Cell Death and Differentiation (2006) 13, 160–162

www.nature.com/cdd

Letter to the Editor

Extensive regions of the FADD death domain are required for binding to the TRAIL receptor DR5

Cell Death and Differentiation (2006) **13,** 160–162. doi:10.1038/sj.cdd.4401714; published online 8 July 2005

Dear Editor,

Fas-associated death domain (FADD) is an adaptor molecule involved in the recruitment and activation of caspases in response to stimulation of at least four death receptors; Fas (CD95), TNFR1 and the TNF-related apoptosis inducing ligand (TRAIL) receptors DR4 and DR5. FADD consists of two protein interaction domains, a death domain (DD) and a death effector domain (DED). Binding of TRAIL to DR5 results in the recruitment of FADD to DR5 to form a complex called the death-inducing signaling complex (DISC) through interactions that require intact DDs of FADD and DR5.[1,2] Caspase-8 and -10 are then recruited to the receptor complex via DED interactions.[3] The induced proximity of caspase-8 at the receptor complex leads to its dimerization and activation.[4] These initiator caspases then cleave and activate effector caspases such as caspase-3. Similar to the TRAIL receptors, FADD binds directly to the Fas receptor [5] while the recruitment of FADD to TNFR1 is mediated by the adaptor molecule TRADD.[6] Since TRAIL can selectively induce apoptosis in tumor cells without affecting normal cells,[7,8] there is much interest in using TRAIL for the treatment of cancer. The mechanisms used to recruit FADD during TRAIL receptor-induced apoptosis is therefore of considerable interest.

The structures of the FADD death domain (FADD DD) and DED have been solved independently of one another.[9–12] Both consist of six antiparallel alpha helices tethered together by a small linker peptide. DD interactions are mediated through charge–charge interactions whereas DED interactions appear to occur mostly through hydrophobic residues. Previous structural studies focused on the Fas/FADD binding surface and suggested that helices two and three of the FADD DD are responsible for mediating this interaction. More recently however, the Fas-binding surface of FADD's DD was recognized to include the loop between helices 5 and 6.[13] In addition, although the isolated DD of FADD is sufficient for binding, regions surrounding helix 5 of the FADD DED also contribute to the interaction of FADD with both Fas and DR5.[14,15] Together, these data indicate that FADD binding to Fas is not mediated just by the residues in helices 2 and 3 of the DD but instead involves a much larger region of the DD and regions in the DED.

Previously we identified a mutation in the FADD DD, valine 108 to glutamate, which abolished interaction with Fas but did not alter binding to DR5 suggesting that despite the similar role of DED residues in binding both Fas and DR5, the surfaces of the FADD DD used for interaction with these two receptors might be distinct.[15] To address this question, we mapped the DR5-binding surface of the FADD DD and identified residues within helices 2–6 of the DD that when mutated, prevented binding to DR5.

Binding of FADD to death receptors is primarily mediated by interactions between charged residues.[12,13] We therefore examined charged surface residues in the DD of FADD by making mutations to alanine or charge reversals (Figure 1a). Several of these residues have previously been shown to be necessary for FADD binding to Fas and TRADD.[9,10,12,13] In addition we made mutations that were in regions not previously shown to be important for FADD binding to death receptors. To determine which mutations prevented FADD binding to the DR5 receptor, we performed a functional assay using a dominant negative version of FADD (FADD-DD). FADD-DD, which consists of the FADD DD fused to yellow fluorescent protein (YFP) with no DED sequences, is able to inhibit apoptosis by all known death ligands including TRAIL.[16] When overexpressed, FADD-DD competes with endogenous FADD for binding to an activated death receptor, but because there is no DED, caspases are not recruited. Each of the DD mutations was made in FADD-DD and transfected into HeLa cells. Since HeLa cells express both DR4 and DR5, the transfected cells were treated with an agonistic DR5 antibody (αDR5) to induce apoptosis. As shown in Figure 1b, of the 18 mutations examined, eight showed a decrease in protection against DR5-induced death. D106A, R135E, R140A and R166E showed approximately a 50% decrease while R113E, R117A, D123R and D175R showed almost complete lack of protection, similar to cells expressing YFP only. Rather than localization to a specific region of FADD such as helices 2 and 3, the mutations were spread out though the entire FADD DD (Figure 1a, residues marked with an asterisk).

