Molecular Microbiology (2000) **35**(6), 1540–1549

Death effector domain of a mammalian apoptosis mediator, FADD, induces bacterial cell death

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Summary

FADD is a mammalian pro-apoptotic mediator consisting of the N-terminal death effector domain (DED) and the C-terminal death domain (DD). The N-terminal 88-residue fragment of murine FADD was defined as the stable structural unit of DED, as determined by proteolytic digestion and conformational analysis. This domain induced bacterial as well as mammalian cell death, whereas the full-length or DD of FADD did not. The *Escherichia coli* cells expressing FADD-DED showed elongated cell morphology and an increased level of nicked chromosomal DNA and mutation. The lethality of FADD-DED was abolished by co-expression of thioredoxin and superoxide dismutase or relieved by the addition of vitamin E as a reducing agent and under anaerobic growth conditions. The toxicity of FADD-DED was genetically suppressed by various oxidoreductases of *E. coli*. All these results suggest that the death effector domain of mammalian FADD induced bacterial cell death by enhancing cellular levels of reactive oxygen species (ROS).

Introduction

Apoptosis is considered as an active self-destructive process working in multicellular higher eukaryotes (Steller, 1995; Ameisen, 1996). However, this process has also been reported in unicellular eukaryotic organisms, such as *Trypanosome* (protozoa), slime mould *Distyostelium and* free-living ciliate *Tetrahymena* (Wheatley *et al*., 1993; Cornillon *et al*., 1994; Welbum *et al*., 1999) and even in prokaryotic organisms (Naito *et al*., 1995; Hochman, 1997). Conservation of apoptosis throughout different phylogenetic lines implies that this process may have evolved from a common origin.

Bax is a mammalian pro-apoptotic protein. Expression of this factor generates reactive oxygen species (ROS) and decreases mitochondrial membrane potential (Xiang *et al*., 1996). A trace amount of human Bax in *Escherichia coli* induced cell death with physiological changes similar to those in human cells (Asoh *et al*., 1998; Ishibashi *et al*., 1998). These results implied that at least some mammalian molecules involved in apoptosis may affect the viability of bacterial cells in a manner similar to their functions in mammalian cells.

In the present work, we used a well-known key mediator of mammalian apoptosis, FADD, to investigate whether this molecule causes bacterial cell death and, if so, what is the cause of death. FADD consists of two functional domains that have been determined by deletion mapping (Chinnaiyan *et al*., 1995). Its C-terminal domain is called the death domain (DD) and recruited to DD of FAS that is oligomerized by Fas ligand (Chinnaiyan *et al*., 1996). The N-terminal death effector domain (DED) of FADD is then associated with its homologous domain, DED1, of FLICE (procaspase-8), triggering the downstream pro-apoptotic cascade (Cohen, 1997; Medema *et al*., 1997; Juo *et al*., 1998).

DED of FADD contains six antiparallel, amphipathic α-helices (Eberstadt *et al*., 1998) and shows an overall structural similarity to DDs of FADD (Jeong *et al*., 1999) and FAS (Huang *et al*., 1996). Overexpression of FADD-DED alone induces mammalian cell death independently of an apoptotic signal through Fas (Muzio *et al*., 1996; Medema *et al*., 1997). We thought that mammalian pro-apoptotic molecules could trigger a bacterial cell death that is similar to mammalian cell death. If this is the case, bacterial cells may be a useful system for investigating mammalian apoptosis and for screening the molecules related to apoptosis. As the first step in exploring this possibility, we determined the structural domains of FADD by proteolytic mapping and tested whether these domains can trigger bacterial cell death. The results suggest that the DED of the mammalian pro-apoptotic molecule, FADD, induced bacterial cell death by enhancing the cellular level of ROS.

Results

Proteolytic and conformational analyses of the death effector domain of FADD

Deletion analysis suggested that DED and DD of FADD

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span from Phe-4 to Leu-79 and from Val-104 to Val-177 respectively (Chinnaiyan *et al*., 1995). However, it is not known whether these domains are equivalent to their exact structural units. Here, we subjected murine FADD to subtilisin digestion to define the structural domains of FADD. The peptides forming a compact conformation would be resistant to the proteolysis of subtilisin.

The subtilisin-digested peptide fragments of FADD were separated by electrophoresis to determine their N-terminal amino acid sequences and molecular weights. The two peptide fragments remained intact after 2 h digestion, suggesting that these peptides would form stable folding units resistant to proteolysis (Fig. 1A). These peptides were isolated by high-performance liquid chromatography (HPLC), and their N-terminal amino acid sequences and molecular weights were determined. The results indicated that the upper and lower bands in the gel are the peptides spanning the C-terminal 95 residues (residues Ala-89 to Ser-183) and the N-terminal 88 residues (residues Met-1 to Thr-88) respectively (data not shown). We have already shown that the C-terminal peptide forms a stable structure of the DD (Jeong *et al*., 1999) and thus thought that the subtilisin digestion defined the structural domains of FADD well. We then investigated the conformation of the isolated FADD-DED by circular dichroism (CD). The CD spectrum obtained from 190 nm to 260 nm was indicative of α-helical structure (Fig. 1B), consistent with its three-dimensional structure determined by nuclear magnetic resonance (NMR) (Eberstadt *et al*., 1998). The melting temperature of this domain was determined to be $\approx$60°C (data not shown). Based on these results, FADD-DED appears to form a stably folded structure.

