In vitro assembly complex formation of TRAIP CC and RAP 80 zinc finger motif revealed by our study

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ABSTRACT

Background: Tumor necrosis factor interacting protein (TRAIP/TRIP) is an important cell-signaling molecule that prevents the TNF-induced-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation via direct interaction with TRAF 2 protein. TRAIP is a crucial downstream signaling molecule, implicated in several signaling pathways. Due to these multifunctional effects, TRAIP is more related to cellular mitosis, chromosome segregation, and DNA damage response. Tumor necrosis factor interacting protein is a downstream signaling molecule that contains a RING domain with E3 ubiquitin ligase activity at the N terminal side followed by coiled-coil and C terminal leucine zipper domain. Human TRAIP is constituted of 469 amino acids with 76% sequence similarity with the mouse TRAIP protein. Although, the main inhibitory function of TRAIP has been known for decades, however, in vitro interaction of TRAIPCC domain with RAP80 Zinc finger motif has not been reported yet. Besides, RAP80, the binding partner of TRAIPCC protein has been implicated in DNA damage response.

Results: Our in vitro study shows that the TRAIP CC (64–166) associates with the RAP80 zinc finger of corresponding amino acid 490–584. However, TRAIP CCLZ (66–260) and TRAIP RINGCC (1 = 157) failed to interact with the RAP80 zinc finger of corresponding amino acid 490–584. The current study reinforces TRAIP CC (64–166) and RAP80 zinc finger of corresponding amino acid 490–584 associates to form a complex. Moreover, SDS PAGE arbitrated the homogeneity of RAP80 Zinc finger and TRAIP CC of corresponding amino acid 490–584 and 64–166, respectively.

Conclusion: In vitro, a specific interaction was observed between the TRAIP CC (64–166) and the RAP80 zinc finger of corresponding amino acid 490–584 and a specific binding area of the RAP80 zinc finger motif were investigated. The TRAIPCC region is required for the complex to bind to the RAP80-Zn finger motif. This strategy may be necessary for the RAP80 zinc finger activity to the TRAIP CC protein.

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many other important signaling pathways (Bhat et al., 2018). The negative effect of TRAIP in TNF- induced NF-κB activation was introduced a few decades ago (Jackson and Bartek, 2009).

RAP80 (receptor-associated protein 80), a ubiquitin-binding protein of 719 amino acids, has two tandem ubiquitin-interacting domains that preferentially detect and bind to Lys-63-linked polyubiquitin chains, allowing the BRCA1-1 complex to reach regions of DNA damage (Besse et al., 2007; Kim et al., 2017). RAP80 localizes to sites of DNA damage to induce the DNA-damage response (DDR) (Besse et al., 2007). RAP80 plays a crucial role in maintaining genomic stability and tumor suppression (Kim et al., 2017; Kim et al., 2007). In DDR pathways, protein-protein interaction and post-translational modification have a key role in it (Besse et al., 2007; Lee et al., 2016). Ubiquitin-interacting motif (UIM) of RAP80 specifically recognizes Lys 63-linked histone ubiquitination, H2A, and H2AX, at sites of DNA damage. The translocation of the BRCA1-A complex to DNA-damage sites has been shown to regulate the G2/M checkpoint and DNA-damage repair and is required for cell survival (Lee et al., 2016; Lee and Choi, 1997; Nasreena et al., 2019). In our present study, different constructs of TRAIIPCC, TRAIP CCLZ, TRAIP RINGCC and RAP80 Zn finger were designed to identify the best overexpression protein. An overexpressed and well-purified construct of TRAIIPCC with corresponding amino acid 66–164 was used for in vitro study. Similarly, the well overexpressed and purified construct of the RAP80 zinc finger motif with corresponding amino acid 490–584 was used for complex association formation. Our in vitro study shows that the TRAIIPCC domain is critical for interaction with the RAP80 zinc finger motif and forms a complex with RAP80 Zinc finger motif. However, TRAIP RINGCC and TRAIP CCLZ of corresponding amino acid 1–157 and 66–260 failed to interact with the RAP80 zinc finger of corresponding amino acid 490–584 respectively. The purity and homogeneity of proteins were analyzed by SDS-PAGE gel. Furthermore, Size-exclusion chromatography confirmed the complex formation.

