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Death domain complex of the TNFR-1, TRADD, and RIP1 proteins for death-inducing signaling

Young-Hoon Park [1], Mi Suk Jeong [1], Se Bok Jang *

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Jangjeon-dong, Geumjeong-gu, Busan 609-735, Republic of Korea

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ABSTRACT

Apoptosis can be induced by an extrinsic pathway involving the ligand-mediated activation of death receptors such as tumor necrosis factor receptor-1 (TNFR-1). TNFR-1-associated death domain (TRADD) protein is an adapter molecule that bridges the interaction between TNFR-1 and receptor-interacting serine/threonine-protein kinase 1 (RIP1). However, the molecular mechanism of the complex formation of these proteins has not yet been identified. Here, the binding among TNFR-1, TRADD, and RIP1 was identified using a GST pull-down assay and Biacore biosensor experiment. This study showed that structural characterization and formation of the death-signaling complex could be predicted using TNFR-1, TRADD, and RIP1. In addition, we found that the structure-based mutations of TNFR-1 (P367A and P368A), TRADD (F266A), and RIP1 (M637A and R638A) disrupted formation of the death domain (DD) complex and prevented stable interactions among those DDs.

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1. Introduction

Tumor necrosis factor alpha (TNF-α) is a cytokine that pertains to the TNF-ligand superfamily, which is important in regulating cell death and cell survival [1,2]. Each factor of the TNF superfamily interacts with at least one receptor of the TNFR superfamily. Some TNF factors bind several receptors [3,4]. For example, TNF-α is known to interact with two distinct cell surface receptors, TNFR-1 and TNFR-2 [5]. The TNFR-1 interaction site of the extracellular position has four characteristic cysteine-rich domains (CRDs) that bind directly to TNF-α trimer [6,7]. The interaction between these two proteins has been shown to mediate protein activation of the TRADD, RIP1, Fas-associated death domain (FADD), and TNFR-associated factor 2 (TRAF2), which is a critical step in the TNF signal transduction pathway [8–12]. A subgroup of the TNF receptor superfamily can be defined by the ability of its members to induce cell death with the crucial involvement of about 80 amino acid hexahelical bundle homology domain. The intracellular death domain (DD) of TNFR-1 that has been activated by TNF-α has been shown to associate with TRADD through homotypic DD interaction [13,14].

Protein kinase RIP1 is another DD molecule found in the TNFR-1 signaling complex. Although RIP1 contains DD, is appears to require TRADD as an adapter to indirectly associate with TNFR-1 [15,16]. The ubiquitination of protein kinase RIP1 determines function as a kinase that promotes cell death by FADD and caspase-8 [17,18]. Following internalization of the TNFR-1 receptor, the DD proteins dissociate from the death receptor of the TNF signal transduction complex and the deubiquitination of RIP1 is mediated by deubiquitination enzymes (cylindromatosis (CYLD) and tumor necrosis factor alpha-induced protein 3 (TNFAIP3)), which eliminates the K63-linked polyubiquitin chains [19,20]. Deubiquitination of RIP1 in the cytosol is crucial to programmed cell death of the TRADD-dependent complex (complex IIA) and TRADD-independent complex (complex IIB) [21]. Complex IIA necessitates a TRADD-FADD scaffold to recruit caspase-8, which has a death effect domain (DED) and DD site, implying an apoptotic pathway [22]. In addition, caspase-8 is inhibited by cytokine response modifier A (CrmA), while pharmacological agents interact with deubiquitinated RIP1-receptor-interacting serine/threonine-protein kinase 3 (RIP3), which binds to the RIP homotypic interaction motif (RHIM) [23–29]. When TRADD does not exist, the formation of complex IIB involves FADD mediated recruitment and activation of caspase-8 for RIP1 and RIP3 cleavage [30].

In this study, we investigated the interactions among the recombinant DDs of TNFR-1, TRADD, and RIP1 and demonstrated direct protein interactions among TNFR-1, TRADD, and RIP1 *in vitro*. We also found that the structure-based mutations of TNFR-1 (P367A and P368A), TRADD (F266A), and RIP1 (M637A and R638A) disrupted formation of the DD complex and prevented stable interactions among the aforementioned DDs.

* Corresponding author. Fax: +82 51 581 2544.
E-mail address: sbjang@pusan.ac.kr (S.B. Jang).
[1] These authors contributed equally to this article.

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2. Materials and methods

2.1. Protein cloning, expression and extraction

TNFR-1 DD (345–455) was subcloned into the His-tagged fusion protein vector pET-28a. The TRADD (160–308) and RIP1 DDs (583–663) were subcloned into His-tagged fusion protein vector pET-26b for purification. The TNFR-1 and RIP1 DDs were subsequently subcloned into a glutathione S-transferase (GST)-fused protein vector pGEX-4T1 for the pull-down experiment.

The TNFR-1, TRADD, and RIP1 DDs were transformed into the overexpression competent cell, *Escherichia coli* BL21(DE3). Each colony was then inoculated in 5 ml of Luria Bertani (LB) medium enriched with 10 μg/ml kanamycin at 37 °C overnight, after which the cells were incubated in 2 L of LB containing 10 μg/ml antibiotics at 37 °C until the OD_{600} reached 0.5–0.6. Next, expression of the protein was induced by 0.5 mM isopropyl-thio-β-D-galactopyranoside (IPTG) at 25 °C overnight, and the bacterial cells were then harvested by centrifugation at 3660×*g* for 25 min at 4 °C. For analysis, the TNFR-1 cell pellets were resuspended with lysis buffer [50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 6 M Urea], TRADD in buffer [50 mM sodium acetate (pH 4.2), 50 mM magnesium sulfate, and 5 mM DTT], and RIP1 [50 mM Tris–HCl (pH 7.5) and 200 mM NaCl] and then sonicated on ice to disrupt the cells using a Branson Sonifier 450 sonicator. Finally, the cell suspensions were centrifuged at 20,170×*g* for 45 min to remove supernatant, after which the TNFR-1 and TRADD inclusion bodies were resuspended in the same buffer on ice and centrifuged at 20,170×*g* for 45 min to remove the supernatant.

