



Molecular basis of dimerization of initiator caspase was revealed by crystal structure of caspase-8 pro-domain

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Abstract

The assembly of death-inducing signaling complex (DISC) for activation of initiator caspase is a key step for the receptor-mediated apoptosis signaling. Many death effector domain (DED)-containing proteins are involved in DISC assembly and controlling. One of the main DISC component, caspase-8, contains DED and DED-mediated dimerization and oligomerization in the DISC is critical for the activation of this initiator caspase. There have been intensive studies to understand DED-mediated dimerization and oligomerization for the DISC assembly but no clear answer has been provided and there are many controversial arguments. Here, we suggested novel dimerization process of tandem DED of caspase-8 with crystallographic study.

Introduction

Caspases, a family of cysteine proteases that cleave specifically after aspartic acid residues, are involved in the apoptosis and inflammation signaling pathways [1–3]. Based on their roles, caspases can be divided into two classes: initiator caspases, including caspase-1, -8, and -10, and effector caspases, including caspase-3 and -7. Initiator caspases are synthesized as zymogens, which possess pro-domains, and are auto-processed upon dimerization through recruitment to oligomeric signaling complexes [4]. Caspase-8 is the main initiator caspase in death receptor-mediated apoptosis. It was discovered as a component of the death-inducing signaling complex (DISC), which contains a death receptor, FADD (Fas-associated protein with death domain), and caspase-8 [5]. Zymogen of caspase-8, also called procaspase-8, consists of a pro-domain harboring tandem death effector domains (DEDs) followed by a

caspase domain. DISC formation for caspase-8 activation depends on the protein interaction domain called the death domain superfamily, which contains four subfamily members, including death effector domain (DED), death domain (DD), caspase-recruiting domain (CARD), and Pyrin domain (PYD) [6]. During the extrinsic apoptosis process, procaspase-8 is recruited to the death receptor through the bipartite adaptor protein FADD [7]. The cytosolic component of the death receptor contains DD. FADD contains DD at the N terminus and DED at the C terminus. Death receptor interacts with FADD via DD: DD interactions, and caspase-8 interacts with FADD via DED: DED interactions. Upon DISC formation, caspase-8 acquires proximity that can induce dimerization and auto-proteolytic processing [8]. Although the main function of procaspase-8 is to induce apoptosis through death receptors, many studies have shown that it can regulate necroptosis and nuclear factor- κ B signaling through various receptor signaling events, including Fc and Toll-like receptors in T, B, and natural killer cells [9–12]. Although the enzymatic function of caspase-8 is critical for apoptosis, the adaptor function has also been emphasized in immune cell signaling [12].

Caspase-8 tandem DEDs and the process of dimerization or oligomerization for DISC assembly have been intensively studied and discussed [13–17]. In addition, studies have investigated how DED-containing proteins, such as FADD, FLIP, PEA-15, DEDD, and caspases, can interact with each other via DED–DED or DED–tandem DED interactions [18]. Despite these studies, there is no clear

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evidence, and many controversial oligomerization models have been proposed [16, 19]. Therefore, in this study, we introduced the structure of intact tandem DEDs of caspase-8. Our structure showed that tandem DEDs of caspase-8 dimerized via a novel domain-swapped strategy, which support the previous finding that the FL motif at DED2, not in DED1, is critical for the dimerization and further activation of caspase-8 in DISC.

Experimental procedures

Sequence alignment

The amino acid sequences of DED domains were analyzed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Protein expression and purification

Pro-domain of caspase-8 (amino acid residue: 1–178) was expressed in *Escherichia coli*. Full-length human caspase-8 (Genebank ID: BAB32555.1) was amplified by PCR using gene-specific primers containing *NdeI* and *XhoI* sites. PCR fragments were subsequently digested and ligated into the pET24a vector containing the C-terminal hexa-histidine tag. The sequences of cloned genes were verified by DNA sequencing. The resulting plasmid was transformed into BL21 (DE3) *E. coli*-competent cells. After this, the cells were plated onto LB (Luria-Bertani) media and incubated for 24 h at 310 K. Next, individual colonies were inoculated into 5 mL LB media and incubated overnight at 310 K with shaking. Cultured cells were then moved to 1 L LB media and incubated for 4 h at 310 K until optical density reached to 0.7 with shaking. After this, the expression was induced by treating the bacteria with 0.25 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 18 h at 293 K. Following induction, the bacteria were collected, resuspended, and lysed by sonication in 50 mL lysis buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 10 mM imidazole). The bacterial lysate was subsequently centrifuged at 16,000 rpm for 30 min at 277 K. After this, the supernatant was applied to a gravity-flow column (Bio-Rad) packed with 1.5 mL of Ni-NTA affinity resin (Qiagen). The unbound bacterial proteins were subsequently removed from the column using 50 mL washing buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 25 mM imidazole). The target protein was subsequently eluted from the column using an elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 250 mM imidazole). Fractions containing more than 90% homogenous proteins upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were then selected and combined. After this, the protein purity was further improved using a Superdex 200 gel-filtration column 10/30 (GE Healthcare)

pre-equilibrated with a solution of 20 mM Tris-HCl pH 8.0 and 150 mM NaCl.

