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Review

Multifunctional Role of Fas-Associated Death Domain Protein in Apoptosis

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Introduction

The Fas-associated death domain protein (FADD) was discovered as a protein that interacts with the Fas receptor (Chinnaiyan *et al*., 1995; Zhang and Winoto, 1996). FADD consists of two distinct domains called the 'death effector domain' and the 'death domain' at its N- and C-terminus, respectively. The death domain of FADD (FADD-DD) interacts with the death domain of the Fas receptor, and the death effector domain of FADD (FADD-DED) interacts with the death effector domain of procaspase-8 (Boldin *et al*., 1996; Muzio *et al*., 1996). In Fas-mediated apoptosis, FADD is recruited to the Fas receptor, and recruits procaspase-8 in turn. This results in the initiation of apoptotic processes (Medema *et al*., 1997; Muzio *et al*., 1998). However, FADD is also involved in T-cell proliferation (Zhang *et al*., 1998; Walsh *et al*., 1998) and necrosis (Kawahara *et al*., 1998; Matsumura *et al*., 2000), independent of known apoptosis mechanisms. In this article, the suggested roles of FADD in various pathways will be reviewed.

FADD in apoptosis

FADD is recruited by the Fas receptor upon the binding of the Fas ligand. It is known that the Fas ligand is a trimer, and induces the trimerization of receptors (Ashkenazi *et al*., 1998). The ligand-receptor complex structures of the tumor necrosis factor (TNF) receptor family show that two ligand monomers interact with a receptor, which indicates that the ligands play a critical role in the formation of the stable receptor trimer (Banner *et al*., 1993; Hymowitz *et al*., 1999; Cha *et al*., 2000). However, the association of receptors without ligand binding has been observed by the fluorescence energy transfer experiment. Furthermore, the apoptotic signal transduction is prevented if the pre-association domain is cleaved off the receptor, which indicates that pre-association is critical for the biological function. Chemical cross-linking of free receptors showed that the receptors exist as a trimer, even without the binding of the ligand. FADD was also observed in the chemically cross-linked receptor complex, but procaspase-8 was not recruited nor activated (Siegel *et al*., 2000b). The chimeric receptor, which is generated by combining the extracellular domain of the platelet-derived growth factor (PDGF) and intracellular domain of TNF receptor I (TNFRI), was responsive to PDGF. The function that is elicited by PDGF by binding to the chimeric receptor was indistinguishable from that induced by the wild-type TNF receptor, even though PDGF induces the dimerization of receptors (Adam *et al*., 1995). This indicates that simple trimerization, or multimerization, may be insufficient to elicit a function, but a specific conformational change of the receptor is needed for the functional signal transduction. The assembly of Fas monomers into trimers without ligand is mediated by a pre-ligand assembly domain (PLAD) in the amino terminal region of Fas (Papoff *et al*., 1999; Siegel *et al*., 2000a). However, PLADs in the complex structure of TNFRI and TNF-β have no interaction with one another (Banner *et al*., 1993). This indicates that either the ligand binding induces the conformational change of the receptor or pre-associated receptors interact with trimeric Fas ligand forming a super-cluster. In any case, pre-association of the receptors is insufficient to induce an effective death-inducing signaling complex (DISC) until the trimeric ligand binds to the receptor. Interestingly, the extra-cellular domain of free TNFRI forms a dimer in the crystal structure (Naismith *et al*., 1996). The dimerization interface was far from the TNF binding region. This suggests the possible multimeric association by TNF ligation, but stoicheometry of chemical cross-linking of free receptors could not be explained.

