

## Full-length Fas-associated Death Domain Protein Interacts with Short Form of Cellular FLICE Inhibitory Protein

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Fas-associated death domain protein (FADD) recruits and activates procaspase-8 through interactions between the death effector domains of these two proteins. Cellular FLICE-inhibitory protein (c-FLIP) was identified as a molecule with sequence homology to caspase-8. It has been postulated that c-FLIP prevents formation of the competent death-inducing signaling complex in a ligand-dependent manner, through its interaction with FADD and/or caspase-8. However, the interaction of FADD and c-FLIPs (short form) in apoptosis signaling has been controversially discussed. We show the purification and the characterization of human full-length FADD and c-FLIPs expressed in *Escherichia coli*. The purified FADD and c-FLIPs are shown as homogeneity, respectively, in SDS-PAGE analysis and light-scattering measurements. The folding properties of the  $\alpha$ -helical structure of FADD and the super-secondary structure of c-FLIPs proteins were characterized by circular dichroism spectroscopy. Furthermore, we report here a series of biochemical and biophysical data for FADD-c-FLIPs binding *in vitro*. The binding of both FADD and c-FLIPs proteins was detected by BIAcore biosensor, fluorescence measurement, and size-exclusion column (SEC).

**Key Words :** Overexpression, Purification, Full-length FADD, c-FLIPs (short form), Interaction

### Introduction

Apoptosis, or programmed cell death, is critical for tissue homeostasis in multicellular organisms. Many diseases are associated with either too much or too little apoptosis, such as AIDS, cancer, and autoimmunity.<sup>1</sup> Apoptosis is initiated by a variety of stimuli, including growth factor withdrawal, UV or  $\gamma$ -irradiation, chemotherapeutic drugs, and death receptor signals. The death receptors contain an intracellular death domain (DD), which is essential for transduction of the apoptotic signal. Six subfamily members of death receptors are known so far, TNFR1 (also called CD120a), CD95 (APO-1/Fas), DR3 (APO-3, LARD, TRAMP, and WSL1), TRAIL-R1 (APO-2 and DR4), TRAIL-R2 (DR5, KILLER, and TRICK2), and DR6.<sup>2</sup> Death receptors are activated by their natural ligands, which have coevolved as a death ligand family, called the tumor necrosis factor (TNF) family.

Fas-associated death domain protein (FADD) is an adaptor molecule that bridges the interactions between membrane death receptors and initiator caspases. Caspases are a family of aspartate-specific cysteine proteases that are necessary for execution of apoptosis. The clustering of Fas receptors, FADD, and procaspase-8, termed death-inducing signaling complex (DISC), is essential for Fas-mediated apoptosis and caspase-8 activation.<sup>3,4</sup> In death-inducing signaling complex formation, FADD mediates signals from Fas receptors to procaspase-8 with its C-terminal death domain (DD) and N-terminal death-effector domain (DED). FADD also participates in signaling other members of the tumor necrosis factor receptor (TNFR) family.

TNF-related apoptosis-inducing ligand (TRAIL) is a type II membrane protein that is processed proteolytically at the

cell surface to form a soluble ligand.<sup>5-8</sup> Due to the tumor selectivity of FADD-induced apoptosis and its ubiquitous expression, it has been postulated that the apoptosis pathway induced by TRAIL is tightly regulated by several mechanisms to prevent spontaneous cell death. One such mechanism involves the cellular inhibitor of apoptosis, cellular FLIP (c-FLIP), which is also known as I-FLICE, FLAME, CASPER, or CASH.<sup>9-15</sup> c-FLIP molecules have several alternative splicing variants at the transcriptional level, but at the protein level, two isoforms of c-FLIP, the long form (c-FLIP<sub>L</sub>, 55 kDa) and the short form (c-FLIP<sub>S</sub>, 28 kDa), exist.<sup>11,16</sup> c-FLIP<sub>L</sub> contains an additional caspase-like domain at its C-terminal part that is deficient in c-FLIP<sub>S</sub>. c-FLIP is a human homolog of viral FLICE-inhibitory proteins (v-FLIPs), which are components of  $\gamma$ -herpesvirus and have anti-apoptotic activity.<sup>9</sup> A short form of c-FLIP structurally resembles v-FLIP.<sup>11</sup> Conflicting data exist about the direct interaction of FADD and c-FLIP in apoptosis signaling.<sup>11,12,14,15</sup>

Herein, we show the methods of efficient overexpression and purification that generate homogeneous full-length FADD and c-FLIPs proteins. Furthermore, we report a series of biochemical and biophysical data for FADD-c-FLIPs binding. Until now, there have been no reports of full-length FADD and c-FLIPs structures. This study could be preliminary studies for the structural bases and identification of the apoptotic signaling pathways of FADD and c-FLIPs proteins.

