The adapter protein FADD consists of two protein interaction domains and is an essential component of the death inducing signaling complex (DISC) that is formed by activated death receptors of the tumor necrosis factor (TNF) receptor family. The FADD death domain binds to activated receptors such as Fas or other adapters such as TRADD, whereas the FADD death effector domain binds to procaspase 8. Each domain can interact with its target in the absence of the other domain, and this has led to the idea that the two domains function independently. FADD death domain interactions with Fas and TRADD are thought to occur on the same surface; however, the regulation of these interactions is poorly understood. We developed a modified reverse two-hybrid method that can identify mutations, which inhibit some protein-protein interactions without affecting other interactions. Using this method, we identified mutations in FADD that prevent binding to Fas but do not affect binding to TRADD. Surprisingly, these mutations were in the death effector domain rather than the death domain. To test whether the mutants function in mammalian cells, we expressed wild type or mutant FADD molecules in FADD-deficient cells. Wild type FADD rescued both Fas ligand- and TNF-dependent signaling, whereas the FADD death effector domain mutants rescued only TNF signaling. These data indicate that in contrast to current models, the death effector domain of FADD is involved in interaction with Fas.

The six identified death receptors of the TNFR family induce apoptosis by forming a complex called the DISC with intracellular proteins (1). Procaspase 8 is then cleaved and activated through interactions with various proteins leading to apoptosis. The adapter protein FADD (2) consists of two protein interaction domains. FADD binds to receptors or other adapters such as TRADD, whereas the FADD death effector domain binds to procaspase 8 through its death effector domain. The recruitment of procaspase 8 to the DISC is thought to result in the autoactivation of the caspase. FADD binds directly to Fas to activate caspase 8 in response to Fas ligand and binds the adapter protein TRADD to activate caspase 8 in response to TNFα (1). Thus, the important interaction for caspase activation by Fas is between FADD and Fas death domains, whereas the corresponding interaction responsible for TNFα-induced caspase activation is between FADD and TRADD death domains. The solution structures of the death domain (amino acids 96–208) and the death effector domain (amino acids 1–81) of FADD have been solved (3–5). Both domains are globular structures consisting of six α-helices that are tethered together by a linker, which is thought to be flexible because it is sensitive to proteases (3). These studies suggest that binding between death domains occurs through charge interactions. By contrast, binding between the death effector domains of FADD and procaspase 8 is the result of hydrophobic interactions (6). Site-directed mutagenesis experiments suggest that the Fas-FADD and TRADD-FADD interactions occur on the same surface of the FADD death domain. Indeed, the mutations in helices 2 and 3 of the FADD death domain abolish interactions with both Fas and TRADD, although one mutation, FADD (R117A), seems to prevent binding to Fas only (7). Current models (3, 4, 8) are based on the idea that the two domains function independently of each other (i.e. that the death domain does not affect death effector domain interactions and vice versa). This view is supported by experiments showing that each domain in isolation can interact with its partner. For example, the isolated death domain can inhibit apoptosis by binding to activated Fas (8). However, the mechanisms through which FADD interactions are regulated in the context of the full-length protein are incompletely understood.

