FADD Is Required for Multiple Signaling Events Downstream of the Receptor Fas

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Abstract
To identify essential components of the Fas-induced apoptotic signaling pathway, Jurkat T lymphocytes were chemically mutagenized and selected for clones that were resistant to Fas-induced apoptosis. We obtained five cell lines that contain mutations in the adaptor FADD. All five cell lines did not express FADD by immunoblot analysis and were completely resistant to Fas-induced death. Complementation of the FADD mutant cell lines with wild-type FADD restored Fas-mediated apoptosis. Fas activation of caspase-2, caspase-3, caspase-7, and caspase-8 and the proteolytic cleavage of substrates such as BID, protein kinase C, and poly(ADP-ribose) polymerase were completely defective in the FADD mutant cell lines. In addition, Fas activation of the stress kinases p38 and c-Jun NH2 kinase and the generation of ceramide in response to Fas ligation were blocked in the FADD mutant cell lines. These data indicate that FADD is essential for multiple signaling events downstream of Fas.

Introduction
Fas (APO-1/CD95) is a transmembrane receptor belonging to the TNF family of death receptors (1, 2). Cross-linking of Fas with Fas ligand or agonistic antibody (FasAb) results in the initiation of an apoptotic signal that ultimately leads to the demise of the cell (3, 4). Fas plays an important role in the immune system. The Fas ligand-Fas system is involved in the deletion of autoreactive T cells, the down-regulation of the immune response, and the maintenance of sites of immune privilege (2, 5). The importance of Fas in the immune system is underscored by the fact that mice and humans with mutations in Fas exhibit symptoms of autoimmune diseases (6–8).

The intracellular domain of Fas contains a protein-protein interaction motif of about 100 amino acids called the death domain. Using yeast two-hybrid and other techniques, several proteins have been shown to interact with the intracellular domain of Fas, including FADD (9, 10), DAXX (11), RIP (12), FAP-1 (13), FAP-1 (14), and Sensem (15). In addition, the adaptor FADD was biochemically shown to be recruited to the death-inducing signaling complex upon receptor cross-linking (16). FADD is also capable of interacting with the cysteine protease caspase-8, which resides at the apex of the cysteine protease cascade and is required for Fas-induced apoptosis (1, 16–21). FADD and RIP have recently been homozogously deleted in mice (22–24). RIP does not appear to be required for Fas-mediated apoptosis because RIP-deficient cells are completely sensitive to Fas-induced death. However, RIP is involved in TNF activation of nuclear factor-kB (22, 25). FADD-deficient mice die in utero and exhibit abdominal hemorrhage and defects in heart development (23). FADD null cells are completely resistant to Fas-induced death, indicating that FADD plays an important role in Fas-mediated apoptosis (23, 24). Studies from FADD-deficient mice and transgenic mice expressing a dominant-negative allele of FADD also indicate that FADD is required for T-cell activation-induced proliferation (24, 26, 27). Other Fas-interacting proteins, such as DAXX, FAP-1, and FAP-1, have also been implicated in Fas-mediated apoptosis (11, 13, 14); however, the physiological relevance of these proteins is not yet clear because gene knockouts or somatic cell mutants have not been generated.

We used an unbiased forward genetic approach to identify essential components of the Fas-induced apoptotic signaling pathway. We randomly mutagenized Jurkat T lymphocytes and selected for cell lines resistant to Fas-mediated apoptosis. We obtained cell lines with defects in Fas, FADD, or caspase-8. Previous studies of T cells from FADD null mice or mice expressing dominant-negative FADD did not biochemically characterize FADD-regulated signal transduction. In this study, we used the FADD-deficient Jurkat cells to investigate the role of FADD in multiple signaling events downstream of Fas.

Received 7/7/99; revised 9/7/99; accepted 9/8/99.