2. Results

2.1. TRAIP CC (66–164aa) and RAP 80 zinc finger (490–584 aa) forms a single homogenous trimeric peak

The TRAIP (53 kDa) consists of 469 amino acids with an N-terminal RING motif followed by coiled-coil (CC) and leucine zipper (LZ) domain (Fig. 1A). The RING domain is known to possess E3 ubiquitin ligase activity (Bartek et al., 2007; O’Driscoll and Jeggo, 2006; Reganey et al., 2003). The corresponding amino acids 211–470 of TRAIP has been involved in a complex with CYLD and prevented the inhibitory activity of TRAIP (Su, 2006; Thompson and Schild, 2002). The RAP80 protein constituted 719 amino acids with the N-terminal UIM domain followed that two Zinc finger motif at the C-terminal end (Fig. 1B). RAP80 is a key signaling molecule in the DNA damage response (Wallace et al., 2014). C-terminal of RAP80 possesses a Zinc finger motif that has been known to interact with TRAIP CC protein (Besse et al., 2007; Wang et al., 2007).

For biochemical studies in vitro, we produced the overexpression construct of each protein viz, TRAIP CC (66–164 aa), TRAIP RINGCC (1–517), TRAIP CCLZ (60–280 aa) and RAP 80 (490–584 aa). Each of one protein was overexpressed and purified by Ni affinity followed by size exclusion chromatography. The size exclusion chromatography showed that the TRAIP CC domain of corresponding amino acid 66–164 was eluted at approximately 17 ml (Fig. 2). The gel filtration chromatography of the TRAIP RINGCC was eluted between 9 and 18 ml which shows RING mediated oligomerization of CC domain (Fig. 3). The gel filtration chromatography of the CCLZ domain was eluted at 17 ml (Fig. 4). The size exclusion chromatography of RAP 80 zinc-finger of corresponding amino acid 490–584 showed that it was eluted at 17 ml which suggests the trimeric in solution (Fig. 5).

To analyze the stoichiometry of TRAIP CC of corresponding amino acid 66–164 in solution by calculating absolute molecular mass, we performed analysis through MALS. The C terminal containing Hexa His tag of TRAIP CC of corresponding amino acid 66–164 with calculated molecular weight 12325 Da, and the major molecular weight 34,254 Da (0.9% fitting error) based on our MALS result, with a polydispersity of 1.012 as shown in (Fig. 6). The results of size exclusion chromatography and MALS showed TRAIP CC is a trimer in solution.

To investigate the complex assembly of TRAIP CC (66–164 aa), TRAIP RINGCC and TRAIP CCLZ (60–280 aa) with RAP 80 zinc finger (490–584 aa) using structural and biochemical assays in vitro, we designed different constructs of TRAIP CC domain shows in Table 1, which is known to interact with the RAP 80 containing zinc finger domain (490–584 aa). TRAIP RINGCC as shown in Table 2 and TRAIP CCLZ as shown in Table 3 has also designed different constructs to find the best-overexpressed construct. Similarly, different constructs of RAP80 Zn finger motif was designed as shown in Table 4. The gel chromatography profile revealed that TRAIP CC (66–164 aa) domain eluted at 17 ml, indicating the formation

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**Fig. 1.** Schematics of TRAIP and RAP80. (A) The domain boundary of TRAIP with the number of amino acids from TRAIP RING, TRAIP CC, TRAIP Leucine zipper. (B) The domain boundary of RAP80 with the N-terminal end contains UIM motif and C-terminal side ZF motif shown.

