Biochemical and Biophysical Research Communications 463 (2015) 864–869

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dimerization of TRAF-interacting protein (TRAIP) regulates the mitotic progression

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ARTICLE INFO

Article history:
Received 31 May 2015
Accepted 4 June 2015
Available online 18 June 2015

Keywords:
TRAIP
Dimerization
Coiled-coil domain
Mitotic progression

ABSTRACT

The homo- or hetero-dimerization of proteins plays critical roles in the mitotic progression. The TRAF-interacting protein (TRAIP) is crucial in early mitotic progression and chromosome alignment defects in the metaphase. The TRAIP is a 469 amino acid protein, including the Really Interesting New Gene (RING), coiled-coil (CC), and leucine zipper (LZ) domain. In general, the CC or LZ domain containing proteins forms homo- or hetero-dimerization to achieve its activity. In this study, a number of TRAIP mutants were used to define the TRAIP molecular domains responsible for its homo-dimerization. A co-immunoprecipitation assay indicated that the TRAIP forms homo-dimerization through the CC domain. The cells, expressing the CC domain-deleted mutant that could not form a homo-dimer, increased the mitotic index and promoted mitotic progression.

1. Introduction

The eukaryotic cell cycle is composed of a series of G1, S, G2, and M phases, which are tightly regulated by several proteins, leading to the division and duplication of cells that produce the daughter cells [1]. The DNA is replicated during the S phase. The mitotic phase is the final step of the cell cycle and is the process, where a eukaryotic cell separates the chromosomes and the mother cell is divided into two daughter cells. A number of protein modifications, including SUMOylation, phosphorylation, and ubiquitination, as well as protein dimerization, are key regulatory mechanisms of mitotic progression [2–5]. The TRAIP (TRAF-interacting protein) interacts with the TRAF (tumor necrosis factor receptor-associated factor) signaling complex and functions as a negative regulator in the signaling transduction of TRAF2-mediated nuclear factor-kappa B (NF-κB) activation [6,7]. The TRAIP is also important for embryo development in drosophila that is encoded by NOPO (no poles) [8]. Recently, an unknown protein was shown to be ubiquitinated when the BEN interacts with the NOPO complex, and this combination leads to genomic integrity in an early drosophila embryo [8]. Furthermore, embryonic lethality occurs in TRAIP-deficient mice [9]. In addition, its knockdown in HeLa cells by RNA interference (RNAi) decreased the time of early mitosis progression from the nuclear envelope breakdown (NEB) to the anaphase onset and increased the percentage of chromosome alignment defects in the metaphase [10]. However, the functions of TRAIP dimerization during mitosis are totally unclear.

In this study we showed that the TRAIP forms a homo-dimer through its coiled-coil (CC) domain. It was further confirmed that the cells expressing the CC domain-deleted mutant, which could not form a homo-dimer, increased the mitotic index. This is the first study to demonstrate the importance of the TRAIP homo-dimerization during mitosis; furthermore, it suggests that the TRAIP homo-dimerization may be very important for the mitotic progression in eukaryotic cells.

2. Materials and methods

2.1. Plasmids and antibodies

A TRAIP gene was purchased from a South Korean human gene bank. An Myc-tagged TRAIP expression plasmid was cloned into a Myc-tagged mammalian expression vector, and a GFP-tagged TRAIP expression plasmid was cloned into a GFP-tagged mammalian expression vector. The TRAIP deletion or siRNA resistant mutants were generated by mutagenesis. An anti-TRAIP antibody was raised

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http://dx.doi.org/10.1016/j.bbrc.2015.06.026
0006-291X/

by immunizing rabbits with the GST-TRAIP fusion protein. The anti-Flag, -GFP and -β-actin antibodies were purchased from Sigma Inc. An anti-Myc antibody was purchased from Roche.

2.2. Cell culture, siRNAs, and transfection

HeLa and human embryonic kidney (HEK) 293T cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc.), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The control siRNA has been previously described in the literature [11]. The TRAIP siRNA sequences were as follows: TRAIP#1: 5′ GCAGCAGGAUGAGACCAAAUU 3′; TRAIP#2: 5′ GCAAGUUGCAGACAGUCUAUU 3′. The siRNAs were transfected into HeLa cells using DharmaFECT 1 (Dharmacon, Inc.). The DNA transfection was performed using Lipofectamine 2000 (Invitrogen, USA), as per the instructions of the manufacturer.