The N-terminal DED of FADD induces bacterial cell death

Overexpression of the full-length or DED of FADD induced mammalian cell death, while its DD did not (Chinnaiyan *et al*., 1995). Here, we tested whether the full-length and proteolytically determined DED and DD of murine FADD affect the viability of *E. coli*. The cDNAs encoding the full-length DED and DD of FADD were cloned to the *E. coli* expression vector containing T7 promoter. All these constructs did not affect cell viability when the plasmids were maintained in *E. coli* DH5α in which T7 polymerase was not present (data not shown). We then introduced these constructs into *E. coli* BL21(DE3) in which T7 polymerase is expressed by IPTG induction. The transformants of *E. coli* BL21(DE3) containing pET3d-FADD-DED were not obtained, whereas the cells containing pET3d-FADD or pET14b-FADD-DD were generated (Fig. 2A). Although *E. coli* BL21(DE3) is not supposed to express T7 polymerase unless ITPG is added, it may contain a low level of T7 polymerase resulting from leaky expression. We thought that the cells containing pET3d-FADD-DED were killed by a trace amount of FADD-DED that was expressed by a low level of T7 polymerase.

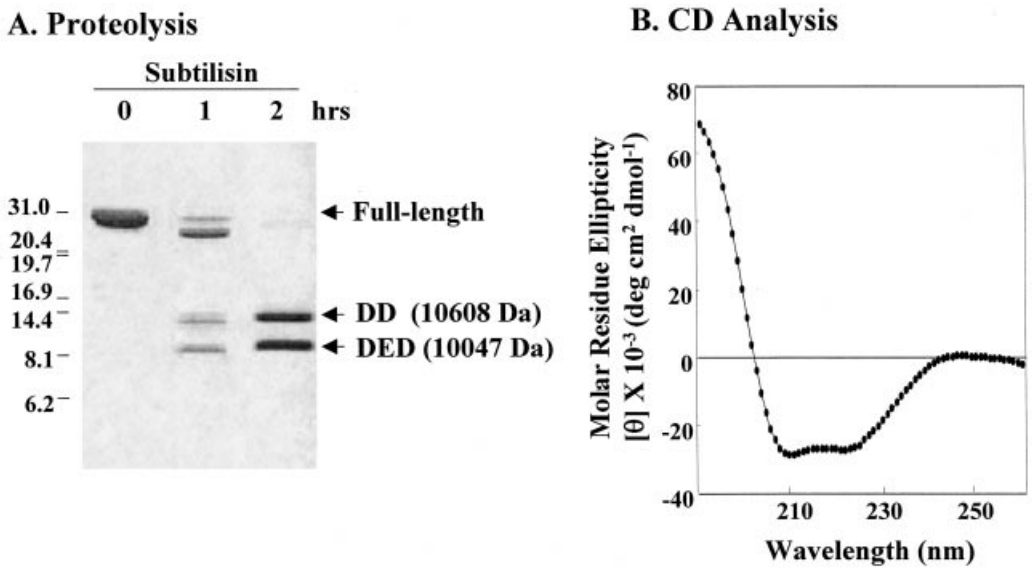

Fig. 1. Proteolytic digestion of the purified recombinant murine FADD and conformational analysis of FADD-DED.
A. The purified murine FADD was digested with subtilisin, and the digested protein was sampled in after 1 h and 2 h. The resulting peptides were resolved by SDS–PAGE. The two peptides resistant to subtilisin digestion were generated after 2 h digestion. The N-terminal amino acid sequences and molecular weights of the peptides were determined by Edman degradation and mass spectroscopy respectively. The upper and lower bands in the gel were determined to be the death effector domain (DED) from Met-1 to Thr-88 and the death domain (DD) from Ala-89 to Ser-183 respectively.
B. The conformation of the isolated FADD-DED was determined by circular dichroism. The CD spectrum is shown as molar residue ellipticity versus wavelength.

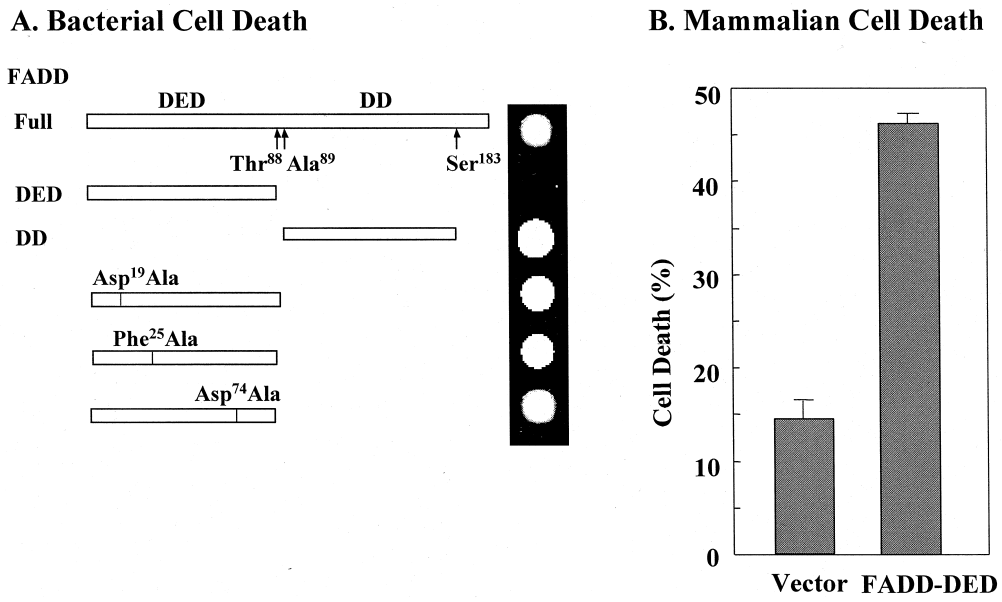

Fig. 2. The death effector domain of FADD induces bacterial and mammalian cell death.
A. Plasmids expressing the full length, DED and DD of FADD were transformed into *E. coli* BL21(DE3). Their effect on cell viability was determined by colony formation on LB plates containing kanamycin. Three substitution mutants were generated and also tested for their lethality.
B. Mammalian vector pcDNA3 containing the gene encoding FADD-DED was transfected into HeLa cells, and the apoptotic cells were counted as described in *Experimental procedures*.

