an array of diverse pro-inflammatory and immunoregulatory activities (29). Distinct from CD95, TNFR-1 is an inducer of nuclear factor-κB (NF-κB) (30). We therefore investigated whether expression of FADD-DN modulated TNF-induced NF-κB activity. MCF7 vector and MCF7-sFADD-DN cells were transfected with an NF-κB-dependent reporter gene (5) and relative NF-κB activity assessed (Fig. 2B). In both cell lines, NF-κB was activated equally well, suggesting that FADD-DN was identified in activated sFADD-DN cells by immunoblotting using anti-AU1 antibody (lower right panel).
TNFR-1 utilizes FADD to transduce the death signal and activates NF-κB by a different mechanism.

To determine the mechanism by which the FADD derivative exerts its dominant negative effect, co-immunoprecipitation of FADD and FADD-DN with CD95 or TNFR-1 was assessed. 293T cells were co-transfected with AU1 epitope-tagged FADD constructs and FLAG epitope-tagged CD95, FLAG-TNFR-1, or FLAG-B94 (31). Cell lysates were immunoprecipitated with anti-FADD antibody and subsequently immunoblotted with anti-FLAG antibody. TNFR-1 and CD95, but not a control cytoplasmic protein, B94, co-immunoprecipitated with FADD-DN (Fig. 3A). The association of FADD and FADD-DN with CD95 was 10-fold greater than with TNFR-1, correlating with the relative apoptotic potential of the two death receptors (32). Thus, our data suggest that FADD-DN exerts its inhibitory action by directly or indirectly forming a complex with the death receptors, preventing recruitment of endogenous FADD.

It remains formally possible, however, that FADD-DN functional action by directly or indirectly forming a complex with the death receptors, preventing recruitment of endogenous FADD.

TNFR-1 DISC, which is formed upon TNFR-1 engagement of FADD is both a necessary and sufficient mediator of CD95 and TNFR-1-induced apoptosis as overexpression of FADD engages the cell death machinery (9), while a truncated derivative acts as a potent dominant-negative regulator. Future studies will hopefully elucidate downstream components of the pathway, thus linking FADD to the apoptotic proteases of the ICE/ced-3 family.

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FADD/MORT1 Is a Common Mediator of CD95 (Fas/APO-1) and Tumor Necrosis Factor Receptor-induced Apoptosis

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