Once FADD is recruited, procaspase-8 or procaspase-10 is recruited and activated by self-cleavage initiating apoptosis. The fact that free receptors already interact with FADD implies that a certain conformational change of FADD is necessary for efficient procasapse-8 recruitment. In the yeast two-hybrid system, FADD interacts with Fas-DD (Chinnaiyan *et al*., 1995) and procaspase-8 (Boldin *et al*., 1996; Muzio *et*

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al., 1996). If their respective binding affinities are the same, procaspase-8 should be recruited to the free receptor, but procaspase-8 is not recruited. This may indicate that Fas-DD and FADD are interacting in vivo, as observed in the yeast two-hybrid system. However, FADD and procaspae-8 are not interacting strong enough unless the conformational change of FADD into a high affinity form is elicited, or an effective concentration of FADD is increased. Otherwise, the third molecule is interfering with FADD to interact with procaspase-8. An active caspase-8 activates the executioner caspase such as caspase-3 that triggers various apoptotic processes (Ashkenazi *et al.*, 1998), or activates BID to initiate a mitochondria-driven cell death (Li *et al.*, 1998; Gross *et al.*, 1999). The strong activation of caspase-8 at DISC is observed in type I cells (BJAB). Only a small amount of DISC was formed in type II cells (Jurkat). Although two types of cell death are interconnected (Scaffidi *et al.*, 1998; Scaffidi *et al.*, 1999b), caspase-8 activated cell death is mainly observed in the type I cells, and mitochondria-driven cell death is predominantly observed in the type II cells.

In the TNF-induced apoptosis, FADD is recruited to TNFRI through the TNFRI-associated death domain protein (TRADD). TRADD recruits FADD, receptor-interacting protein (RIP), as well as the TNF receptor-associated factor 2 (TRAF2) to TNFRI, or to the death receptor 3 (DR3). The recruitment of FADD to TRADD induces caspase-8 activation and apoptosis, while TRAF2 recruitment activates the NF-κB signal that leads to cell survival (Ashkenazi *et al.*, 1998). The recruitment of RIP can activate both the apoptosis and NF-κB signal. Once RIP is cleaved by caspase-8, the C-terminal domain of RIP enhances the caspase-8 activation by enhancing the FADD recruitment to TRADD (Kim *et al.*, 2000). FADD and RIP can also interact with each other through death domains (Grimm *et al.*, 1996).

In the TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death, the death-associated protein 3 (DAP3) (Kissil *et al.*, 1999) is reported to bridge the TRAIL receptor and FADD (Miyazaki *et al.*, 2001). The TRAIL receptor binds to the N-terminus of DAP3, and FADD binds to the C-terminus of DAP3. FADD-DED is shown to interact with the C-terminus of DAP3. DAP3 has a nucleotide-binding motif in the N-terminal end. The binding of GTP to the nucleotide-binding motif enhances the recruitment of FADD, and the subsequent caspase-8 activation. Interestingly, DAP3 was also observed in DISC that was induced by the Fas ligand. The Fas receptor and DAP3 do not interact directly, but FADD interacts with DAP3. This indicates that DAP3 is recruited to the Fas receptor via FADD. However, there is a conflicting report showing that DAP3 is not a member of DISC (Kissil *et al.*, 1999). FADD-DED binds to both DAP3 and procasapse-8, but it is unknown whether or not DAP3 enhances the association of procasaspe-8 with FADD. The death effector domain of FADD or procaspase-8 also interacts with the FADD-like IL-1β-converting enzyme (FLICE)-inhibitory proteins (FLIPs). The FLIP is known to regulate the apoptosis by interfering with the activation of caspase-8 (Irmler *et al.*, 1997; Scaffidi *et al.*, 1999a).