### Materials and Methods

**Subcloning, Expression, and Purification.** In the present study, full-length human FADD (1-208 amino-acids) and c-

FLIPs (short form, 1-221 amino-acids) were subcloned into non his-tagged vectors, pQE-30 and pET-21b, respectively. FADD and c-FLIPs were expressed in *Escherichia coli* BL21(DE3) strains. Cells were initially grown in 5 mL of LB-ampicillin (50  $\mu$ g/mL) overnight and maintained at 37 °C. Inoculums were added to 2 L of LB-ampicillin. The large cultures were immediately moved to an incubator at 37 °C. When the culture reached an  $A_{600}$  of 0.5-0.7, isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce FADD as well as c-FLIPs. After 4-6 hrs, the cells were harvested and stored frozen at -70 °C. FADD and c-FLIPs bacterial lysates were prepared by sonication in buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 5% Glycerol, and 1 mM DTT). Up to 20% ammonium sulfate for FADD was added to the soluble fraction of the cell extract. The precipitated FADD cell pellets were resuspended two times in buffer A (30 mM ammonium acetate [pH 4.0], 300 mM NaCl, 0.1% Triton X-100, and 1 mM DTT). The pH in the protein solution was changed to protect the aggregation by 30 mM sodium citrate (pH 5.0-5.5). The supernatant for the FADD was used in purification, but c-FLIPs was used as the pellet in purification. The inclusion body for c-FLIPs was resuspended more than five times in buffer A. All of the purification methods required a superdex 75 column (Amersham-Biosciences). The full-length FADD and c-FLIPs protein solutions were concentrated to 3-5 mg/mL using a Vivaspin 20 (Satorius).

**Refolding.** To obtain more soluble and stable proteins of FADD and c-FLIPs for crystallization, a refolding process was performed. The full-length FADD and c-FLIPs genes were subcloned into expression vectors, his-tagged pET15b and pET-21b, respectively. The constructs were transformed into the expression host *Escherichia coli* BL21(DE3)RIL strains. The cells were disrupted in buffer B (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, and 1 mM DTT) by sonication on ice at a high setting for five cycles of 20 s with a 5 min gap between each cycle, and then centrifuged at 14,500 rpm for 15 min at 4 °C. The inclusion bodies of the FADD and c-FLIPs were resuspended under protein denaturing conditions in buffer B containing 6 M urea. The resuspended cells were then centrifuged at 14,500 rpm for 45 min at 4 °C in a refrigerated high speed centrifuge. The supernatant was collected and loaded onto a column containing 7 ml pre-equilibrated Ni-NTA resin. The loaded protein was refolded by gradient against refolding buffer B. The resin was washed with buffer B containing 20 mM imidazole and then eluted with buffer B containing 200 mM imidazole. The eluted fractions were dialyzed against buffer B to remove the imidazole. The FADD and c-FLIPs proteins were purified as (His)<sub>6</sub>-tagged fusion proteins. All purifications required the nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen), the resource Q anion exchange column (Amersham Biosciences), and a Superdex 75 column (Amersham-Biosciences). The purity of purified FADD and c-FLIPs was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% (w/v) stacking and 15% (w/v) resolving gels. The protein solutions were concentrated to 6-10

mg/ml using a Vivaspin 20 (Satorius).

The purified FADD and c-FLIPs were mixed in 1 : 1 molar ratio. After incubation for 12 hrs at 4 °C, the mixture was loaded on Superdex 200 HR 10/30 size-exclusion column (SEC) (Amersham Pharmacia Biotech). The fractions containing the FADD-c-FLIPs complex were collected and boiled at 90 °C for 10 min to elute the proteins followed by loading on SDS-PAGE. The native gel electrophoresis (12.5%) was carried out at 4 °C using 1 $\times$  gel buffer.

**Circular Dichroism (CD) spectroscopy.** Data were collected on a JASCO J-715 spectropolarimeter equipped with a thermoelectric temperature controller. CD spectra were recorded with protein samples (5-10  $\mu$ M) in a 20 mM phosphate buffer [pH 5.0] at 25 °C over the range of 200-260 nm in a nitrogen atmosphere. Each spectrum was the accumulation of three scans corrected by subtracting signals from the buffer control. The resulting spectrum was smoothed and corrected for mean residue weight concentration and cell pathlength using JASCO data analysis software.

