FADD is known to function as a common signaling conduit in Fas- and tumor necrosis factor (TNF)-mediated apoptosis. The convergent death signals from the Fas receptor and TNF receptor 1 are transferred to FADD by death domain interactions triggering the same cellular event, caspase-8 activation. In this work, we investigated whether the same binding surface of FADD is used for both signaling pathways by using FADD death domain mutants. Mutations in helices α2 and α3 of the FADD death domain, the interacting surface with the Fas death domain, affected TNF-mediated apoptosis to various extents. This indicated that TNF-mediated apoptosis uses the same binding surface of the FADD death domain as Fas-mediated apoptosis. The binding specificity is not the same, however. Some mutations affected the binding affinity of the Fas death domain for the FADD death domain, but did not influence TNF-mediated apoptosis and vice versa. Interestingly, all mutants tested that affected TNF-mediated apoptosis have structural perturbations, implying that the structural integrity, involving helices α2 and α3 in particular, is critical in TNF-mediated apoptosis. Our results suggest that different signaling molecules use a similar structural interaction to trigger the same cellular event, such as caspase-8 recruitment, which could be typical in convergent signal transduction.

The tumor necrosis factor (TNF) receptor family consists of type I transmembrane proteins characterized by cysteine-rich repeats in their extracellular ligand-binding domains (1). A subset of the family, comprising CD95/Fas, TNFR1, p75 nerve growth factor receptor, DR3 (death receptor 3), and the two TRAIL (TNF-related apoptosis-inducing ligand) receptors (DR4/TRAIL receptor 1 and DR5/TRAIL receptor 2), shares a conserved motif in the cytoplasmic regions known as the death domain, which is essential for transducing the apoptotic signal (2–4). Upon receptor ligation, the death domain acts as the docking site for homotypic interaction with death domain-containing cytoplasmic proteins such as FADD (Fas-associated death domain protein) (5, 6) and TRADD (TNFR1-associated death domain protein) (7). FADD binds directly to Fas and is also recruited to TNFR1, DR3, and possibly other related receptors via TRADD (3, 8). The death effector domain at the N-terminal end of FADD then interacts with a related motif in the prodomain of caspase-8 (5, 9) or caspase-10 (10). Activation of these upstream cysteine proteases is thought to trigger a proteolytic cascade, which apparently constitutes the execution of apoptosis. Under certain environments, however, Fas and other members of the TNFR family that contain death domains can stimulate alternative signaling pathways, which exert positive effects on cell survival and proliferation rather than triggering apoptosis (1).

Endogenous FADD associates with Fas in an activation-dependent fashion (11), and TRADD and RIP (receptor-interacting protein) have been found in the activated TNFR1 complex (8). It has been postulated that TRADD acts as an adapter molecule for TNFR1, mediating the interaction of TNFR1 with FADD, whereas RIP is involved in nuclear factor κB signaling. Low levels of FADD were found in the TNFR1 complex when FADD, TRADD, and TNFR1 were coexpressed, raising the question of whether FADD is involved in TNF-mediated signaling. Apoptosis mediated by TNF and Fas is similar; both signaling cascades are initiated by death domains and end in activation of interleukin 1β converting enzyme-like proteases. However, clear differences in these two cell death pathways have been observed (12, 13). Fas-mediated cell death occurs much more rapidly than that triggered by TNF (14). A dominant-negative version of FADD blocks TNF- and Fas-induced apoptosis, indicating that FADD functions as the common signaling conduit for cytokine-mediated cell death (15, 16). It is not known yet whether the different signaling molecules use the same interaction surface of the molecule in the common conduit to trigger the same cellular event, such as caspase-8 recruitment, in this convergent signaling pathway.

The homotypic interactions of the death domains, death effector domains, and caspase recruitment domains are critical to the signaling pathways in apoptosis. These interactions are specific, as not all death domains interact with each other. The three-dimensional structures and site-directed mutagenesis of the death domains of Fas and FADD revealed that electrostatic interactions might be responsible for molecular recognition between the death domains (17, 18). Previously, we characterized several mutant FADD-DD proteins that do not interact with Fas-DD (18). Here, we investigated the effect of those mutations in FADD-DD on the TNF-mediated apoptosis of a CHANG liver cell. Several mutations that affected the binding
of Fas-DD to FADD-DD also affected TNF-mediated apoptosis, suggesting that the binding surface of FADD is used for the signal transduction of Fas- and TNF-mediated apoptosis. Our results suggest a possible structural mechanism for convergent signal transduction.