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A variety of techniques were used to demonstrate that 23 of the Fas-resistant mutant cell lines contained mutations in the receptor Fas (data not shown). However, five cell lines contained mutations in the adaptor protein, FADD. RT-PCR analysis using primers specific for FADD indicated that all of the mutant cell lines contained correctly sized FADD RNA products (data not shown). Western blot analysis with antibodies to FADD indicated that in contrast to wild-type A3 cells, which express FADD, five mutant cell lines, I2.1, I5, I6.2, E1, and S4, do not express FADD at the protein level (Fig. 2A). As a control, we show that Fas-resistant cell line I4.1, which contains a mutation in the death domain of Fas, expresses wild-type levels of FADD (Fig. 2A and data not shown). In addition, all five FADD mutant cell lines express wild-type levels of caspase-8, DAXX, bcl-2, bcl-xL, and MAPK (Fig. 2A). The FADD mutant cell lines all express wild-type levels of Fas as determined by surface FACS analysis and RT-PCR (data not shown). Wild-type cells (A3), FADD, or Fas mutant cells were treated with FasAb for various amounts of time, and the extent of cell death was determined using trypan blue dye exclusion and DNA ladders (Fig. 2B and data not shown). Whereas wild-type cells died over time and generated DNA ladders, the FADD and Fas mutant cell lines were completely resistant to Fas-mediated apoptosis (Fig. 2B and data not shown). To characterize signaling downstream of FADD, we focused on two FADD mutant cell lines, I2.1 and I6.2.

**Complementation of the FADD-deficient Cell Lines.** To investigate whether the FADD mutation is the only defect in the Fas pathway in these mutant cell lines, we complemented the mutant cell lines with wild-type FADD. Transfection of wild-type FADD but not empty vector into the FADD mutant cells rescued the ability of FasAb to induce apoptosis (Fig. 2C). FasAb induced apoptosis in the wild-type cells whether they were transfected with empty vector or wild-type FADD (Fig. 2C). These data suggest that mutant FADD is the sole genetic lesion in the Fas pathway in these mutant cells and provides genetic evidence that FADD is required for Fas-induced death.

**FADD Is Required for Fas Activation of Multiple Caspases.** We used the FADD-deficient cell lines to dissect signaling events downstream of Fas. Through its death effector domain, FADD is capable of recruiting and oligomerizing the cysteine protease caspase-8, which can subsequently initiate the caspase cascade (18–21, 30–33). DAXX may also be recruited to the Fas death domain, providing a FADD-independent mechanism of coupling Fas to the cysteine protease cascade (11). Although our study and data from others indicate that FADD is required for Fas-induced death (23, 24), careful biochemical characterization of signaling downstream of FADD has not yet been carried out. Wild-type, FADD, or Fas mutant cell lines were treated with FasAb for various amounts of time, and cell extracts were prepared. We tested the ability of Fas-activated proteases in wild-type and mutant cell extracts to cleave PARP and pro-caspase-2 in vitro. PARP and pro-caspase-2 were proteolytically cleaved in extracts from FasAb-treated wild-type but not FADD or Fas mutant cells (Fig. 3A). We next examined the ability of FasAb to activate multiple caspases in vivo by
Fig. 2. Isolation and initial characterization of FADD mutant cell lines. 
A, five mutant cell lines do not express FADD protein. Lysates from wild-type (A3) and mutant cell lines were Western blotted for expression of FADD, caspase-8, DAXX, bcl-2, bcl-xL, and MAPK. A3, wild-type; I4.1, Fas mutant. B, wild-type (A3), FADD (I2.1, I5, I6.2, E1, and S4), or Fas (I4.1) mutant cell lines were either left untreated (○, □) or treated with FasAb (●) for various amounts of time. Cell death was monitored by trypan blue dye exclusion and DNA laddering (data not shown). Bars, SD. C, complementation of FADD mutant cell lines I2.1 and I6.2. Wild-type (A3) or FADD mutant (I2.1 and I6.2) cells were transfected with a surface marker, CD8, and empty pRSV vector (1:3 ratio) or CD8 and pRSV-FADD (1:3 ratio). Cells were either left untreated or treated with FasAb for 12 h. Cells were stained with anti-CD8-FITC and propidium iodide. Transfected, viable cells were identified by FACS on the basis of CD8 expression and propidium iodide exclusion. The number of viable, CD8-positive cells is expressed as a percentage relative to the untreated controls. Data represent the means; bars, SD; n = 3.
using immunoblot analysis. FasAb treatment of wild-type cells but not FADD or Fas mutant cells resulted in the proteolytic cleavage and presumed activation of caspases with long prodomains, such as caspase-2 and caspase-8, and the caspase-8 substrate BID (Refs. 34, 35; Fig. 3B). Furthermore, FasAb activated proteases capable of cleaving the
effector proteases caspase-3, caspase-7, and substrates PKC-\(\delta\) and PARP in wild-type cells but not in FADD or Fas mutant cells (Fig. 3C). These data suggest that FADD is essential for Fas activation of multiple caspases and that a parallel pathway linking Fas to the caspase cascade through DAXX does not appear to function in Jurkat cells in the absence of FADD.