The GST-tagged plasmid TNFR-1 and RIP1 were transformed into BL21(DE3), after which individual colonies were inoculated in 5 ml of LB medium enriched with 50 μg/ml ampicillin overnight at 37 °C. The cells were then incubated in 1 L of LB containing 50 μg/ml antibiotics and maintained at 37 °C until the OD600 reached 0.5–0.6. The expression of the protein was induced by 0.5 mM IPTG at 25 °C for 5 h.

2.2. Point mutations of the TNFR-1, TRADD, and RIP1 DDs

Double-stranded oligonucleotides were used for site-directed mutagenesis of the four different TNFR-1 residues to alanine (N365A, P367A, P368A, and C395A). Additionally, double-stranded oligonucleotides were used for site-directed mutagenesis of the two different TRADD residues to alanine (Y262A and F266A). Double-stranded oligonucleotides were used for site-directed mutagenesis of the two different RIP1 residues to alanine (M637A and R638A).

2.3. Purification

The soluble supernatant of the His-tagged TNFR-1 fusion protein was loaded onto a Ni–NTA (Amersham-Pharmacia Biotech, Orsay, France) column and pre-equilibrated with buffer A [50 mM Tris–HCl (pH 7.5) and 200 mM NaCl], after which the bound protein was eluted using buffer A containing 20–200 mM imidazole. The concentrated fractions of TNFR-1 from the Ni–NTA column were subsequently purified by gel filtration chromatography using a Superdex 200 10/300 GL fast protein liquid chromatography (FPLC) column equilibrated in buffer A.

The supernatant of GST-RIP1 was loaded onto a glutathion-sepharose column that had been pre-equilibrated with PBS buffer at a flow rate of 2 ml/min. The bound protein was eluted in buffer [50 mM Tris–HCl (pH 7.5)] containing 5–30 mM glutathione.

2.4. Western blotting

Separated TNFR-1, TRADD, and RIP1 proteins by 15% SDS–PAGE were electrophoretically transferred onto an immobilon-P membrane at 105 V for 1 h, then blocked with 5% skim milk in PBS buffer containing 0.1% Tween 20 (PBS-T) for 45 min. After blocking, the membrane was incubated in primary antibody [His-probe (G-18) diluted to 1:2500 (Santa Cruz Biotechnology, Inc.), and GST (B-14) diluted to 1:5000 (Santa Cruz Biotechnology, Inc.)] for 1 h. After washing with PBS-T for 30 min, the bound antibodies were detected by His secondary antibody [goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc.)] and with GST secondary antibody [goat anti-mouse IgG-HRP] diluted to 1:10,000 in blocking buffer for 1 h.

2.5. GST pull-down assay

A GST pull-down assay was performed by mixing the purified His-TNFR-1, His-TRADD, with purified GST, GST-TNFR-1, and GST-RIP1 proteins, which were incubated with glutathione-sepharose 4B beads and binding buffer A. After 3 h of reaction at 4 °C the bound proteins and beads were centrifuged at 3660×*g* for 3 min and then washed three times with binding buffer A. The binding proteins were then eluted with buffer [50 mM Tris–HCl (pH 7.5) and 30 mM glutathione] and analyzed by 15% SDS–PAGE. Finally, the proteins were visualized by immunoblotting assay using anti-His and anti-GST.

2.6. Structural modeling

The TNFR-1, TRADD, and RIP1 DDs models were constructed using the SWISS-MODEL software, which is a relative three-dimensional (3D) protein modeling system [31]. The 3D models of TRADD and RIP1 DDs with TNFR-1 DD were used as templates for the homology protein models of PIDD (PDB ID: 2OF5), FADD (PDB ID: 2GF5), and TNFR-1 (PDB ID: 1ICH) [32–34]. The TNFR-1-RIP1 DDs complex and TRADD DD were bound and the most stable complex structure was selected from among the top 20 complexes obtained from each docking.

2.7. Biacore biosensor analysis

Measurements of the apparent dissociation constants (K_D) among TNFR-1, TRADD, and RIP1 DDs were carried out using a Biacore 2000 biosensor (Biosensor, Sweden). Each TNFR-1, TRADD, and RIP1 DD (20 μg/mL in 10 mM sodium acetate with a pH of 5.0) was covalently bound to the carboxylated dextran matrix at a concentration corresponding to 3300, or 1000 response units (RU) using the amine-coupling method suggested by the manufacturer. For kinetic measurements at room temperature, TNFR-1, TRADD, and RIP1 DDs samples with concentrations ranging from 125 to 2000 nM were prepared by dilution in HBS buffer (150 mM of NaCl, 3 mM of EDTA, 0.005% surfactant P20 and 10 mM of HEPES) with a pH of 7.4.

3. Results and discussion

The domain structures of full-length TNFR-1, TRADD, and RIP1 are shown in Fig. 1A. The TNFR-1 sequence has N-terminal cysteine-rich repeats (Cys 1–4, 43–196) in its extracellular domains and the C-terminal death domain (DD, 356–441). The TRADD was divided into the N-terminal TRAF binding site (51–144) and the C-terminal death domain (DD, 215–304). The RIP1 was composed of the N-terminal kinase domain (KD, 17–289) and the C-terminal death domain (DD, 583–669), which were connected

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