MATERIALS AND METHODS

Estimation of the Electrostatic Interaction—The interaction between the death domains of Fas and FADD was measured by an enzyme-linked immunosorbent assay. Fas-DD protein (1 μg/well) in carbonate buffer (0.1 M, pH 9.8) was incubated overnight in a 96-well cell culture plate. The surface of the plate, which was coated with Fas-DD, was treated with blocking buffer (PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.47 mM KH2PO4) containing 5% skim milk and 0.01% Antifoam, pH 7.4) and washed six times with PBS containing 0.2% Tween 20, pH 7.4 (PBST). The plate was then incubated with 3.5 μM His-tagged FADD-DD protein dissolved in different salt concentrations in PBs (2.7 mM KCl, 4.3 mM Na2HPO4, and 1.47 mM KH2PO4, pH 7.4) for 1 h at 25 °C. After washing with PBST six times, the bound His-tagged FADD-DD protein was detected by successive treatment with anti-His antibody (in PBs; wash buffer; 20 ng/well) and horseradish peroxidase-conjugated goat anti-mouse IgG diluted in blocking buffer. The plates were washed again before 100 μl of substrate buffer (100 mM Na2HPO4, 50 mM citric acid, 0.1% o-phenylenediamine, and 0.003% H2O2) was added to each well. After 20 min, the reaction was stopped by adding 100 μl of 2 M H2SO4/well. The intensity was measured by absorbance at 490 nm using a Spectra Max 340 spectrophotometer (Molecular Devices). All experiments were carried out in triplicate. The Fas-DD and FADD-DD proteins were produced as described previously (18).

CD Analysis of Mutant FADD-DD Proteins—The FADD-DD proteins were dissolved in 10 mM sodium phosphate buffer, pH 7.4, at a concentration of 0.1 mg/ml, and the CD spectra were recorded on a Jasco 710 instrument. Each scan was recorded between 195 and 280 nm at 10 °C, and five scans were accumulated.

NMR Spectroscopy—The 15N-labeled samples for heteronuclear NMR experiments were prepared by culturing the recombinant cells in M9 minimal medium supplemented with 1 g of 15NH4Cl/liter. All NMR data were acquired at 30 °C on a Varian UNITYplus 600 spectrometer (Advanced Analysis Center of the Korea Institute of Science and Technology) using ~2 μM protein dissolved in 90% H2O and 10% 2H2O. Typical carrier frequencies employed in the NMR experiment were 4.7 ppm for 1H and 118.5 ppm for 15N. Each amide proton and nitrogen of the FADD death domain was assigned based on previously reported data (18). All of the NMR spectra were processed using NMRPipe (19) and analyzed with the program PIPP (20).

TNF-induced Apoptosis—The FADD-DD mutants were generated by overlap polymerase chain reaction as described previously (18). The 284-base pair fragment extracted from the amplified polymerase chain reaction products was ligated into the pET-15b expression vector containing NdeI and BamHI restriction enzyme sites. To construct the mammalian expression system, pET-15b harboring the FADD-DD gene was digested with NdeI and then filled in by Klenow fragment (Promega). The resulting fragments were cut by BamHI and ligated into the mammalian expression vector pcDNA3.1 (Invitrogen).

CHANG liver cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) with 10% fetal bovine serum (Life Technologies, Inc.) before transfection. For transient expression experiments, 2–8 × 106 CHANG liver cells were grown to 60% confluence in a well and washed with DMEM prior to treatment with transfection mixtures containing DNA (0.9 μg) of the wild-type or mutant FADD-DD plasmids and 0.3 μg of LactZ and 3 μg of LipofectAMINE (Life Technologies, Inc.) in 1.0 ml of serum-free DMEM. After 4 h of incubation, DMEM was substituted with serum-supplemented DMEM and incubated for an additional 24 h. Cells were then exposed to TNF-α (30 ng/ml) and cycloheximide (4 μg/ml) for 16 h. To determine cell viability, cells were stained for 4-galactosidase activity as described by Kumar et al. (21). Briefly, cells were washed with PBS; fixed with 0.5% glutaraldehyde; and stained with PBS containing 0.1% X-gal and 1.0 mg/ml X-gal for 3 h. Viability was then determined based on the morphology of X-gal-positive cells. All Fas and TRADD proteins were of human origin, and FADD was of mouse origin (22).

RESULTS

Salt Effect on the Interaction between Fas and FADD Death Domains—Mutations of charged residues in helices α2 and α3 of FADD-DD alleviated the binding affinity for Fas-DD (Fig. 1). It is believed that charged residues in helices are involved in death domain interactions, but that structural perturbation of helices α2 and/or α3 induced by mutations could break interactions. If electrostatic interactions between charged residues in death domain interactions were responsible for death domain interactions, the interactions would be dependent on salt concentration. To investigate the salt effect on death domain interactions, an enzyme-linked immunosorbent assay has been designed to detect the interaction between Fas-DD and FADD-DD in vitro. The binding affinity of FADD-DD for Fas-DD up to an additional 50 mM NaCl was not affected, but a higher concentration of salt significantly reduced the binding affinity (Fig. 2). This result indicates that electrostatic interactions play a role in death domain interactions between FADD and Fas.