Reverse two-hybrid screens identify mutations in proteins that result in a loss of protein-protein interactions (10, 11). In this method, one typically uses in vitro mutagenesis to create a library of mutants of one of the components in a two-hybrid screen, either the DNA-binding domain (DBD) fusion plasmid or the activation domain (AD) fusion plasmid. The investigator then screens for the loss of two-hybrid interaction. The major problem with current reverse two-hybrid methods is that one commonly identifies mutations that prevent stable expression of the two-hybrid protein or that affect gross protein folding. Such mutants are often not useful for mechanistic studies. To help dissect FADD signaling, we developed a reverse two-hybrid system that identifies mutations, which specifically abolish interactions among particular partner proteins while requiring that the mutated protein still interacts with a different protein partner; thus, demanding that the mutant protein
is stably expressed in its native conforming. Using this method, we identified mutations in FADD, which suggest that in contrast to current models, the FADD death effector domain regulates the interaction between FADD and Fas.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The GAL1-TetR expression cassette was constructed by cloning the Tn10 Tet Repressor with two copies of the SV40 nuclear localization signal (one at the N-terminal and one C-terminal) between the GAL1 promoter and the ADH1 terminator. This GAL1-TetR cassette was then cloned into plasmid pYYYY-kanMX4-HO (9), which allows it to be targeted into the HO locus. To construct the TetO-ADE2 reporter, the promoter of the ADE2 gene was deleted and replaced with a promoter fragment containing TetO sites made by PCR amplification from strain Y1584 (Bio101, Carlsbad, CA). Plasmid Gal4-DBD-(Yip-TRP1) was constructed by inserting a cassette with the ADH1 promoter, Gal4 DNA-binding domain, and ADH1 terminator from plasmid pAS2-1 (10) into pRS306 (11). Plasmid LexA-DBD-(Yip-URA3) was constructed by first inserting the ADH1 promoter Gal4-DBD-ADH1 terminator cassette into plasmid pRS306 (11) and then replacing the Gal4 DBD with an EcoRI-HindIII fragment from pBTM116 (12) containing the LexA DBD. Plasmid pACT3, a YEp vector with a LEU2 marker, was constructed by modifying the polylinker of the pACT2 activation domain vector (CLONTECH, Palo Alto, CA). Plasmid Gal4-DBD-FAS containing amino acids 191–313 of human Fas was made by PCR amplifying nucleotides 571–939 of human Fas and inserting it into the EcoRI and BamHI sites of Gal4-DBD-(Yip-TRP1). The full-length TRADD cDNA was inserted into LexA-DBD-(Yip-URA3) at the EcoRI and BamH1 sites to give LexA-DBD-TRADD. For pACT3-FADD, a PCR fragment with full-length FADD was inserted into the EcoRI and BamH1 sites of pACT3. pCDNA3.1 Puro was constructed by replacing the Hygro gene of pcDNA3.1 (+) with the Puro gene. FADD and FADD mutant cDNAs were cut from the pACT3 versions with EcoRI and XhoI and inserted into the same sites of pCDNA3.1 Puro. A detailed description of plasmid construction, maps, and sequences is available upon request.

**Yeast Strains, Transformations, and Media**—The genotype of SFY526 (13) is Mata ade2 can1 his3 leu2 lys2 trp1 gal1 galo80 URA3::GAL1-lacZ. The genotype of DY6877 is Mata ade2 can1 his3 leu2 lys2 trp1 URA3::LexA(op)-lacZ. The genotype of LY62 is Mata can1 his3 leu2 met15 trp1 ural3 gal4::hisG gal80::hisG LYS2::LexA(op)-HIS3 TetO-ADE2 hoc::KanMX::GAL1-TetR. The GAL80 gene in DT7088 (ade2 can1 his3 leu2 met15 trp1 ural3 LexA(op)-HIS3) was disrupted with a galo80::hisG-GAL1 his3::galo80::hisG disruption and then converted to gal4::hisG. A detailed description of LY62 construction is available upon request. The medium was prepared as described previously (11). The medium was prepared as described previously (11).

**Reverse Two-hybrid Screening**—We modified the yeast split hybrid system (21) to include reporters for two DBD fusion proteins (sometimes called "baits"), similar to dual bait systems (Fig. 1) (24, 25). The first bait fused to the Gal4 DNA-binding domain is used to detect loss of interaction via a dual reporter system. A two-hybrid interaction between the Gal4-DBD fusion and the AD fusion results in expression of the reporter, which blocks transcription of ADE2 from the TetO-ADE2 reporter. Thus, the two-hybrid interaction with the Gal4-DBD fusion results in no ADE2 expression and an Ade− phenotype. A mutation that disrupts this interaction removes ADE2 inhibition, and the yeast are able to grow in the absence of Ade+ phenotype. Thus, we can select for the loss of two-hybrid interaction by selecting for Ade− yeast. The second bait protein, a LexA-DBD fusion, is used to eliminate mutations in the AD fusion that affect expression or stability of the AD protein fusion. Two-hybrid activation between the prey and the LexA-DBD fusions will activate the LexA(op)-HIS3 reporter, resulting in an His+ phenotype. Thus, specific mutations in the AD fusion that block interaction with partner 1 (the Gal4-DBD fusion) but maintain overall protein integrity, allowing interaction with partner 2 (the LexA-DBD fusion), can be selected as Ade− His+ transformants (Fig. 1C). Importantly, the sensitivity of both two-hybrid interactions can be titrated either with tetracycline, which modulates the activity of the Tet repressor (21), or with 3-amino triazole, which increases the amount of HIS3 expression required for histidine prototrophy (10). This allows the identification of strong and weak mutant alleles for both interactions.

