Saudi Journal of Biological Sciences 27 (2020) 1562–1565

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Molecular cloning, expression, overproduction and characterization of human TRAIP Leucine zipper protein

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ARTICLE INFO

Article history:
Received 24 December 2019
Revised 1 March 2020
Accepted 5 March 2020
Available online 13 March 2020

Keywords:
TNF interacting protein
NF-κB
TRAF2
TNFR
Apoptosis
Proliferation

ABSTRACT

The TRAIP interacting protein is known as a negative regulator of TNF-induced-nuclear factor, kappa-light-chain-enhancer of activated B cell (NF-κB) by direct interaction with the adaptor protein TRAF2, which inhibits the function of TRAF2 via the RINGCC domain protein. The TRAIP protein is composed of 469 amino acids with an N-terminal RING motif that is followed by a coiled coil (CC) and leucine zipper domain. TRAIP proteins are critical in programmed cell death, cell proliferation and differentiation, and embryonic development. The critical functions of TRAIP together with the molecular inhibitory mechanism effect of TRAIP have been reported by two different studies and have opened up new research into the field of TRAF biology. In this study, we designed different constructs of the Leucine zipper domain to find the over –expressed construct for further studies. We successfully cloned the C-terminal TRAIP containing the leucine zipper domain. In addition, we have over-expressed and purified the TRAIP LZ for their biochemical characterization.

1. Introduction

The 53 KDa TRAF-interacting protein (TRAIP, TRIP, and RNF206) comprises an N-terminal RING finger, leucine zipper regions and coiled-coil that bind TRAF-family (Lee and Choi, 1997; Besse et al., 2007). The (TNF/TRAF--interacting protein (TRIP/TRAIP) from RING-type E3 ubiquitin ligase family undergoes autoubiquitination, however, in *in vivo*, its substrate(s) has not been identified yet (Besse et al., 2007). Historically, TRAIP has been reported by many authors to interact with TNF/TRAFs (Lee and Choi, 1997). TRAIP is expressed at low levels in most tissues (Chapard et al., 2012; Su et al., 2004), mainly found in the nucleolus of interphase mammalian cells (Zhou & Geahlen, 2009). It has been reported that NF-κB activation mediated by TRAF-2 is negatively regulated by a handful of binding partners including member of TRIP/TRAIP TRAF family associated with NF-κB activator (TANK/I-TRAF) proteins. Of all these binding partners, TRAIP plays main role in regulating the activation of NF-κB mediated by TRAF2. In Eukaryotics, several proteins are associated in regulation of cell cycle, thereby helping in producing daughter cell from parent cells (Karin and Gallagher, 2009). During S phase, DNA gets replicated and, during the final mitotic phase the chromosomes divide and shift toward poles, leads the division of parent cell into two daughter cells. Several modifications that regulate the progression of mitosis are controlled by, but not limited to ubiquitination, SUMOylation, and phosphorylation. (Wan et al., 2012; Hunt, 2013; Fournane et al., 2012; Kaseda et al., 2008). TRAIP along with TRAF signaling complex, plays an important role in TRAF2 mediated NF-kB activation (Boisvert et al., 2007; Karin and Gallagher, 2009). TRAF domain exhibits mushroom like trimeirc structure in solution (Kim et al., 2016). Recently, it was seen that an unknown protein shows ubiquitination when the BEN (ubiquitin –conjugating enzyme E2 N) interacts with the NOPO complex, and this mechanisms of association between proteins depicts genomic integrity in pre-mature