Crystallization and data collection

The initial conditions for crystallization were screened at 293 K by the hanging-drop vapor-diffusion method using screening kits from Hampton Research and the deCODE Biostructure Group. Initial crystals were grown on a siliconized cover slip by equilibrating a mixture containing 1 μ L of protein solution (6–7 mg/mL protein in 20 mM Tris pH 8.0 and 150 mM NaCl) and 1 μ L of reservoir solution containing 2.0 M ammonium sulfate, 0.2 M NaCl, and 0.1 M cacodylate pH 6.5 against 0.4 mL of reservoir solution. The initial crystals from the screening were sufficient for collecting the data set. Crystals reached to maximum dimensions of 0.3 \times 0.3 \times 0.3 mm in 3 days. The diffraction data set at 2.1 Å resolution was collected at Pohang Accelerator Laboratory (PAL) 5C beamline in South Korea and processed using the HKL2000 package [20] (Table S1).

Structural determination and analysis

The molecular replacement (MR) phasing method was conducted with Phaser [21] using the F122G/L123G double mutant form of caspase-8 (PDBid: 5JQE) [17] as a search model. The initial solution model was extended and completed by iterative manual building and refinement in Coot [22] and PHENIX Refine [23], respectively. The quality of the model was checked using PROCHECK [24]. Molecular structure images were generated using the PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

Multi-angle light scattering (MALS)

The absolute molar mass of the caspase-8 tandem DEDs (1–178) was determined by MALS. Briefly, the target protein was loaded onto a Superdex 200 h 10/30 gel-filtration column (GE Healthcare) that had been pre-equilibrated in buffer containing 20 mM Tris-HCl pH 8.0 and 150 mM NaCl. The Acta chromatography system (GE Healthcare) was coupled to a MALS detector (mini-DAWN Treos) and a refractive index detector (Optilab DSP) (both from Wyatt Technology).

Results

Crystal structure of caspase-8 tandem DEDs revealed domain-swapped dimerization

In an effort to shed light on the mechanism of DISC assembly via oligomerization of caspase-8, FADD, death

