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In-vitro Phosphorylation of Recombinant Human Tim23 and its Fragments: Post Translational Modification

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ABSTRACT: The present study aimed to investigate the post-translational modification of recombinant human Tim23 and its fragments. Recombinant human TIM23 fragments were cloned into the pET28a vector and analyzed by agarose gel electrophoresis. Overexpression of the proteins and their fragments was achieved in BL21DE3 cells followed by affinity purification and validated by SDS-PAGE. *In-vitro* phosphorylation of the purified proteins was carried out using mitochondrial extract as a kinase source. A specific band corresponding to recombinant human Tim23 after phosphorylation followed by immunoblotting confirmed the phosphorylation of human Tim23. Molecular cloning of human TIM23 was confirmed by the detection of specific bands (gene sizes: 219 bp, 288 bp, 435 bp, and 582 bp) on an agarose gel. Overexpression, purification, and phosphorylation were confirmed by single discrete bands (fragment 2: ~12 kDa, fragment 3: ~18 kDa, and fragment 4: ~22 kDa) on SDS-PAGE. The study highlights the use of indigenously designed primers in the amplification, of Tim23. Further, in the phosphorylation studies, rat liver mitochondrial extract was used as kinase source and Tim23 proteoliposomes were employed to mimic the membrane environment. Overall, the present investigation reports the phosphorylation of Tim23 protein and its fragments under *in vitro* conditions which may influence the protein transport mechanism and add to the literature on mitochondrial biogenesis.

Keywords: Mitochondria, Recombinant protein, Cloning, Phosphorylation, Tim23, Anti *r*human Tim23.

INTRODUCTION

Translocases of mitochondrial outer (TOM) and inner membranes (TIM) consist of distinct complexes. These channel proteins regulate the protein entry into mitochondrial organelle which in turn involves the mitochondrial biogenesis of eukaryotes. A crucial protein of the mitochondrial inner membrane receptor channel is Tim23 which associates with several partner proteins. The C-terminal domain of Tim23 is responsible for anchoring in the inner membrane of mitochondria, the intermediary domain serves as a pre-sequence receptor in the intermembrane gap and the N-terminal part of Tim23 has been reported to be protruding from the surface of the outer membrane. In the field of membrane biology, a remarkable property of Tim23 is that it has a topology that spans two membranes. Tim23 is responsible for the formation of connections between both membranes i.e., outer, and inner membranes of the mitochondria by simultaneously integrating into two membranes. Tethering the inner membrane channel to the outer membrane makes it easier for precursor proteins to be transferred from the TOM protein channel to the TIM23 complex (Donzeau *et al.,* 2000), thus increasing the efficiency of protein import.

Numerous distinct import processes work towards the mitochondrial proteins to be sorted from the cytosol into the distinct compartments of the mitochondria. The TOM complex, also known as the translocase of the outer membrane of mitochondria, functions as the entrance gate for mitochondrial proteins in general (Dekker *et al.,* 1998). The receptors that are responsible for the recognition of precursors on the outer membrane surface of mitochondria include Tom20 and Tom22, which are exposed to the cytosol. These Tom receptors are functionally supported by additional pre-sequence-binding TOM subunits, including Tom5, Tom70, and the pore-forming Tom40 (Melin *et al*., 2014). The intermembrane space (IMS) domain of Tom22 is where the trans-binding site for the pre-sequence may be found, which is located at the exit of the Tom40 channel (Esaki *et al*., 2004).

The precursor proteins must be transferred from the TOM complex of the mitochondrial outer membrane to the TIM23 complex located on the inner membrane for inner membrane translocation to take place. To promote precursor transfer across the IMS to the TIM23

complex, it is assumed that a complicated network of protein interactions between the IMS domains of Tim23, Tim50, Tom22, and Tim21, as well as interactions with the pre-sequence of the incoming precursor, take place. There is, however, a lack of a molecular understanding of these activities in the context of the translocation machinery. The Tim50 IMS domain functions as the principal pre-sequence receptor at the inner membrane, where it is responsible for precursor recognition. $Tim50^{IMS}$ and $Tim23^{IMS}$ are two other IMS proteins that, along with $Tom22^{IMS}$, interact with the pre-sequence of proteins in the intermembrane space of mitochondria (de la Cruz *et al.,* 2010).