To determine whether the toxicity of FADD-DED is specific to its structural integrity, we introduced substitution mutations to FADD-DED and tested their effect on the lethality of FADD-DED. Phe-25 and Asp-74 are located in helices 2 and 6, respectively, and Asp-19 is located between helices 1 and 2. All these residues are located on the surface of the protein and are conserved among DEDs and DDs of various death molecules, implying their functional importance (Eberstadt *et al.*, 1998). None of these substitution mutants induced cell death (Fig. 2A), although they were stably expressed, as determined by immunoblotting (data not shown). Further deletion of FADD-DED or tagging of heterologous peptide to this domain also abolished the lethal activity (data not shown). Its extreme toxicity in trace amounts and the loss of its toxicity by various mutations strongly suggest that the lethal effect of FADD-DED requires its native conformation and is related to its intrinsic biological function, but is not a result of its misfolding.

We then tested whether FADD-DED can also induce mammalian cell death. The HeLa cells transfected with pcDNA3-FADD-DED and the vector alone showed about 45% and 15% cell death respectively (Fig. 2B). Thus, FADD-DED consisting of the N-terminal 88 amino acids induced bacterial as well as mammalian cell death.

Expression of FADD-DED induced cell morphology change

As we thought that the lethal effect of FADD-DED was a result of its leaky expression, the structural gene of FADD-DED was transferred into another expression vector, pET28a, which contains the *lacI* gene, to control the expression of T7 polymerase more tightly. As expected, the *E. coli* BL21(DE3) containing pET28a-FADD-DED was viable on LB plates in the absence of IPTG but not in the presence of IPTG (data not shown). *E. coli* BL21(DE3) containing pET28a-FADD-DED was grown in LB broth without IPTG, and the expression of FADD-DED was then induced with different concentrations of IPTG. Cell growth was more severely inhibited according to the amount of IPTG added (Fig. 3A). We then added 500 μM IPTG to the *E. coli* cells containing pET28a and pET28a-FADD-DED and compared the number of viable cells at various time intervals. Almost all the *E. coli* cells containing pET28a-FADD-DED lost viability after IPTG induction, while those containing pET28a alone were still viable, although the two *E. coli* stains were both viable at the same level at the time of IPTG induction (Fig. 3B). The cellular level of FADD-DED was increased according to the time course after IPTG induction (Fig. 3C). These results indicate that the expression of FADD-DED was responsible for cell death in *E. coli*.

We then investigated whether the morphology of the *E. coli* cells is changed by FADD-DED expression. Expression of Bax generated ROS, decreased mitochondrial membrane potential in mammalian cells (Xiang *et al.*, 1996) and also gives a similar effect in *E. coli* cells (Asoh *et al.*, 1998). The production of ROS caused elongation of

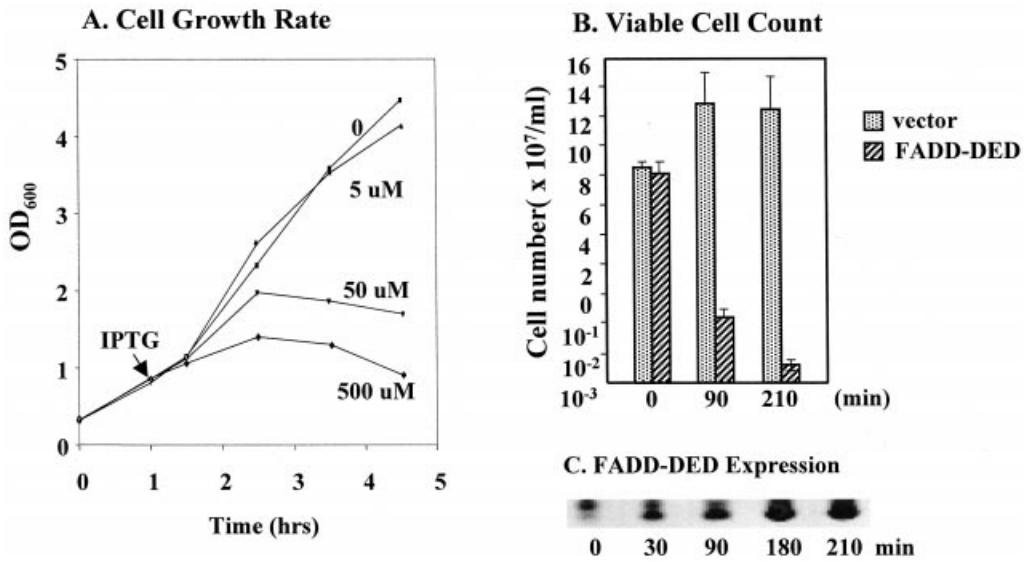

Fig. 3. *E. coli* cell death depends on expression of FADD-DED.
A. *E. coli* BL21(DE3) containing pET28a-FADD-DED were grown in LB broth, and expression of FADD-DED was induced by adding the indicated amounts of IPTG. Cell growth was monitored by OD_{600}.
B. *E. coli* BL21(DE3) containing pET28a-FADD-DED were grown in LB broth in the presence of 0.5 mM IPTG, and the cells were sampled at various time intervals and plated on LB to count the viable cells.
C. Expression of FADD-DED was induced from *E. coli* BL21(DE3) containing pET28a-FADD-DED and determined by immunoblotting with anti-FADD-DED antibody.

the *E. coli* cells (Brandi *et al*., 1989a). If FADD-DED induces ROS production in *E. coli*, we thought that it might cause a similar effect to Bax on the morphology of *E. coli*. The cells were gradually elongated, and the frequency of the elongated cells was increased according to the time course after IPTG induction (Fig. 4). In contrast, the *E. coli* cells containing vector alone showed normal morphology. Although a similar morphological change has also been reported by other toxic agents (Phillips *et al*., 1967; Suzuki *et al*., 1967; Hrebenda *et al*., 1985; Brandi *et al*., 1989b), we thought that cell elongation might have resulted from the increased ROS based on the functional analogy of FADD and Bax in apoptosis.