2.3. Immunoprecipitation

For immunoprecipitation, the cells were washed with ice-cold PBS and then lysed in an NETN buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM DTT, and 50 μg/ml PMSF), at 4 °C for 10 min. The crude lysates were cleared by centrifugation, at 14,000 rpm at 4 °C for 5 min, and the supernatants were incubated with the protein A agarose-conjugated primary antibodies. The immunocomplexes were washed three times with NETN buffer and subjected to SDS-PAGE. And then western blotting was performed using the antibodies, which are denoted in the Figure Legends (below).

2.4. Immunofluorescence

HeLa cells, on coverglasses, were fixed with methanol at −20 °C for 30 min. Alternatively, the cells were extracted with a BRB80-T buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA, and 0.5% Triton X-100) and then fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were then permeabilized and blocked with PBS-BT (1 × PBS, 3% BSA, and 0.1% Triton X-100) for 30 min at room temperature. The coverslips were then incubated in primary and secondary antibodies that were diluted in PBS-BT. The images were acquired using an AxioVision 4.8.2 (Carl Zeiss) under a Zeiss Axiovert 200M microscope with a 1.4 NA plan-Apo 100× oil immersion lens and an HRm CCD camera. The deconvolved images were obtained using an AutoDeblur v9.1 and AutoVisualizer v9.1 (AutoQuant Imaging). All the images are maximum projections from z stacks of representative cells that were stained for the