**Dynamic Light Scattering (DLS).** All of the samples were degassed and dust-free. The dynamic light-scattering spectra of the FADD and c-FLIPs were obtained to identify the distributions of species of differing molecular masses, degree of aggregation, shape, and branching (SEC-mode). Light-scattering spectra were recorded with protein samples (0.1-0.2  $\mu$ M). The molecular weight range was measured from  $\sim 10^3$  to  $\sim 10^9$  Daltons, and the molecular size range was measured from 10 to 500 nm.

**BIAcore Biosensor Analysis.** Measurements of the apparent dissociation constants ( $K_D$ ) between FADD and c-FLIPs were carried out using a BIAcore 2000 biosensor (Biosensor, Sweden). FADD (30  $\mu$ g/ml in 10 mM MES [pH 6.0]) was covalently bound to the carboxylated dextran matrix at a concentration corresponding to -1,200 response units (RU) by an amine coupling method, as suggested by the manufacturer. A flow path involving two cells was employed to simultaneously measure the kinetic parameters from one flow cell containing the FADD-immobilized sensor chip to the other flow cell containing an underivatized chip. For kinetic measurements at room temperature, c-FLIPs samples, ranging from 50 to 500 nM, were prepared by dilution with an HBS buffer (150 mM NaCl, 3 mM EDTA, 0.005% polysorbate, and 10 mM HEPES [pH 7.4]). Each sample was injected with 150  $\mu$ L of c-FLIPs solution into the flow cells (association phase) at 30  $\mu$ L/min. Between cycles, the immobilized ligand was regenerated by injecting 30  $\mu$ L of 100 mM glycine hydrochloride [pH 2.0] at 10  $\mu$ L/min.

**Fluorescence Spectroscopy.** Fluorescence emission spectra were obtained on a Quantamaster spectrofluorimeter model C-61SE (Photon Technology International) using 1 cm path length cuvettes with excitation and emission slit widths of 5 nm. The fluorescence emission spectra of the FADD and c-FLIPs were obtained to identify characteristic structures, namely double bonds and aromatic groups, in molecules of varying complexities. The emission intensity was measured from 280 to 400 nm with an excitation at 295 nm. All of the spectra were taken at a protein concentration of 50  $\mu$ g/mL at

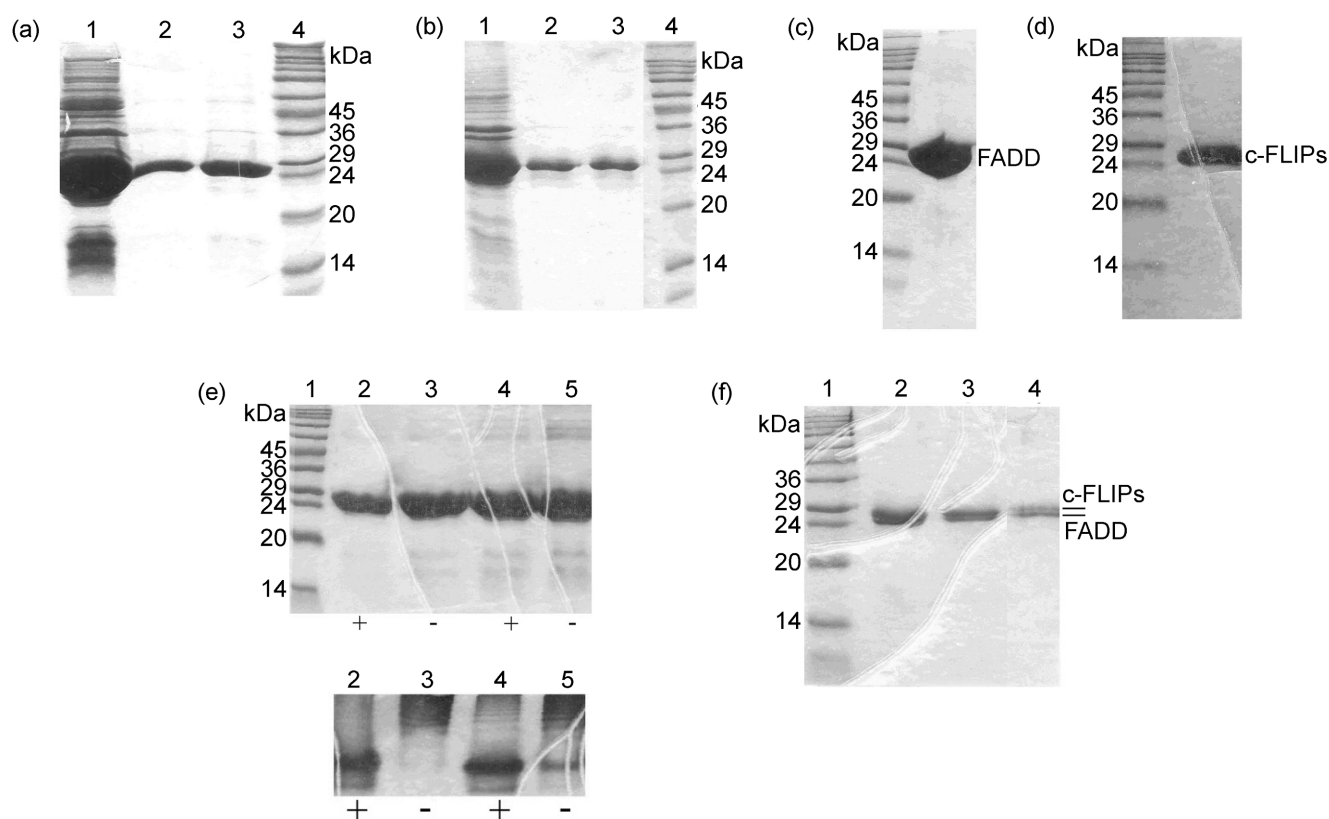
24 °C. Ten spectra of each protein sample were accumulated, averaged, and subjected to baseline correction by subtracting the buffer spectrum.