To establish that the system works, plasmids with Gal4-DBD-FAS and LexA-DBD-TRADD were integrated into the yeast genome. Three activation domain plasmids, the empty AD vector, the AD-FADD (wild type) fusion, and the AD-FADD (R117A) mutant, were transformed and tested for two-hybrid interaction with Gal4-DBD-FAS and LexA-DBD-TRADD (Fig. 1D). When assayed on selective media, yeast carrying the empty AD vector do not activate the LexA(op)-HIS3 reporter and display an His− phenotype. Similarly, yeast expressing the Gal4-DBD (wild type) fusion have two-hybrid activation of the GAL1-TetR reporter, resulting in the repression of the TetO-ADE2 reporter and an Ade− phenotype. The FADD (R117A) mutant binds to TRADD but is not able to bind to Fas in vitro (7) or in yeast (22). When tested in the dual bait two-hybrid strain, the AD-FADD (R117A) fusion interacts with LexA-DBD-TRADD, resulting in an His− phenotype. Failure of AD-FADD (R117A) to interact with Gal4-DBD-FAS leads to no expression of TetR, resulting in Ade2 expression and an Ade+ phenotype. Thus, the differential
interaction of the AD-FADD (R117A) mutant with the two baits results in an Ade\textsuperscript{*}/H11001 phenotype.

Identification of Mutations That Prevent FADD-Fas but Not FADD-TRADD Interactions—To identify other FADD mutants that discriminate between Fas and TRADD, a library consisting of the GAL4 activation domain fused to a randomly mutated FADD molecule was generated through mutagenic PCR followed by gap repair in yeast. A screen of 23,500 mutant FADD molecules yielded 120 Ade\textsuperscript{*}/H11001 transformants. These colonies potentially represent mutations in FADD that do not bind to Fas but retain interaction with TRADD. Each transformant was mated to a strain carrying either a GAL1-lacZ or LexA-lacZ reporter, and the two-hybrid activation of these reporters was used both to eliminate false positives and to determine the strength of each interaction. Several plasmids showing strong binding phenotypes were assayed for their ability to bind to a GAL4-caspase 8 bait to ensure that the mutation was specific for the loss of Fas binding only.

Plasmids encoding FADD molecules that show decreased binding to Fas but still bind to TRADD (Fig 2A) were sequenced. As the FADD-Fas interaction is mediated through death domain interactions, we expected to find mutations in the death domain of FADD. Surprisingly, the mutations mapped to the death effector domain (Fig. 2B). Histidine 59 was mutated to tyrosine (H59Y), arginine 71 was mutated to tryptophan (R71W), and arginine 72 was mutated to either cysteine (R72C) or histidine (R72H). These mutations are in surface amino acids that flank helix 5 of the FADD death effector domain. R72E mutation showed intermediate levels as measured using a β-galactosidase reporter gene in the two-hybrid assay (Fig. 2D).

All of the mutations bound to TRADD and caspase 8 about as well as the wild type FADD protein. We used site-directed mutagenesis to investigate other amino acid substitutions at these positions. When Arg-72 was changed to either an alanine (R72A) or a glutamate (R72E) and was tested for interaction with Fas in yeast, both mutations prevented interaction (Fig. 2D). However, when the same substitutions were made at position 71 (R71A and R71E), only a change to alanine prevented interaction. All of the mutations interacted with TRADD and caspase 8 similar to wild type FADD with the exception of the R72E mutation, which bound to TRADD only.