FADD in necrosis

Fas-mediated apoptosis is initiated by caspase-8 activation, so casapse-8 is indispensable for the Fas-mediated apoptosis. However, oligomerization of FADD could kill Jurkat cells that are deficient in caspase-8, or the wild-type cells that were treated with the broad-spectrum caspase inhibitor. The dying Jurkat cells by this pathway showed a necrotic morphological change (Kawahara *et al.*, 1998; Matsumura *et al.*, 2000). It is also reported that FADD-DED kills *E. coli* by inducing reactive oxygen species (Lee *et al.*, 2000). These results imply that the death effector domain is related to necrotic cell death. Artificial oligomerization, using the FK506 binding protein fusion (Nagata group), showed that FADD-DED is responsible for the FADD-mediated necrotic cell death. The oligomerization of the death-effector domain, but not the death domain of FADD, was able to induce necrosis in caspase-8 deficient cells. This process was accompanied by a loss of mitochondrial membrane potential, but not by the release of cytochrome c from mitochondria. The reduction of membrane potential can be inhibited by pyrolidine dithiocarbamate, a metal chelator and antioxidant (Matsumura *et al.*, 2000). The killing of *E. coli* by FADD-DED was also alleviated by adding antioxidants, which indicates that a similar pathway exists in *E. coli* (Lee *et al.*, 2000). The broad-spectrum caspase inhibitor, zVAD-fmk, only weakly blocked cell death in the resting T cells, and had no effect on activated T cells in the FasL-induced cell death. This suggests that the FasL-induced cell death of the activated T cell is caspase-independent (Holler *et al.*, 2000). The caspase-independent cell death showed the necrotic morphological change, and is completely resistant in FADD-deficient cells. The necrotic cell death is also completely resistant in RIP-deficient, or in the cells that have defective RIP activity. This indicates that the RIP kinase activity is essential for the necrotic cell death. In FasL- and TRAIL-induced necrotic cell death, FADD-DED is required, but FADD is not required in the TNF-induced necrotic cell death. It is interesting that RIP was directly recruited to Fas in the FADD-deficient cell, although the necrotic cell death is not observed. The authors, therefore, suggest that an unidentified protein is recruited by FADD and phosphorylated by the receptor-bound kinase RIP in order to initiate necrotic cell death. However, further work is necessary to explain why FADD is not required in the TNF-induced necrotic cell death if indeed FADD plays such a critical role in recruiting another factor by the death effector domain. In the TNF-induced apoptosis, no protein other than FADD is known to have the death effector domain.

FADD in cell proliferation

FADD-deficient mice are embryonic lethal with heart

developmental problems, and die around 10 days after gestation (Zhang *et al.*, 1998). The inactivation of FADD by the expression of a FADD dominant negative molecule, or by gene targeting, leads to the impairment of activation-induced T-cell proliferation. This suggests a role for FADD in T-cell development and activation, as well as in embryonic development (Newton *et al.*, 1998; Walsh *et al.*, 1998). In pursuing the role of FADD in T-cell development, two forms of FADD were identified. It was discovered that one of them was phosphorylated at serine 194, but it was unrelated to the Fas-mediated apoptosis. The phosphorylation of FADD that correlated with the cell cycle, and a 70-kDa cell-cycle-regulated kinase that specifically binds to the C-terminal half of FADD, was identified. Because Fas-mediated apoptosis is independent of the cell cycle, it is possible that FADD regulates proliferation through its interaction with the cell cycle-regulated kinase (Scaffidi *et al.*, 1998). In the absence of FADD, cell cycle abnormality was reported (Zhang *et al.*, 2001).

FADD is also involved in acid sphingomyelinase activation (Adam-Klages *et al.*, 1998; Schwandner *et al.*, 1998). Activation of acid sphingomyelinase releases ceramide, a metabolite that triggers cell death when administered exogenously. Stimulation of Fas blocks T lymphocyte calcium channels through sphingomyelinase and sphingolipids (Lepple-Wienhues *et al.*, 1999; Hueber *et al.*, 2000). FADD is required for the activation of sphingomyelinase, but the over-expression of caspase-8 does not lead to enhanced acid sphingomyelinase activation. This implies that the activation of sphingomyelinase is different from the apoptotic pathways. However, broad-spectrum caspase inhibitors reduce the sphingomyelinase activity (Schwandner *et al.*, 1998). An influx of calcium ions into the T cell is needed to activate the T cell, and allow it to proliferate in response to the antigen. However, a dominant negative mutant of FADD (FADD-DN) inhibits proliferation and leads to impaired calcium mobilization in both T-cells and fibroblasts, which indicates that the effect of FADD-DN on proliferation and calcium mobilization is not T-cell specific (Hueber *et al.*, 2000). Blocking the FADD function by FADD-DN or FADD-deficient cells affected the cell cycle, and prevented its entry into the S phase of T cells. However, the regulation of cell cycle proteins appears to be different in the FADD-deficient cell (Zhang *et al.*, 2001), and in the FADD-DN transgenic T cell (Hueber *et al.*, 2000). Also, blocking the FADD function by FADD-DN could cause lymphoid malignancy, indicating that FADD represents a tumor suppressor (Newton *et al.*, 2000).