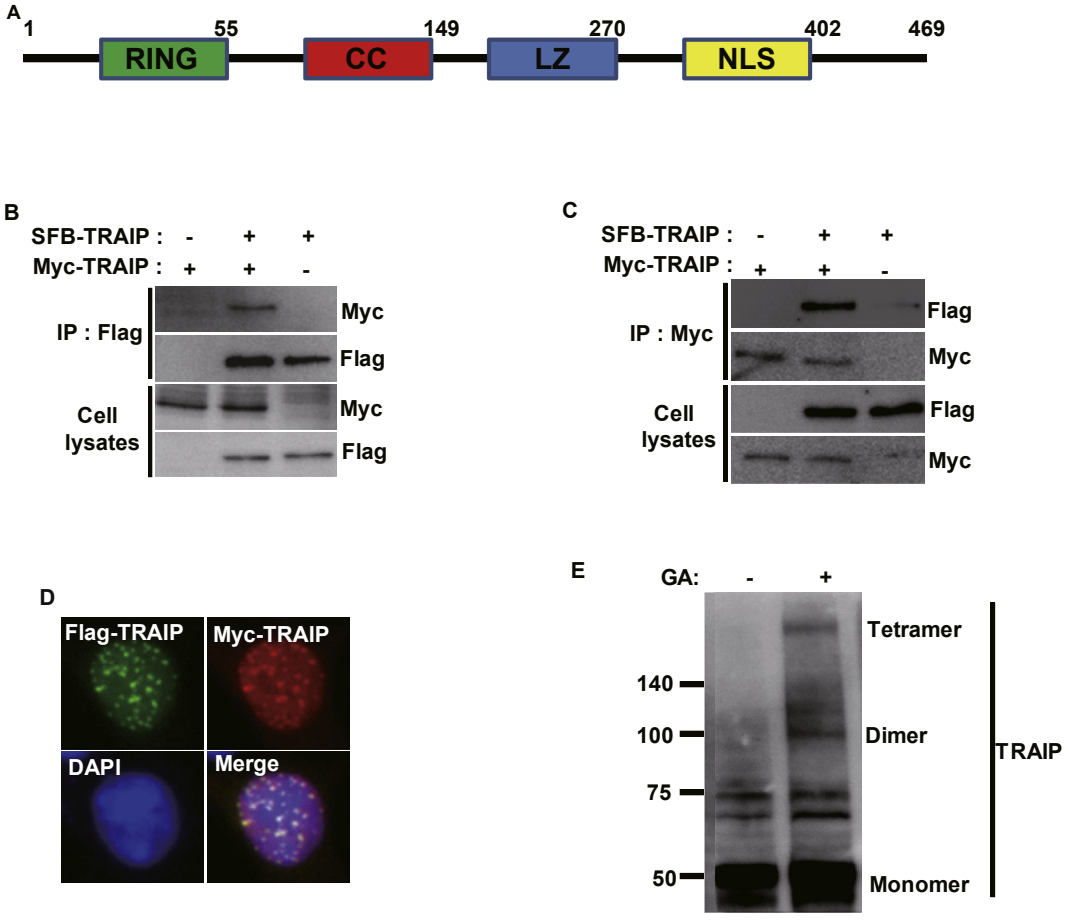

Fig. 1. Detection of the TRAIP dimerization. (A) The domain organization of TRAIP. RING, really interesting new gene; CC, coiled-coil; LZ, leucine zipper; NLS, nuclear localization sequence. (B and C) Myc-TRAIP binds to SFB-TRAIP in HEK 293T cells. Twenty-four hours after transfection with indicated expression plasmids, the lysates from transfected 293T cells were immunoprecipitated with anti-Flag (B) or anti-Myc (C) antibody and subjected to western blot analysis using the indicated antibodies. (D) Colocalization of Myc-TRAIP and SFB-TRAIP in HEK 293T cells. Twenty-four hours after transfection with indicated expression plasmids, immunofluorescence assays were performed using the transfected cells with the indicated antibodies. DAPI was used as an indicator for the nucleus. (E) Endogenous TRAIP forms the oligomer. The HEK 293T cells were treated with/without 0.01% glutaraldehyde and western blotting analysis was performed using TRAIP antibody.

