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Biochemical and Biophysical Research Communications 324 (2004) 432–439
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The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination

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Received 3 September 2004
Available online 25 September 2004

Abstract

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a crucial signaling transducer that regulates a diverse array of physiological processes, including adaptive immunity, innate immunity, and bone metabolism. Importantly, it is essential for activating NF-κB signaling pathway in response to interleukin-1 and Toll-like receptor ligands. Previously, we characterized TRAF6 to be a ubiquitin ligase. In combination with the ubiquitin conjugating enzyme complex Ubc13/Uev1A, TRAF6 could catalyze the formation on itself of unique Lys-63 linked polyubiquitin chain that positively regulated NF-κB signaling pathway. However, it remains unknown how this auto-ubiquitination process is regulated. In this study, we found that the coiled-coil domain of TRAF6 was essential for its auto-ubiquitination and activating NF-κB signaling pathway. This domain served not as the specific target where the polyubiquitin chain was linked, but as a specific bridge to recruit Ubc13/Uev1A.

Keywords: TRAF6; Coiled-coil domain; Nuclear factor-κB; Ubiquitin

Ubiquitously expressed and highly conserved in all eukaryotes, ubiquitin (Ub) is a 76-amino-acid protein that can be linked covalently onto target protein and regulates the activity or stability of the latter. Implicated in different aspects of immune response [1], the process of ubiquitination is carried out by an enzymatic cascade composed of Ub activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub protein ligase (E3). Ub is first activated by E1 in the presence of ATP to form a high-energy thioester bond. The activated Ub is then transferred to a member of the E2 family, which, in conjunction with E3, conjugates Ub onto a lysine residue of the target protein to form an isopeptide bond [2]. Conjugated Ub itself can be further ubiquitnated through one of its seven lysine residues to form a polyubiquitin (poly-Ub) chain. Two types of poly-Ub chains have been reported previously. A typical poly-Ub chain is linked through the lysine-48 (K48) of one Ub and the carboxyl terminus of another Ub. Proteins thus modified are recognized by proteasome, where they are degraded [3]. Previously, we identified the non-classical lysine-63 (K63) linked form of poly-Ub chain on TRAF6, which influenced the function of signal adaptors [4].

TRAF6, as a member of the TNF-receptor-associated factor (TRAF) family, was isolated by screening of an EST expression library [6] and by yeast two-hybrid screening using CD40 cytoplasmic tail as bait [5]. It acts as the signaling mediator for both the TNF receptor superfamily and the IL-1R/TLR superfamily. TRAF6 plays a crucial role in various cellular signal transductions. Previous study had shown that TRAF6 deficiency resulted in defects in interleukin-1, CD40, and LPS signaling [14]. TRAF6 was recruited into the signaling complex via its association with IRAK on exposure of cells to proinflammatory IL-1 [6,20,25]. Following formation of the complex, the dimerization of TRAF6

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.070

triggered a K63 poly-Ub chain formation on itself, which required a heterodimeric Ub-conjugating enzyme (E2) composed of Ubc13 and Uev1A [4]. The ubiquitination of TRAF6 had an important role both in the activation of TAK1 and phosphorylation of IKK but not in inducing its degradation through proteinsome-dependent pathway [7]. It has been recently reported that inactivation of TRAF6 was regulated by deubiquitination instead of de novo protein synthesis [11,21]. Additionally, TRAF6 also could lead to the ubiquitination of TAB2 and TAB3 following IL-1 stimulation [15]. However, the role of these two ubiquitinations is still controversial.

Like other TRAFs, TRAF6 has a conserved carboxyl-terminal TRAF-C domain, a TRAF-N domain and, with the exception of TRAF1, an amino-terminal Ring finger [6,24]. It has been shown that TRAF-C domain mediated both homo- and heterodimerization of TRAF proteins and interactions with the adapter proteins from distinct cell surface receptors [5,8,9,22]. Ring finger domain is required for TRAF6 to catalyze the synthesis of unique polyubiquitin chains, which is essential for TRAF6 downstream signaling [4,7]. TRAF-N domain was predicted to form a coiled-coil structure [10], but the role of the structure is still unknown.

Here we report that the coiled-coil domain of TRAF6 is essential for its auto-ubiquitination and activating NF-κB signaling pathway. In addition, we show that this domain serves not as specific target where the polyubiquitin chain is linked, but as a specific bridge to recruit Ubc13/Uev1A. Our investigation uncovers a new interaction between TRAF6 and its cognate ubiquitin conjugating enzyme E2 and shed new light on how TRAF6 initiates its auto-ubiquitination process.

