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Deletion Mutagenesis of TAK1: Role of TAB1 as an Activator

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Abstract: **TGF-β Activated kinase 1, a serine threonine kinase, was first discovered as a member of the MAPK kinase kinase (MAP3K) family, named as MAP3K7, and is activated by TGF-β1. TAK1 is an extensively expressed kinase, which, as the name implies, was originally spotted as a TGF*β*-activated enzyme. In addition to TGF-β1, TAK1 can be activated by various other stimuli encompassing lipopolysaccharides, pro-inflammatory cytokines like interleukin (IL)-1, Tumor Necrosis Factor (TNF)-α and environmental stress. TAK1 is an intracellular hub molecule that regulates a wide array of cellular processes encompassing apoptosis, embryonic development, cellular differentiation and cell survival. Apart from these processes TAK1 plays a very important role in regulating the mTOR pathway, which is a central pathway in the regulation of cellular proliferation and autophagy. Activation of TAK1 requires its binding with certain binding partners, called as the TAK1 binding proteins. The binding of these proteins to TAK1 is stimuli dependent. We successfully generated various truncation mutants of TAK1 in mammalian expression system and assessed their activity in presence and absence of TAB1 (TAK 1 binding partner) using MKK6 as a substrate. We report that TAB1 is indispensable for the activity of TAK1.**

Keywords: **apoptosis, autophagy, kinase, lipopolysaccharides, pro-inflammatory.**

I. INTRODUCTION

Transforming growth factor β (TGF-β) activated kinase 1 (TAK1), a serine threonine kinase also known as MAP3K7 (mitogen activated protein kinase kinase kinase) is a key signaling molecule activated in response to a number of stimuli involving pro-inflammatory cytokines like TNF-α, IL-1, lipotoxic molecules like ceramide and ligands of Toll-like receptors, T-cell receptors and B-cell receptors [1], [2], [3], [4], [5], [6]. TAK-1 regulates a wide array of cellular processes encompassing apoptosis, inflammation, embryonic development and innate immunity. TAK-1 also serves a role in the suppression of tumor development in liver and prostate tissues [7], [8]. Distortion of TAK-1 signaling in mice results in the disruption of tissue homeostasis and evokes tissue damage [9]. TAK1 regulates a diverse number of cellular processes encompassing cell survival, differentiation and inflammation [10]. TAK-1 has recently been found to be a central molecule in the regulation of autophagy as indicated by the increased conversion of LC3BI to LC3BII [11], [12], [13]. In addition to autophagy TAK-1 has also been shown to be involved in apoptosis [7].

TAK1, as a member of MAP3K family behaves differently in that its activity requires the binding of certain binding partners known as the TAK1 binding proteins. These proteins include TAK1 binding protein 1, TAK1 binding protein 2, and TAK1 binding protein 3 (TAB1, TAB2, and TAB3). These proteins bind at specific sites within the TAK1 sequence. TAK1 requires the constitutive binding of TAB1, as the binding of TAB1 mediates the autophosphorylation of TAK1, which is crucial for its activation. Among the TAK1 binding proteins TAB2 and TAB3 share 48% of amino acid sequence homology, however TAB1 is structurally distinct from TAB2 and TAB3. TAB1 binds to the N-terminal catalytic domain of TAK1, however, the binding sites of TAB2 and TAB3 span through the C-terminus of TAK1. TAB1 (TAK1 binding protein 1) is one of the regulatory subunits of TAK1. The formation of the functional complex of TAK1 requires its interaction with three binding partners TAB1, TAB2 and TAB3. TAB2 and TAB3 function redundantly in TAK1 mediated signaling. The formation of TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3 complex is mandatory for the

occurrence of TAK-1 autophosphorylations and hence these proteins are important in activation of TAK-1. The TAK1 binding domain present at the carboxy terminus of TAB's is responsible for binding to TAK-1 [14], [15], [16], [17]. The TAK1 binding domain (TAK-1 BD) is composed of α-helices that are important in mediating its interaction with TAK1. The TAK1 binding domain in different TAB's shows a little sequence homology. TAB1 constitutively binds to the amino terminus of TAK1 where as the binding of TAB2 and TAB3 to the carboxy terminus of TAK1 is context dependent. The amino acid residues 480–495, located at the carboxy terminus of TAB1, play an indispensable role in its binding to TAK1 [18], [19]. The TAK1 binding region of TAB1 is also located within the carboxy terminal 68 amino acid residues [19], [20], [21].