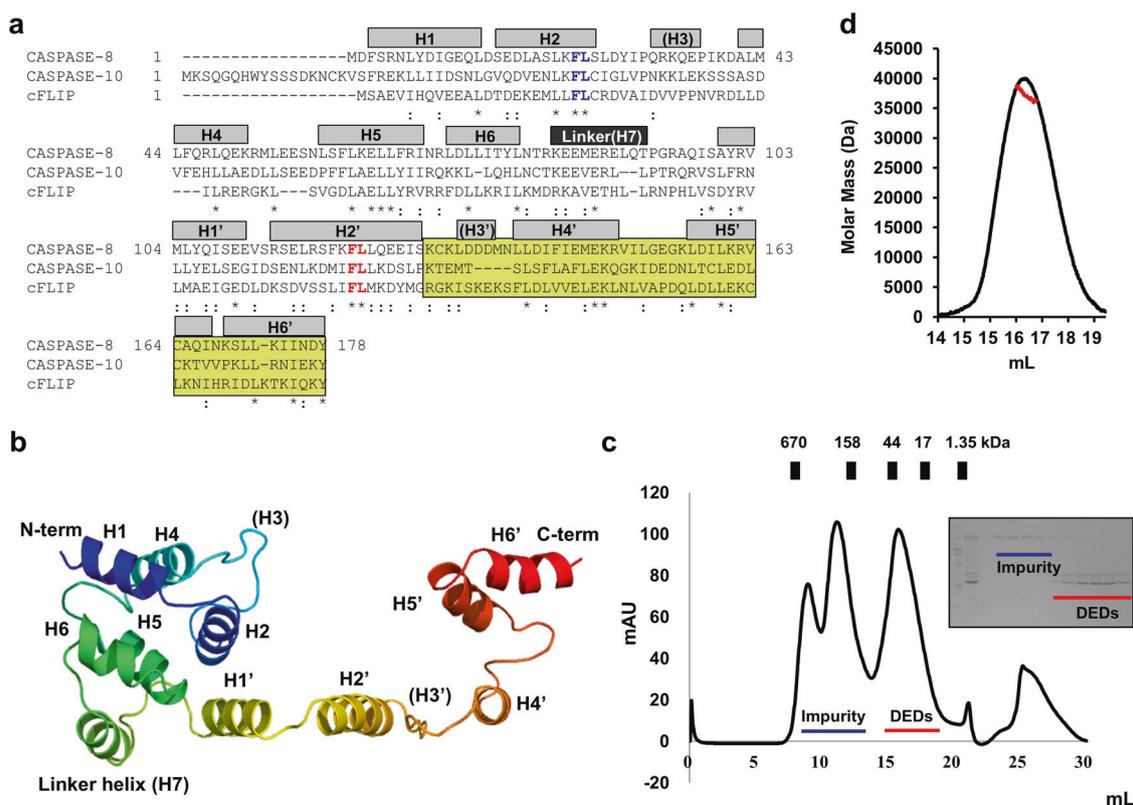


Fig. 1 The crystal structure of the tandem DEDs of wild-type caspase-8. **a** Sequence alignment of tandem DEDs. Functionally important conserved FL motif on DED2 and another FL motif on DED1 are shown in red and blue color, respectively. Secondary structures that were observed in previous studies are indicated above the corresponding sequences (H1–H6 for DED1 and H1′–H6′ for DED2). Partially unfolded region in DED2 due to the formation of a domain-swapped dimer is highlighted in yellow color. **b** An illustration of the caspase-8 tandem DED structure. The structure from the N terminus

(N-term) to the C terminus (C-term) is colored blue to red. Secondary structures of α -helices are labeled in H1–H6 (DED1), H7 (linker), and H1′–H6′ (DED2). **c** Tandem DED of caspase-8 was detected as a dimer in solution. The profile of size-exclusion chromatography. Among the three eluted peaks, the last peak eluted around 16–17 mL was a tandem DED based on SDS-PAGE (indicated as red bar). The other two peaks that eluted earlier were impurities (indicated as blue bar). **d** MALS profile. Red line indicates the experimentally calculated molecular weight

receptor, and their specific interaction, we determined the crystal structure of the tandem DEDs (DED1–DED2) of caspase-8 at 2.1 Å resolution (Fig. 1b and Table S1). The structure of the F122G/L123G double mutant form of caspase-8 (PDBid: 5JQE) [17], which has been designed for increased solubility, was used as a search model for MR. Our intact tandem DEDs of caspase-8 were insoluble in solution as previously reported [17]. However, we successfully purified soluble caspase-8 tandem DEDs by collecting tiny soluble fractions produced from 2 L bacterial cell cultures. We repeated small-scale 2 L purification steps 50 times to obtain 3 mg of soluble caspase-8 tandem DEDs (Fig. 1c). The structure was refined to an $R_{\text{work}} = 22.0\%$ and $R_{\text{free}} = 26.9\%$. The data collection and refinement statistics are summarized in Table S1. During the model-building step, we realized that one molecule was located in the asymmetric unit and the second DED part of the molecule was partially unfolded and extended to the opposite direction (Fig. 1b). The high-resolution structure of the intact

caspase-8 tandem DEDs revealed that it was comprised of 13 helices, H1 to H6 for DED1, H7 for linker helix, and H1′ to H6′ for DED2 (Fig. 1b). A typical six-helical bundle fold of DED1 formed an eight helical bundle with linker helix H7 and H1′ from DED2. H2′–H6′ of DED2 were completely unfolded and extended to the opposite direction of DED1 (Fig. 1b). Previous biochemical and structural studies showed that tandem DEDs of caspase-8 and its mutants could exist as monomers, dimers, or even higher oligomeric forms in solution [17, 25]. To confirm the stoichiometry of caspase-8 tandem DEDs in solution, we conducted size-exclusion chromatography followed by MALS (SEC-MALS). Tandem DEDs of caspase-8 were eluted at around 16 mL, which corresponded to ~40 kDa, indicating that it existed as a dimer in solution (Fig. 1c). This result was confirmed by MALS. The theoretically calculated molecular weight of caspase-8 tandem DEDs with a C-terminal His-tag was 18.41 kDa, and the experimental molecular weight from MALS was 38.19 kDa (1.72% fitting error), with a