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.03.011
1319-562X/

embryo of drosophila (Wallace et al., 2014). NOPO (No poles) contains RING dependent E3 ubiquitin that is necessary for early embryogenesis. Furthermore, embryonic lethality occurs in TRAIP-deficient mice (Brummelkamp et al., 2003). In addition, the time of early mitosis progression was decreased by RNA interference (RNAi) in HeLa cells by from the NEB to the anaphase onset and in the metaphase, the percentage of chromosome alignment defects was increased (Cheng and Baltimore, 1996). The current understanding related to recognition of TRAIP with TRAFs and associated diseases marks the TRAIP critical signaling molecule (Nasreena et al., 2019), and is tangled in different pathways related with signaling likewise RAP80 signaling pathway, DNA damage response, mitosis or cell cycle process, SyK-binding partner and also inhuman diseases (Bhat and Rather, 2018). Recently, the inhibitory molecular mechanism effect of TRAIP has been reported, the study showed that a TRAIP RINGCC hijacks the dimeric TRAF2CC domain. Moreover, it showed that a high oligomerized RING domain was required for the interface of the TRAF2CC domain as reported by Bhat et al. (2018). Here, we successfully cloned the human TRAIP protein containing the leucine zipper domain. Further, it was overexpressed and purified in two rapid steps by exploring Size exclusion chromatography (SEC) and Ni-NTA affinity chromatography. The main peak of TRAIP Leucine zipper domain eluted at 16 mL, which suggests that it forms the trimer, and was highly homogeneous as seen by SDS-PAGE, which was further confirmed by a Multi-Angle Light Scattering (MALS). The purity or homogeneity of protein was analyzed by SDS-PAGE.

2. Material and methods

2.1. TRAIP cloning, expression and purification

For polymerase chain reaction (PCR), cDNA of Human TRAIP (1–280 amino acids) was used as a template. NdeI and XhoI restriction enzymes (Enzynomics) were used to digest the PCR product. The pET24a plasmid was digested with the same enzymes. The generated construct leucine zipper corresponding amino acid 198–280, were then sub-cloned using plasmid vector pET24a purchased from Novagen (Daegu, South Korea) with C-terminal His-tag His6. The plasmids were then transformed into BL21 cell (DE3) competent cells and each clone from the generated constructs was speckled on Luria-Bertani (LB) agar plates encompassing the applicable antibiotics (50 μl/mL), then the plates were incubated for 17 h at 37 °C. Single colonies from different constructs were picked with micro-loop and inoculated in 5 mL of LB medium, incubated overnight at 37 °C in shaking incubator. A 1000 mL of LB media in 2000 mL flask was inoculated with 5 mL of overnight culture, and was incubated in shaking incubator at 37 °C for 4 hrs. Further, the optical density (O.D) was checked at 600 nm, after the OD reached between 0.6 and 0.7 nm, overexpression was induced with 0.25 mM isopropyl-β-D thiogalactopyranoside (IPTG) treatment at 20 °C overnight. Each protein expressing bacteria was pelleted by centrifuging at 10,000 × g, re-suspended and sonicated in 50 mL of lysis buffer which was supplied with phenylmethanesulfonyl fluoride (PMSF). Next, centrifugation of all lysates was done at 4 °C for 30 min at 12,600 × g After centrifugation, the supernatant collected were divided into small fractions, and each fraction was subjected to separation using gravity column, the column was packed with Ni-NTA affinity resin. Furthermore, 60 mL of washing buffer was used to remove the non-specific proteins and subsequently the proteins of target were eluted with elution buffer. A 4* 0.5 mL eluted fractions being collected over a total of 2 mL. After checking on SDS-PAGE gel, fractions containing more than 90% homogeneous TRAIP Leucine zipper domain protein were collected and combined. Each sample were loaded onto a size exclusion chromatography column HR 10/30 of superdex 200 that was pre-equilibrated with a solution of 20 mM Tris-HCl at pH8.0 and 150 mM NaCl in the final purification step. Fractions containing the TRAIP Leucine zipper domain protein were then pooled and stored at 4 °C for crystallization. SDS-PAGE gel determined the homogeneity of the TRAIPLZ protein domain.