Research studies revealed that the Tim23 channel consists of many proteins (Mokranjac *et al.,* 2010) that aid in the transport of proteins into mitochondria and most of these findings have been carried out in yeast (i.e., in lower eukaryotes). The studies showed that 9,400 phosphorylation sites distributed throughout 972 proteins (~70%) that make up yeast mitochondria (Frankovsky *et al*., 2021). In this process, phosphorylation of the TOM complex proteins is known in this transport mechanism (Walter *et al*., 2021). These studies further suggest that there could be a molecular mechanism behind the binding and assembly of the complex proteins during the biogenesis of mitochondria and the sorting of nuclear-encoded proteins into mitochondria. Mitochondrial DNA (mtDNA) sequencing studies have also contributed in understanding the origin and phylogeny of goats (Sepehri and Seyedabadi 2015). Their studies based on PCR demonstrated that the sequences of the Cyt B gene were conserved among goats from different regions and some of them exhibited short distancing from the Khalkhali goat of Iran. Further, Ramya and Bhoominathan (2018) in their studies correlated the similarity in 16s RNA sequences among metal resistant bacteria to their multi heavy metal resistances and due applications. However, mitochondria protein complexity is higher in humans comparatively. The role of TIM complexes of mitochondria in humans needs to be studied thoroughly as mitochondrial dysfunction may lead to several diseases in humans. In this perspective and to understand the exact role of human Tim23, if any, in assembly and binding with pre-sequence proteins in sorting of mitochondrial proteins from cytosol through TIM23 channel the present study involved in *in-vitro* phosphorylation of full-length human Tim23, cloning expression and purification of human Tim23 fragments. Further, *in-vitro* phosphorylation of recombinant (*r*human) Tim23 fragments.

MATERIALS AND METHODS

The present study was carried out in the Department of Biochemistry, University College of Science, Osmania University, Hyderabad.

A. Materials

Acrylamide, agarose, sodium dodecyl sulphate (SDS), tetramethyl ethylene diamine (TEMED), tween-20, imidazole, ethylene diamine tetra acetic acid (EDTA), lysozyme, ammonium per sulphate (APS) and phenyl methane sulphonyl fluoride (PMSF) were procured from Sigma-Aldrich (USA). DNA ladder, dNTPs (dATP, dGTP, dCTP, and dTTP), restriction enzymes, T4 DNA ligase, and protein markers were purchased from ThermoFisher Scientific, (USA). Agar powder, ethidium bromide, and kanamycin were procured from Himedia (India). Radio labeled ATP (γ ^{32}P) was purchased from BARC, Mumbai, India. All other chemicals and reagents were of analytical grade.

B. Culture media for bacteria

Culture media was prepared using yeast extract (1%), tryptone (2%), and sodium chloride (2%). The Ph was adjusted to 7.0.

C. Vector

Bacterial vector, pET28a was used for the cloning of human TIM23 fragments.

Primers and restriction enzymes

Primers were designed and used for amplification. Forward primer: 5'-ATGGGAAGGAGGCGGGGGAAGCGGCAACAAA-3' and reverse primers: 5'- TTTA AAGCTT TCA AAA TCT GCC CCG GGT TTT -3' (fragment1), 5'- TTTA AAGCTT TCA AAT CTG TAC ATT TCT TGG -3' (fragment 2), 5'- TTTA AAGCTT TCA AAT GAT GAC ACC AAA TGC -3' (fragment 3) and 5' TTTA AAGCTT TCA ATA TAG TGC ATA GAG GCT 3' (fragment 4) were used. *EcoR I* and *Hind III* restriction enzymes were used.

D. Mitochondrial extract preparation

Rats (Wistar) were procured from ICMR-National Institute of Nutrition, Hyderabad. The animals were sacrificed and the liver tissues were collected. The mitochondria were then isolated from rat liver by using differential centrifugation (Tammineni *et al.,* 2013). The liver tissue was homogenized in cold centrifugation buffer (200 mM Mannitol, 2 mM HEPES, pH 7.4, 70 mM sucrose, 0.2 mM EDTA, and added BSA 0.36 mg mL^{-1}). The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was collected and again centrifuged at 1000 rpm for 10 min. Then the pellet was washed with H-medium and finally dissolved in import buffer. Isolated mitochondria were solubilized in 0.2% detergent (Triton × 100) and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was then collected and used as a protein kinase source.