Structural basis of multiple functions of FADD

FADD is a 208 amino acid long protein that consists of two domains-the death effector domain at its N-terminus and the death domain at its C-terminus. In addition to these two domains, the peptide of about 20 amino acids is in the C-terminal end, which is the first degraded in the protease digestion, indicating that the C-terminal peptide is not structured (Lee *et al.*, 2000). Ser^{194} in the C-terminal peptide is phosphorylated by the cell-cycle-regulated kinase (Fig. 1). Although the phosphorylation of FADD correlates with the

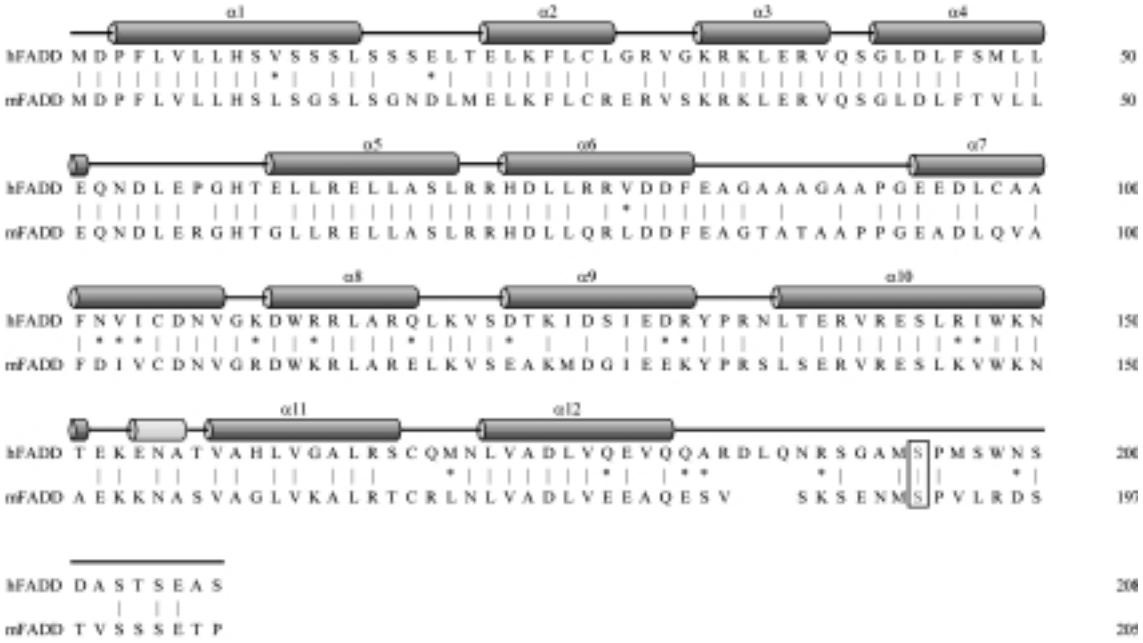

Fig. 1. Sequences of human and mouse FADD. Secondary structures of FADD are from the structure of human FADD-DED (M^{1}-E^{83}) and from the structure of mouse FADD-DD (A^{89}-S^{183}). The helix number is given, assuming the full length FADD. The structure of each domain has 6 helices in a determined structure. The phosphorylation site (S^{194} of human FADD) of FADD is shown in red. A 3^{10} helix is located between α10 and α11 helix.