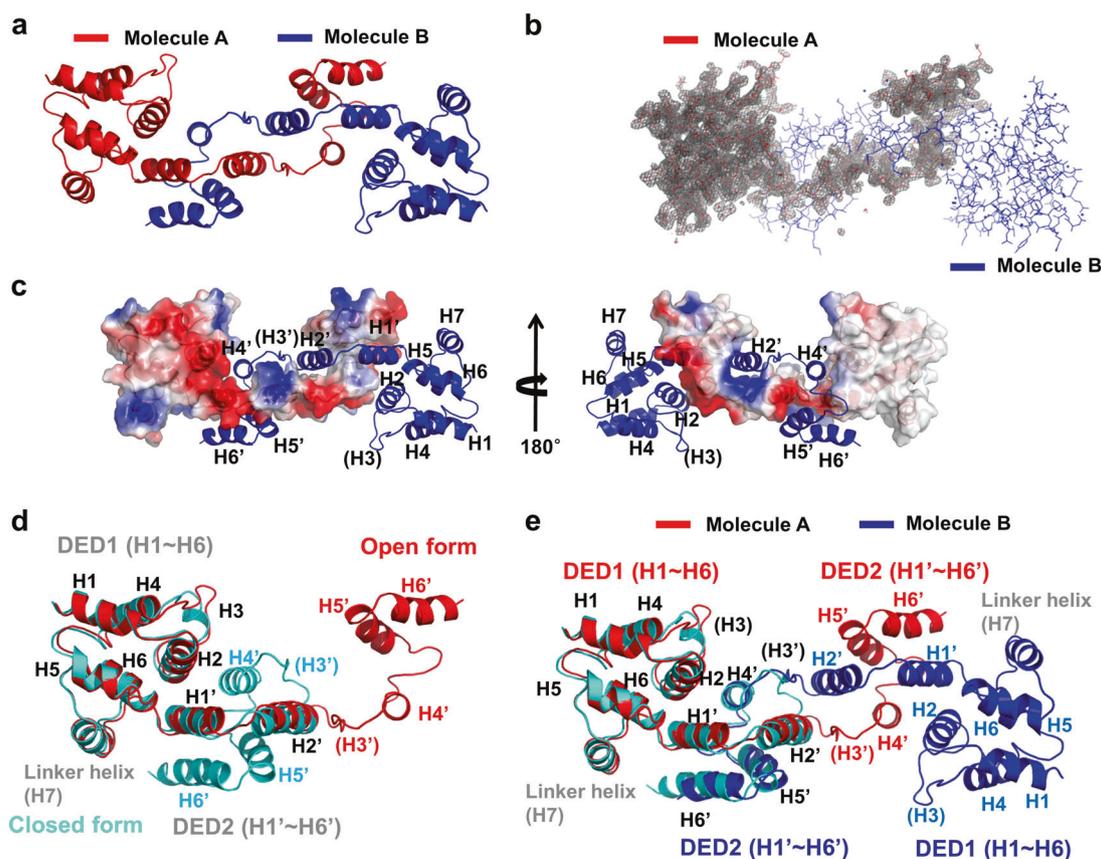


Fig. 2 Domain-swapped dimer of the caspase-8 tandem DEDs. **a** Structure of the domain-swapped dimer. **b** 2Fo-Fc electron density map contoured at 1.0 σ of molecule A. **c** Representation of electrostatic surface of caspase-8 tandem DEDs showing the interface of domain-swapped dimer. Secondary structures, from H1 to H7 (DED1) and H1' to H6' (DED2), are indicated on the corresponding helices.

d Structural comparison with monomeric, closed form of caspase-8 tandem DEDs. Current wild-type structure (red color) was superimposed with previously solved monomeric mutant structure (cyan color) (PDBid: 5JQE). **e** Structural comparison of dimeric caspase-8 tandem DED structure with monomeric, closed form of caspase-8 tandem DEDs

polydispersity of 1.0 (Fig. 1d). Based on our analysis using SEC-MALS, we concluded that tandem DEDs of caspase-8 existed as dimers in solution.

By searching for the symmetry molecule, second molecule, which interacts with the first molecule by domain swapping, was found (Fig. 2a). The electron density clearly showed that the helix bundle fold of the second DED (DED2) of caspase-8 was partially unfolded and used for domain swapping (Fig. 2b). Surface representation showed that the extended DED2 was well-fitted in the surface cleft formed by the extended DEDs from the opposite molecule (Fig. 2c). When compared to the monomeric structure of the caspase-8 FL motif mutant DED (F122G/L123G double mutant, PDBid: 5JQE), dislocation of the helix bundle fold started from H3' to H6' (Fig. 2d). For the domain swapping, displaced H4', H5', and H6' of molecule A were placed to another molecule (molecule B) to form a complete six helix bundle fold with H1', H2', and H3' of molecule B, which is the typical structure of the death domain superfamily, including DED (Fig. 2d).