DNA damage and mutation frequency are increased by the expression of FADD-DED

The enhanced ROS may also increase chromosomal damage and mutation (Moody and Hassan, 1982; Ames and Gold, 1991). The chromosomal damage in *E. coli* cells containing pET28a-FADD-DED and the vector alone was determined by measuring the nicked DNAs. The chromosome was alkaline denatured, and the nicked DNAs were isolated and resolved by gel electrophoresis. The amount of nicked DNA was determined by hybridization using random *E. coli* genomic fragments as a probe. The nicked DNA became apparent 210 min after induction of FADD-DED, whereas almost no nicked DNA was detected in cells containing the plasmid alone (Fig. 5A). The DNA damage induced by FADD-DED was also determined by comparing the mutation frequency between the *E. coli* cells containing pET28a-FADD-DED and pET28a alone. After incubating these cells on LB plates, they were pooled and respread on LB plates with and without rifampicin, which was chosen as a marker of mutation (Asoh *et al*., 1998). The rifampicin-resistant cells were generated at $\approx 3.2 \times 10^{-9}$ and 4×10^{-10} from the cells containing pET28a-FADD-DED and pET28a respectively

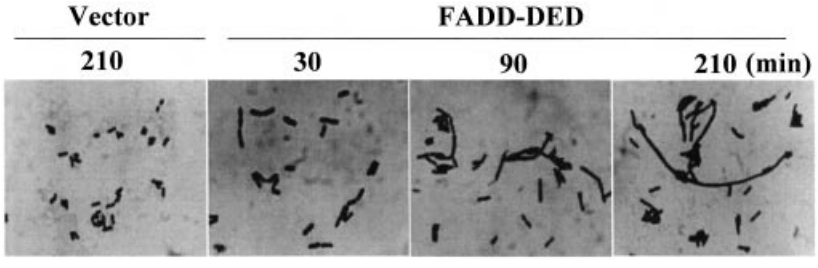

Fig. 4. Morphological change in *E. coli* induced by FADD-DED. Expression of FADD-DED was induced with 0.5 mM IPTG, and the morphological change was monitored by microscopy.

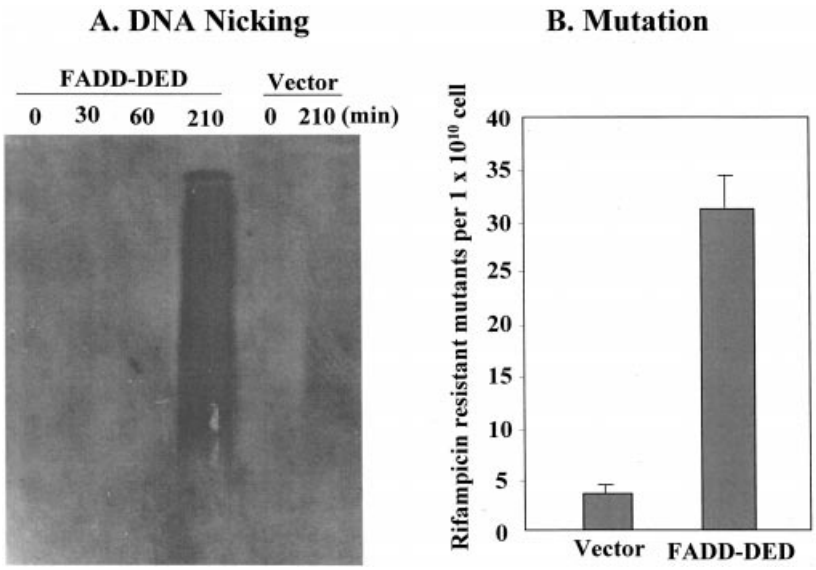

Fig. 5. Chromosomal damage and mutation frequency are increased by expression of FADD-DED in *E. coli*.
A. Expression of FADD-DED was induced by 0.5 mM IPTG in *E. coli* BL21(DE3) containing pET28a-FADD-DED or the vector alone. The nicked chromosomal DNAs were separated from the cells at the indicated times after IPTG induction and detected by Southern blotting with random *E. coli* genomic fragments, as described in *Experimental procedures*.
B. *E. coli* BL21(DE3) cells containing pET28a-FADD-DED were incubated on LB plates until they formed colonies. They were then pooled and spread on LB plates with and without rifampicin (Rifampicin resistance was used as a marker for mutation.) The rifampicin-resistant colonies were counted and divided by the number of colonies formed on LB plates without rifampicin.

(Fig. 5B). This result suggests that the occurrence of rifampicin-resistant cells was increased about 10-fold even by a trace level expression of FADD-DED.