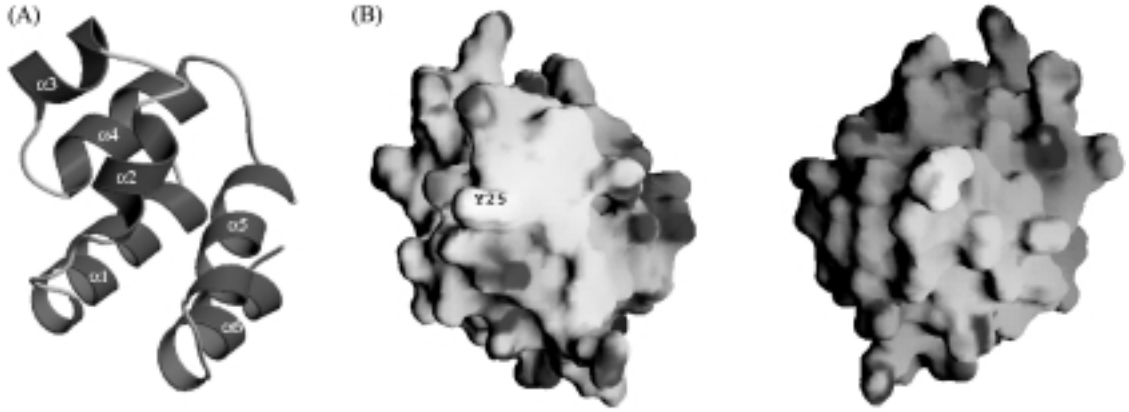

Fig. 2. (A) Ribbon drawing of the human FADD-DED F25Y. (B) The electrostatic potential surface of FADD-DED is shown in the same view as in A, and the functionally critical residue Tyr25 is labeled (*left*). Residue 25 is Phe in the wild-type protein. The opposite side of the hydrophobic surface is shown at the *right*. The negatively-charged surface is in red, and the positively-charged surface is in blue. The hydrophobic surface is shown in white (Eberstadt *et al.*, 1998).

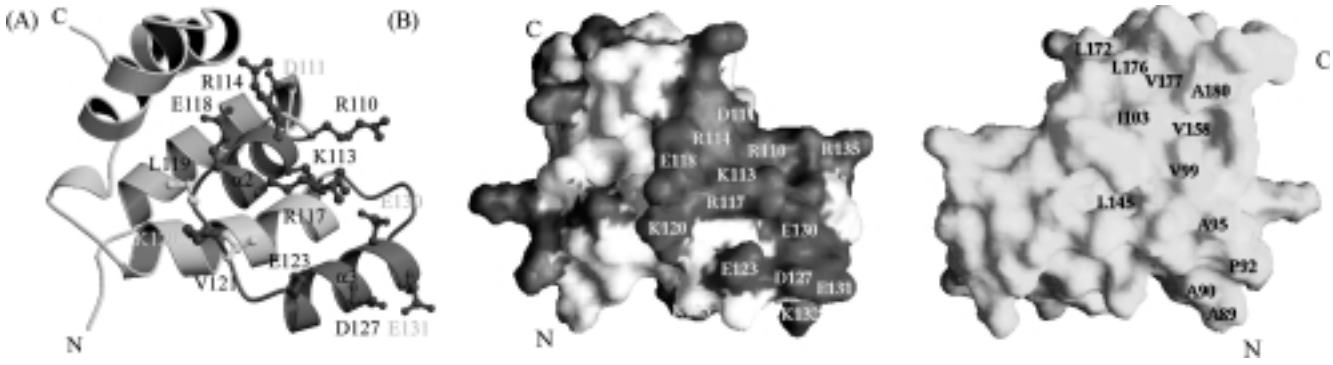

Fig. 3. (A) A ribbon drawing of the mouse FADD-DD with side chains in the suggested interaction site. (B) The electrostatic potential surface of FADD-DD is shown in the same view as in A (*left*). The colors of the potential surface are the same as in Figure 2. The hydrophobic residues on the opposite side are shown in *sky blue* (*right*) (adapted from Jeong *et al.*, 1999)

cell cycle, an additional function of the phosphorylated FADD has not yet been identified (Scaffidi *et al.*, 2000). The phosphorylation of FADD may affect the binding of FADD to DISC, or recruit another factor that is related to other functions of FADD such as cell proliferation.