Materials and methods

Plasmids and reagents. TRAF6 truncations were created by PCR amplification of the relevant fragments and then cloning into pcDNA3.1 mammalian expression vector in-frame with the Flag coding sequence. T6RZC and T6RZ expression plasmids were described previously [4]. T6RZC (1–340) and T6RZC (1–330) cDNA were also generated by PCR and then cloned into pEF-IRSEP mammalian expression vector in-frame with the HA coding sequence. The lysines of the coiled-coil domain of TRAF6 were mutated to arginine individually and in combination using the QuikChange site-directed mutagenesis method (Stratagene) and confirmed by sequencing. The coiled-coil domain of TRAF6 was cloned into pGEX4T-3 bacterial expression vector in-frame with the GST coding sequence, and the GST fusion protein was purified from *Escherichia coli* using glutathione–Sepharose 4B resin (Amersham Biosciences). The proteins for in vitro ubiqutination system were purified as described previously [4]. The antibodies against TRAF6, Ub (P4D1), HA, and His were purchased from Santa Cruz Biotech. Anti-Flag M2 affinity Gel, anti-Flag M2 monoclonal antibodies, and coumermycin A1 were purchased through Sigma.

Cell culture, transfection, and stimulation. Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) plus 10% fetal calf serum (FCS, Gibco) supplemented with antibiotics. Transfection of HEK293T cells was carried out according to the calcium phosphate precipitation method as preciously described [4]. The 293T cells in 100 mm plates were transfected with the expression plasmids (10 μg). After transfection of T6RZC or (T6RZC truncation mutants) into HEK293T cells for 24 h, cells were treated with coumermycin A1 (1 μM) for 45 min to induce the dimerization of TRAF6. Then the cell extracts were analyzed by immunoblotting with HA antibody.

NF-κB reporter gene assays. The HEK293T cells (2×10^5 cells/well) were seeded into six-well (35 mm) plates. Cells were transfected by the calcium phosphate precipitation method 24 h after seeding with the NF-κB-Luc reporter gene plasmid, along with each expression vector as indicated. The reporter consisted of three NF-κB response elements upstream of firefly luciferase. The total DNA concentration (550 ng) was kept constant by supplementing with empty vector DNAs. GFP vector (20 ng) was used for normalizing transfection efficiencies. At 24 h after transfection, luciferase activity was determined with the Luciferase Assay System (Promega). The values shown are averages of three independent experiments in which each transfection was performed in duplicate.

Immunoprecipitation and immunoblotting. HEK293T cells in 10 cm-diameter dishes were transfected with various pcDNA3.1-N-Flag-TRAF6 constructs. After 48 h, cells were washed using PBS before application of 600 μl lysis buffer containing 50 mM Tris–HCl, pH 7.5, 0.5% Nonidet P40, 150 mM NaCl, 20 mM NaF, 0.5 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM DTT, and 0.2 mM EGTA. After centrifugation for 5 min at 13,000*g*, the supernatant was removed. One hundred microliters of various Flag-TRAF6 cell lysates was incubated with 2 μl anti-flag M2 affinity Gel (Sigma) for 2 h incubation at 4 °C. Then the beads were washed three times using IP buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 and then twice using TBS buffer. The beads were subjected to in vitro ubiquitination assay. For immunoblotting, the immunoprecipitates after ubiquitination assay or whole-cell lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to PVDF membranes (Bio-Rad). The membranes were immunoblotted with appropriate antibodies, and the bound antibodies were visualized with AP-conjugated antibodies against mouse immunoglobulin G (IgG) by using NBT/BCIP Western Blotting System (Promega).

Ubiquitination assay. TRAF6 auto-ubiquitination activity was measured in a reconstituted system (10 μl) containing an ATP buffer (50 mM Tris–HCl, pH 7.5, 5 mM MgCl2, and 2 mM ATP), purified E1 (50 nM), His-Ubc13-Uev1A (0.3 μM), ubiquitin or KO (0.1 mM) and immunoprecipitated Flag-TRAF6 using anti-FLAG affinity Gel (see above). After incubation at 30 °C for 1 h, the resin was washed three times with IP buffer and twice with TBS buffer. Then the resin was analyzed by immunoblotting with TRAF6 antibody and ubiquitin antibody, respectively.

In vitro phosphorylation assay. To measure IKK activity, HEK293T cells were transfected with various TRAF6 constructs. After 24 h, cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 30 mM NaF, 0.1 mM Na_3VO_4, and 1 mM DTT. Then anti-IKKβ immunoprecipitates were incubated in 20 μl reactions containing 50 mM Tris (pH 7.5), 0.5 mM DTT, 5 mM MgCl2, 15uM ATP, 2 μg GST-IκBα, and 5 μCi [γ-^{32}P]ATP at 30 °C for 30 min. Reactions were terminated by the addition of SDS–PAGE sample buffer and resolved on a 12% polyacrylamide gel. The gel was dried and exposed to X-ray film.

In vitro binding assay. Equal amounts of His-tag purification proteins from *E. coli* were mixed, respectively, with 2 μg of a GST fusion protein, and then incubated with 2 μl of glutathione–Sepharose resin (Amersham Biosciences) for 2 h at 4 °C in 500 μl binding buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100. The pelleted resin was washed three times with the wash buffer (the same as binding buffer), then resuspended in 10 μl of 2× SDS loading buffer, and subjected to SDS–PAGE.