The lethal effect of FADD-DED is abolished by reducing agents

We then tested whether the lethality of FADD-DED is abolished by proteins that can quench ROS. Thioredoxin is a protein that can control cellular redox potential by the formation of a disulphide bond between two cysteines located in the active site. We introduced pET28a-FADD-DED into *E. coli* BL21(DE3) overexpressing thioredoxin, and the lethal effect of FADD-DED was tested by IPTG induction. The *E. coli* cells overexpressing thioredoxin grew well on the ITPG-containing LB plate, while the same strain without thioredoxin did not (Fig. 6A). This result suggests that the lethal effect of FADD-DED is abolished by a high level of thioredoxin. To see whether the reduced form of thioredoxin is important for this quenching effect, pET28a-FADD-DED was transformed into the *E. coli* AD494(DE3) strain in which thioredoxin reductase is defective. As this strain lacks thioredoxin reductase, the reduced form of thioredoxin is not made. As expected, this cell did not form a colony on the IPTG-containing LB plate, indicating that the reducing potential

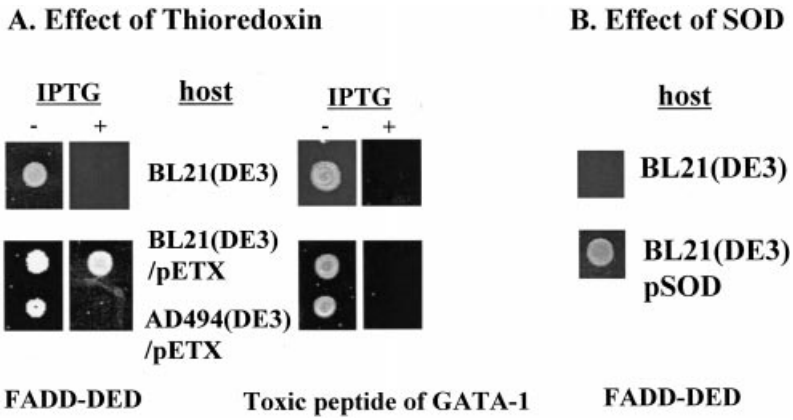

Fig. 6. The effect of thioredoxin and SOD on the cell growth of *E. coli* expressing FADD-DED.
A. The chloramphenicol-resistant plasmid pETX expressing thioredoxin was transformed into *E. coli* BL21(DE3) and AD494(DE3) (Novagen), which is a thioredoxin reductase-defective mutant of BL21(DE3). Plasmid pET28a-FADD-DED was then transformed into each of these strains, and the transformants were incubated on LB plates containing the selected antibiotics in the absence and presence of IPTG. The plasmid expressing a toxic peptide derived from mouse transcription factor GATA-1 was also transformed into the same tester strains, and its expression was also induced by IPTG.
B. The effect of SOD on the lethality of FADD-DED was tested. The FADD-DED gene was cloned in pACYC184 and transformed into *E. coli* BL21(DE3) with and without pSOD (the plasmid expressing SOD of *Aquifex pyrophilus*), and the transformants were detected on each of the selected plates.

of thioredoxin is essential for the suppression of FADD-DED lethal activity.

We also tested the lethal effect of the domain derived from mouse transcription factor GATA-1 to see whether this peptide also induces ROS (Trudel *et al*., 1996). The peptide consisting of a zinc finger and basic amino acids binds to the bacterial replication origin to give a toxic effect. The *E. coli* cells expressing this peptide did not form a colony on the IPTG-containing plate as reported previously. However, the lethal effect of this peptide was not neutralized by co-expression of thioredoxin (Fig. 6A). This result means that the induction of ROS is specific to FADD-DED. To confirm further the involvement of the FADD-DED-induced ROS in cell killing, we also expressed FADD-DED in *E. coli* BL21(DE3) expressing superoxide dismutase (SOD) of *Aquifex pyrophilus* (Lim *et al*., 1997). Expression of this enzyme also quenched the killing effect of FADD-DED (Fig. 6B).

Lethality of FADD-DED is relieved by depletion of oxygen

If ROS increased by FADD-DED is a real cause of cell death, the lethal effect of FADD-DED would be relieved by scavenging ROS or by the depletion of oxygen. To explore this possibility, we first cultivated *E. coli* BL21(DE3) expressing FADD-DED in the presence of a reducing agent, vitamin E. While the cells ceased growth 1 h after IPTG induction in the absence of vitamin E, growth continued in the presence of vitamin E (Fig. 7A). The same cells were cultivated under partially anaerobic conditions. The cells continued to grow after the induction of FADD-DED, although growth was retarded (Fig. 7B). All these results support the hypothesis that ROS induced by FADD-DED is responsible for cell death.

Genetic suppression of the lethal effect of FADD-DED

ROS appears to be a common mediator for both mammalian and bacterial cell death (Hochman, 1997; Storz and Imlay, 1999). Fas activation increases ROS via NADPH oxidase (Suzuki *et al*., 1998). FADD is the immediate downstream effector of Fas and interacts with the homologous domain of FLICE (pro-caspase-8) (Cohen, 1997; Medema *et al*., 1997; Juo *et al*., 1998). The activated caspase-8 then cleaves BID, which is translocated into mitochondria, damaging its membrane potential (Li *et al*., 1998). These results support the notion that ROS may be involved in FADD-mediated mammalian cell death.

The results of the experiments we carried out suggest that FADD-induced ROS is responsible for bacterial cell death as well. If this is the case, the *E. coli* proteins that can control the cellular level of ROS may suppress the lethal effect of FADD-DED. To explore this possibility, we co-expressed the *E. coli* genomic library with FADD-DED to select genes that can restore cell viability. The majority of the selected genes encoded proteins involved in the