The observed interface generated by domain swapping

Procaspase-8 dimerized through domain swapping of caspase-8 tandem DEDs (Fig. 3a–c). The interface was formed by massive H-bonds and salt bridges (Fig. S1), which buried $\sim 3079 \text{ \AA}^2$ among $13,017 \text{ \AA}^2$ total accessible surface area. PISA (Proteins, Interfaces, Structures and Assemblies) interface analyzing server scored 1.000 in the complex formation significance score, indicating that the domain-swapped dimer was a reasonable structure of the caspase-8 tandem DEDs. FL motif-containing helix at DED2 (H2'), in the middle of the domain-swapped dimer, mediated major interactions between the two molecules. Residue F122 on H2' formed a hydrophobic patch with F122 from the opposite molecule (Fig. 3b). Residue L133 on (H3') contributed to form this patch. Residue L133 on H2' formed another hydrophobic patch with residues V163 and I167 on H5' from the opposite molecule (Fig. 3b). The position of residues F122 and L123 were located in the

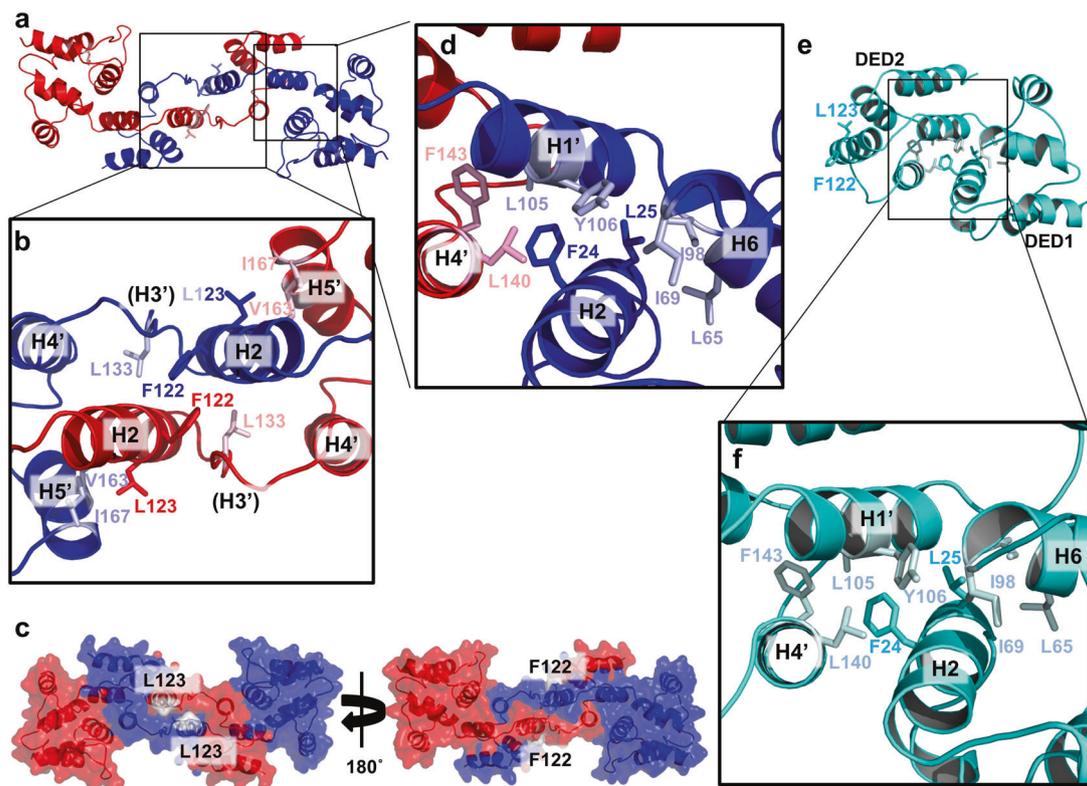


Fig. 3 The observed interface on the domain-swapped dimer of the caspase-8 tandem DEDs. **a** The interface of the domain-swapped dimer. **b, c** Magnified view of the interface formed by the domain-swapped dimer. Interaction interface formed in the middle (**b**) and side part (**c**) is magnified. **d** The monomeric structure of the FL motif mutant DED of caspase-8 (F122G/L123G double mutant, PDBid:

5JQE). The mutated FL motif is labeled. **e** Magnified view of the intramolecular interaction interface formed by two DEDs (DED1 and DED2) of caspase-8. **f** The location of the FL motif on DED2, which is critical for the dimerization-mediated activation of caspase-8, on the surface of the domain-swapped dimer of caspase-8 tandem DEDs

middle of the interface surface formed by the domain-swapped dimer (Fig. 3c). These residues, F122 and L123 on DED2, were largely conserved in other tandem DED-containing proteins (Fig. 1a). FL motif on DED1 was involved in the formation of intermolecular hydrophobic patches with neighboring hydrophobic residues, including L105 and Y106 on H1', and L65 and I69 on H6. Dislocated H4' by domain swapping was re-located in between H1' and H2 of the opposite molecule and was involved in the hydrophobic interaction (Fig. 3d). According to the monomeric mutant tandem DEDs of caspase-8, FL motif on DED1 was located inside of the tandem DEDs and mediated formation of the hydrophobic core formed in between DED1 and DED2. In contrast, FL motif on DED2 was located at the end of one side portion on the surface (Fig. 3e, f). The hydrophobic core, which was formed by the FL motif on H2 and other hydrophobic residues on H6, H1', and H4' and is critical for the correct folding of the caspase-8 tandem DED structure by stabilized DED1 and DED2, was the same as that detected at the domain-swapped dimer. H4' from the same molecule was replaced

by H4' from the opposite molecule by domain swapping (Fig. 3d–f).

Beside the hydrophobic cores formed by FL motifs on DED1 and DED2, massive salt bridges and H-bonds were newly formed through domain swapping. R102, R118, E127, D135, E147, and R162 from molecule A formed salt bridges with the same residues as the oppositely located molecule B (Fig. S1). This combination of massive interactions made the dimeric complex stable and lowered B-factors (Fig. S2).

FL motif on DED2 is critical for caspase-8 dimerization via domain swapping

The importance of the FL motif on DED2 for dimerization, oligomerization, and further activation of caspase-8 has been reported by various groups [16, 18, 26]. Additionally, FL motif mutation to glycine or alanine increased solubility of caspase-8 tandem DEDs and improved possibility for structural studies [17, 25]. To determine why the FL motif on DED2 is critical for caspase-8 function, we investigated our

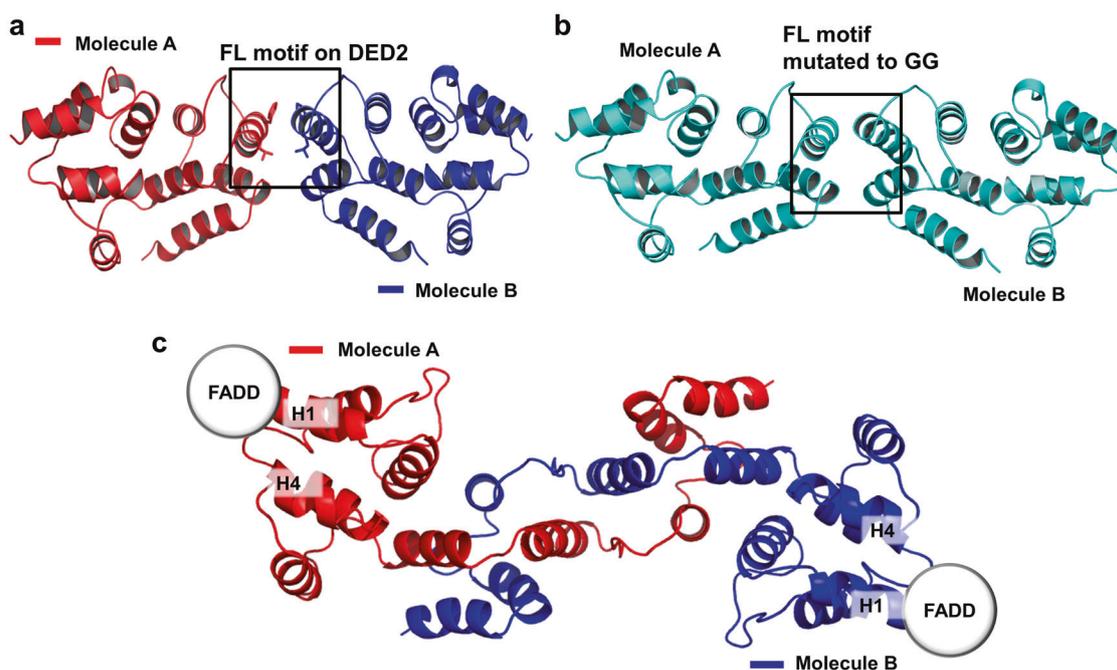


Fig. 4 FL motif on DED2 is critical for caspase-8 dimerization via domain swapping. **a** The location of the FL motif on DED2. Closely located FL motif on two different tandem DEDs from caspase-8 are boxed. **b** The structure of FL motif mutants of caspase-8 tandem