Results

Coiled-coil domain of TRAF6 is involved in its activation

It has been shown that auto-ubiquitination of TRAF6 played a key role in the activation of NF-κB [7]. To determine whether the coiled-coil domain is involved in the activation of NF-κB and ubiquitination of TRAF6, a series of truncation constructs (Fig. 1A) were generated and transfected into HEK-293T cells to analyze their capabilities of stimulating NF-κB responsive reporter gene encoding luciferase. The reason to use a fragment of the bacterial gyrase B instead of the TRAF-C domain was that the former was able to mimic the oligomerization function when cells were treated with coumermycin A1 and it was easy to reveal the function of the coiled-coil domain in this context [7]. Following transfection with different T6RZC truncation plasmids for 24 h, the cells were treated with coumermycin A1 for 45 min before luciferase assay. As shown in Fig. 1B, we found that NF-κB reporter gene was strongly activated by the transfection of HEK 293T cells with an expression vector encoding T6RZC, whereas reporter gene was not activated by transfection of cells with an expression vector encoding T6RZ, which has no coiled-coil domain. In addition, transfection of T6RZC (1–340) or T6RZC (1–330), which, respectively, contained a portion of coiled-coil domain, impaired the activation of reporter gene in a significant degree.

Previously, we demonstrated that the oligomerization of T6RZC catalyzed the formation of a polyubiquitin chain with the treatment of coumermycin A1 [7]. To investigate whether the coiled-coil domain also functions in the ubiquitination of T6RZC, various T6RZC truncation plasmids were transfected into HEK-293T cells. After transfection for 24 h, cells were treated with coumermycin A1 for 45 min. Consistent with the results of luciferase assay, T6RZC formed a clear polyubiquitin chain (Fig. 1C, lane 2), whereas the degree of ubiquitination was drastically reduced for both the T6RZC (1–340) and T6RZC (1–330) truncations (Fig. 1C, lanes 3–4). Most importantly, T6RZ, lacking the coiled-coil domain, abolished its ubiquitination (Fig. 1C, lane 5). Taken together, the NF-κB reporter gene assay and in vivo ubiquitination data suggested that the coiled-coil domain is involved in the activation of TRAF6.

Coiled-coil domain is essential for ubiquitination of TRAF6 and activation of NF-κB pathway

TRAF6, which has been shown to contain a RING domain [6] and possess E3 ubiquitin ligase activity [4,7], catalyzes the formation of polyubiquitin chain on itself. To clarify that coiled-coil domain is indeed essential for ubiquitination of TRAF6, an in vitro recombinant ubiquitination system was used [4,7], which

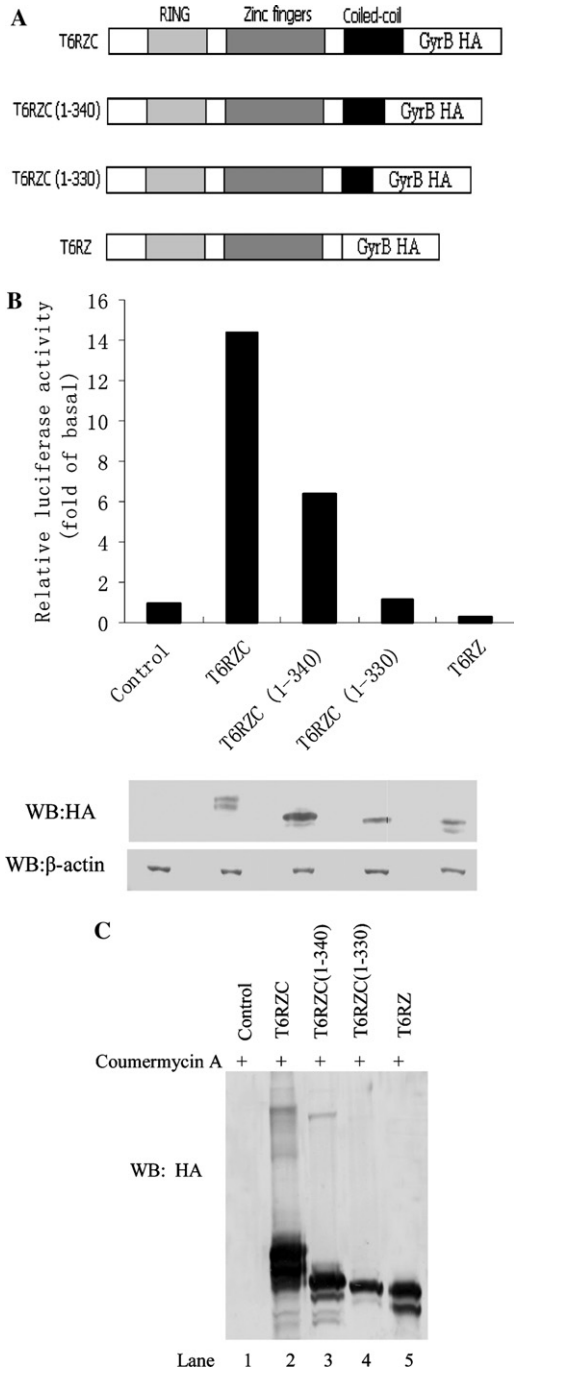

Fig. 1. Coiled-coil domain is involved in activation of TRAF6. (A) Schematic representation of TRAF6-gyrase B chimaeric constructs. In these constructs, the TRAF-C domain is replaced by a fragment of the bacterial gyrase B. (B) Luciferase assay for TRAF6-gyrase B constructs. 293T cells were co-transfected with 25 ng κB-Luc luciferase reporter plasmids and 100 ng various TRAF6-gyrase B constructs as indicated. After transfection for 24 h, cells were treated with coumermycin A1 for 45 min before harvesting. Relative luciferase activity was measured as fold activation. Transfection efficiencies were normalized by co-transfection with GFP as described in the Materials and methods. The bars represent the average of at least three experiments. (C) Analysis of the in vivo ubiquitination of TRAF6-gyrase B constructs. 293T cells transfected with various TRAF6-gyrase B plasmids were treated with coumermycin A1 for 45 min, and cell extracts were collected and immunoblotted with a HA antibody.