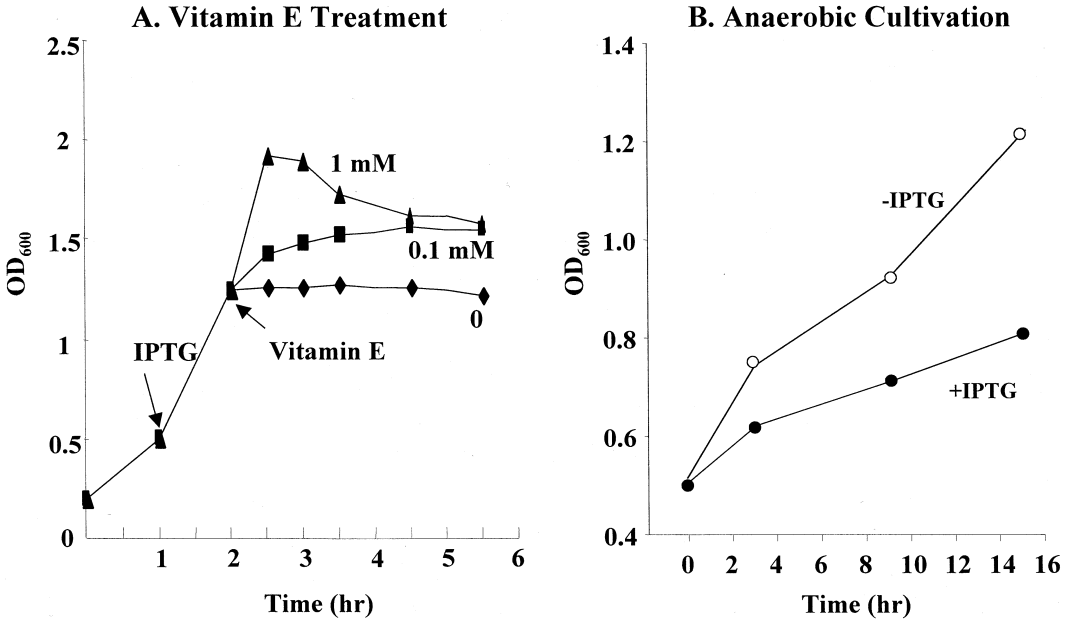

Fig. 7. The effect of vitamin E on the lethal effect of FADD-DED.
A. *E. coli* BL21(DE3) containing pET28a-FADD-DED was cultivated in LB broth containing ampicillin. Expression of FADD-DED was induced by the addition of 0.1 mM IPTG, and then vitamin E (0.1 and 1 mM) was added to the growth media after 1 h. Cell growth was monitored by OD_{600}.
B. The same cells were cultivated in air-sealed tubes without agitation until OD_{600} reached 0.5. Cell growth was then monitored at various time intervals after the addition of IPTG at 0.5 mM.

Table 1. The *E. coli* genes selected to suppress the lethality of FADD-DED.

Genes	Functions	Frequency[a]
cyoB	Cytochrome O ubiquinol oxidase subunit I	3
aldA/gapC	Aldehyde dehydrogenase glyceraldehyde 3-phosphate dehydrogenase	2
nuoK/J/I/H	NADH dehydrogenase I chain K/J/I/H	1
ORF (62 min)	Homologue to 2-keto-3-deoxygluconate oxidoreductase of *B. subtilis*	2
yeeS	*radC* family	1

The *E. coli* genes were co-expressed with FADD-DED to screen those that could suppress the lethality of FADD-DED. The plasmids containing the *E. coli* genes were isolated from the viable cells, and the selected *E. coli* genes were identified.**a.** Number of identical clones selected.

metabolism of oxidation and reduction, such as cytochrome O oxidase, aldehyde dehydrogenase A/glyceraldehyde 3-phosphate dehydrogenase, NADH dehydrogenase I chain and an open reading frame (ORF) encoding a protein homologous to 2-keto-3-deoxygluconate oxidoreductase of *Bacillus subtilis* (Table 1). These oxidoreductases are expected to modulate the cellular redox potential that can buffer the toxic effect of ROS induced by FADD-DED. A *radC* family protein (*yeeS*) was also selected. Although the function of this protein is not completely clear, it may be involved in repairing DNA damaged by ROS (Felzenszwalb *et al*., 1987). In addition, a few genes encoding proteins of unknown functions were also selected (data not shown). Although the detailed mechanisms of the suppression of these genes on the lethal effect of FADD-DED need further investigation, the selection of these suppressors is consistent with the involvement of ROS in bacterial cell death.

Discussion

Although DED and DD are located in the N- and C-terminal regions of FADD, their exact structural boundaries have not been well defined. In the present work, we used proteolytic cleavage to define the structural units for DED and DD of FADD. The subtilisin cleavage of FADD generated two proteolysis-resistant peptide fragments (Fig. 1A). One of the resulting peptides from Ala-89 to Ser-183 formed a stable folding unit of DD whose structure was solved recently (Jeong *et al*., 1999). This result suggested that the other domain spanning from Met-1 to Thr-88 should be a structural unit for DED. Although this domain is a little larger than the size of DED determined previously by deletion mapping (Chinnaiyan *et al*., 1996), it forms a stable α-helical conformation, as determined by CD analysis (Fig. 1B).

Point mutations of FADD-DED abolished its lethal effect (Fig. 2A), implying that the structural integrity of FADD-DED is important for lethal activity. Human FADD-DED consists of six antiparallel helices. The substitution mutation at Phe-25 located in helix α2 abolished its lethal activity in both *E. coli* (Fig. 2A) and mammalian cells (Eberstadt *et al*., 1998). It suggests that the same protein surface of FADD-DED may be used in triggering lethal activity. Two other mutations at Asp-19 and Asp-74 are also expected to affect the interaction surface that is not yet understood clearly. Based on these results, it appears that the lethality of FADD-DED results from its intrinsic biological activity.