We previously demonstrated that the isolated DD of FADD did not bind as well as the full-length protein to DR5.[15] Therefore, full-length versions of each FADD mutation identified in the functional screen were tested for interaction with the cytoplasmic domain of DR5 in a directed yeast two-hybrid assay. Wild-type FADD showed strong interaction with DR5 whereas empty vector and most of the FADD mutations did not (Figure 1c). D106A was the only mutation tested that showed interaction with DR5, but the level was somewhat decreased compared to wild-type FADD. This was consistent with the FADD-DD functional assay in which D106A retained some ability to prevent anti-DR5-induced cell death (Figure 1b). The same mutations were tested for interaction

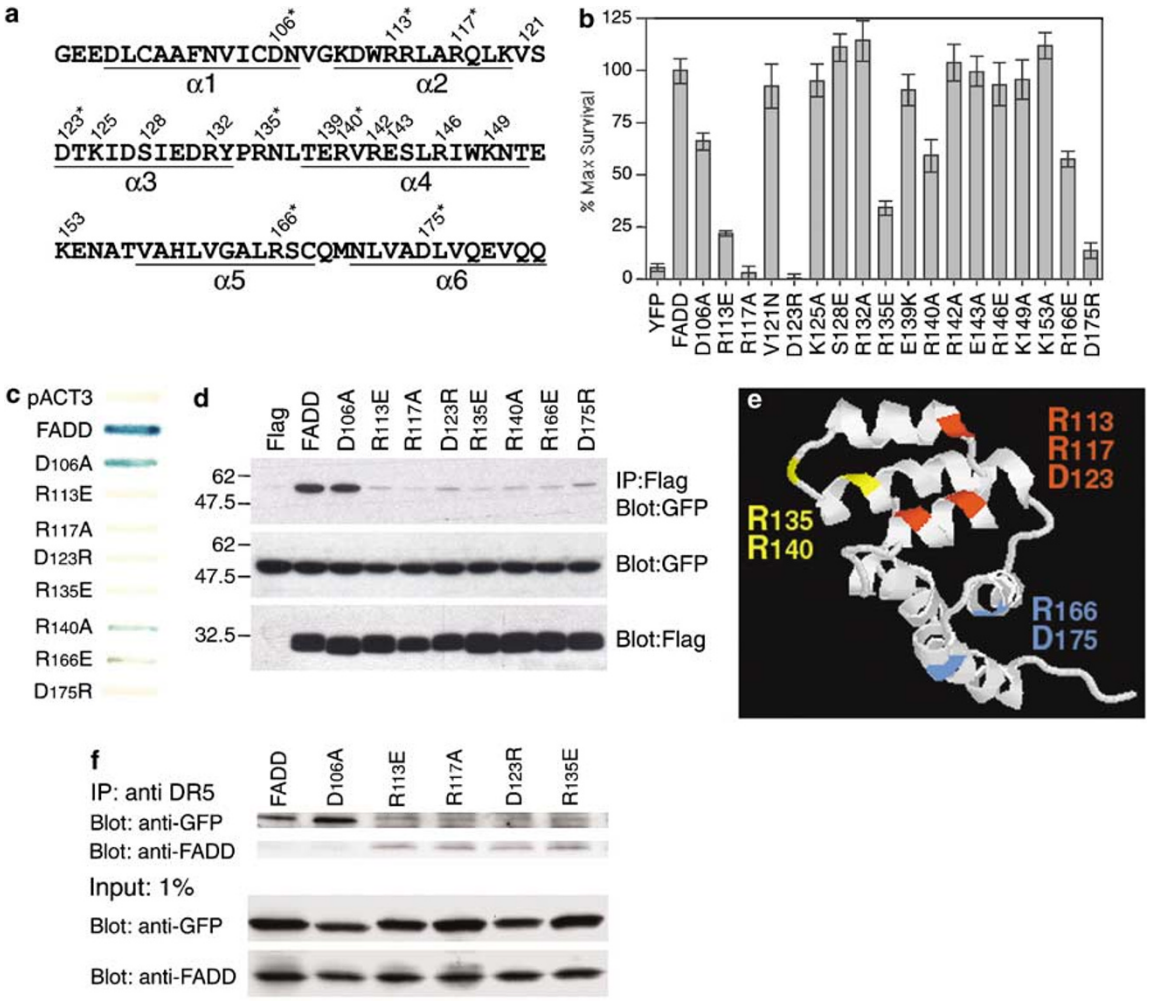

Figure 1 FADD death domain residues that affect binding to DR5. (**a**): The amino-acid sequence of the FADD death domain with the location of residues that were mutated and alpha helices underlined and numbered. Mutations in FADD-DD with at least a 25% decrease in DR5-induced death are marked with asterisk. Mutations that prevented binding to DR5 were identified in all alpha helices except helix 1. (**b**): HeLa cells were transfected with 2 μg of pYFP–FADD-DD or point mutants using Fugene 6 (Roche, Indianapolis, IN, USA) then seeded into 96-well plates. The next day cells were treated in triplicate for 24 h with 2.5 μg/ml cycloheximide and 200 ng/ml of αDR5 (MAb631, R&D Systems, Minneapolis, MN, USA) crosslinked with an equal amount of anti-Fc (Sigma, St Louis, MO, USA). MTS reagent (Promega, Madison, WI, USA) was used to measure cytotoxicity by comparing treated to untreated cells after incubation for 24 h; percent survival was calculated by normalizing each FADD-DD mutant to cells expressing wild-type FADD-DD. (**c**): Mutations in FADD-DD that cannot prevent DR5-induced apoptosis were tested in the context of full-length FADD for interaction with the DR5 cytoplasmic domain in a directed yeast two-hybrid. Strain Y190 (*MATa his3 ade2 trp1 leu2 gal4 gal80 cyh2, LYS2:Gal1-HIS3 URA3:Gal1-LacZ*), was used for directed two-hybrid assays as previously described.[14] Wild-type FADD and FADD (D106A) showed interaction with DR5 as indicated by a change in color. The other DD mutations showed no interaction with DR5. (**d**): Each FADD mutation was tested for interaction with DR5 in mammalian cells by coimmunoprecipitation. HeLa cells were transfected with 2 μg pEGFP–DR5 plus 2 μg of Flag–FADD or FADD mutants using Fugene 6. After 18 h cells were lysed in Triton X-100 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 $\times$ protease inhibitors) and the soluble fraction was incubated with 30 μl M2-Agarose (Sigma) for 4 h a 4°C. The beads were washed four times in TBS and precipitated GFP fusions were detected by immunoblot. Similar to the directed two-hybrid experiment, wild-type FADD and FADD (D106A) showed interaction with DR5, but other death domain mutations did not. Whole cell lysate was immunblotted with GFP and Flag antibodies to show equal loading. (**e**): Mutations in FADD that