contained E1, Ubc13/Uev1A, TRAF6, Ub, and an ATP-regenerating system. In order to easily monitor auto-ubiquitination of TRAF6, we replaced Ub (wt) with Ub (KO), which is a lysine-less ubiquitin mutant [7]. We found that the auto-ubiquitination of TRAF6 was more readily detected with a TRAF6-specific antibody (Fig. 2A, lanes 2 and 4) in this way. Therefore, the usage of Ub (KO) in our system facilitates the detection of auto-ubiquitination of TRAF6.

To answer the question whether coiled-coil domain plays an essential role in auto-ubiquitination of TRAF6, several of TRAF6 truncation constructs were generated (Fig. 2B) and analyzed in recombinant ubiquitination system described above for their capabilities of auto-ubiquitination. After in vitro ubiquitination reaction, immunoblot analysis revealed that TRAF6 (1–358), lacking TRAF-C domain, have the ability to react with Ub (KO) (Fig. 2C, lane 3). It has been reported that TRAF-C domain mediates both homo- and heterodimerization of TRAF proteins and interaction between TRAF proteins and distinct cell surface receptors [5,8,9]. In contrast, TRAF6 (1–291), without both coiled-coil domain and TRAF-C domain, failed to display any ubiquitination shift (Fig. 2C, lane 5), which