II. MATERIALS AND METHODS

A. *Cloning*

pDNR-Dual containing human TAK-1 (Plasmid Harvard Repository) was used as a template to amplify TAK-1 and its various truncation mutants for cloning in pEBG, a mammalian expression vector. The following set of primers was used to amplify the gene.

Full length TAK1 (1-579), FL TAK1

Forward primer: 5' GA**GGATCC**ATGTCTACAGCCTCTGCC 3'

Reverse primer: 5' GC**GGTACC**TGAAGTGCCTTGTCGTT 3'

Amino terminal truncation of TAK1 △(1-30) TAK1, △NH TAK1

Forward primer: 5'GCTG**GGATCC**ATGATCGACTACAAGAAGGAGAT CGAG 3'

Reverse primer: 5'GCTG**GGTACC**CTACCATGAAGTGCCTTGTCGTT3'

Carboxy terminal truncation of TAK1 △(306-579) TAK1, △CT TAK1

Forward primer: 5'GCTG**GGATCC**ATGTCTACAGCCTCTGCCGCC 3'

Reverse primer: 5'GCTC**GGTACC**CTAATCTGAATACTGACAAGG 3'

Truncation of both the amino and carboxy terminal regions of TAK1 △(1-30)/△(306-579) TAK1, △NHCT TAK1

Forward primer: 5'GCTG**GGATCC**ATGATCGACTACAAGAAGGAGAT CGAG 3'

Reverse primer: 5'GCTC**GGTACC**CTAATCTGAATACTGACAAGG 3'

pENTR223 containing human TAB1 was purchased from plasmid harvard repository and was used as a template to amplify TAB1 for cloning in pKMYC, a mammalian expression vector. The following set of primers was used to amplify the gene.

Forward primer: 5' GTTA**GGATCC**ATGGCGGCGCAGAGGAGC 3'

Reverse primer: 5' GCTG**GAATTC**CTACGGTGCTGTCACCACGCTCTG 3'

MKK6 cloned in pCDNA3 was ordered from Addgene and it was used as a template to amplify MKK6 for cloning in pGEX-4T2, a bacterial expression vector. The following set of primers was used to amplify MKK6.

Forward primer: 5' GGC**GTCGAC**ATGGACTATAAGGACGA 3'

Reverse primer: 5' TAT**GCGGCCGC** TTAGTCTCCAAGAATCA 3'

B. *Cell culture*

HEK-293T cells were cultured in Dulbecco's modified Eagles medium (sigma) containing 10% (v/v) fetal bovine serum (sigma) with 50µg/ml penicillin and 100µg/ml streptomycin (sigma). The cells were grown in a humidified incubator with 5% CO_2, 95% air at 37^oC. The cells were passaged routinely by trypsinization.

C. *Transfections*

Transfections were carried out using PEI (Polyethylene Amine) as the transfection reagent. HEK-293T cells were grown to 100% confluency in T75 flasks. The cells were then trypsinised and seeded in 6cm plates. The cells were grown overnight to 70% confluency prior to transfection in a humidified incubator (5% CO_2, 95% air and 37^oC). Transfection mixture was prepared by adding 3µg of DNA to 400µL of serum and antibiotic free media. The mixture was allowed to stand for 5 minutes at room temperature. 15µL of PEI (1µg/mL) was added to the mixture and the mixture allowed to

stand for another 20 minutes at room temperature. Fresh and complete media was added to the cells. After changing the media of the cells, the transfection mixture was added and the cells were again placed in the humidified incubator overnight. After overnight incubation with the transfection mixture, the media was aspirated from the cells and fresh and complete was added again to let the cells grow further.