The expression of FADD-DED induced cell elongation (Fig. 4) and DNA damage (Fig. 5), implying that the enhanced level of ROS was responsible for these results. To confirm this possibility, we used reducing proteins and reagents or anaerobic conditions to test whether these treatments can neutralize the lethal effect of FADD-DED. ROS can be removed by the thioredoxin/thioredoxin reductase system (Fernando *et al*., 1992; Das *et al*., 1997). Thioredoxin is a detoxification agent against oxidative stress and is involved in the oxidative cell death pathway in mammalian cells (Spector *et al*., 1988; Fernando *et al*., 1992). It inhibits oxidative cell death by binding to ASK-1 (apoptosis signal-regulating kinase 1), a kinase leading to cell death. Thioredoxin is reduced by a thioredoxin reductase coupled to NADPH in normal cells (Lennon and Williams, 1996; Prinz *et al*., 1997; Takemoto *et al*., 1998). Likewise, SOD protects from oxidative DNA damage (Keyer *et al*., 1995). We found here that thioredoxin or SOD detoxified FADD-DED (Fig. 6). Vitamin E (Fuentes and Amabile-Cuevas, 1998) or anaerobic conditions partially relieved the killing effect of FADD-DED (Fig. 7). In addition, most of the *E. coli* genes that suppressed the lethality of FADD-DED were related to the redox potential of the cell (Table 1). All these results strongly suggest the involvement of ROS in cell death triggered by FADD-DED.

Alternatively, FADD-DED may induce bacterial cell death via deadly interactions with essential *E. coli* proteins. However, this possibility is not supported for the following reasons. First, if the lethal effect of FADD-DED were blocked by physical interactions with *E. coli* proteins, these proteins would not be metabolically related. However, the selected suppressors were mostly clustered to oxidation/reduction processes (Table 1). Secondly, we used a yeast two-hybrid system (Gyuris *et al*., 1993) to screen the *E. coli* proteins that may interact with FADD-DED. However, no proteins were selected to interact with FADD-DED even after screening of more than 20 000

clones (data not shown). Thirdly, as eukaryotic DED can make homotypic interactions with the homologous domains (Cohen, 1997; Medema *et al*., 1997; Juo *et al*., 1998), FADD-DED may bind to the *E. coli* proteins containing its homologous domain. However, *E. coli* proteins with the homologous domain have not been identified so far (Aravind *et al*., 1999). Based on these observations, a fortuitous lethal interaction between FADD-DED and *E. coli* protein(s) is unlikely.

Combined together, it can be concluded that FADD-DED would induce bacterial cell death by enhancing the level of ROS by an as yet unknown mechanism. As ROS works in both prokaryotic and eukaryotic systems as a death mediator, *E. coli* may be used as a convenient system to investigate the molecular mechanism of cell death.

Experimental procedures

Proteolysis and circular dichroism analysis of FADD-DED

The recombinant murine FADD was induced in *E. coli* BL21(DE3) with 0.4 mM IPTG at 37°C for 3 h from pET3d (Novagen) containing its structural gene. The cells were harvested and disrupted by ultrasonication in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol and 1 mM phenylmethylsulphonyl fluoride (PMSF). The proteins were precipitated by adding ammonium sulphate up to 30% to the soluble fraction of the cell extract. The precipitant was collected by centrifugation, dialysed overnight at pH 4.0 and purified further by reverse-phase HPLC using a C_8 Vydac column. The purified FADD protein was digested with subtilisin for 2 h at 15°C in phosphate buffer at pH 8.0. The digested peptides were resolved in 15% SDS–polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (MSI). The N-terminal amino acid sequence and molecular weight of the peptides were determined using a Procise491 amino acid sequencer (ABI) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy (Hewlett-Packard, HPG2025A) respectively. The conformation of the isolated FADD-DED was determined by circular dichroism (CD) analysis (JASCO 715). FADD-DED generated by subtilisin digestion was isolated as described above and dissolved at 0.1 mg ml^{-1} in 10 mM sodium phosphate (pH 7.0). A CD spectrum was generated by scanning from 190 nm to 260 nm at 25°C.

Construction of FADD mutants

The DNA fragments encoding proteolytically determined DED (Met-1 to Thr-88) and DD (Ala-89 to Ser-183) of FADD were isolated by polymerase chain reaction (PCR) using their specific primers and cloned into pET3d and pET14b, respectively, using *Nco*I and *Bam*HI sites that were artificially designed into the primers. The Asp-19(r)Ala, Phe-25(r)Ala and Asp-74(r)Ala mutants were generated by PCR mutagenesis using the specific mutagenic PCR primers. The primers for the 5′ and 3′ ends of the gene for FADD-DED were paired with each of the mutagenic primers. The two PCR fragments containing the same region of the mutated site were obtained and mixed again for the second PCR. The two fragments were then combined during the second PCR to cover the whole structural region for FADD-DED. The mutated genes for FADD-DED were then cleaved with *Nco*I and *Bam*HI and cloned into the same sites of pET3d. The mutations were confirmed by DNA sequencing.

Determination of cell viability and morphology

The DNA fragment encoding FADD-DED was cleaved with *Bgl*II and *Hin*dIII and transferred into the same sites of pET28a. As pET28a contains the *lacI* gene, the expression of FADD-DED is more tightly repressed in the absence of IPTG. *E. coli* BL21(DE3) cells harbouring pET28a-FADD-DED were grown in LB broth containing kanamycin at 50 μg ml^{-1}. The expression of FADD-DED was induced by adding different concentrations of IPTG, and cell growth was measured by OD_{600}. To determine the number of viable cells, the culture broth was sampled at various time intervals after induction of FADD-DED with 0.5 mM IPTG, and the sampled cells were plated on LB plates containing kanamycin. To monitor cell morphology, the sampled cells were stained with 1% crystal violet and observed by light microscopy at 1200 × magnification.