**Fig. 2.** Gel filtration chromatogram. His tag and gel filtration chromatography of TRAIIPCC (66–164 aa) domain. The SDS-PAGE judged purity of both Ni-affinity and purified fractions of gel filtration chromatography. M# marker, S# supernatant, P# pellet, F# flow through, W# wash and E1-E6 (Elution).
of a trimer as reported previously in a different construct of protein by (Bhat et al., 2018). This is in accordance with previous findings. To confirm these results we performed size exclusion chromatography. The mixtures of different combination did not produce complex peaks (Figs. 7 and 8). Only the mixture of TRAIP CC of corresponding amino acid 66–164 and RAP 80 zinc-finger of corresponding amino acid 490–584 co-migrated in SDS PAGE (Fig. 9). This result showed consistency with our previous results, revealing that TRAIP CC (66–164 aa) specifically binds with RAP 80 zinc finger (490–584 aa) in vitro.

3. Discussion

Human TRAIP is constituted of 469 amino acids with putative domains, include an N-terminal RING domain followed by coiled-coil (CC) and leucine zipper (LZ) domains (Almeida et al., 2011; Bartek et al., 2007). The RING domain has been detected in many E3 ligases and is critical for the activity of ubiquitin ligation. TRAIP protein has E3 ubiquitin ligase activity to TANK-binding kinase 1 has been shown (Wu et al., 2012). TRAIP with corresponding residues 211–470 at the C-terminal known to be interacted directly with CYLD and enhanced the inhibitory activity of TRAIP. TRAIP marks a crucial signaling molecule, implicated with several signaling pathways (Bhat et al., 2018).

A ubiquitin-binding protein, RAP80 (receptor-associated protein 80) is constituted of 719 amino acid is a novel binding partner of Human TRAIPCC proteins (Besse et al., 2007), contains two tandem ubiquitin-interacting motifs that specifically recognize and bind to Lys-63-linked polyubiquitin chains, allows BRCA1-1 complex to sites of DNA damage (Besse et al., 2007; Kim et al., 2017). RAP80 localizes to sites of DNA damage to induce the DNA-damage response (DDR) and plays a crucial role in maintaining genomic stability and tumor suppression (Besse et al., 2007; Kim
During normal cellular processes, such as replication, it is possible as a result DNA lesions occur or prone to various environmental hazards such as ionizing radiation and ultraviolet light (Yan and Jetten, 2008). To prevent the accumulation of DNA insults and genomic integrity or damaged genetic transmission from mother to daughter cells, DNA damage repair mechanisms with sophisticated cell cycle checkpoint pathways are developed (Yan et al., 2007; Yin et al., 2012). The DNA double-strand break (DSB), most deleterious type of DNA damage among various kinds of DNA insults, and subsequently alters genomic stability and induces tumorigenesis. DNA damage response factors (DDR) are frequently recruited at DNA damage and activates cell cycle checkpoints and DNA repair mechanisms (Yoon et al., 2014). Subsequently, genomic instability and tumorigenesis are caused by the loss of these DNA damage response factors (Zhang et al., 2012; Zhang et al., 2012). Large multi subunit protein complexes are often formed by these DNA damage response factors.

Table 1
Different constructs of TRAIP coiled-coil domain protein.

| Name  | Species | Region | Amino acid | DNA | Enzyme | Vector | PCR | Cloning | Expression |
|-------|---------|--------|------------|-----|--------|--------|-----|---------|------------|
| TRAIP-1 | Human 66(L)-164(K) | 99 a.a | 297 bp | NdeI/XhoI | pET24a | successful | successful | over expressed |
| TRAIP-2 | Human 72(N)-167(E) | 96 a.a | 288 bp | NdeI/XhoI | pET24a | successful | successful | expressed |

Table 2
Different constructs of TRAIP RINGCC domain protein.