The structure of FADD-DED consists of 6 α-helices that are arranged in an anti-parallel fashion, and it has two hydrophobic patches on the surface (Eberstadt *et al.*, 1998). The hydrophobic residues from one of these sites are highly conserved among the death-effector domains of procasaspe-8 and FLIPs. Phe25 in the α2 helix, which is located in the middle of this hydrophobic site, is critical for the apoptotic activity and binding to procaspase-8. The substitution of Phe25 by glycine inactivated FADD in apoptosis, but the substitution by tyrosine or tryptophan had little effect on apoptosis and binding to procasapse-8. This indicates that the hydrophobic interaction plays a critical role in the function. However, the other hydrophobic patch is not well characterized, although it may not be critical for apoptotic activity. The interaction site with procasapse-8 appears to be a hydrophobic surface that contains Phe25, and the same site seems to be used in an interaction with FLIP (Fig. 2). However, the interaction site of FADD-DED with DAP3 would be different from the binding site of procaspase-8 since DAP3 and procasapse-8 should bind simultaneously to FADD-DED in the TRAIL-induced apoptosis (Miyazaki *et al.*, 2001). In addition, the unaccounted interaction of FADD-DED in necrotic apoptosis could be different from the binding site of procaspase-8.

The structure FADD-DD also consists of 6 α-helices that are arranged in an anti-parallel fashion that is similar to the death-effector domain (Jeong *et al.*, 1999). The surface of the death domain consists of the hydrophobic and hydrophilic surface. It is known that the hydrophilic surface plays a critical role in protein interaction, and the hydrophobic surface may be involved in domain interaction, which could play a role in the conformational change in signal transduction (Fig. 3). The helices α2 and α3 are involved in the interaction with Fas-DD, but the exact interaction mode is controversial (Jeong *et al.*, 1999; Weber *et al.*, 2001a,b). The complex

structure of the death domains of Pelle/Tube (Xiao *et al.*, 1999), and the caspase recruitment domain of Apaf-1/procaspase-9 (Qin *et al.*, 1999), were determined. However, the binding sites that are observed in the complex structure were different in each complex. The interaction site of Apaf-1 is similar to the suggested binding site of Fas-DD and FADD-DD, but those of other domains in the complex are different from the suggested binding sites of Fas-DD and FADD-DD. From the observed binding sites in the domain, a heterohexameric complex of Fas-DD and FADD-DD was proposed (Weber *et al.*, 2001a,b). In the proposed complex, either a Fas trimer or a FADD trimer is located at the center, which is surrounded by three FADD or Fas molecules in analogy to the TNF-β/TNFRI complex structure (Banner *et al.*, 1993). The Fas ligand and Fas receptor would form at least a hexamer, but they may form a super-cluster. Considering that the dimerization of TNFRI also effectively transduced the signals (Adam *et al.*, 1995), it appears that the conformational change of Fas is required for the effective recruitment of FADD and activation of caspase-8. On the other hand, various binding patterns of the domain may be crucial for the multiple functions that are implicated for FADD. FADD may be involved in many functions without participating in DISC, but directly binding to other molecules. FADD mediates Fas-, TNF- and TRAIL-induced apoptosis. In TRAIL-induced apoptosis, it is unclear yet whether or not FADD-DD plays a role in the signal transduction. However, the binding surface of the FADD death domain plays a critical role in both the Fas- and TNF-mediated apoptosis, which indicates that the Fas- and TNF-induced apoptosis use the same binding surface of FADD to recruit procasapse-8 (Bang *et al.*, 2000). This indicates that different signaling molecules use a similar structural interaction to trigger the same cellular event in the convergent signal transduction.

Concluding Remarks

FADD is involved in many cellular processes. FADD could get involved in various cellular processes either by participating in the DISC formation, or by directly interacting with other molecules. Some functions may operate as a main process in the cell, but others may be operational only under special circumstances. Some functions require the conformational change of FADD, but others may need the FADD molecule itself to elicit functions. Sorting out all of the functions will be pivotal in understanding the complexity of the cellular signal transduction that is related to FADD.

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