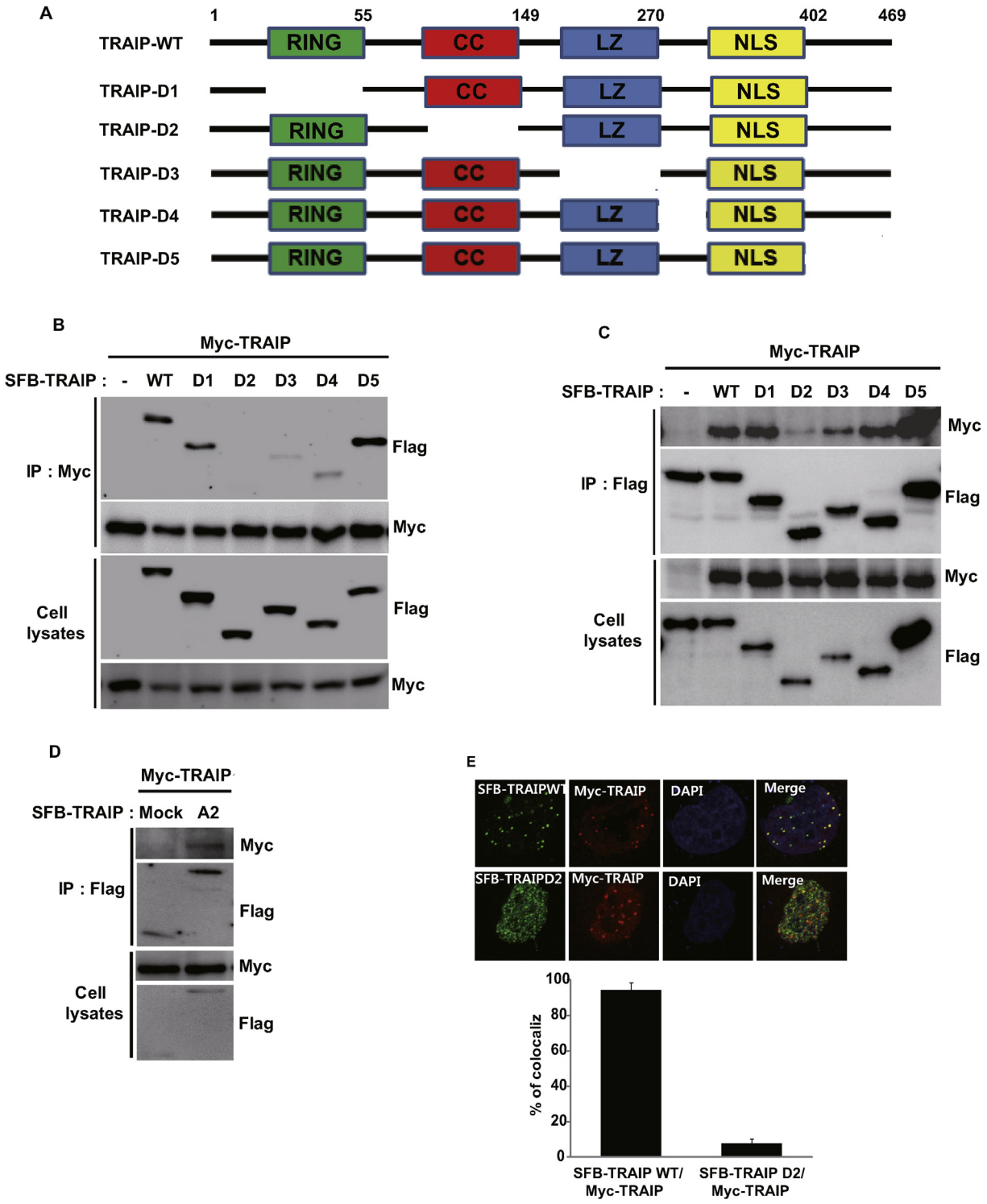

Fig. 2. Identification of the domains involved in TRAIP dimerization. (A) Diagram of the TRAIP wild type and serial deletion mutants. (B and C) 293T cells were transfected with plasmids encoding Myc-TRAIP and the indicated SFB-TRAIP expression plasmids. Cell lysates were subjected to immunoprecipitation (IP) and immunoblotting with indicated antibodies. (D and E) The CC domain is sufficient for TRAIP dimer formation.

indicated antigens. The mages for quantification were acquired under a constant exposure in each channel for all of the cells.

2.5. Cell synchronization

The cells were synchronized at the late G_1 phase using a double thymidine block method [12]. Briefly, the cells were plated in 100-mm diameter Petri dishes and, after cell adherence, thymidine was added to a final concentration of 2 mM. The cells were cultured for 16 h. After the removal of the thymidine and incubation for 10 h in a fresh medium, thymidine was added to a final concentration of 2 mM for an additional 16 h. After removal of the thymidine, the synchronized cells were cultured in a fresh medium and collected at different times for cell cycle analysis and western blotting. The