prevent interaction with DR5 were mapped onto the solved structure of the FADD DD 10. Residues in helices 2 and 3 (Red) as well as the loop region between helices 5 and 6 (blue) are necessary for interaction of FADD with Fas and DR5. A further surface of FADD defined by R135 and R140 (yellow) encompassing the loop region between helices 3 and 4 as well as part of helix 4 appears to contribute to the FADD/DR5 interaction but not the interaction of FADD with other DD-containing proteins. (**f**): Comparison of the association of endogenous DR5 with YFP–FADD-DD mutants or endogenous FADD protein was assessed by DISC immunoprecipitation. In all, 293 cells were transfected with YFP–FADD-DD and various mutants using Fugene 6 reagent (Roche). Cells were treated with 200 ng/ml of anti-DR5 Ab crosslinked with an equal amount of anti-Fc (Sigma) for 1 h, spun down and washed with PBS and then freeze-thawed in 1 ml buffer (20 mM Tris-HCl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGDT, 0.5% NP-40 detergent) with protease inhibitors (1 mM Bestatin, 10 mM Leupeptin, 30 mM PMSF). Insoluble material was removed by 2 $\times$ centrifugation at 15000 $\times$ *g*. Immunoprecipitations were with 80 ml of a 50% slurry of protein G-agarose beads (Roche) for 5 h. Beads were rinsed 5 $\times$ with buffer and proteins were eluted with SDS loading buffer with boiling for 3 min. After running the protein on a 4–20% gradient gel, and transferring to a PDVF membrane, the membrane was cut horizontally and the bottom portion (below 33 kDa) was probed for endogenous FADD with a monoclonal antibody (BD transduction labs, clone1) followed with an anti-mouse Fc-specific secondary HRP (Jackson Labs). The upper portion of the blot was probed with a polyclonal anti-GFP antibody (Santa Cruz) and anti-rabbit secondary-HRP (Calbiochem). Wild-type FADD-DD and the D106A mutant both bound to the activated DR5; the other tested mutants did not. In contrast the endogenous FADD protein was not found in the DISC IP containing FADD-DD or D106A FADD-DD while endogenous FADD was found in the DR5 precipitates from cells transfected with the FADD-DD mutants that cannot bind to DR5. These data show that binding of the FADD-DD mutants to activated endogenous DR5 receptors correlates with inhibition of apoptosis signaling, binding to exogenously expressed receptors and binding in yeast and suggest that the inhibitory effect of the FADD-DD mutants that block apoptosis occurs because of competition with endogenous FADD for binding to the receptor

with DR5 in mammalian cells by coimmunoprecipitation. The DR5 cytoplasmic domain fused to GFP (GFP–DR5) was cotransfected into HeLa cells with versions of Flag–FADD containing each mutation. Flag–FADD complexes were precipitated and interaction of DR5 with each FADD mutation was determined by immunoblotting for GFP. GFP–DR5 coprecipitated with both wild-type FADD and FADD (D106A) but not with any of the other FADD mutations (Figure 1d). Taken together, these data suggest that the DR5-interaction surface of FADD involves residues extending from helices 2 to 6 of the FADD DD.