CD Analysis of FADD-DD—Mutations of charged residues in helices α2 and α3 of FADD-DD might affect the structural integrity of the protein. If the structure of the protein is perturbed by mutation, it is not safe to say that electrostatic interactions play a role in death domain interactions, although mutations of charged residues are responsible for the alleviation of the binding affinity of FADD-DD for Fas-DD. To investigate the effect of mutations on the structural integrity of the protein, CD spectra were obtained for each mutant as well as for wild-type FADD-DD. The CD spectra of the FADD-DD mutants showed that each mutation affected the conformation of FADD-DD to a different extent (Fig. 3). Some mutations induced significant structural change, and others hardly affected the protein conformation, which is almost indistinguishable from the wild-type spectrum. The loss of the secondary structures in the proteins can be observed either by local structural perturbation or by increasing the unfolded fraction of the protein. However, all of the mutant proteins except K113A have unfolding transition temperatures high enough to affect the CD spectrum at the experimental temperature (10 °C), indicating that the structural perturbation observed by CD is correlated with the local structural perturbation rather than the global unfolding. The mutant K113A has a completely unfolded CD spectrum.

NMR Spectroscopy of FADD-DD—Many of the mutants studied by CD showed that the secondary structure was lost by
mutation. However, it is not clear whether the structural change is localized around the mutation site or not. To localize the structural perturbation, we obtained $^1$H-$^{15}$N HSQC spectra of FADD-DD mutants (R110A and E123A) and compared them with the wild-type protein spectrum (Fig. 4). In the case of the mutant protein R110A, a few residues around the mutation site in helix $\alpha_2$ (Gly$^{109}$, Arg$^{110}$, Asp$^{121}$, Trp$^{112}$, and Lys$^{123}$) were perturbed, as shown by chemical shift perturbation. Significant chemical shift perturbation was observed in helices $\alpha_2$ (Arg$^{114}$, Leu$^{115}$, and Arg$^{117}$) and $\alpha_3$ (Val$^{121}$, Ser$^{122}$, Glu$^{123}$, Ala$^{124}$, Asp$^{127}$, Gly$^{128}$, Ile$^{129}$, Glu$^{130}$, Glu$^{131}$, and Ser$^{130}$) in the E123A mutant protein. These results indicate that structural perturbations are localized around the randomization sites in helices $\alpha_2$ and $\alpha_3$ and that the conformations of helices $\alpha_2$ and $\alpha_3$ are highly sensitive to a mutation. Although a significant conformational change in FADD-DD was induced by mutations at Arg$^{117}$, Asp$^{127}$, and Glu$^{130}$ did not perturb the structure, however, the binding affinity of these mutants varied regardless of the extent of structural perturbation. This indicates that a single amino acid substitution can break domain interactions by disrupting electrostatic interactions.

**Mutational Effect of FADD-DD on TNF-mediated Apoptosis**—We previously demonstrated that charged residues in helices $\alpha_2$ and $\alpha_3$ are involved in death domain interactions and that the interacting helices appear to interact in an antiparallel pattern, helix $\alpha_2$ of FADD with helix $\alpha_3$ of Fas and vice versa (18). To elucidate the physiological function of FADD-DD in TNF-mediated apoptosis, FADD-DD and its variants were transiently expressed in a CHANG liver cell line using the pcDNA3.1 expression vector. The effect of mutations in FADD-DD on TNF-mediated apoptosis was investigated by treating the cells with TNF-$\alpha$. Approximately 71% of the cells transfected with a control plasmid (pcDNA3.1) died upon treatment with TNF-$\alpha$ and cycloheximide compared with ~7% without treatment. One the other hand, wild-type FADD-DD transfection induced 43% death with TNF-$\alpha$ compared with 24% without treatment (Fig. 5). This result indicates that FADD-DD inhibits TNF-$\alpha$-mediated apoptosis. The expression of mutants R110A, D111A, R117A, and E130A blocked TNF-$\alpha$-induced cell death, as in the wild type. In contrast, mutants K113A, E118A, K120A, V121N, and E123A did not block TNF-induced cell death. The dominant-negative effect of FADD-DD on cell death induced by TNF-$\alpha$ indicates that FADD participates in TNF-mediated apoptosis. The abrogation of the inhibitor activity of FADD-DD by mutations in helices $\alpha_2$ and $\alpha_3$ indicates that helices $\alpha_2$ and $\alpha_3$ are involved in TNF-induced apoptosis.