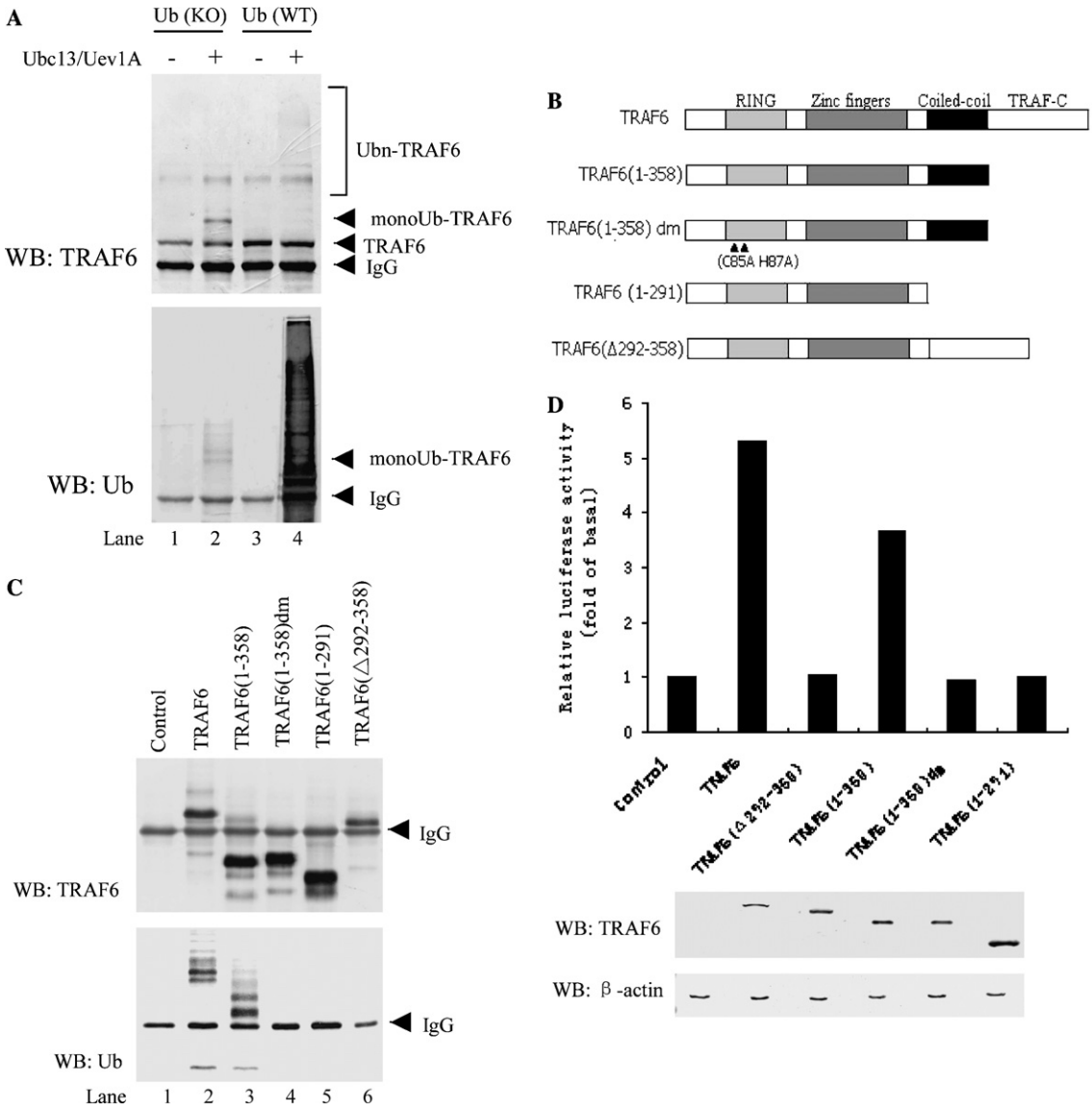

Fig. 2. Coiled-coil domain is essential for ubiquitination of TRAF6 and activation of NF-κB pathway. (A) Advantage of using Ub (KO) instead of Ub (WT) for in vitro ubiqutination reaction. The reaction contains E1, E2 (Ubc13/Uev1A), TRAF6, Ub, and ATP. Auto-ubiquitination of TRAF6 is readily detected by the replacement of Ub (WT) with Ub (KO). KO, ubiquitin mutant containing no lysine. (B) Schematic representation of wild type and truncation mutants of TRAF6. (C) Analysis of auto-ubiquitination capability of TRAF6 truncations. Products of ubiquitination reactions from various TARF6 truncations were immunoprecipitated and then probed by immunoblotting with TRAF6 antibody (top) and ubiquitin antibody (bottom), respectively. (D) Analysis of NF-κB activation in cells transfected with various TRAF6 truncations. 293T cells were co-transfected with 25 ng κB-Luc luciferase reporter plasmids with 250 ng various expression plasmids of TRAF6 truncations as indicated. Relative luciferase activity was measured as fold activation. The bars represent the average of at least three experiments.

indicated that the coiled-coil domain is required for ubiquitination of TRAF6. Furthermore, TRAF6 (Δ292–358), lacking only coiled-coil domain but not TRAF-C domain, also could not be detected by any ubiquitination shift with both TRAF6-specific and ubiquitin-specific antibodies (Fig. 2C, lane 6). As a negative control, TRAF6 (1–358) dm, a RING finger mutant, abolished its ability of ubiquitinaiton (Fig. 2C, lane 4). Thus, these results clearly demonstrated that the coiled-coil domain played a crucial role in auto-ubiquitinaiton of TRAF6.

To determine whether the coiled-coil domain of TRAF6 is required for NF-κB activation in living cells, we transfected these TRAF6 truncations into 293T cells together with a NF-κB reporter gene encoding luciferase. As shown in Fig. 2D, TRAF6 (1–358) lacking only TRAF-C domain was still active in this assay (Fig. 2D), but TRAF6 (1–291) did not stimulate reporter gene expression (Fig. 2D). Furthermore, TRAF6 with an internal deletion of amino acids from 292 to 358, in which the coiled-coil domain lies, did not activate the reporter gene expression either (Fig. 2D). These experiments indicated that the coiled-coil domain is required for ubiquitination of TARA6 and activation of NF-κB.

Activation of IKK needs the coiled-coil domain

IKK complex functions as downstream signal transducer of MyD88, IRAK1, and TRAF6 in the IL-1R signaling pathway [16,17]. Upon stimulation of cells by overexpression of TRAF6, IKK complex is rapidly activated and then phosphorylates IκB proteins [16]. To investigate whether the coiled-coil domain plays a role in the activation of IKK complex, we transfected various TRAF6 truncations into HEK 293T cells to measure the kinase activity of endogenous IKK complex. As expected, the kinase assay revealed that TRAF6 (1–358) induced the phosphorylation of Gst-IκBα just like full-length TRAF6 (Fig. 3, lanes 2 and 5), whereas TRAF6 (1–291) abolished the activation of IKK complex (Fig. 3, lane 4). Importantly, TRAF6 with an internal deletion of coiled-coil domain did not display any stimulation of IKK complex (Fig. 3, lane 3). Thus, the coiled-coil domain (292–358) of TRAF6 is required for the activation of IKK complex.

Lysines in coiled-coil domain are not the ubiquitination sites

Based on the above observations, we wondered what is the possible role of coiled-coil domain in ubiquitinaiton of TRAF6 and activation of NF-κB. Since ubiquitination occurs exclusively on lysine residues [13] and the coiled-coil domain contains four lysine sites, one possibility is that the coiled-coil domain of TRAF6 contains its ubiquitination sites. It has recently been shown that XIAP (X-linked inhibitor of apoptosis protein), which