| Name  | Species | Region | Amino acid | DNA | Enzyme | Vector | PCR | Cloning | Expression |
|-------|---------|--------|------------|-----|--------|--------|-----|---------|------------|
| TRAIP-3 | Human 1(M)-150(K) | 150 a.a | 450 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | No expression |
| TRAIP-4 | Human 1(M)-154(E) | 154 a.a | 462 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | No expression |
| TRAIP-5 | Human 1(M)-157(R) | 157 a.a | 471 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | Over Expression |
| TRAIP-6 | Human 1(M)-162(K) | 162 a.a | 486 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | No expression |

Table 3
Different constructs of TRAIP coiled-coil Leucine Zipper domain protein.

| Name  | Species | Region | Amino acid | DNA | Enzyme | Vector | PCR | Cloning | Expression |
|-------|---------|--------|------------|-----|--------|--------|-----|---------|------------|
| TRAIP-7 | Human 66(L)-280(L) | 215a.a | 645 bp | NdeI/XhoI | Pold | successful | successful | over expressed |
| TRAIP-8 | Human 66(L)-276(E) | 211a.a | 633 bp | NdeI/XhoI | pET24a | successful | successful | expressed |
| TRAIP-9 | Human 66(L)-272(T) | 207a.a | 621 bp | NdeI/XhoI | Pold | successful | successful | expressed |

Table 4
Different constructs of RAP80 Zinc finger motif.

| Name  | Species | Region | Amino acid | DNA | Enzyme | Vector | PCR | Cloning | Expression |
|-------|---------|--------|------------|-----|--------|--------|-----|---------|------------|
| RAP80-1 | Human 490(K)-584(Q) | 95a.a | 285 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | over expressed |
| RAP80-2 | Human 490(K)-590(Q) | 101a.a | 303 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | expression |
| RAP80-3 | Human 490(T)-584(Q) | 89a.a | 267 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | expression |
| RAP80-4 | Human 490(T)-584(Q) | 95a.a | 285 bp | NdeI/XhoI | pET24a/pOKD | successful | successful | expression |

Fig. 7. Gel filtration chromatogram. Size-exclusion chromatography (SEC) profiles of TRAIP RINGCC and RAP80 Zn finger in vitro. TRAIP RINGCC domain did not interact with the RAP80Zn finger in vitro.

Fig. 8. Gel filtration chromatogram. Size-exclusion chromatography (SEC) profiles of TRAIP CCLZ and RAP80 Zn finger in vitro. TRAIPCCLZ domain failed to interact with the RAP80Zn finger in vitro.
Consequently, the plates were incubated at 37 °C for 20 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 a solution containing 20 mM Tris-HCl at pH 8 and 150 mM NaCl. Moreover, the system was connected with three-angle light scattering refractive index detector and mini-DAWN treos MLAS detector (Wyatt Technology, Santa Barbara, CA, USA). After every 0.5 s, the data collected was analyzed by the ASTRA program, suggesting molar mass plus mass distribution of each sample.

4.2. MALs

The protein TRAIP CC corresponding amino acid 66–164 was used to assess the absolute molecular mass by multi-angle light scattering (MALS). TRAIPCC was purified by two rapid steps, Ni-affinity chromatography, and size exclusion chromatography column HR 10/30. The main peak fractions of the purified TRAIP CC were collected. Centrifugation (14000 rpm) was done at 4 °C for 10 min before loading on size exclusion chromatography column HR 10/30 (bed dimensions 10*300 mm), which was pre-equilibrated with a solution containing 20 mM Tris-HCl at pH 8 and 150 mM NaCl. The protein mixture was passed through a Superdex 200 gel filtration column HR 10/30 (GE health care) which was pre-equilibrated with a solution containing 20 mM Tris-HCl at pH8.0 and 150mMNaCl [31].

5. Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

6. Availability of data and materials

The datasets used in the current study are available from publically.

Declaration

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.083.

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