## Results and Discussion

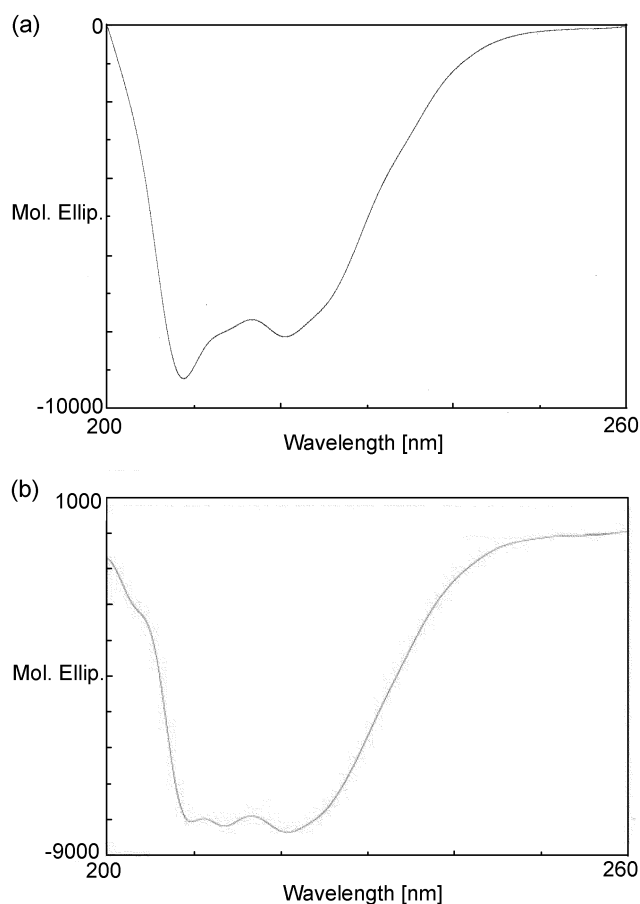
The FADD was expressed mainly as a soluble form at 37 °C in the *E. coli* BL21(DE3) host using the pQE-30 vector (Fig. 1a). The supernatant for the FADD was used in purification, but the c-FLIPs using the pET-21b vector was used as a pellet in purification (Fig. 1b). The inclusion body for the c-FLIPs was resuspended more than five times by the resuspension buffer A (30 mM ammonium acetate [pH 4.0], 300 mM NaCl, 0.1% Triton X-100, and 1 mM DTT). To prevent aggregation the pH of the protein solution was raised to the pH 5.0-5.5 using 30 mM sodium citrate. The recombinant FADD and c-FLIPs were purified in the correctly folded state as indicated by several lines of evidence. To obtain more soluble and stable proteins of FADD and c-FLIPs, the refolding process was performed. The purified FADD and c-FLIPs each showed a homogeneous single band in an SDS-PAGE analysis (Figs. 1c and d). Protein expression as inclusion bodies provides us with a

significant amount of pure protein for further biophysical and structural studies. In these studies, the separated fractions of FADD protein, after using a superdex-75 column, were shown in two types of peaks in the eluted fractions and in two bands in the SDS-PAGE analysis (Fig. 1e). Only the first peak was identified as the homogeneity in the native gels and the second peak was presumed to the aggregates of higher mass. On the other hand, c-FLIPs protein was eluted as a sharp single peak from a superdex-75 column that was employed as a final purification step, which is an indication of structural homogeneity. We have successively obtained soluble proteins to final concentrations of 8 mg/ml for FADD and 6 mg/mL for c-FLIPs, using a quantification kit with bovine serum (Figs. 1c and d).