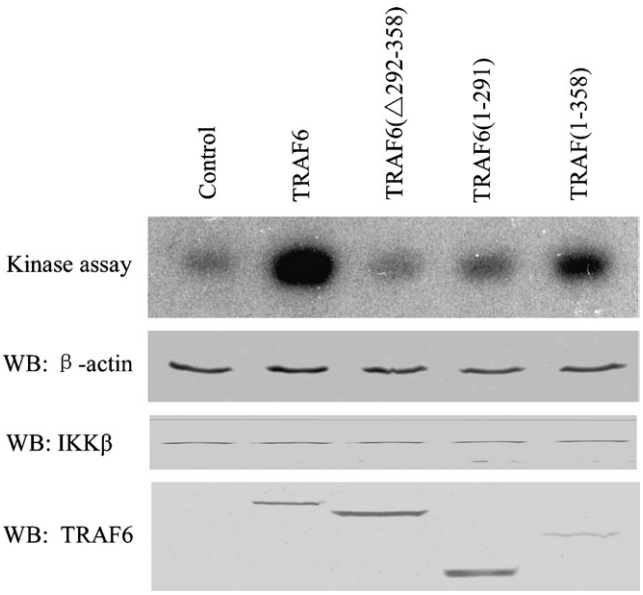

Fig. 3. Activation of IKK needs the coiled-coil domain. 293T cells were transfected with various TRAF6 truncations. Cell extracts were immunoprecipitated (IP) with anti-IKKβ antibody. The immunoprecipitates were subjected to an in vitro phosphorylation using bacterially expressed GST-IκBα as an exogenous substrate (top panel). The immunoprecipitates were analyzed by immunoblotting (IB) with anti-IKKβ antibody (second panel). Whole-cell extracts (WCE) were immunoblotted with anti-TRAF6 antibody (bottom panel).

is a RING finger protein and ubiquitin ligase, catalyzes polyubiquitination on its specific lysine [12]. To investigate whether ubiquitination of TRAF6 also occurs on specific lysine, we mutated the four lysine residues, individually and in combination, to arginine residues (Fig. 4A). First, we tested whether these mutants had any effect on the activation of NF-κB. TARF6 and the respective mutants were transfected into HEK 293T cells together with κB Luc reporter plasmids. The luciferase assay data showed that TRAF6 stimulated the activation of NF-κB, and there was no significant difference observed between wild-type TRAF6 and any of the lysine mutants (Fig. 4B). In addition, wild-type TRAF6, R3 (single lysine mutant), and 4R (four lysine mutant) were subjected to in vitro ubiquitination assay to test whether there existed any difference between wild-type TRAF6 and lysine mutants for their auto-ubiquitination. Similarly, no significant difference was observed in ubiquitination assay (Fig. 4C). Taken together, the luciferase assay and ubiquitination assay data suggested that the coiled-coil domain is not the ubiquitination site.

Coiled-coil domain mediates the interaction between TRAF6 and Ubc13

Since activation of TRAF6 required a heterodimeric Ub-conjugating enzyme (Ubc or E2) composed of Ubc13 and Uev1A, we examined whether the coiled-coil domain contributed to the interaction between TRAF6