E. In-vitro phosphorylation

The *in-vitro* phosphorylation assay was carried out by following the procedure reported by (Tammineni *et al*., 2013). To perform *in-vitro* phosphorylation studies, the purified recombinant human Tim23 proteo-liposomes

were prepared to mimic the membrane environment (Maurya and Mahalakshmi 2013) and was incubated with mitochondrial extract (5 µg) in phosphorylation buffer [20 mM Tris (pH 7.5), 25 µM ATP, 1 mM DTT, 1 mM NaF, 10 mM $MgCl_2$, 40 Mm KCl, and 1 mM Na_3VO_4] containing γ- labeled ^{32}P (15 µCi) ATP at 35 °C for 30 min. The recombinant protein was pulled down with Ni-NTA affinity beads and was analyzed by SDS-PAGE. Further, blotting was done and exposed to phosphor imager scanning. Four truncated mutants (fragments) of recombinant human Tim23 were cloned by using specific primers, expressed, and purified. The *in-vitro* phosphorylation of these fragments was also done along with full-length human Tim23 by using mitochondrial extract and analyzed by SDS-PAGE and western blotting.

F. Polymerase chain reaction

The polymerase chain reaction was carried out to amplify the fragments of human TIM23 with specific primers as mentioned above. The TIM23 fragments were amplified by PCR by using 0.5 µM of forward and reverse primer, 50 µM of each of the four dNTPs, and 0.5 U of DNA polymerase. The reaction was run keeping the lid temperature at 104°C. The number of cycles was 32 which included 3 steps denaturation (94 °C/4 min), annealing (56 °C/1 min), and extension (72 °C/1:30 min). The amplicon was resolved by agarose gel (1%) electrophoresis.

G. Agarose gel electrophoresis

Agarose gel (1%) electrophoresis was used to separate all DNA (human TIM23 fragments) samples.

Restriction digestion of human TIM23 fragments and pET28a vector

Vector DNA and human TIM23 fragment samples were treated with 1 unit (1 µL) each of *EcoR I* and *Hind III* restriction enzymes and the reaction mixture were incubated at 37 °C for 3 hrs.

Ligation of human TIM23 fragments into a bacterial vector

Plasmid DNA and human Tim23 fragments were mixed in a 1:3 ratio with 1 unit of T4 DNA ligase enzyme. The reaction mixture was incubated overnight at 18 °C. Later, the ligated samples were transformed into bacterial-competent cells (Calcium-treated BL21DE3).

H. Transformation of ligation products of human TIM23 fragments into bacterial cells

The ligated product pET28a with human TIM23 fragments (10 µL) was added to competent bacterial cells (BL21DE3) and then incubated for 30 min. The cells were subjected to a 42 °C heat shock treatment for 90 secs, after which they were immediately placed on ice and allowed to stand for 3 min. Cells were then transferred to 1 mL of Luria-Bertani (LB) broth and cultured for 60 min at 37 °C. For 5 min, centrifugation was done at 6000 rpm. The remaining solution was then discarded while an aliquot of the supernatant was stored. The cells were spread on solid agar plates with an antibiotic (kanamycin; 25 µg mL^{-1}) and incubated overnight at 37 °C.

I. Overexpression by IPTG induction of human Tim23 fragments

The overnight culture of transformed BL21DE3 cells with all pET28a-Tim23 fragments was added to 15 mL of LB broth followed by the addition of kanamycin 25 µg mL^{-1} and the mixture was incubated at 37 °C until the cells reached an optical density (O.D.) of 0.6 when measured at wavelength 600 nm, using a UV-VIS spectrophotometer (Hitachi, USA). After that, the cells were supplied with isopropyl thiogalactoside pyranoside (IPTG; 1 mM) and induction was carried out for 3 hrs at 37 °C. The bacterial culture (1 mL) was centrifuged at 6000 rpm for 6 min at room temperature. Following treatment with cell lysis buffer, the cell pellet was freeze-thawed at -20 oC for 60 min. Then sonication with 7 pulses (15 secs/pulse) using a probe sonicator (Labman Scientific Instruments, India) was done to lyse the cells. The cell lysate was subjected to centrifugation at 8000 rpm for 5 min to separate the supernatant and pellet and SDS sample buffer was added to both these samples and then boiled for 10 min at 100 °C. The recombinant human Tim23 fragments overexpression was evaluated by SDS-PAGE.