**FADD and Caspase-8 Are Required for Fas-induced Ceramide Generation.** The sphingolipid ceramide is generated in response to multiple apoptotic stimuli and has been proposed to mediate the death signal (36). Ceramide can activate several signaling proteins such as ceramide-activated protein kinase, PKC-\(\zeta\), and ceramide-activated protein phosphatase; however, the precise mechanism by which ceramide induces apoptosis is not yet clear (36). Fas receptor cross-linking has been shown to result in an increase in sphingomyelinase activity and intracellular ceramide (36–40). Several studies have suggested that ceramide plays an important role in mediating the Fas death signal (36–40). However, the origin of the signal emanating from Fas that leads to increased ceramide levels is not known. We sought to determine whether FADD and caspase-8 are required for Fas-induced increases in endogenous ceramide levels.

FasAb treatment of wild-type but not FADD or caspase-8 mutant cells resulted in an increase in intracellular ceramide (Fig. 4). These results indicate that FADD and caspase-8 mediate the Fas—ceramide signal and are required for the generation of ceramide in response to Fas ligation.

**FADD Is Required for Fas Activation of p38 and JNK.** We and others have shown previously that Fas cross-linking results in the activation of the stress kinases, p38 and JNK, in a caspase-dependent manner (41–45). We determined whether FADD was required for Fas activation of p38 and JNK. Fas cross-linking of wild-type cells resulted in the activation of p38 and JNK. In contrast, Fas cross-linking was unable to activate p38 and JNK in the FADD or Fas mutant cell lines, suggesting that FADD is required for Fas activation of the stress kinases (Fig. 5A). However, cotransfection of FADD and p38 or FADD and JNK did not result in the activation of the stress kinases, suggesting that overexpression of FADD is not sufficient to activate the stress kinases (Fig. 5B). In contrast, osmotic shock was still capable of activating transfected p38 and JNK (data not shown). These data suggest that FADD is required for Fas activation of p38 and JNK but is not sufficient for their activation.
Discussion
We used an unbiased forward genetic approach in mammalian cells to identify essential components of the Fas-mediated apoptotic signaling pathway. We obtained several Fas-resistant cell lines that contained mutations in the receptor Fas, the adaptor FADD, and the cysteine protease caspase-8. Complementation of wild-type FADD (Fig. 2C) or caspase-8 (20) back into the FADD or caspase-8 mutant cell lines, respectively, restored Fas-mediated apoptosis, suggesting that these are the only defects in the Fas pathway in these cells. Because we used an unbiased genetic screen and obtained cell lines with specific mutations in FADD and caspase-8, this study supports recent results from mouse knockouts by providing genetic evidence that FADD and caspase-8 are required for Fas-mediated apoptosis (23, 24). We did not obtain mutations in other proteins reported to associate with Fas. This may be attributable simply to the fact that our mutagenesis was not saturating. However, it is also possible that other proteins implicated in the Fas pathway are not essential for Fas-mediated apoptosis or have redundant functions downstream of FADD and caspase-8.