DEDs. Closely located mutant FL motif on two different tandem DEDs from caspase-8 are boxed. **c** Tentative interaction mode between caspase-8 tandem DEDs and FADD DED. The position of helix 1 and helix 4 on caspase-8 DED1 is labeled as H1 and H4

structure. Since the FL motif on DED2 mediated domain swapping, it might be feasible that two closely located FL motifs on DED2 may trigger domain swapping with two different caspase-8 via strong hydrophobic interactions (Fig. 4a). However, mutation of the FL motif on DED2 to either alanine or glycine lost its capability to interact with each other to trigger domain swapping (Fig. 4b).

Although DED2 of caspase-8 was suggested to bind with the FADD adaptor for DISC assembly [7, 27], recent biochemical and computational studies indicated that FADD binds to the H1 and H4 regions on DED1 of caspase-8 [15, 16, 18, 26], which is supported by our structure. Because FL motif and most on the helices on DED2 are involved in the domain swapping-mediated homodimerization, FADD should bind to available H1 and H4 regions on DED1 of caspase-8 (Fig. 4c and Fig. S3).

A model of DISC assembly followed by caspase-8 activation via dimerization

Based on our novel dimer structure of caspase-8, a model of DISC assembly followed by caspase-8 activation was proposed. Caspase activation may proceed in several steps (Fig. 5a). At a basal state, without recruiting FADD to the death receptor, monomeric caspase-8 is freely available in the cell. Upon apoptosis induction triggered by interaction of the death ligand to the trimeric death receptor (steps 1 and 2), adaptor FADD is recruited to the death receptor via a DD–DD

interaction (step 3). Subsequently, FADD recruited at cell surface can recruit caspase-8 via a DED–DED interaction (step 4), leading to procaspase-8 proximity to meet another procaspase-8. This DISC assembly mediates accessibility of FL motif on procaspase-8 DED2 (step 5). Closely located FL motif on DED2 causes domain swapping and dimerization of procaspase-8 (step 6). The dimerization via tandem DED domain swapping causes the proximity-mediated self-activation of caspase-8. Because trimeric death receptor association is known to be critical for DISC assembly, the complete massive DISC complex is expected to form a cluster in lipid rafts (Fig. 5b, c).

This new model derived from our novel structure can explain why FL motif on DED2 is critical for the dimerization, oligomerization, and activation of caspase-8. In addition, this model can also explain the increased local concentration of initiator caspase by DISC formation, which is critical for the activation of initiator caspase.

Discussion

Caspases are a family of cysteine proteases that work in the apoptosis and inflammation signaling pathways. Caspases are divided into two classes, initiator and effector, and caspase-8 is a representative initiator caspase. Initiator caspases are auto-activated when they obtain proximity through recruitment to oligomeric signaling complexes. For