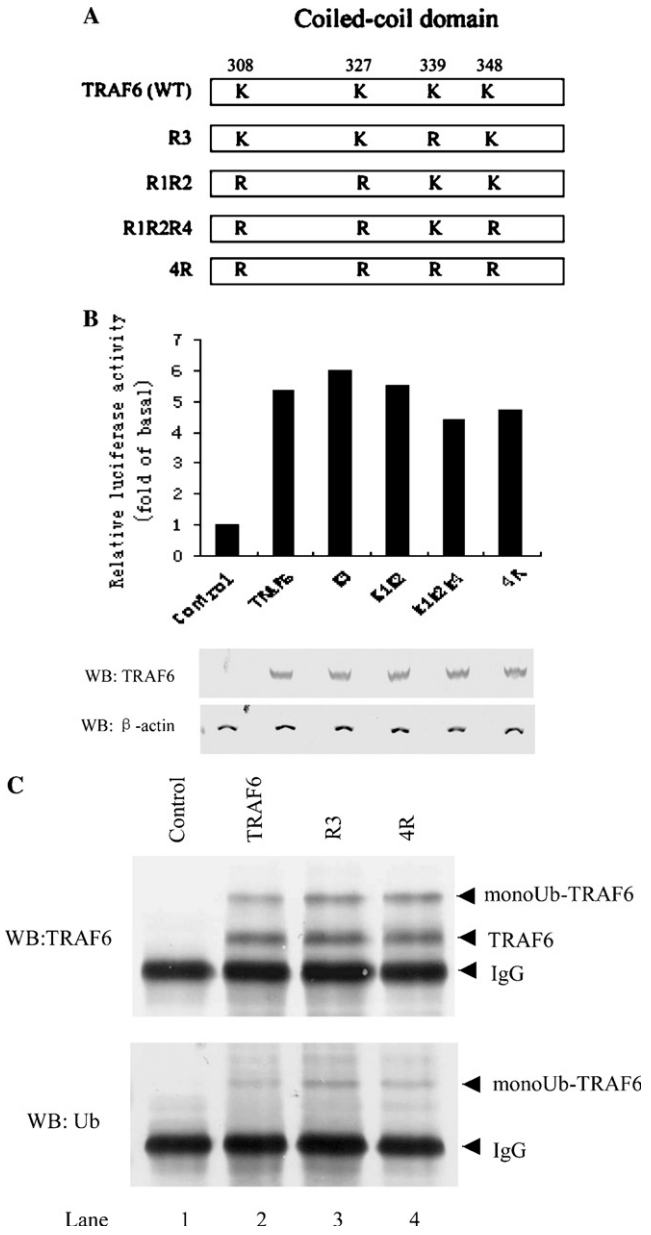

Fig. 4. The coiled-coil domain is not the ubiquitinaiton site. (A) Schematic representation of wild type and lysine site mutants of TRAF6. Four lysine residues of the coiled-coil domain of TRAF6 were mutated to arginine in various combinations. (B) Effect of lysine to arginine mutants of TRAF6 on NF-κB activation in cells. 293T cells were co-transfected with 25 ng κB-Luc luciferase reporter plasmids and 250 ng expression plasmids of TRAF6 point mutants as indicated. Relative luciferase activity was measured as fold activation. The bars represent the average of at least three experiments. (C) Analysis of auto-ubiquitination of TRAF6 point mutants. Two forms of TRAF6 point mutants were used for in vitro ubiquitination assay and the products were immunoprecipitated and detected by immunoblotting with TRAF6 antibody (top) and ubiquitin antibody (bottom), respectively.

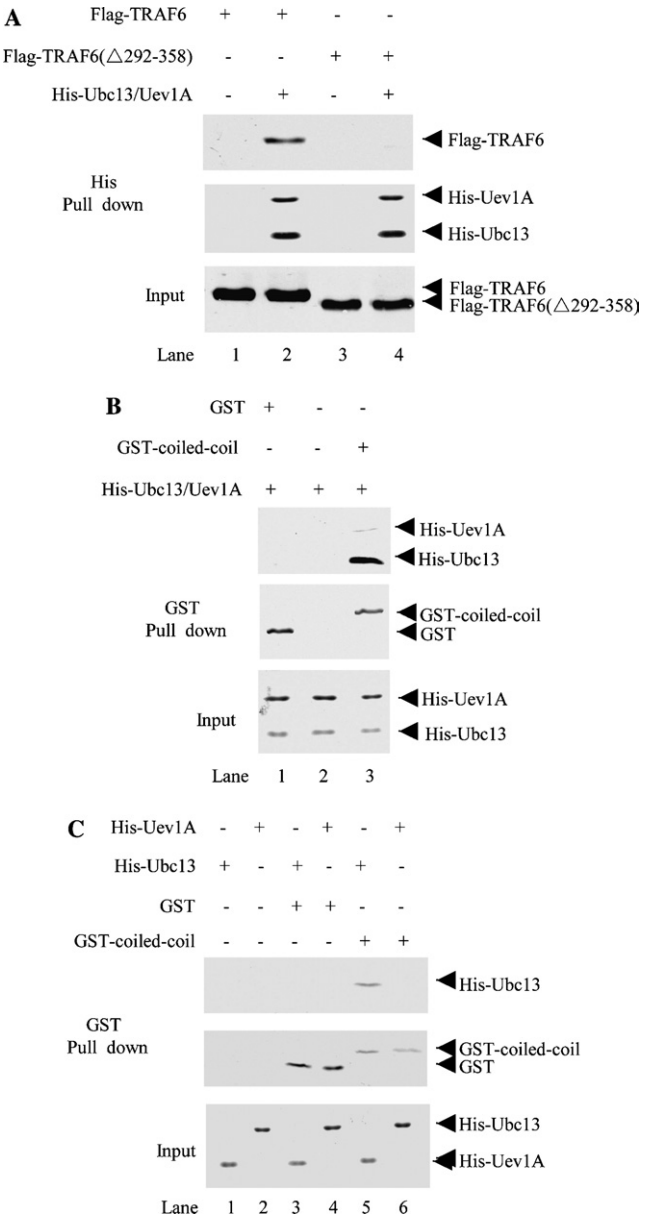

Fig. 5. Coiled-coil domain of TRAF6 mediates interaction with Ubc13 in vitro. (A) Analysis of the interaction between TRAF6 (Δ292–358) and Ubc13/Uev1A. Cell extracts expressing Flag-TRAF6 or Flag-TRAF6 (Δ292–358) were incubated with His-Ubc13/Uev1A in vitro and pulled down by Ni–NTA beads. The beads were probed with anti-Flag and anti-His antibody, respectively. (B) The coiled-coil domain interacts with Ubc13/Uev1A. Purified GST or GST-coiled-coil was incubated with His-Ubc13/Uev1A in vitro and pulled down by GST beads. The beads were probed with anti-GST and anti-His antibody, respectively. (C) The coiled-coil domain interacts with Ubc13 directly. Purified GST or GST-coiled-coil was incubated with His-Ubc13 or His-Uev1A in different combinations and analyzed as in (B). Top panel, GST pull-down co-precipitate; middle panel, GST protein bound to GST bead; and lower panel, input proteins for GST pull-down.

and Ubc13/Uev1A (Fig. 5). The HEK 293T cell lysates, which have been transfected with Flag-TRAF6 and Flag-TRAF6 (Δ292–358), respectively, were mixed with His-Ubc13/Uev1A. Then pull-down experiment of His-Ubc13/Uev1A with Ni–NTA beads was performed, followed by immunoblot analysis of the precipitate with anti-FLAG antibody M2. Interestingly, the full-length