J. Purification of recombinant human Tim23 fragments

Affinity beads were used to purify the protein. Initially, the Ni-NTA affinity beads (Qiagen, Germany) were equilibrated with washing buffer and spun for 2 min at 5000 rpm. The supernatant was discarded. Later the protein samples were added to the affinity column. The flow-through was collected and stored. The affinity column was washed with buffer (1 mM imidazole) and purified protein using elution buffer (0.4 M imidazole). Protein concentration was estimated by using the Bradford method (Bradford, 1976). Affinity-purification of human Tim23 fragments was evaluated by carrying out SDS-PAGE. Further *in-vitro* phosphorylation was carried out as mentioned earlier.

K. Polyacrylamide gel electrophoresis

Prepared 12% and 15% polyacrylamide gels and electrophoresis were performed after loading samples in wells for 3 hours at 100 v. Gels were removed from plates and placed in a staining solution for 2 hrs. Then the gels were destained until bands were visualized clearly. Images were obtained by scanning and used for analysis.

L. Western blotting

The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by using a mini transfer pack (BIO-RAD, USA). The membrane was blocked with milk powder (5%). The blot was probed with primary antibody (anti-human Tim23) and HRP conjugate secondary antibody at room temperature for 2

hours. Then, the blot was developed by using ECL (Enhanced chemiluminescence) reagents (GE HealthCare, USA) on VersaDoc gel doc system (BIO-RAD, USA).

RESULTS AND DISCUSSION

The Tim23 is a mitochondrial protein present in the inner membrane of mitochondria a cell organelle in almost all eukaryotes. The transport of proteins from cytosol remains one of the early developments in mitochondrial evolution (Avendaño-Monsalve *et al*., 2020). Numerous subunit components make up the mitochondrial protein import system i.e., TOM and TIM complexes. The TOM is present on the outer membrane and is the entry gate for pre-sequences of mitochondria (Dekker *et al*., 1998), whereas the TIM complex is present in the inner membrane of mitochondria. These complexes coordinate with each other and transport proteins (Donzeau *et al*., 2000).

The cytosol is the site of synthesis for a significant proportion (~90%) of mitochondrial proteins. Signal sequences are present on proteins and target these proteins to the different compartments of mitochondria through transport protein complexes or receptor channels (Habib *et al*., 2007). The major function of the TIM23 complex involves the transport of mitochondrial matrix proteins which consist of N-terminal presequence that contribute to the biogenesis of mitochondria.

The mitochondria perform several functions in addition to producing ATP, such as metabolic processes, calcium homeostasis, apoptosis, cancer, and aging. Numerous neurological illnesses, including Parkinson's disease and Alzheimer's disease, have been demonstrated to be caused by the dysregulation of mitochondrial proteins as a result of their mutations. In this regard, studies on mitochondrial biogenesis is gaining attention in the past few decades and most of the findings in the last decade focused on TIM and TOM complexes of yeast models. Recently also (Im Sim *et al*., 2021) showed the structural basis of TIM complex-mediated import in yeast mitochondria by cryo-EM analysis. In this context, specific investigations are required to understand the regulatory mechanism behind mitochondrial biogenesis in higher eukaryotes. Along the similar lines, Jehle *et al*. (2022) revealed the phosphorylation-dependent protein-protein interactions in yeast through *in-silico* studies. So, the present study focused on -post-translational modification, particularly for the possibility of phosphorylation of inner membrane complex protein of mitochondria *i.e*., human Tim23.