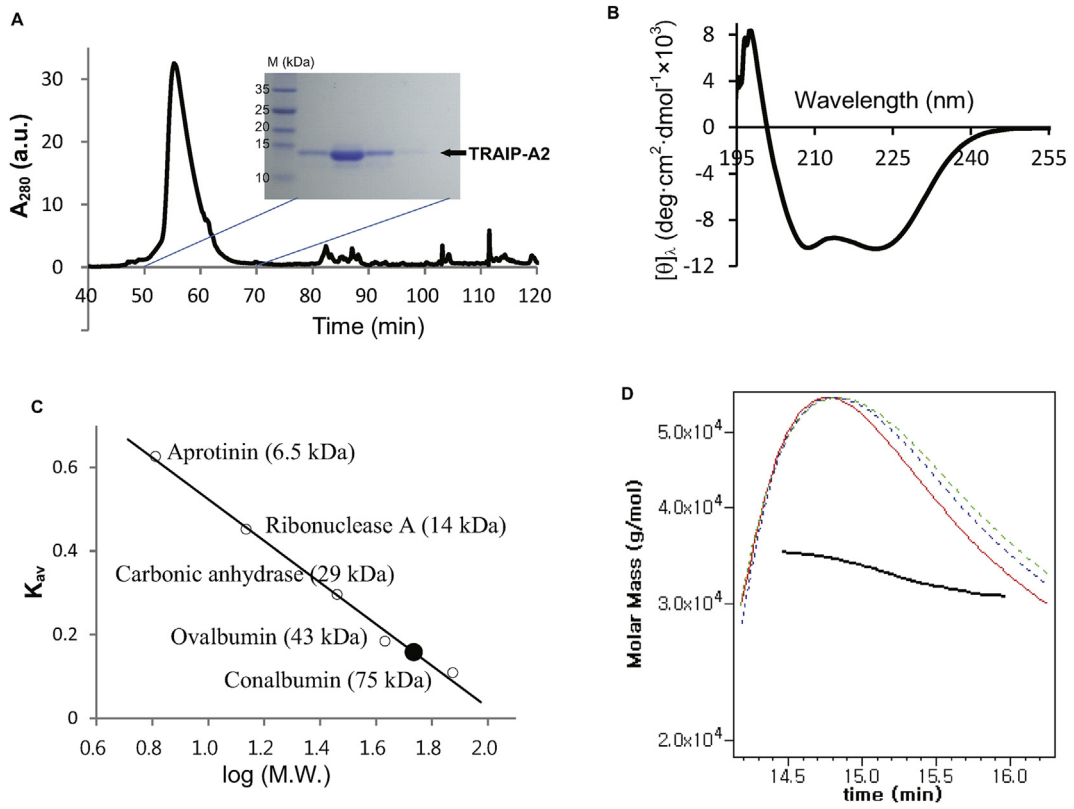

Fig. 3. In vitro verification of the coiled-coil dimerization. (A) Gel-filtration elution profile of the purified TRAIP-A2. Inset shows the SDS-PAGE results of the indicated fractions (M, molecular size marker). (B) Far-UV CD spectrum of the purified TRAIP-A2. (C) Hydrodynamic size estimation of TRAIP-A2, by gel-filtration analysis. Open circles indicate the protein standards used. Filled circle designates the gel-phase distribution coefficient (K_{av}) value of TRAIP-A2, of which the apparent molecular weight was calculated using a standard curve. (D) MALS analysis of the purified TRAIP-A2. The protein elution from the SEC column was detected by UV (green), MALS (red), and RI (refractive index; blue), which was fitted to calculate the molecular mass (black line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells were synchronized in the prometaphase with 17 h of nocodazole treatment, and then released into a fresh medium for further incubation.

2.6. Cell cycle analysis by flow cytometry

The double thymidine- or nocodazole-synchronized cells were collected at different times after its release from a G_1/S boundary. After washing twice with PBS, the cells were fixed with chilled 70% alcohol at 20 °C for 24 h. The fixed cells were collected by centrifugation at 2000 rpm for 5 min, washed twice with PBS, incubated with 30 mg/ml RNase A for 30 min at 37 °C, stained with 50 μg/ml propidium iodide (Sigma-Aldrich) for 30 min at room temperature, and then analyzed by flow cytometry.

2.7. Recombinant protein preparation

In order to generate the predicted CC domain as a recombinant protein (TRAIP-A2), the corresponding region (residues 65–182) was subcloned into the pGEX-4T1 vector, thereby yielding an N-terminally GST-tagged protein. The *Escherichia coli* BL21(DE3) pLysS cells, transformed with the constructed plasmids, were grown in a Luria–Bertani (LB) media at 37 °C, and the protein expression was induced by adding IPTG. The harvested cells were disrupted by sonication, and from the supernatant, the GST-tagged TRAIP-A2 was purified via a sequential application of GST-affinity (GSTrap FF column, GE Healthcare), ion-exchange (Q-FF, Q-HP column, GE Healthcare), and gel permeation (HiLoad 16/60 Superdax 75, GE Healthcare) chromatography. The tagged GST was then cleaved with thrombin, followed by the removal of a thrombin, GST, and other impurities via the sequential application of the aforementioned chromatography. The purified protein concentration was estimated by the typical Bradford and BCA assays.

2.8. Gel-filtration analysis

The purified TRAIP-A2 protein solution, concentrated at around 150 μM, was loaded (2 ml of injection volume) onto a HiLoad 16/60 Superdex 75 (GE Healthcare) column with a total bed volume of 120 ml, which was pre-equilibrated with a 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl. The protein was eluted at a flow rate of 1 ml/min, and detected by measuring the absorbance of the eluates at 280 nm. The hydrodynamic size was represented by the apparent molecular weight, which was deduced from the elution volume [13].

2.9. MALS (multi-angle light scattering) analysis

The purified protein solutions were loaded onto a size-exclusion chromatography (SEC) column (WTC-015S5, Wyatt Technologies), which was connected with a MALS detector (DAWN HELEOS-II, Wyatt Technologies) and a differential refractive-index detector (Optilab T-rEX, Wyatt Technologies). The column was pre-equilibrated with a 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl, at a flow rate of 0.5 ml/min. The weight averaged molar masses were calculated from the elution data, using ASTRA software (Wyatt Technologies).

2.10. Circular dichroism (CD) spectroscopy

A far-UV CD spectrum of the purified TRAIP-A2 protein (10 μM) was recorded in the 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM NaCl, at 20 °C, using a 0.4 cm path-length cell on a Jasco J-710 spectropolarimeter that was equipped with a temperature controller. Three individual scans were taken in the range of 260 nm–190 nm, with a 0.1 nm step resolution, a 1 nm bandwidth, and a 1 s response time; and then were summed and averaged, followed by the subtraction of the solvent CD signal. The CD intensity at a wavelength λ was normalized as the mean residue molar ellipticity, $[\theta]_\lambda$ [14].