Five mutant cell lines do not express FADD by immunoblot analysis but appear to express wild-type levels of several other proteins, including DAXX and caspase-8 (Fig. 2A). Recent studies have suggested that DAXX can bind Fas and mediate a FADD-independent apoptotic signal (11, 46). All five FADD-deficient cell lines are completely resistant to Fas-mediated apoptosis, indicating that in Jurkat cells, there are no other major parallel pathways. FADD null lymphocytes and embryonic fibroblasts from homozygously deleted mice are completely deficient in Fas-induced death, supporting the conclusion that there are no other major parallel Fas-death pathways (23, 24). DAXX has also been implicated in mediating Fas activation of JNK and p38 (11, 46). We show that Fas activation of p38 and JNK is defective in our FADD-deficient cell lines and that transfection of FADD alone is not sufficient to activate p38 and JNK (Fig. 5). These results are consistent with other studies that have shown that FADD is not sufficient to activate the stress kinases (11, 47). However, our data indicate that although FADD is not sufficient, it is required for Fas activation of p38 and JNK. Hence, DAXX may function downstream of FADD and caspase-8, cooperate with FADD, or be more relevant in other cell types.

In this study, we have used our FADD-deficient cell lines to dissect signaling events downstream of Fas. To analyze FADD-dependent signaling, other groups have overexpressed a dominant-negative version of FADD consisting of the death domain alone (47–51). Use of this reagent is complicated by the fact that the FADD death domain may bind the Fas death domain and block the recruitment of other Fas death domain-interacting proteins. Experiments from FADD null cells showed that FADD was required for Fas-induced death and heart development (23, 24). However, detailed biochemical characterization of signaling events downstream of Fas were not performed in these cells. We now show that FADD is required for Fas activation of multiple caspases, including caspase-2, caspase-3, caspase-7, and caspase-8 and for cleavage of substrates BID, PKC-δ, and PARP (Fig. 3). These results suggest that in Jurkat cells, parallel signaling pathways that might emanate from Fas are not sufficient to activate caspases.

FADD binds and recruits caspase-8 to the receptor complex, which ultimately results in its activation (18, 19, 21). We provide direct evidence that FADD is required for Fas activation of caspase-8 because Fas-induced processing of caspase-8 and cleavage of the caspase-8 substrate BID are defective in the FADD mutant cell lines (Fig. 3B). Fas cross-linking has been shown to result in an increase in intracellular ceramide levels that may potentially mediate apoptosis, but the origin of this signal emanating from the receptor complex has remained obscure (37–40). We now show that FADD and caspase-8 are required for Fas-induced increases in intracellular ceramide (Fig. 4), suggesting that these proteins mediate the Fas→ceramide signal. The increase in ceramide may be mediated by acid sphingomyelinases because a recent study showed that TNF-induced activation of acid sphingomyelinases requires FADD (52). However, the mechanism by which caspase-8 regulates ceramide levels remains unclear.

In conclusion, the isolation of cell lines containing mutations in FADD in a genetic screen provides strong evidence that FADD is required for Fas-induced death, supporting recently published data from FADD knockout mice (23, 24). Furthermore, we have used these FADD-deficient cell lines to show that FADD is required for multiple signaling events downstream of Fas. We show that FADD is required for Fas activation of caspase-2, caspase-3, caspase-7, and caspase-8, suggesting that other signaling pathways emanating from Fas are not sufficient to activate these caspases. We also show that increases in intracellular ceramide in response to Fas ligation require FADD and caspase-8. Finally, we show that although FADD is not sufficient to activate the stress kinases, p38 and JNK, it is required for Fas activation of these kinases. These Fas-, FADD-, and caspase-8-deficient cell lines provide useful reagents for studying the physiological role of these proteins in the absence of overexpression. In particular, it will be interesting to determine the role of FADD and caspase-8 in apoptosis induced by other members of the death receptor family and other receptor-independent inducers of apoptosis.