**DISCUSSION**

FADD is both a necessary and sufficient mediator of Fas- and TNF-induced apoptosis, as overexpression of FADD engages the cell death machinery (6), whereas a truncated derivative acts as a potent dominant-negative regulator (6). It has been proposed that electrostatic interaction is responsible for death domain interactions by mutation work (17, 18). Here, we further confirmed this by investigating the salt effect on death domain interactions. A high salt concentration virtually abolished the binding affinity of FADD-DD for Fas-DD. The CD and $^1$H-$^{15}$N HSQC spectra of FADD-DD mutants indicate that mutations in helices $\alpha_2$ and $\alpha_3$ induce structural perturbation to various extents, but most of the perturbation is limited to helices $\alpha_2$ and $\alpha_3$. The binding specificity is not the same, however. Some mutations affected the binding affinity of Fas-DD for FADD-DD, but did not influence TNF-mediated apoptosis and vice versa. Interestingly, all mutants

![FIG. 2. Effect of salt on the interaction between Fas and FADD. Plates were coated with Fas-DD (1 µg/well); and the next day, the plates were incubated with 3.5 µM His-tagged FADD-DD in PBS base buffer containing different concentrations of NaCl (8.5, 50, 150, 200, and 500 mM and 1 M). The bound fraction of FADD-DD was then determined with anti-His antibody as described under "Materials and Methods." Binding was normalized to the absorbance value in 150 mM NaCl.](image)

![FIG. 3. Circular dichroism spectra of FADD-DD and its variants. Spectra were obtained at 10 °C with FADD-DD proteins (wild-type (+), R110A (●), K113A (◇), R117A (△), E118A (□), K120A (■), V121N (×), E123A (▲), D127A (○), and E130A (▲)) in 10 mM phosphate buffer, pH 7.4. deg, degrees.](image)
tested that affected TNF-mediated apoptosis have structural perturbations, implying that the structural integrity, involving helices α2 and α3 in particular, is critical in TNF-mediated apoptosis (Table I and Fig. 3). The interaction observed between the death domains of TRADD and FADD (16) suggested the possibility that TRADD might be able to recruit FADD to TNFR1. If the death domain of TRADD interacts with the death domain of FADD, the death domain of TRADD should have a unique pattern of interactions since the death domains of TNFR1 and RIP also interact with the TRADD death domain. Our results indicate that the binding surface of FADD for Fas is also involved in TNF-mediated apoptosis, suggesting...
that TRADD interacts with FADD at the same surface that Fas does (Table I). FADD recruits a caspase-8 upon binding to Fas or TRADD death domains. This suggests that the conformational change in FADD required for caspase-8 recruitment is triggered in a similar pattern, although the interacting molecules are different. TNFR1 recruits TRADD by binding to part of the death domain and enables TRADD to recruit FADD to the other part of the death domain. Amino acid sequence alignment of death domains shows that TRADD has a longer loop between helices α2 and α3 than other death domains (18). Considering that helices α2 and α3 are involved in death domain interactions between Fas and FADD, the long loop might be involved in other roles, assuming that helices α2 and α3 of TRADD are also involved in death domain interactions. It is interesting that the mutations in the aforementioned loop do not affect cell death induced by the TRADD death domain and that they fail to activate nuclear factor κB (25), implying that the loop is involved in RIP-activated cell death. Considering that other death domains such as Pelle and Tube (26) have a different interaction mode from the Fas and FADD death domains, TRADD would have at least two distinctive surfaces for TNFRI and FADD interactions. However, a detailed picture of the pattern of binding of TRADD to different death domains must await further structural work.

In the cell, many interacting molecules bind to a single molecule and trigger diverse cellular events or a single cellular event. One molecule can have different or similar binding surfaces for each interacting molecule. In divergent signal transduction, where the diverse cellular events are triggered, each interacting molecule is likely to have a different binding surface. In this way, they are not competitive in signal transduction. In convergent signal transduction, however, the interacting molecules may have the same binding surface. If an interaction is required in creating a new binding surface, the specific conformational change in the molecule is necessary. To trigger a specific conformational change in the molecule, the interacting molecules should bind to the same surface. In the case of FADD, our results indicate that Fas or TRADD is required for creating a new binding surface for caspase-8. This indicates that the different signaling molecules bind to the same surface of the molecule to trigger convergent signal transduction.

In conclusion, we showed that helices α2 and α3 of the FADD death domain are critical in the death domain interaction in Fas- and TNF-mediated apoptosis. The death domain interaction in Fas- and TNF-mediated apoptosis is mediated by electrostatic interactions, but the integrity of the structure is also important. The convergent signals from the Fas receptor and TNFRI to FADD are transferred using the same surface of FADD. This result suggests that the different signaling molecules use the same structural interaction to trigger the same cellular event, such as caspase-8 recruitment in convergent signal transduction.

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