3. Results and discussion

3.1. TRAIP forms the homo-dimer through its coiled-coil domain

Because the TRAIP contains a coiled-coil (CC) and leucine zipper (LZ) domain (Fig. 1A), the possibility was considered that the TRAIP might dimerize through those domains. To demonstrate the dimerization of TRAIP, we generated the differently tagged TRAIP-expression plasmids, SFB-TRAIP and Myc-TRAIP. Using these expression plasmids, an immunoprecipitation assay was performed. The Myc-TRAIP protein specifically associated (Fig. 1B and C) and colocalized (Fig. 1D) with the SFB-TRAIP protein in HEK 293T cells. Next, we tested the dimerization of endogenous TRAIP, by treating it with glutaraldehyde (a chemical commonly used to cross-link proteins) [15]. The dimer and tetramer species of the endogenous TRAIP were detected as resultants of the chemical cross-linking (Fig. 1E). To map the region(s) responsible for the dimerization of TRAIP, we performed an immunoprecipitation using Myc-TRAIP with/without the SFB-tagged wild type or serial deletion mutants (Fig. 2A). The D2 deletion mutant that didn't contain the CC domain completely lost its binding capability to the wild type (see Fig. 2B and C). In addition, the A2 mutant, which contains only the CC domain, bound to the wild type TRAIP (Fig. 2D). The D2 deletion mutant could not colocalize with the wild type TRAIP to the nuclear dots (Fig. 2E). As a whole, all these data indicated that the TRAIP formed the homo-dimer predominantly through its CC domain. Finally, the homo-dimerization of the predicted CC domain was validated in vitro, using its recombinant protein encompassing residues 65–182 (TRAIP-A2; 14.1 kDa). Consistent with an experimental hypothesis about a secondary structure on the CC domain (Fig. S1), the purified TRAIP-A2 protein (Fig. 3A) appeared to adopt a predominantly helical conformation, as its far-UV CD spectrum showed the obvious double minima at 208 and 222 nm (Fig. 3B). Although its apparent hydrodynamic size, which can be increased by both the oligomeric organization and elongated helical shape, was extensively large (approximately 54.3 kDa) in the gel-filtration analysis (Fig. 3C), the actual molecular weight (approximately 33 kDa) estimated by MALS analysis (Fig. 3D) indicated a dimeric behavior of the protein.

3.2. TRAIP dimerization regulates the mitotic progression

A recent study showed that the TRAIP protein is crucial in early mitotic progression and chromosome alignment defects in the metaphase [10]. It also showed an increased mitotic index in the