2.2. MALS

The protein TRAIP Leucine zipper corresponding amino acid 198–280 domain was used to determine the absolute molecular mass by multi angle light scattering (MALS). Target proteins were purified by Ni- affinity chromatography using SEC column HR

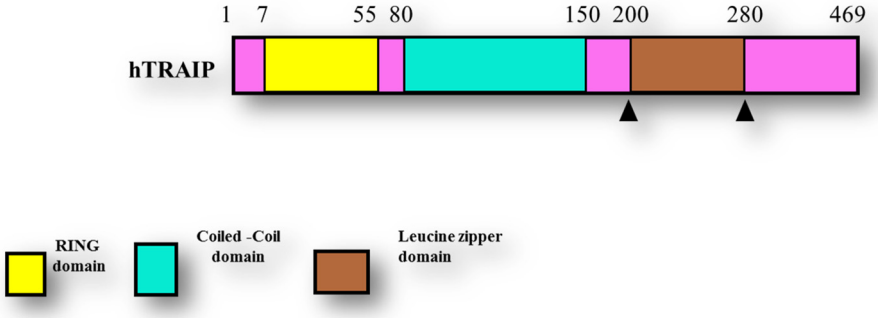

Fig. 1. TRAF-interacting protein (TRAIP). The domain boundary of TRAIP. The number of amino acids of TRAIP RING, TRAIP CC and TRAIP Leucine zipper shown.

Table 1
Different constructs of TRIP leucine zipper protein.

Name (LZ domain)	Species	Region	Amino acid	DNA	Enzyme	Vector	PCR	Cloning	Expression
TRAIP-1	Human	186(D)-280(L)	95 a.a	285 bp	NdeI/XhoI	pET24a	Successful	Successful	Expression
TRAIP-2	Human	186(D)-276(E)	91a.a	273 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression
TRAIP-3	Human	186(D)-272(I)	87a.a	261 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression
TRAIP-4	Human	190(G)-280(L)	91a.a	170 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression
TRAIP-5	Human	193(A)-280(L)	88a.a	261 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression
TRAIP-6	Human	196(Q)-280(L)	85a.a	252 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression
TRAIP-7	Human	198(A)-280(L)	83a.a	246 bp	NdeI/XhoI	pET24a	Successful	Successful	Over-expression
TRAIP-8	Human	203(S)-280(L)	78a.a	231 bp	NdeI/XhoI	pET24a	Successful	Successful	No expression

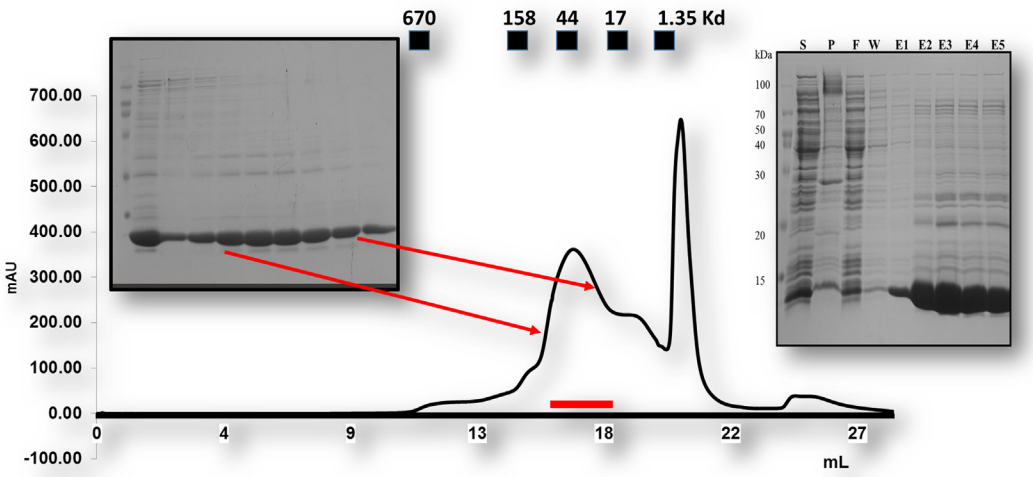

Fig. 2. Gel filtration chromatogram. His tag and Gel filtration chromatography of TRAIP Leucine zipper (198–280 a.a) domain with SDS-PAGE shows both Ni—affinity and fractions of Size exclusion chromatography Gels. the S# supernatant, P# pellet, F# flow through, W# wash and the red arrows shows main peak fractions on SDS-PAGE.