A, In-vitro phosphorylation of rhuman Tim23

Phosphorylation of *r*human Tim23 under *in-vitro* conditions has been carried out using radiolabelled ^{32}P(γ)-ATP. The proteoliposomes were prepared by adding urea-denatured Tim23 to liposomes to mimic the membrane environment and were used for this *in-vitro* phosphorylation study. Total mitochondrial protein extract was used as a kinase source. Reaction samples were exposed to phosphorimaging after the phosphorylation assay. Blot was analyzed by autoradiography as seen in Fig. 1. Phosphorylation of proteins was observed in the sample containing mitochondrial extract with radio-labeled ^{32}P ATP as shown in (lane 3). No bands were observed without kinase source (mitochondrial extract) and radiolabeled ^{32}P ATP (lanes 2 and 5). Phosphorylated recombinant human Tim23 protein (~23 kDa) bands were observed with mitochondrial extract (different concentrations) and with radio-labeled ^{32}P ATP (lanes 4, 6, and 7). These results confirmed the phosphorylation of Tim23 under *in-vitro* conditions. For further validation a purified Tim 23 protein is needed hence the protein separated on SDS-PAGE was subjected to affinity purification, followed by autoradiography and immunoblotting.

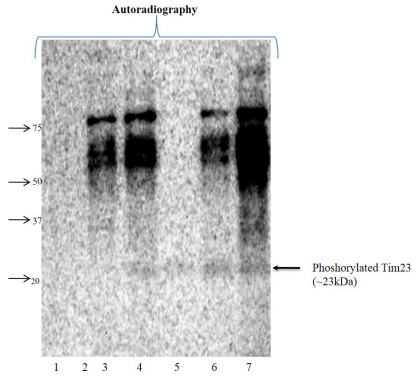

Fig. 1. Autoradiography analysis of phosphorylated of full length *r* human Tim23. Lane 1: indicates protein marker; Lane 2: total mitochondrial extract alone; Lane 3: mitochondrial extract (5 µg) with radio labeled ^{32}P ATP; Lane 4: mitochondrial extract (10 µg) with radio labeled ^{32}P ATP and *r*human Tim23; Lane 5: radio labeled ^{32}P ATP and *r*human; Lane 6: mitochondrial extract (5µg) with radio labeled ^{32}P ATP and *r*human Tim23; Lane 7: mitochondrial extract (15 µg) with radio labeled ^{32}P ATP and *r* human Tim23.

B. Pull down assay after phosphorylation.

All the samples after phosphorylation were subjected to pull down on Ni affinity beads, followed by SDS-PAGE (12%) analysis, and transferred onto nitrocellulose (NC) membrane by western blotting. Then the blots were analyzed by autoradiography as shown in Fig. 2. The phosphorylation of proteins in the sample containing mitochondrial extract with radio-labeled ^{32}P ATP is depicted in (lane 2). No bands were observed without radio-labeled ^{32}P ATP and kinase source (mitochondrial extract) (lanes 3 and 5 respectively). Specific phosphorylated recombinant human Tim23 protein (i.e., ~23 kDa) bands were observed with mitochondrial extract and with radio-

labeled ^{32}P ATP (lanes 4, 6, and 7) after pull-down as indicated by the right arrow mark. This experiment confirmed the phosphorylation of Tim23 under *in-vitro* conditions. Interestingly, some interacting partner proteins bands were also observed along with the Tim23 in lanes 4, 6, and 7 even after pull-down with Ni-NTA affinity beads.

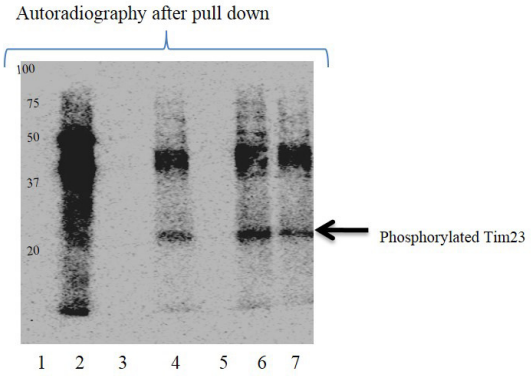

Fig. 2. Autoradiography analysis after pull down assay of full length *r* human Tim23. Lane 1: indicates protein marker; Lane 2: total mitochondrial extract (10µg) with ^{32}P ATP; Lane 3: mitochondrial extract alone (10µg); Lane 4: mitochondrial extract with ^{32}P ATP and *r*human Tim23; Lane 5: ^{32}P ATP and *r* Human Tim23; Lane 6: mitochondrial extract (5µg) with ^{32}P ATP and *r*human Tim23 (2 µg); Lane 7: mitochondrial extract (10µg) with ^{32}P ATP and *r*human Tim23 (1 µg).