Materials and Methods
Antibodies and Reagents. Antibodies to caspase-7, caspase-8 (rat monoclonal), and BID were kindly provided by Junying Yuan (Harvard Medical School). Antibodies to DAXX (M112), PKC-δ (C20), caspase-2 (Ich-1, C20), bcl-xL (S-18), and bcl-2 (100) were purchased from Santa Cruz Biotechnology. Caspase-3 antibodies were purchased from PharMingen. Anti-Fas monoclonal antibody (CH11) was purchased from Kamiya Biomedicale Co. (Thousand Oaks, CA), Antibodies to FADD were purchased from PharMingen and Transduction Labs. Antibodies to PARP (C2-10) were obtained from G. G. Poirier (CHUL Research Center, Quebec, Canada), [35S]Methionine and [γ-32P]ATP were obtained from Amer sham (Cleveland, OH) and New England Nuclear (Boston, MA), respectively.

Mutagenesis. For EMS mutagenesis, Jurkat subclone A3 was either left untreated (1×10⁶) or treated (1×10⁶) with EMS (Sigma) at 200 μg/ml for 24 h (53). Cells were allowed to recover for 5 days prior to selection in FasAb. To obtain one cell/well, cells were plated in serial dilutions into 96-well plates in the presence of FasAb (CH11; Kamiya). After 2–4 weeks, surviving clones were expanded and further characterized. ICR191 mu-
tagenesis was performed as described (28, 29). Briefly, Jurkat subclone A3 was either left untreated (1 × 10⁶) or treated (1 × 10⁶) with ICR191 (Polysciences, Inc.) at 2 μg/ml for 2 h. Cells were exposed to ICR191 for 3 cycles to increase the frequency of mutagenizing both alleles (28, 29). After the initial treatment, mutagen was washed out, and cells were allowed to recover for 15 days. Cells were then treated again with ICR191 and allowed to recover for 2 days before the final treatment. Cells were then plated in serial dilutions into 96-well plates in the presence of FasAb as described above (CH11; Kamiya).

RT-PCR and in Vitro Transcription and Translation. RNA was isolated from cells using Trizol (Life Technologies, Inc.). Reverse-transcription was performed using oligo(dT) primers (Superscript; Life Technologies, Inc.), followed by PCR using primers specific for Fas. The 5′ primers also contained a T7 RNA polymerase promoter overhang (54). To visualize the protein products, the PCR products were purified using Gene Clean (Bio 101), transcribed (T7 polymerase), translated, and labeled with [35S]methionine using TNT (Promega). Labeled proteins were separated by SDS-PAGE.

Transfection and Complementation. Jurkat cells were transfected as described (20). FADD was subcloned into pRusV using HindIII and XbaI after PCR using pcDNA3-FADD as a template (gift from V. Dixit, University of Michigan Medical School, Ann Arbor, MI). After 1-h recovery, cells were spun over a Ficoll-Paque gradient (Pharmacia) to remove dead cells attributable to the transfection procedure. Transfections were divided in half and either left untreated or treated with FasAb (ascites, 7C11 at 1:1000; gift from M. Robertson, Indiana University School of Medicine) for 12 h. Cells were then washed in FACS wash (0.5% BSA in PBS) and stained with anti-CD8-FITC (Collaborative Labs) at a 1:50 dilution. Propidium iodide (Sigma) was added 5 min prior to FACS analysis at 40 μg/ml to detect dead cells. Cells were analyzed on a FACCaliber (Becton Dickinson) using CellQuest software.

Western Blot Analysis. This was performed as previously described (20).

In Vitro Protease and Protein Kinase Assays. Protease assays were performed as described by Li et al. (55). Briefly, Jurkat cells were concentrated to 2 × 10⁷ cells/ml in complete medium and either left untreated or treated with 250 ng/ml anti-FasAb (CH11) for various times. Cells were washed twice with ice-cold RPMI and resuspended at 4 or treated with 250 ng/ml anti-FasAb (CH11) for various times. Cells were then plated in serial dilutions into 96-well plates in the presence of FasAb as described above (CH11; Kamberi).

Acknowledgments

We thank Michael J. Robertson, Junying Yuan, Gerry Crabtree, Vishva M. Dixit, Vincent Cryns, and Richard Konz for technical assistance in FACS analysis and members of the Blenis lab for critical reading of the manuscript.

References