D. *Immuno-precipitation*

After 48 hours of transfection, cells were harvested in ice-cold PBS at 5000rpm for 5 minutes at 4^{o}C. The cells were then lysed on ice in a buffer containing 50mM Tris-Cl (pH 7.5), 10mM MgCl2, 5mM EDTA, 2mM DTT, 50mM β-Glycero-phosphate, 0.5% Triton X-100 and Protease inhibitor cocktail (Sigma). The cells were resuspended in the lysis buffer and incubated on ice for 30 minutes. After incubation the cells were centrifuged at 14000rpm for 30 minutes to clear the cell lysates. The lysates were then incubated with anti-GST antibody immobilised on protein G-Agarose beads (Genscript) overnight on a 360^{o} rotor at 8rpm. The beads were then washed thrice with the lysis buffer containing 500mM NaCl. A final wash with kinase buffer was given to remove the salt.

E. *Western blotting*

Proteins run on SDS-PAGE were transferred to PVDF membrane using wet blotting procedure. The transfer was performed at a constant voltage of 70V for 90 minutes. After transfer the membrane was blocked in 5% non-fat dry milk in 1X PBS overnight. The membrane was then washed thrice in 1X PBST (PBS with 0.05% Tween 20) for 10 minutes each. After washing, primary antibody in the ratio of 1:1000 in 1% BSA was added to the blot. The primary antibodies used include rabbit anti TAK1 (Cell Signaling Technology), mouse anti GST (Sigma), rabbit anti actin (Sigma). The blot was then incubated overnight with primary antibody at 4^{o}C on a rocking plate. The blot was again washed thrice in 1X PBST for 10 minutes each. It was followed by the addition of secondary antibody, 1:10,000 dilution of Infrared dye conjugated goat anti-rabbit secondary antibody (800CW) or 1:10000 dilution of Infrared dye conjugated goat anti-mouse secondary antibody (680CW). The blot was then incubated with the secondary antibody for 1 hour at room temperature on the rocking plate. The blot was then washed thrice with PBST. The antibody binding was then visualized by imaging on LICOR ODYSSEY infrared system. For reprobing, the blots were first stripped in the stripping buffer (10 mM glycine, 2% SDS, pH 2.0) at room temperature with constant agitation. Secondary antibody was then added again to check for the presence of any bound primary antibody. After proper stripping the blot was again blocked and reprobed with the desired antibody.

F. *Immuno-complex kinase assay*

TAK1 and its various truncation mutants immobilised on anti-GST immobilised protein G- Agarose beads were incubated with 1μg GST-MKK6 and 5μCi P^{32} ATP in a kinase reaction buffer containing 50mM Tris-Cl (pH 7.0), 10mM $MgCl_2$, 0.5mM DTT, 50mM β-Glycero-phosphate, and 1mM ATP for 20 minutes at 37°C. The reaction was stopped by adding 5X lamelli buffer to the reaction mixture. The mixture was then run on 12% SDS-PAGE, followed by the transfer of proteins to the PVDF membrane. After transfer of the proteins to the PVDF membrane, autoradiography was done. After autoradiograph, the blots were probed with desired antibody. After that the blots were analyzed using the Odyssey infrared imager (LI-COR).

III. RESULTS AND DISCUSSION

A. *Cloning and expression of TAK1 and its various truncation mutants of in pEBG:* pDNR-Dual containing human TAK1 was used as a template to amplify TAK1 and its various truncation mutants for cloning in pEBG. The PCR mixtures were loaded on 1% agarose gel. The PCR products were excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen as per manufacturer's protocol. The purified PCR products were digested using KpnI (Thermo Scientific) and BamHI (Thermo Scientific). pEBG was also digested using the same set of enzymes. The digestion mixtures were incubated at 37°C for 1 hour. After digestion the mixtures were loaded on 1% agarose gel and the digested products were excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen. The double digested and purified PCR products were then ligated with double digested and purified pEBG using T4 DNA ligase from fermentas along with religation control. A ratio of 1:3 (vector:insert) was using to perform the ligation reaction. The ligation and religation mixtures were incubated overnight at 16°C and transformed in DH5α competent cells and grown on ampicillin positive (100μg/mL) LB agar plates at 37°C overnight. The colonies from the ligation plate