TRAF6 could directly interact with the His-Ubc13/Uev1A complex (Fig. 5A, lane 2), whereas no interaction was observed between TRAF6 (Δ292–358) and His-Ubc13/Uev1A (Fig. 5A, lane 3). Moreover, the interaction between Ubc13/Uev1A and TRAF6 (1–358) but not TRAF6 (1–291) was detected by a similar experiment (data not shown). To further demonstrate that the coiled-coil domain is required for the interaction with Ubc13/Uev1A, we constructed GST-coiled-coil (292–358), which only contains the coiled-coil domain of TRAF6. GST pull-down experiment was carried out between GST-coiled-coil (292–358) and His- Ubc13/Uev1A. As shown in Fig. 5B, GST-coiled-coil could directly interact with His- Ubc13/Uev1A complex (Fig. 5B, lane 3). These results indicate that the coiled-coil domain is sufficient for the interaction between TRAF6 and Ubc13/Uev1A.

We next investigated further the interaction between GST-coiled-coil and Ubc13, Uev1A, respectively, by GST pull-down experiment. As shown in Fig. 5C, Ubc13 but not Uev1A could interact with the coiled-coil domain of TARF6 (lanes 5 and 6). Taken together, these results demonstrated that the coiled-coil domain of TRAF6 mediate, directly interaction with Ubc13 but not Uev1A.

Discussion

TRAF6 is a signal transducer that activates IKK and JNK in response to pro-inflammatory mediators such as IL-1 and LPS [14,18]. The proliferation of thymocyte and the activation of NF-κB and JNK of embryonic fibroblasts in response to IL-1 were absent in TRAF6$^{-/-}$ mice [14]. Upon receptor stimulation, TRAF6 is recruited into the signaling complex via its association with IRAK [6,19] and modified by ubiquitin. Ubiquitination of TRAF6 is an essential event for the activation of NF-κB pathway induced by IL-1 [4,7]. It has been shown that TRAF6 contains a RING finger domain and functions as ubiquitin ligase catalyzing the synthesis of polyubiquitin chain. Using in vitro auto-ubiquitination system, we found that the coiled-coil domain played a crucial role in auto-ubiquitination of TRAF6. Mutational analysis revealed that TRAF6 lacking TRAF-C domain only partially attenuated the intensity of TRAF6 polyubiquitination, whereas TRAF6 lacking the coiled-coil domain abolished the capability of ubiquitination (see Fig. 2A, lanes 3 and 6). Consistent with the results of ubiquitination assay, both NF-κB reporter gene assay and in vitro IKK kinase assay had shown in this investigation that the coiled-coil domain was required for the activation of NF-κB.

We next investigated how the coiled-coil domain affected the ubiquitination of TRAF6. Based on the previous studies showing that ubiquitination could occur on specific lysines [12,23], we wondered if the coiled-coil domain harbored the ubiquitination sites of TRAF6. To investigate the possibility, various Lys to Arg mutants in coiled-coil domain were generated and analyzed by in vitro ubiquitination assay and NF-κB reporter luciferase assay. Our results showed that these lysine mutants behaved similarly to wild-type TRAF6. Then we proposed another possibility that the coiled-coil domain was important in the interaction between TRAF6 and Ubc13/Uev1A complex. It turned out that deletion of the coiled-coil domain of TRAF6 made it unable to interact with Ubc13/Uev1A (Fig. 5A, lanes 2 and 4), which suggested that coiled-coil domain mediated this specific interaction. To further support this model, GST fusion protein containing only the coiled-coil domain could interact specifically with Ubc13/Uev1A too (Fig. 5B). In addition, we demonstrated that the coiled-coil domain interacted directly with Ubc13 but not Uev1A, (Fig. 5C). These results strongly suggested that TRAF6 recruited Ubc13/Uev1A through its coiled-coil domain, which in turn catalyzed the formation of K63 linkage polyubiquitin chain on TRAF6.

In conclusion, this investigation found that the coiled-coil domain played an essential role for the auto-ubiquitination of TRAF6 and the activation of NF-κB. This domain was not the ubiquitination site. Instead, the coiled-coil domain performed its function by mediating the specific interaction between TRAF6 and Ubc13. Our investigation uncovers a new interaction between TRAF6 and its cognate ubiquitin conjugating enzyme E2 and sheds new light on how TRAF6 initiates its auto-ubiquitination process.

Acknowledgments

We thank Professors Allan Weissman (National Institute of Health, USA), Mike Ellison (University of Alberta, Canada), and James Chen (University of Texas Southwestern Medical Center, USA) for providing plasmids in this study. We are grateful to Professor Lin Li for technical help. Chen Wang was a scholar of "The Rising Star Program" from Shanghai Municipal Government and of "The Hundred Talents Program" from Chinese Academy of Sciences. This work was supported in part by "The Distinguished Young Scholars Program" from National Natural Science Foundation of China (30225013), CAS renovation program, and 973 Project (2002CB513003).

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