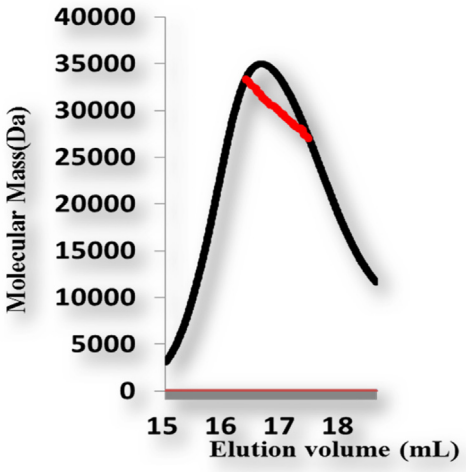

Fig. 3. MALS result of TRAIP Leucine zipper domain (198–280 a.a) with 2% fitting error.

10/30. The main peak fractions of the leucine zipper domain protein corresponding amino acid 198–280 was collected. Subsequently, each sample was centrifuged (10,000 × g), at 4 °C for 10 min to remove the precipitate before loading on size exclusion chromatography column HR 10/30 (bed dimensions 10* 300 mm) which was pre-equilibrated with solution containing 20 mM Tris-HCl at pH8.0 and 150 mM NaCl. In addition, the system was tied with three-angle light scattering refractive index detectors. After every 0.5 s, the date collected was analyzed by ASTRA program, suggesting molar mass plus mass distribution of each sample.

3. Results and discussion

The TRAIP (53 kDa) consists of 469 amino acids with N-terminal RING motif followed that coiled coil (CC) and leucine zipper (LZ) domain (Fig. 1). A human homologous TRAIP present in mice contained 470 amino acids and showed 76% sequence similarity. The N-terminal RING domain of human TRAIP possesses the E3 ubiquitin ligase activity. The C-terminal part of TRAIP (residues 211–470) has been involved to form a complex with CYLD and prevented the inhibitory activity of TRAIP (Regamey et al., 2003). For the *in vitro* biochemical characterization of TRAIP leucine, different constructs of TRAIP Leucine zipper domain were designed to find the best expression protein (Table 1). Among the constructs, only one construct was over- expressed which was used further in this study for biochemical characterization. The construct of the leucine zipper at the C-terminal end was eluted from rapid two-step chromatography, the affinity chromatography followed by the size exclusion chromatography. The sample from the Ni-affinity chromatography and gel filtration chromatography was pooled and analyzed by SDS-PAGE and the results are shown in (Fig. 2). In the figure, it is clear that the TRAIP Leucine zipper domain corresponding amino acid [(198–280) (from lane E1-E5)] migrates to a position in the gel close to the 15 kDa band with a calculated molecular weight with His-tag (HHHHHH) of 10,561 Da. Furthermore, the stoichiometry changes were analyzed by MALS. The calculated molecular weight of the monomeric TRAIP-leucine zipper domain of TRAIP including the C-terminal his-tag were 10,561 Da, and the experimental molecular weights from MALS were 29,530 Da (2% fitting error), with a polydispersity of 1.04 for the TRAIP LZ domain (Fig. 3). Based on SEC and MALS results, TRAIP Leucine zipper is a trimer in solution. Further studies for understanding the *in vivo* molecular inhibitory mechanism will provide the answers to the unanswered questions in the field of TRAF-mediated biology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. (DF-700-130-1441). The authors, therefore, gratefully acknowledge DSR technical and financial support.

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