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were then inoculated in LB broth containing ampicillin (100µg/mL). Plasmids were then isolated from these cells using a mini prep kit from Qiagen as per manufacturer's protocol. The plasmids were digested using KpnI and BamHI to check for the presence of insert. The presence of inserts at appropriate positions confirmed the recombinants. Also, the expression of different truncation mutants was monitored using the technique of western blotting. Anti-GST antibody was used to probe the blot for confirming the expression of the clones and anti-actin antibody was used as a loading control.

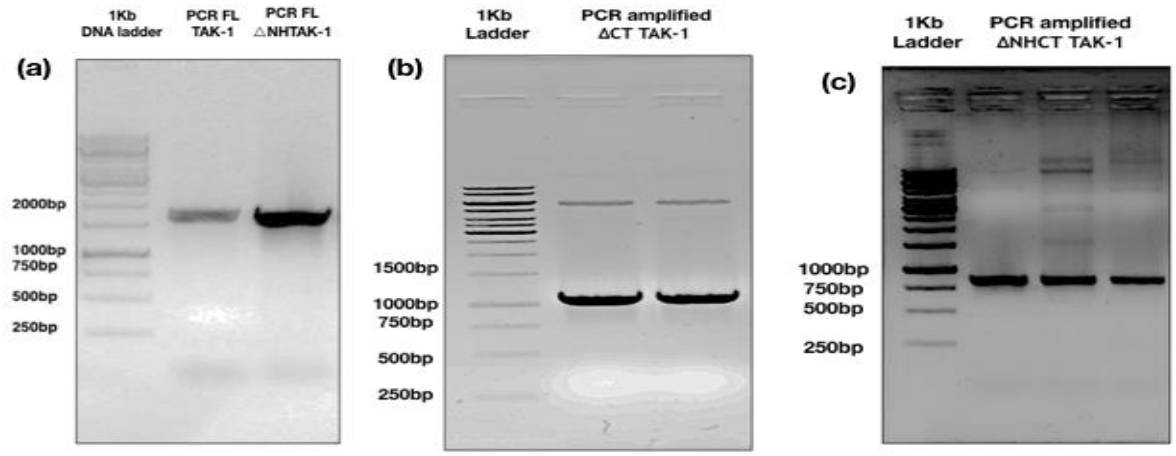

Fig 1: PCR amplification of different constructs of TAK1: Agarose gel pictures showing the PCR amplification of (a)FL TAK1, ΔNH TAK1, (b) ΔCT TAK1 and (c) ΔNHCT TAK1