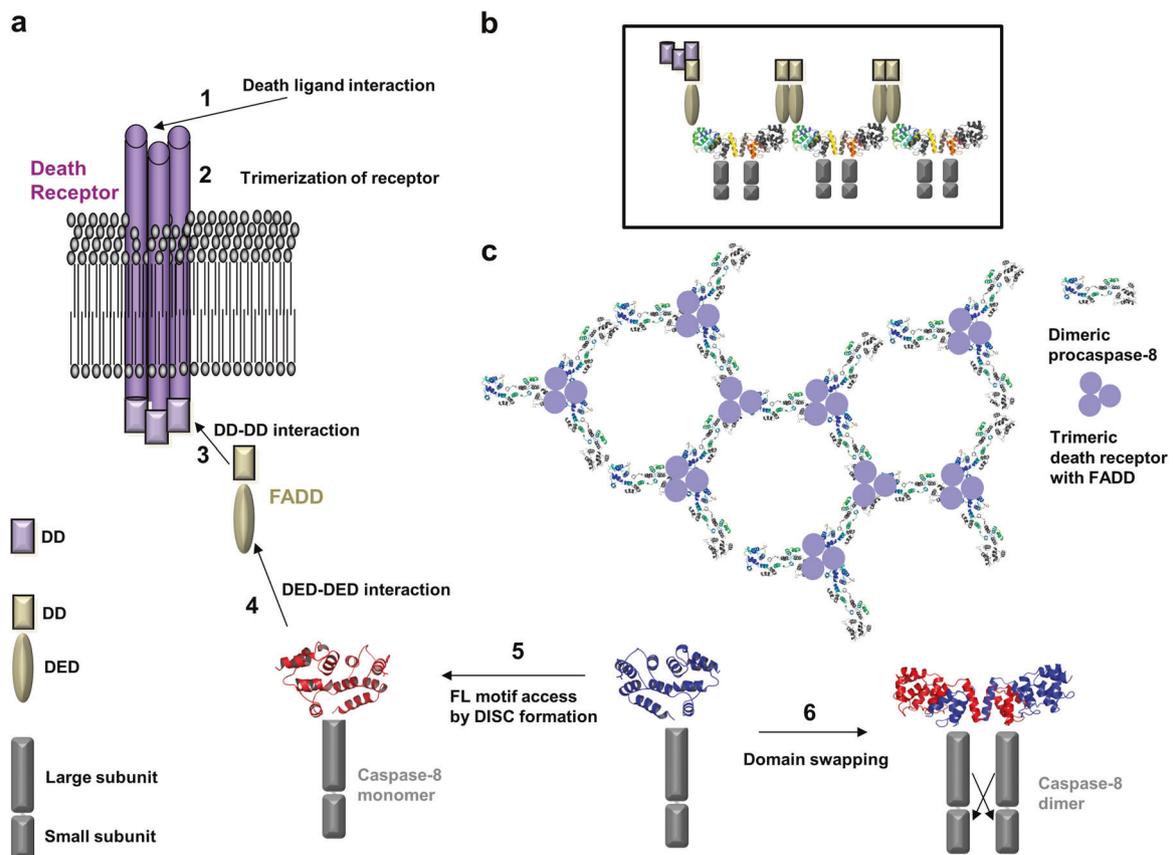


Fig. 5 A model of DISC assembly followed by dimerization-mediated caspase-8 activation. **a** Proposed procedure of caspase-8 activation via domain-swapped dimerization. **b, c** Massive DISC chain assembly via

interaction of the trimeric death receptor, adaptor FADD, and domain-swapped caspase-8. Side view (**b**) and top view (**c**) are proposed

the activation of caspase-8, DISC, which is composed of Fas, FADD, and caspase-8, is formed to recruit caspase-8. Caspase-8 contains pro-domains at the N terminal, also called tandem DEDs, which are critical for DISC formation and the protein interaction.

Although intensive studies have been performed during several decades, the recruitment process of initiator caspase in DISC and further activation is still controversial. To unveil how caspase-8 is adapted at the trimeric death receptor, oligomerized, and further activated by gaining proximity, we determined the structure of intact tandem DEDs of caspase-8. Surprisingly, our structure revealed novel domain-swapped dimerization that has not been reported previously for any death domain superfamily, including other DEDs despite extensive structural pursuit. Caspase-8 dimerization is known to be critical for activation. Domain-swapped dimerization strategy detected in current study can explain why the FL motif on DED2 is critical for dimerization, further oligomerization, and activation of caspase-8. Our structure can also explain the increased local concentration-dependent activation of initiator caspase. For the domain swapping of tandem DEDs and further activation, two procaspase-8 have to be closely

related. FADD-binding site on the caspase-8 tandem DEDs, which is known to be the H1 and H4 regions of DED1, is also explained with our structure. Therefore, our studies uncovered a novel tandem DED-mediated dimerization mechanism, which is critical for DISC assembly followed by initiator caspase activation.

Taken together, our domain-swapped dimer structure of caspase-8 tandem DEDs is the first structure of unmodified wild-type caspase-8 DEDs, which can explain dimerization and further cluster formation using the FL motif on DED2 as critical for oligomerization. Because caspase-8 can form many different oligomeric complexes with various signaling molecules during apoptosis, necroptosis, and other immune signaling pathways by acting as an enzyme and sometimes scaffolding adaptor [12, 28], it is not surprising that several different forms of procaspase-8, including current domain-swapped dimer form and previously reported filament form [17], can be found in the cell. A similar case was also detected in the study of Fas DD and FADD DD complexes, whose structures were reported as two different forms, helical oligomeric complex [29] and structurally altered Fas DD-mediated complex [30]. Structural alteration of DD superfamily, including DD and DED, might regulate

assembly of homo- or hetero-oligomeric complexes in cellular signaling. The controlling points and effectors that can determine the different forms of DD superfamily complexes, especially caspase-8 tandem DEDs during various signaling events, must be studied further in the future.

Accession number

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with PDB ID: 6AGW.

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Author contributions HHP designed the project, performed experiments, and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

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