To confirm that binding of the mutants occurred to the endogenous DR5 protein, we performed DISC immunoprecipitation experiments in cells expressing YFP-tagged FADD-DD or mutant FADD-DD molecules and compared recruitment of the exogenous FADD-DD molecule or the endogenous FADD protein to the endogenous DR5 DISC. If FADD-DD acts as an inhibitor of apoptosis by competing with the wild-type FADD protein for binding to the receptor, FADD-DD and point mutants that can bind to DR5 should be precipitated in the DR5 DISC while endogenous FADD is not. Conversely, FADD-DD point mutants that prevent DR5 binding should not be precipitated in the DISC and, in this case, endogenous FADD should be found in the complex. Figure 1f shows that both wild-type FADD-DD and the D106A mutant were indeed bound to the endogenous DR5 receptor after activation with an agonistic antibody and that this complex did not contain significant amounts of the endogenous FADD protein. In contrast, four FADD-DD point mutants (R113E, R117A, D123R and R135E) that did not bind to DR5 in our previous assays and did not inhibit DR5-induced apoptosis were not recruited to the endogenous DR5 DISC; instead these complexes contain endogenous FADD proteins.

Together, with our previous studies showing that the same mutations in the FADD DED prevent binding to both Fas and DR5, the data shown here suggest that the regions of FADD for binding to Fas and DR5 are very similar. Both involve the DED and an expanded binding surface on the DD that encompasses more than just helices 2 and 3 as previously thought.[9,10] Several residues in FADD identified as preventing interaction with DR5 (R113, R117A and D123) are essential for interaction with Fas.[9] Additionally R166, Q169, L172 and D175, which form a loop between helices 5 and 6 of FADD, were recently shown to contribute to the interaction with Fas.[13] FADD also uses this loop for binding to DR5 because mutations in two of these residues (R166E and D175R) inhibited binding to DR5. Finally, we identified a unique region of FADD that is necessary for binding to DR5; mutations of R135 and R140, which are near the loop between helices 3 and 4 prevented binding to DR5 (Figure 1e). These data show that FADD binding to activated DR5 involves extensive surfaces of the FADD protein. Although FADD binding is similar to different receptors, the subtle differences in the binding (e.g. R142A prevents Fas binding [13] but does not alter interaction with DR5) provide a potentially useful way to experimentally discriminate between different death receptor signaling pathways.

LR Thomas[1,3], LM Bender[1], MJ Morgan[2] and A Thorburn[*,1]*

[1] Department of Cancer Biology, Wake Forest University School of Medicine, Medical Center Blvd. Winston-Salem, NC 27157, USA
[2] Department of Cell and Cancer Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[3] Current address: Department of Microbiology and Immunology, Vanderbilt Medical Center, Nashville, TN 37232, USA.
* Corresponding author: A Thorburn, Current address: Department of Pharmacology and University of Colorado Cancer Center, University of Colorado Health Sciences Center, UCHSC at Fitzsimons, L18-6100, RC1 South Tower, PO Box 6511, Mail Stop 8303, Aurora, CO 80045-0508, USA. Tel: 303 724 3290; Fax: 303 724 3663; E-mail: Andrew.Thorburn@uchsc.edu

1. Sprick MR *et al.* (2000) Immunity 12: 599–609
2. Kischkel FC *et al.* (2000) Immunity 12: 611–620
3. Kischkel FC *et al.* (2001) J. Biol. Chem. 276: 46639–46646
4. Boatright KM *et al.* (2003) Mol. Cell. 11: 529–541
5. Chinnaiyan AM *et al.* (1995) Cell 81: 505–512
6. Hsu H *et al.* (1996) Cell 84: 299–308
7. Ashkenazi A *et al.* (1999) J. Clin. Invest. 104: 155–162
8. Walczak H *et al.* (1999) Nat. Med. 5: 157–163
9. Bang S *et al.* (2000) J. Biol. Chem. 275: 36217–36222
10. Berglund H *et al.* (2000) J. Mol. Biol. 302: 171–188
11. Eberstadt M *et al.* (1998) Nature 392: 941–945
12. Jeong EJ *et al.* (1999) J. Biol. Chem. 274: 16337–16342
13. Hill JM *et al.* (2004) J. Biol. Chem. 279: 1474–1481
14. Thomas L *et al.* (2002) J. Biol. Chem. 277: 34343–34348
15. Thomas LR *et al.* (2004) J. Biol. Chem. 279: 32780–32785
16. Wajant H *et al.* (1998) Curr. Biol. 8: 113–116