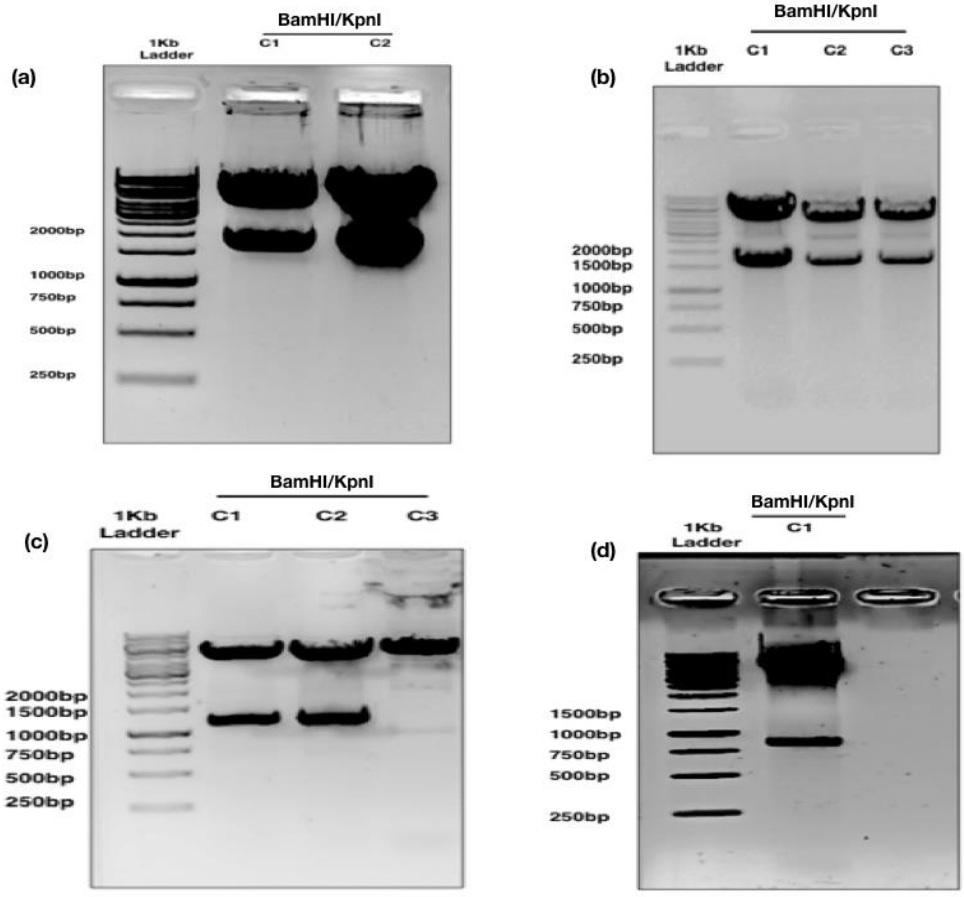

Fig 2: Confirmation of the clones using restriction digestion: Agarose gel pictures showing successful cloning of (a) FL TAK1, (b) ΔNH TAK-1, (c) ΔCT TAK-1 and (d) ΔNHCT TAK1 in pEBG using different plasmids recovered from various colonies grown on ligation plates.

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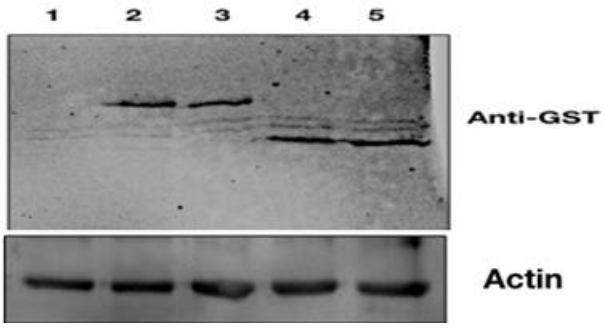

Fig 3: Immunoblot showing the expression of TAK1 and its various truncation mutants cloned in pEBG: HEK-293T cells were transfected with recombinant plasmids corresponding to FL TAK1 (2), ΔNH TAK1 (3), ΔCT TAK1(4) and ΔNHCT TAK1 (5). HEK-293T cells in which no plasmid was transfected was used as a control (1). After 48 hours of transfection the cells were harvested and subsequently lysed. Loading dye (lamelli buffer) was added to the samples, and the samples were boiled in the loading dye for 5 minutes. Samples were then loaded on 15% SDS-PAGE, blotted at 70V for 90 minutes, blocked and probed with the indicated antibodies.