The effect of FADD-DED on the mammalian cells was tested using HeLa cells. The structural gene for FADD-DED was obtained by PCR using the specific primers. The PCR product was cleaved with *Eco*RI and *Sal*I that were designed into the primers and cloned into the mammalian expression vector, pcDNA3 (Invitrogen), cleaved with *Eco*RI and *Xho*I. The resulting plasmid was then transfected into HeLa cells using GenePorter (Gene Therapy System). The β-galactosidase gene cloned in the same vector was co-transfected to identify the transfected cells. The cells were fixed with 0.2% glutaraldehyde and 2% formaldehyde 24 h after transfection, and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside for 3–5 h. The apoptotic cells were determined by apoptotic morphology among the transfected cells (Yang *et al*., 1997). At least 500 β-galactosidase-positive cells were scored for each transfection in triplicate, and the mean percentages of apoptotic blue cells and their standard errors were determined.

Analysis of nicked DNA

The DNA nicking induced by FADD-DED was analysed as described previously (Asoh *et al*., 1998). *E. coli* BL21(DE3) cells containing pET28a-FADD-DED or pET28a vector were cultured in LB broth at 37°C until OD_{600} reached 0.8. Expression of FADD-DED was induced by adding 0.5 mM IPTG. The cells were harvested at different time intervals and lysed with 1 N NaOH. Chromosomal DNA was alkaline denatured, and the nicked DNA was separated from intact DNA by ultracentrifugation at 100 000 *g* for 1 h. The single-stranded DNA in the supernatant was precipitated with ethanol and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The contaminating RNA was removed by RNase A treatment, and the remained DNA was precipitated with isopropanol again. The pellet was redissolved in TE buffer

and resolved in a 1% alkaline agarose gel (50 mM NaOH, 1 mM EDTA). The single-stranded DNA was then transferred onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). The transferred DNAs on the membrane were hybridized with digoxigenin-labelled probe prepared by PCR with random primer using *E. coli* genomic DNA as a template.

Determination of mutation frequency

To see whether FADD-DED expression increases mutation, the frequency of rifampicin-resistant cells was determined as described previously with a slight modification (Asoh *et al.*, 1998). *E. coli* BL21(DE3) cells containing either pET28a-FADD-DED or the vector alone were incubated on LB plates containing kanamycin for 16 h. The colonies on each plate were pooled, and $1 \infty 10^{10}$ cells were spread on kanamycin-containing LB plates with and without rifampicin (50 μM), which was chosen as an indicative marker to monitor spontaneous mutation frequency. The colonies on each plate were counted to determine the frequency of rifampicin-resistant cells.

Immunoblotting of FADD-DED

E. coli BL21(DE3) cells expressing FADD-DED were lysed, and the proteins were separated by SDS–PAGE. The proteins in the gel were transferred to PVDF membrane (Millipore), and FADD-DED was bound by immunoblotting with anti-human FADD mouse antibody (Pharmingen). The antibody complex was then detected using a enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Co-expression of thioredoxin or SOD with FADD-DED

The thioredoxin gene of *E. coli* was isolated by PCR with the specific primers and cloned at the *Bam*HI site of pACYC184 to generate pETX (K.-S. Kim, unpublished results). This recombinant plasmid was transformed into *E. coli* BL21(DE3) and AD494(DE3) *(Δara-leu7967, ΔlacX74, ΔphoApvuII phoR ΔmalF3,* F′[*lac*$^+$*(lacIq)pro*]*trxB::kan*(DE3)), which is a defective mutant of thioredoxin reductase derived from BL21(DE3). The transformants were selected on LB plates containing chloramphenicol. pET28a-FADD-DED was introduced to each of the transformed cells, and the transformants were selected on LB plates containing chloramphenicol and kanamycin. Expression of FADD-DED was then induced on LB plates containing 0.5 mM IPTG. To compare the killing effect of FADD-DED with other toxic peptides, another plasmid, pGATA (CPC USA), expressing a toxic peptide derived from mouse transcription factor GATA-1, was also transformed into the same tester strains. The gene encoding Fe-superoxide dismutase (SOD) of *Aquifex pyrophilus* was cloned at the *Nco*I and *Bam*HI sites of pET3c to generate pSOD (Lim *et al.*, 1997). The FADD-DED gene cloned at *Bgl*II/*Hin*dIII was cut from pET3d and cloned into pACYC184. Expression of FADD-DED from pACYC184-FADD-DED was also induced by IPTG in *E. coli*.

Cell growth in the presence of vitamin E or in anaerobic conditions

The *E. coli* BL21(DE3) containing pET28a-FADD-DED was cultured in LB broth containing kanamycin, and 0.5 mM IPTG was added to induce FADD-DED. Vitamin E was added 1 h after IPTG induction, and cell growth was monitored by OD_{600}. The same cells were distributed into 1.5 ml Eppendorf tubes completely filled with LB broth and cultivated without agitation until OD_{600} reached 0.5. Then, after IPTG was added at 0.5 mM, cell growth was monitored.

Genetic suppression of FADD-DED with E. coli genes

The *E. coli* chromosome was digested with *Bam*HI, and the 3–5 kb genomic fragments were ligated into the *Bam*HI site of pBR322. The ligation mixture was transformed into *E. coli* DH5α, and the transformants were pooled to isolate the plasmids. The plasmids were retransformed into *E. coli* BL21(DE3). Then, pACYC184-FADD-DED was additionally transformed into the cells containing pBR322 with each of the *E. coli* genes. The transformants forming colonies were selected on LB plates containing ampicillin and chloramphenicol (30 μg ml^{-1} each). The plasmids were isolated from the viable cells to determine the *E. coli* genes cloned in pBR322.

Acknowledgements

This work was supported in part by a grant from National Creative Research Initiatives (S.K.), by Critical Technology 21 (K.-S.K.) of the Ministry of Science and Technology and by KIST2000 (K.-S.K.). We are grateful to Dr W. G. Seol for critical reading of the manuscript.

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