FADD Mutants Reconstitute TNFR Signaling but Not Fas Signaling—To test whether the mutations in FADD identified in yeast have a phenotype in mammalian cells, wild type FADD and two of the mutants (R71W and H59Y) isolated in yeast were expressed in FADD-deficient Jurkat I2.1 cells (23). To verify stable expression, cell extracts were Western blotted with an anti-FADD antibody. Cell lines transfected with FADD or FADD mutants but not green fluorescent protein (GFP) or the parental FADD-deficient cells (I2.1) had a unique band.
with an apparent molecular mass of 26 kDa corresponding to FADD protein (Fig. 3A). The FADD mutants were expressed at similar levels, which were slightly lower than those of endogenous FADD in wild type Jurkat A3 cells. Cell lines expressing the FADD cDNAs with mutations at amino acid 72 produced very little FADD protein and were not examined further. A FADD mutant that can bind to TRADD but not Fas should reconstitute TNFR1 signaling but not Fas signaling in the I2.1 cells. Because the I2.1 cells were made by treating with a mutagen and selecting for growth in high levels of Fas ligand (23), they may contain other mutations that affect downstream Fas signaling. This appeared to be the case because even when we expressed wild type FADD in these cells, the cytotoxicity in response to Fas or TNF

We treated the Jurkat cell lines with Fas ligand, which should result in the recruitment of FADD and activation of caspase 8 followed by activation of caspase 3. Wild type Jurkat A3 cells and cells expressing wild type FADD exhibited Fas ligand-dependent caspase 8 cleavage, whereas cells expressing GFP or R71W mutant version of FADD did not (Fig. 3B). The mutation from histidine to tyrosine at position 59 weakens but does not eliminate the interaction with Fas in yeast (Fig. 2D). Consistent with this finding, Jurkat cells expressing the H59Y mutant still showed some caspase 8 processing in response to Fas ligand. We next tested whether the mutants could mediate caspase 8 activation in response to TNFα. Caspase 8 was cleaved in wild type Jurkat cells and cells expressing wild type FADD and both FADD mutants but not in the parental I2.1 cells and the cells expressing GFP (Fig. 3B). Similar results were obtained when we monitored caspase 3 processing. TNFα treatment caused caspase 3 activation as determined by the appearance of the active cleaved form of the enzyme in parental cells and in FADD-deficient cells expressing wild type FADD or either of the point mutants. Conversely, caspase 3 processing in response to Fas ligand was observed only when wild type FADD was expressed. These data indicate that the FADD R71W and H59Y mutants are compromised in their ability to mediate Fas signaling but not TNFR signaling. This finding is consistent with the mutants retaining native conformation to allow interaction with TRADD but not with Fas.

To directly test whether mutations in the FADD death effector domain prevented binding to Fas in mammalian cells, DISC immunoprecipitation assays were performed using I2.1 cells expressing GFP, wild type FADD, or FADD mutants were Western blotted for FADD. B, protein samples from the same cells were probed with anti-caspase 8 or caspase 3 after treatment with or without Fas ligand or TNFα. Blots were stripped and reprobed with anti-β-actin to test for equal loading.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Mutations in FADD that do not bind Fas but still bind TRADD. A, yeast expressing FADD mutants were grown on -Trp-Leu-Ade-His + 1 mM 3-amino triazole media. Yeast expressing FADD molecules with mutations at positions 59, 71, or 72 are able to grow on selective media, indicating loss of interaction with Fas but retention of interaction with TRADD. B, linear map of FADD with the location of mutations identified in our screen. C, NMR structure of the death effector domain of FADD (5). The three mutations that disrupt Fas binding are on either side of helix 5. D, empty vector, wild type FADD, or FADD mutants were tested for interaction with Fas, TRADD, or caspase 8 using a LacZ reporter in yeast.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** FADD mutants can reconstitute TNFR1 signaling but not Fas signaling. A, wild type (A3), FADD-deficient (I2.1), or Jurkat cells stably expressing GFP, wild type FADD, or FADD mutants were Western blotted for FADD. B, protein samples from the same cells were probed with anti-caspase 8 or caspase 3 after treatment with or without Fas ligand or TNFα. Blots were stripped and reprobed with anti-β-actin to test for equal loading.
FIG. 4. Mutations in FADD prevent binding to Fas in mammalian cells. A, Jurkat cells stably expressing GFP, wild type FADD, or the Tyr-59 and Trp-71 FADD mutants were treated with cross-linked FLAG-FasL. The DISC was precipitated and Western blotted for the Tyr-59 and Trp-71 FADD mutants were treated with cross-linked cells. A we treated the Jurkat cell lines with TNFα interaction with FADD leads to caspase 3 activation. Therefore, we treated the Jurkat cell lines with TNFα to further test whether TRADD-dependent signaling via FADD was functional in these cells. Cells expressing wild type FADD and both FADD mutants but not cells expressing GFP displayed caspase 3 activity (Fig. 4B). The FADD mutants were as effective (R71W) or more effective (H59Y) than the wild type protein at activating caspase 3 in response to TNFα.