B. *Cloning and expression of TAB1, a TAK1 binding protein in pKMYC:*

pENTR223 containing human TAB1 was purchased from plasmid harvard repository and was used as a template to amplify TAB1 for cloning in pKMYC. The PCR product was excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen as per manufacturer's protocol. The purified PCR product was digested using EcoRI (Thermo Scientific) and BamHI (Thermo Scientific). pKMYC was also digested using the same set of enzymes. The digestion mixtures were incubated at 37°C for 1 hour. After digestion the mixtures were loaded on 1% agarose gel and the digested products were excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen. The double digested products (TAB1 and pKMYC) were ligated using T4 DNA ligase from fermentas along with the religation control. A ratio of 1:3 (vector:insert) was using to perform the ligation reaction. The ligation and religation mixtures were incubated overnight at 16°C and transformed in DH5α competent cells and grown on ampicillin positive (100μg/mL) LB agar plates at 37°C overnight. The colonies from the ligation plate were then inoculated in LB broth containing ampicillin (100μg/mL). Plasmids were then isolated from these cells using a mini prep kit from Qiagen as per manufacturer's guidelines. The plasmids were digested using EcoRI and BamHI to check for the presence of insert. The presence of insert corresponding to 1512 bp confirmed the recombinant plasmid of TAB1.

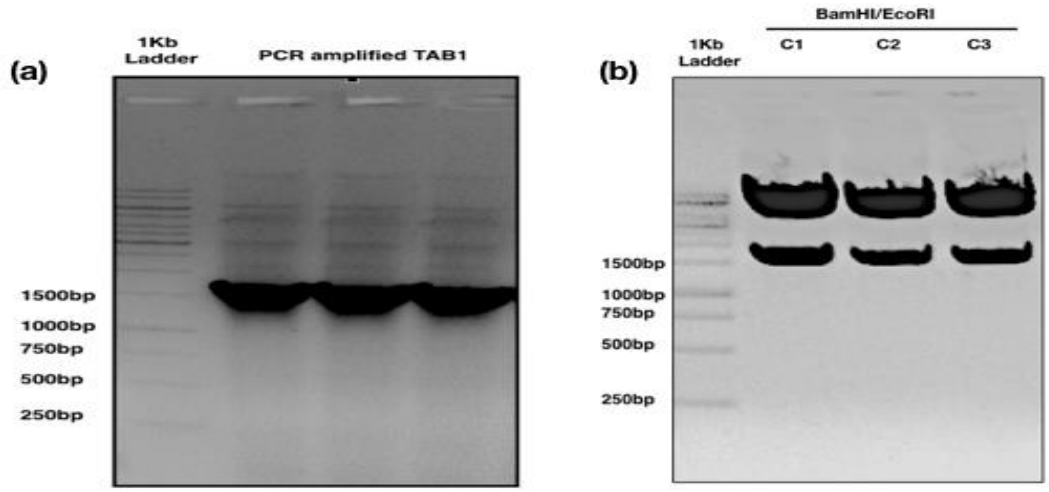

Fig 4: Agarose gel electrophoresis confirming successful cloning of TAB1 in pKMYC: (a) 1% Agarose gel picture showing PCR amplification of TAB1, (b) 1% agarose gel showing digestion of plasmids recovered from three colonies (C1, C2 and C3) from the ligation plate for confirming the successful cloning TAB1 in pKMYC.

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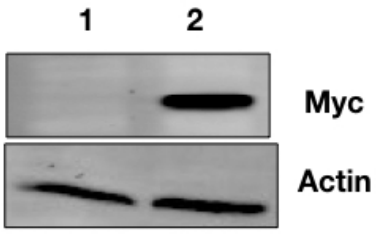

Fig 5: Immunoblot showing the expression of TAB1 cloned in pKMYC in HEK-293T cells: HEK-293T cells were transfected with recombinant plasmid of pKMYC with TAB1 cloned in it (2). HEK-293T cells in which no plasmid was transfected was used as a control (1). After 48 hours of transfection, the cells were harvested and subsequently lysed. Loading dye (Lamelli buffer) was added to the samples, and the samples were boiled in the loading dye for 5 minutes. Samples were then loaded on 10% SDS-PAGE, blotted at 70V for 90 minutes, blocked and probed with the indicated antibody.