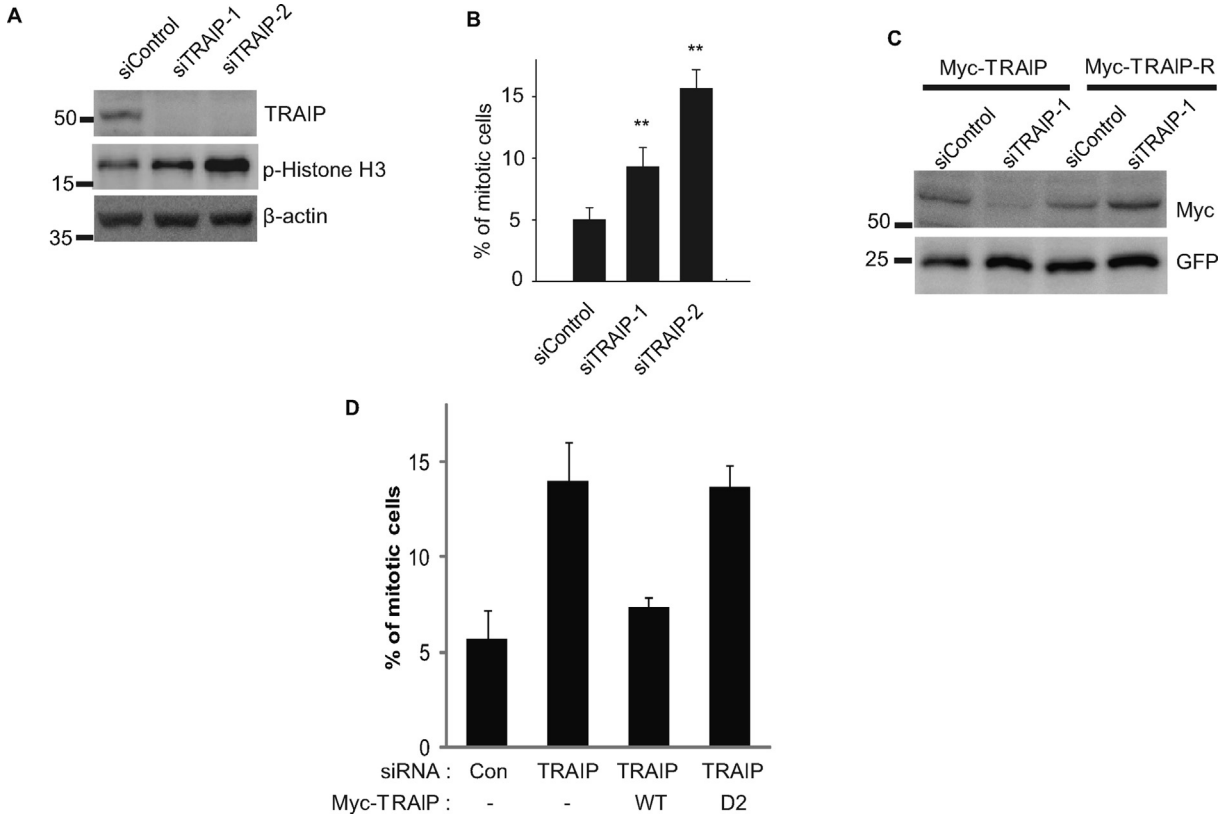

Fig. 4. TRAIP dimerization is important for the mitotic progression. (A) Seventy-2 h after siRNA transfection, the cells were immunoblotted with indicated antibodies. (B) The graph shows the percentage of the mitotic population. The results represent the average of the three independent experiments. Error bars, SEM. (C) Seventy-2 h after siRNA transfection, the cells were transfected with WT TRAIP (TRAIP-WT) or siRNA resistance (TRAIP-R) plasmids. The lysate from transfected cells were immunoblotted with the indicated antibodies. GFP was used to demonstrate the specific depletion of TRAIP. (D) The graph indicates the percentage of the mitotic population of cells transfected indicated with siRNA or expression plasmids. The results represent the average of the three independent experiments. Error bars, SEM.

TRAIP-knockdown cells by transfecting TRAIP siRNA. We also observed an increased mitotic index in the TRAIP-knockdown cells by transfecting TRAIP siRNA in this study (Fig. 4A and B). Hence, we tested the functions of the TRAIP dimerization in the mitotic index and mitotic progression. The overexpression of the siRNA-resistant wild type in the TRAIP-knockdown HeLa cells rescued the increased mitotic index, but the overexpression of siRNA-resistant D2 deletion mutant did not, as evaluated by the p-Histone H_3 staining (Fig. 4C and D).

Although it has been previously shown that the TRAIP is a regulator of mitotic progression through the spindle assembly checkpoint, the function of its dimerization in the mitotic progression has never been studied. The present data demonstrated that the TRAIP forms a homo-dimer. An immunoprecipitation assay and in vitro assay, using purified protein encompassing residues 65–182 of TRAIP, indicated the homo-dimerization of the TRAIP through its CC domain. Based on these results, it is reasonable to suggest that the homo-dimerization of the TRAIP through its CC domain could be a novel mechanism that functions in mitotic progression. However, the data in this study also showed that the TRAIP dimer form did not increase in the mitotic phase (Fig. S2). This suggests that the TRAIP dimer may have functions other than that in mitotic progression. The exact molecular mechanism of mitotic progression control by the TRAIP dimerization will be further studied. We conjecture that mutations of the CC domain that block dimerization may have a potential role in tumorigenesis to regulate the mitotic progression. Future studies will be done to elucidate the molecular mechanisms of the TRAIP dimerization-dependent regulation of mitotic progress.

Acknowledgments

We thank the staff at Korea Basic Science Research Institute (KBSI; Ochang, Korea) for assisting the CD and MALS operations. This work was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (HI11C1596) and National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2012R1A1A2038415).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.026.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.026.

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