We next performed a screen for second-site mutations that compensate for loss of binding to Fas. We randomly mutagenized the R71A mutant, which cannot interact with Fas (Fig. 2D) and performed a forward two-hybrid screen to identify mutants that could now bind to Fas. Fig. 5A shows three second-site mutations, which allow the R71A mutant to bind to Fas as well as the wild type FADD protein. Interestingly, these mutations (E61K, L62F, and E65K) were in helix 5 of the death effector domain (Fig. 5B). Thus, a mutation in the loop between helices 5 and 6 of the death effector domain abolishes interaction between FADD and Fas, but further mutations in helix 5 rescue the interaction. These data further indicate that the death effector domain plays a role in mediating interaction between FADD and Fas and suggest that helix 5 is important in this response.

**DISCUSSION**

In this paper, we describe a modified reverse two-hybrid system that permits the facile identification of mutations that inhibit some protein-protein interactions without affecting overall protein structure or binding to other proteins. Using this approach, we identified mutations in the death effector domain of FADD that prevent binding to Fas but do not alter binding to TRADD. FADD mutants that were identified using the reverse two-hybrid system showed the expected ability to reconstitute TNFR1 signaling but not Fas signaling when they were expressed in FADD-deficient Jurkat cells. These data indicate that the binding characteristics in yeast were mirrored in mammalian cells. The fact that mutations in the death effector domain of FADD prevent interaction with Fas suggests that current models for the formation of the DISC (24) in which the FADD death domain functions independently of the death effector domain to bind Fas are oversimplified. Rather than functioning as two separate entities, we suggest that the death domain and the death effector domain of FADD work together to regulate Fas binding.

A simple explanation for our results is that the FADD death effector domain directly participates in binding to Fas. Alternatively, mechanisms include an inhibitory effect of the death effector domain that is promoted by our mutations or an allosteric modification of the death domain by the death effector domain. Our results do not conclusively discriminate between these possibilities. However, the fact that we rescued the binding of the R71A mutant by introducing new mutations in helix 5 of the death effector domain suggest that it is the death effector domain itself and not an effect by the death effector domain on the death domain that is important. By performing structural studies using mutants similar to those identified with our screening method, it may be possible to determine how the death effector domain regulates death domain interactions. The death effector domain of FADD may also be involved in interaction with the TRAIL receptors where it is thought that DAP3, which binds to the death effector domain, is the adapter responsible for FADD recruitment (25). Our data also suggest that it may be possible to design drugs that specifically interfere with some but not all FADD interactions by searching for molecules that disrupt death domain interactions through an effect on the death effector domain. The yeast system might be a useful screening method for such molecules, which could be used to selectively inhibit signaling by some death receptors without affecting signaling from the other receptors that use FADD.

The reverse two-hybrid system can be used to study any protein interactions that occur in yeast and should be generally useful for the identification of mutants that have specific protein binding characteristics. This may allow the generation of dominant negative mutants that selectively inhibit specific activities of a particular protein. In addition, the system may be generally useful for the identification of mutants that provide useful insights into the structural requirements for specific protein-protein interactions.

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Regulation of Fas-associated Death Domain Interactions by the Death Effector Domain Identified by a Modified Reverse Two-hybrid Screen

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