C. *Cloning and expression of MKK6, an invitro substrate of TAK-1*

MKK6 cloned in pCDNA3 was ordered from Addgene and it was used as a template to amplify MKK6 for cloning in pGEX-4T2. The PCR product was excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen as per manufacturer's protocol. The purified PCR product was digested using High fidelity SalI (Thermo Scientific) and High fidelity NotI (Thermo Scientific). pGEX-4T2 was also digested using the same set of enzymes. The digestion mixtures were incubated at 37°C for 3 hour. After digestion the mixtures were loaded on 1% agarose gel and the digested products were excised off the gel under UV illumination and purified using the gel extraction kit from Qiagen. The double digested products (MKK6 and pGEX-4T2) were ligated using T4 DNA ligase from fermentas along with the religation control. A ratio of 1:3 (vector:insert) was used to perform the ligation reaction. The ligation mixture was incubated overnight at 16°C and transformed in DH5α competent cells and grown on ampicillin positive (100µg/mL) LB agar plates at 37°C overnight. The colonies were then inoculated in LB broth containing ampicillin (100µg/mL). Plasmids were then isolated from these cells using a mini prep kit from qiagen as per manufacturer's guidelines. The plasmids were digested using EcoRI and BamHI to check for the presence of insert.

5µL of the positive clone was transformed in 50 µL of BL21-DE3 competent cells and grown overnight on ampicillin positive (100µg/mL) LB agar plates at 37°C. A single colony was picked up at random and inoculated in 5ml of LB broth containing ampicillin (100µg/mL) and grown overnight at 37°C and 220rpm using orbital shaking incubator. The cultures were regrown in 5ml LB broth containing ampicillin (100µg/mL) to log phase and induced using 1mM IPTG. The induction was performed overnight. After induction the cells were harvested at 5000rpm for 5 minutes, lysed using bacterial lysis buffer containing (Tris-Cl (50mM), EDTA (5mM), NaCl (50mM), lysozyme (0.2mg/mL) and protease inhibitor cocktail (Sigma) at pH 8.0. The extracts were run on 10% SDS-PAGE to check for the induction of the recombinant GST fusion protein.

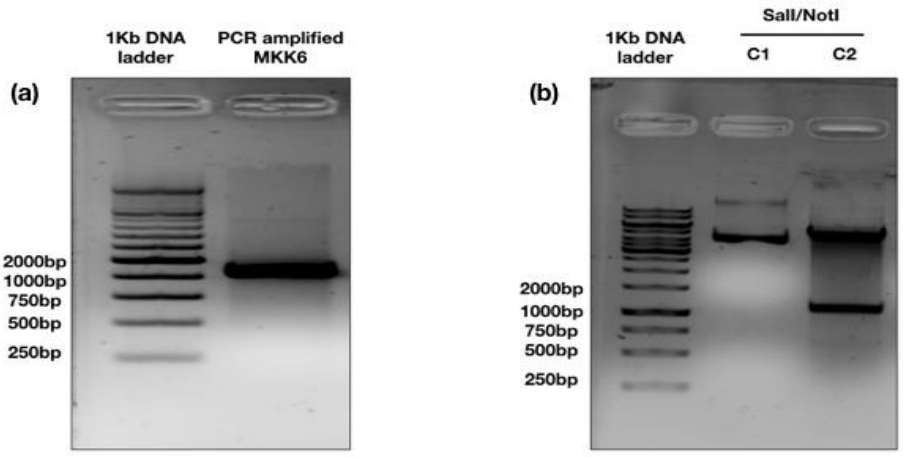

Fig 6: Agarose gel electrophoresis confirming successful cloning of MKK6 in pGEX-4T2: (a) 1% Agarose gel picture showing PCR amplification of MKK6, (b) 1% agarose gel showing digestion of plasmids recovered from two colonies (C1 and C2) from the ligation plate, for confirming the successful cloning of MKK6 in pGEX-4T2.