The folding properties of both the FADD and c-FLIPs proteins were characterized by CD spectroscopy and light-scattering measurements (Figs. 2 and 3). To determine the nature of the secondary structural elements of FADD and c-FLIPs, far-UV CD-spectra were recorded and analysed. The secondary structural element and conformational property of FADD were somewhat different from those of c-FLIPs. The CD spectrum of the purified FADD exhibited two negative maxima at 208 and 222 nm, and that of the purified c-FLIPs



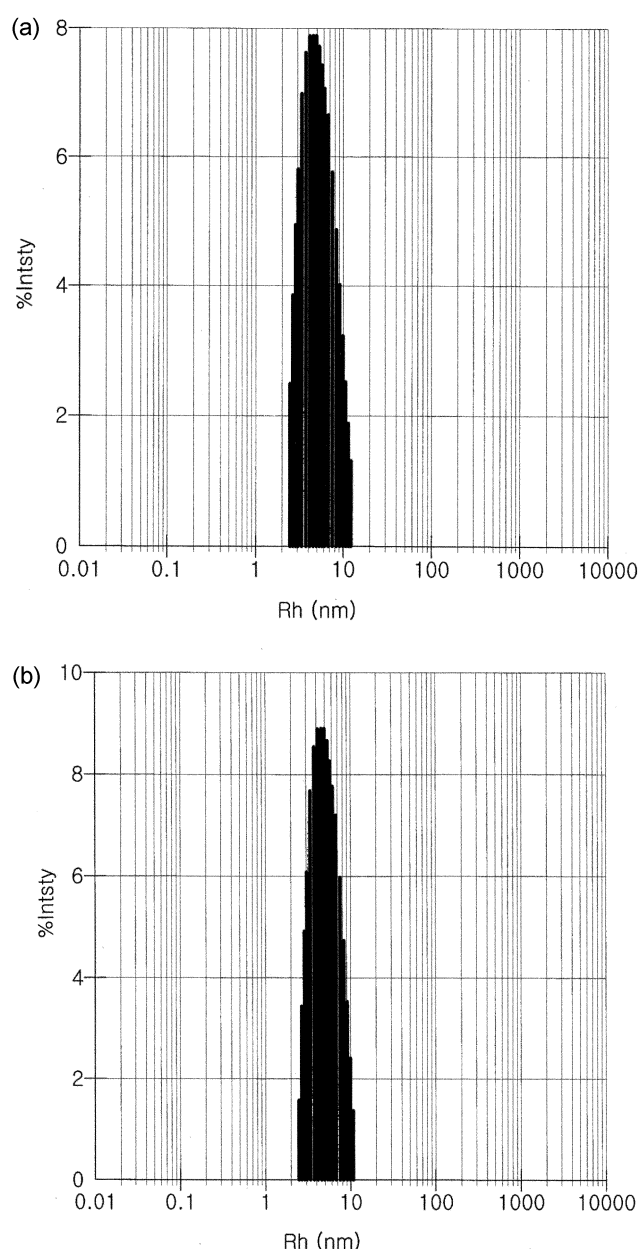
**Figure 1.** SDS-PAGE and native gels of purified FADD and c-FLIPs proteins. (a) Fractions of FADD expressed in *E. coli* BL21(DE3). Lane 1: supernatant of lysed bacteria after induction by IPTG; Lanes 2 and 3: supernatants after resuspension of ammonium sulfate precipitate; Lane 4: molecular weight markers (b) Fractions of c-FLIPs expressed in *E. coli* BL21(DE3). Lane 1: supernatant of lysed bacteria after induction; Lanes 2 and 3: supernatants after resuspension; Lane 4: molecular weight markers (c) Final concentrated FADD protein (26 kDa). (d) Final concentrated c-FLIPs protein (28 kDa). (e) Comparison of SDS-PAGE (top) and native (bottom) gels after using a superdex 75 column; Lanes 2 and 4: purified FADD; Lanes 3 and 5: inactive proteins. (+): homogeneous protein; (-): non-homogeneous protein and the aggregates of higher mass. (f) Binding of FADD and c-FLIPs complex shown in SDS-PAGE analysis from the size-exclusion column binding Lanes 2-4: different volumes (5, 3, and 2 ul) of FADD (bottom) and c-FLIPs (top) complex.



**Figure 2.** Far-UV CD spectra of FADD and c-FLIPs. CD spectra were measured from 200 to 260 nm using a 0.1 cm pathlength cell, and the CD signals were merged using the CDNN. (a) FADD; (b) c-FLIPs. Spectra were typically recorded as an average of three scans at 25 °C.

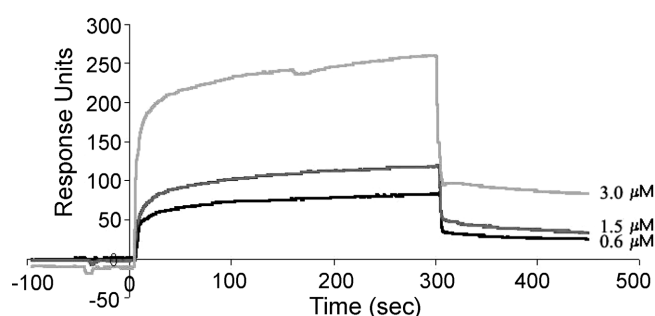
exhibited three negative maxima at 208, 214, and 222 nm (Figs. 2a and b). FADD protein is composed mostly of alpha-helical structure and c-FLIPs protein is presumed to super-secondary structure, which is motifs consisting of no more than three secondary structure elements. The dynamic light-scattering spectra of the FADD ( $R_h$  [nm] = 5.681,  $C_p$  [nm] = 2.532 and  $C_p/R_h$  [%] = 44.4) and c-FLIPs ( $R_h$  [nm] = 5.250,  $C_p$  [nm] = 1.710 and  $C_p/R_h$  [%] = 32.3) were obtained to identify the distributions of species of differing molecular masses, the degree of aggregation, shape, and branching (SEC-mode). Dynamic light-scattering spectra were recorded with 0.1–0.2  $\mu$ M protein samples. The proteins were purified to homogeneity as shown in the regularization process, and an aggregation peak was not found (Figs. 3a and b).

The extrinsic pathway is initiated by ligation of trans-membrane death receptors (CD95, the TNF receptor, and the TRAIL receptor) to active membrane-proximal (activator) caspases (caspase-8 and -10), which in turn cleave and activate effector caspases such as caspase-3 and -7. This pathway can be regulated by c-FLIP, which inhibits upstream activator caspases, and inhibitors of apoptosis proteins (IAPs), which affect both activator and effector



**Figure 3.** Light-scattering spectra of the FADD (a) and c-FLIPs (b). The light-scattering spectra were recorded with 0.1–0.2  $\mu$ M protein samples. The regularization histograms (the sharp peak in the middle) are shown as the homogeneity of purified FADD and c-FLIPs proteins in solution.

caspases. To investigate the interaction between the FADD protein and c-FLIPs, the binding affinity of FADD for the c-FLIPs was estimated by surface plasmon resonance spectroscopy (BIAcore) (Fig. 4). Sensorgrams of FADD binding to c-FLIPs were used to calculate the kinetic binding constants. Background sensorgrams were then subtracted from the experimental sensorgrams to yield representative specific binding. The kinetic parameters of the binding of FADD to c-FLIPs are shown in Table 1. It was measured twice to observe the reproduction of the experiment at different concentrations. We found that FADD indeed binds c-FLIPs strongly with an apparent  $K_D$  of 178 nM for the 1<sup>st</sup> experi-



**Figure 4.** BIAcore biosensor analysis of binding of FADD to c-FLIP<sub>S</sub> at 25 °C. The sensorgrams for 0.6, 1.5, and 3.0 μM human c-FLIP<sub>S</sub> are shown, which were used to calculate the dissociation constants. The FADD was immobilized to the dextran matrix, and c-FLIP<sub>S</sub> samples at the three indicated concentrations were injected for 5 min. The kinetic parameters of the binding reactions were determined using the BIAevaluation version 2.1 software provided by the manufacturer. A control experiment using BSA did not show a detectable binding response to either of the enzymes.