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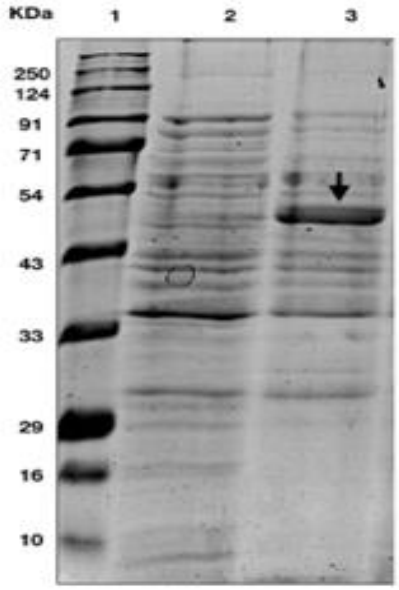

Fig 7: SDS-PAGE showing the expression of MKK6 in BL-21 DE3 cells: BL-21 DE3 cells were transformed with recombinant plasmid containing MKK6. The culture of these cells was then grown in LB broth containing ampicillin (100μg/mL) and induced using 1mM IPTG. After induction the cells were harvested and lysed using SDS sample buffer (Lamelli buffer) along with the uninduced controls. The lysates were then loaded on 10% SDS-PAGE which was subsequently stained and destained, lane 1 contains the standard protein marker, lane 2 contains uninduced MKK6, lane 3 induced MKK6.

D. *Invitro kinase assay of different truncation mutants of TAK1:*

TAK1 and its different truncation mutants were analyzed for kinase activity using GST-MKK6 as a substrate. TAK1 and its different truncation mutants were transfected in HEK-293T cells with and without the TAK1 binding partner TAB1. After 48 hours of transfection the cells were harvested and lysed and an anti GST- immunoprecipitate was obtained and analyzed for kinase activity using invitro kinase assay. The results decipher that TAK1 is moderately active in the absence of TAB1, however, in presence of TAB1, the activity is increased approximately three fold as can be seen from the blots suggesting the requirement of TAB1 in the activation of TAK1.

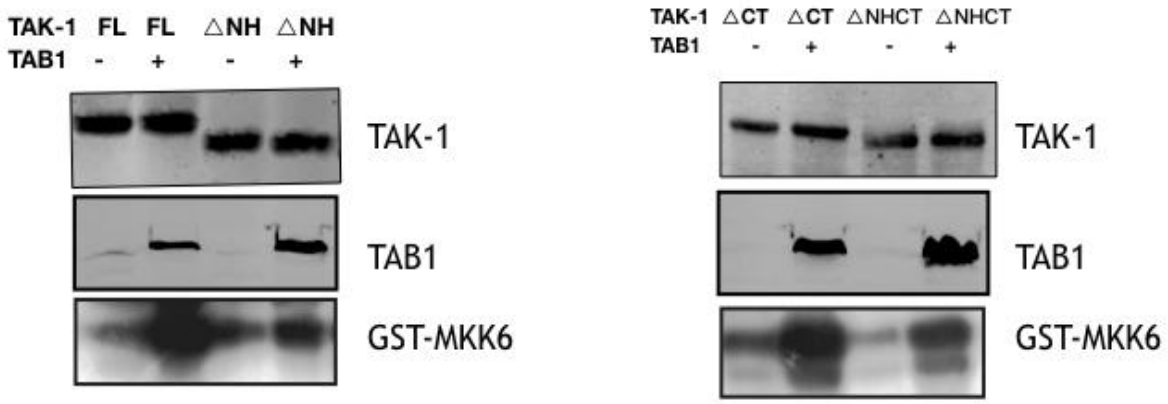

Fig 8: TAB1 increases the kinase activity of various truncation mutants of TAK1: HEK-293T cells were cotransfected with TAK1 and TAB1. After 48 hours of transfection the cells were harvested in ice-cold PBS and lysed. After lysis, a anti- GST immunoprecipitate was obtained and kinase assay was done using GST-MKK6 as a substrate.

IV. CONCLUSION

We achieved successful cloning of various truncation mutants of TAK1 in mammalian system. The clones were expressed in HEK-293T cells. Also, in-vitro kinase assay using all the truncation mutants as kinases to phosphorylate GST-MKK7, an invitro substrate of TAK-1, revealed the need of TAB1 as an indispensable partner for activation of TAK1.

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Conflict of interest:

The authors declare that there is no conflict of interest regarding the publication of this paper.

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