**Table 1.** Kinetic parameters of binding of FADD to c-FLIP<sub>S</sub><sup>a</sup>

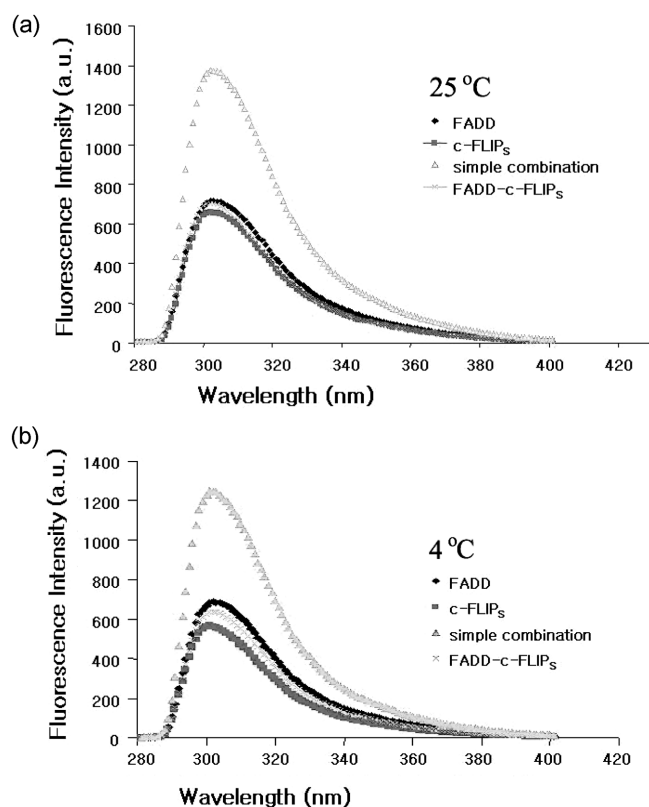
Numbers of experiment	Concs of analyte (nM)	$k_a$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_d$ (s <sup>-1</sup> )	$K_D$ (M)
1 <sup>st</sup>	600	$9.57 \times 10^3$	$9.97 \times 10^{-4}$	$1.04 \times 10^{-7}$
	1500	$4.76 \times 10^3$	$8.24 \times 10^{-4}$	$1.73 \times 10^{-7}$
	3000	$3.62 \times 10^3$	$9.25 \times 10^{-4}$	$2.56 \times 10^{-7}$
$K_D$ (nM) <sub>avg</sub>				178
2 <sup>nd</sup>	1000	$4.38 \times 10^3$	$6.22 \times 10^{-4}$	$1.42 \times 10^{-7}$
	2500	$2.75 \times 10^3$	$8.22 \times 10^{-4}$	$2.98 \times 10^{-7}$
	5000	$5.19 \times 10^3$	$6.96 \times 10^{-4}$	$1.34 \times 10^{-7}$
	5000 <sup>b</sup>	$4.35 \times 10^3$	$6.96 \times 10^{-4}$	$1.60 \times 10^{-7}$
$K_D$ (nM) <sub>avg</sub>				191

<sup>a</sup>The association rate constant ( $k_a$ ) was determined from a plot of  $\ln[\text{Abs}(dR/dt)]$  versus time, where  $R$  is the intensity of the surface plasmon resonance signal at time  $t$ . The dissociation rate constant ( $k_d$ ) was determined from a plot of  $\ln(R_0/R)$  versus time, where  $R_0$  is the resonance signal intensity at time zero. The apparent  $K_D$  was calculated from the kinetic constants  $K_D$  (M) =  $k_d/k_a$ . <sup>b</sup>In a repeat of the experiment, the value was shown in the same concentration.

ment and 191 nM for the 2<sup>nd</sup> experiment. As compared with both of the experimental results, the average dissociation value of the 1<sup>st</sup> experiment is very similar to that of the 2<sup>nd</sup>, and the association rate constant ( $k_a$ ) between FADD and c-FLIP<sub>S</sub> is gradually decreased at high analyte concentrations in the range between 3,000 and 5,000 nM. This might cause the reproduction of aggregation according to the high analyte concentration, as well as a different pH according to the buffer change in solution. From the binding of FADD and c-FLIP<sub>S</sub>, we presume that c-FLIP<sub>S</sub> has the characteristics of an inhibitory of apoptosis and that it blocks caspase-8 activation. No association between c-FLIP and caspase-8 or FADD could be detected without receptor triggering.<sup>17</sup> This demonstrates that no stable preformed complex between FADD, caspase-8, and c-FLIP exists in cytoplasm *in vivo*. There are conflicting reports about whether c-FLIP interacts with FADD and/or caspase-8. In this study, we found direct interaction of FADD with c-FLIP<sub>S</sub> without receptor trig-

gering, and estimated the actual binding affinity between the two proteins by employing surface plasmon resonance spectroscopy (Fig. 4). Also, in order to confirm the interaction between FADD and c-FLIP<sub>S</sub>, FADD protein was mixed with c-FLIP<sub>S</sub> protein in size-exclusion column at 4 °C for 12 hrs. The mixed protein solution was loaded to a gel filtration column, and the FADD-c-FLIP<sub>S</sub> complex revealed itself in an SDS-PAGE analysis (Fig. 1f).

The fluorescence emission spectra of the purified FADD and c-FLIP<sub>S</sub> proteins were checked, and  $\lambda_{\text{max}}$  curve was found at 301 nm or 305 nm, respectively, suggesting a similarity in conformation of the FADD and c-FLIP<sub>S</sub> proteins. The spectra of the FADD and c-FLIP<sub>S</sub> proteins represented the unusual emission of a tryptophan with  $\lambda_{\text{max}}$  at 301 nm or 305 nm, consistent with the solvent-inaccessible environment of the tryptophan residue. The fluorescence intensity of the FADD was somewhat increased by the exposed tryptophan residues, suggesting that the tryptophan fluorescence of the FADD mainly originates from aromatic amino acids. FADD has C-terminal DD and N-terminal DED. A signal of Fas-mediated apoptosis is transferred through an adaptor protein, FADD. A death effector domain of FADD has three tryptophan residues (Trp112, Trp148, and Trp198), whereas those of c-FLIP<sub>S</sub> have no tryptophan residues. The death domain of the FADD has no tryptophan residues. The FADD DED has two hydrophobic patches for binding to c-FLIP<sub>S</sub> and for apoptotic activity. One of these patches contains a conserved set of hydrophobic residues that is important for its apoptotic activity and binding to the DEDs of procaspase-8.<sup>18-20</sup> The FADD appears to undergo some degree of temperature-dependent conformational change, because the fluorescence spectrum of the protein exhibits an appreciable change as a function of temperature, as shown in Figure 5. The fluorescence intensity of the FADD is about 630 a.u. at 25 °C, but at 4 °C it is 550 a.u. This is in contrast to c-FLIP<sub>S</sub>, which displays a smaller spectral difference. At 25 °C, an increase in the fluorescence intensity took place when the FADD and c-FLIP<sub>S</sub> were mixed together in 1 : 1 molar ratio, both at 5 μM (Fig. 5a). These results show that the structural stability and activity of the FADD-c-FLIP<sub>S</sub> complex at 25 °C were increased. However, no appreciable fluorescence change was detected at 4 °C under the same experimental conditions, which is consistent with the result from the BIAcore biosensor analysis in a prolonged incubation (Figs. 4 and 5b). The simple combination of the spectra of the FADD and c-FLIP<sub>S</sub> are not identical to the spectrum of the FADD-c-FLIP<sub>S</sub> complex. Probably, the tight interaction of the two proteins is accompanied by more significant conformational changes of one or both of the two proteins, which is likely to be greatly facilitated at room temperature, because the residues of aromatic groups are buried in the three-dimensional structure of protein and the spectrum of the FADD-c-FLIP<sub>S</sub> complex is much more decreased than in the simple combination. Also, the less rigid and hydrophobic environment needed by the conformational change of FADD-c-FLIP<sub>S</sub> can be caused by the decreasing of fluorescent intensity.



**Figure 5.** Temperature effect on the binding of FADD to c-FLIPs. Fluorescence spectra of the 1 : 1 mixture of FADD and c-FLIPs and the sum of the spectra of individual FADD and c-FLIPs. The FADD and c-FLIPs were preincubated together for 25 min at 25 °C (a) and 4 °C (b), respectively. The fluorescence spectra were recorded with a wavelength of 295 nm to excite tryptophan and tyrosine. The concentration of each of the two proteins was 5  $\mu$ M. The sample buffer contained 50 mM Tris-HCl [pH 7.5], 200 mM NaCl, and 1 mM DTT.

The majority of protein-protein interactions that thus far identified in death receptor signaling have been through the use of isolated domains involving homotypic interaction between death domains or death effector domains.<sup>21</sup> For example, the DD of FADD has been shown to interact with the DD of Fas or of TRAIL receptors. The DED domain of FADD interacts with the DED domain of caspase-8. However, in a screen for FADD mutants that are deficient in interaction with the DD of Fas, the majority of the identified mutations were within the DED domain of FADD, which indicates that interactions other than homotypic associations are also involved in mediating signals from death receptors.<sup>22,23</sup>

In the present study, we show the purification and characterization of the full-length FADD and c-FLIPs proteins in *E. coli*. Also, we confirm that FADD interacts with c-FLIPs *in vitro* by a series of biochemical and biophysical measurements. These results indicate that c-FLIPs potentially blocks FADD-mediated cell death by interfering with caspase-8 activation.

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