C. Immunoblotting assay after autoradiography

Nitrocellulose (NC) membrane incubated with polyclonal antibodies (Anti-human Tim23 raised in rabbit) after autoradiography for specific identification of human Tim23 is depicted in Fig. 3.

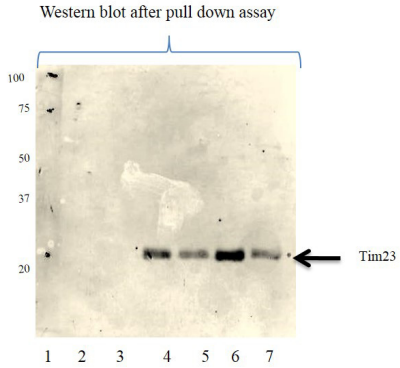

Fig. 3. Immunoblotting analysis of full-length *r* human Tim23 after *in-vitro* phosphorylation. Lane 1: indicates protein marker; Lane 2: total mitochondrial extract (10 µg) with ^{32}P-ATP; Lane 3: mitochondrial extract alone (10 µg); Lane 4: mitochondrial extract with ^{32}P-ATP and *r*humanTim23; Lane 5: ^{32}P-ATP and *r*human Tim23; Lane 6: mitochondrial extract (5 µg) with ^{32}P ATP and *r*human Tim23 (2 µg); Lane 7: mitochondrial extract (10 µg) with ^{32}P-ATP and *r* human Tim23 (1 µg).

The recombinant human Tim23 is clearly shown in lanes 4, 5, 6, and 7 corresponding to molecular weight (i.e., ~23 kDa). Phosphorylated protein bands (indicated in lanes 4, 6, and 7 in Fig. 2) were also observed in the immunoblotting assay (indicated in lanes 4, 6, and 7 in Fig. 3). Although the non-phosphorylated protein band was identified by immunoblotting no signal was found in autoradiography (lane 5 in Fig. 2). Autoradiography and immunoblotting results together (after affinity purification with Ni beads) confirmed the specificity of phosphorylated recombinant human Tim23 under *in-vitro* conditions.

Phosphorylation is one of the important post-translational modifications of proteins. Many proteins undergo phosphorylation and dephosphorylation by the reversible process. Several *in-vitro* assays were performed in this regard including Wong *et al.* (1983) who showed the phosphorylation of peptide analogs of angiotensin, by a specific enzyme, Cao and Lin (2008) proved zinc finger motif 36 in Tristetraprolin (TTP) phosphorylation by various kinases (ERK1, cdc2, GSK3, PKA, PKC, P38 MAPK and PKCµ) based on autoradiography studies and Utepbergenov *et al*. (2016) who demonstrated phosphorylation of full length recombinant ribosomal s6 kinase2. According to studies on proteomic research, ~40% of the organellar proteome is phosphorylated (Kruse *et al*., 2017; Giorgianni *et al*., 2014; Padrao *et al*., 2013). Gene regulation and gene expression may be achieved by either phosphorylation (by kinases) or dephosphorylation (by phosphatases) depending on the substrate and the pathways. Strong speculation was made on the possibility of phosphorylation of Tim 23 and revealed in the present study. de la Cruz *et al*., 2010, studies suggested that Tim50 and Tim23 inter-membrane space domains interact with pre-sequences in the intermembrane space of mitochondria. Schmidt *et al*. (2011) have shown TOM complex protein phosphorylation by cytosolic kinases while the phosphorylation of TIM complex proteins of mitochondria is not yet known. Generally, proteins get phosphorylated at specific sites. In this regard, the characterization of human Tim23 phosphorylation sites is essential. To address this aspect, in the present study, recombinant Tim23 fragments were generated by cloning, expression, and purification as depicted in Fig. 4 to 13.

D. Amplification of human TIM23 fragments

The fragments of human TIM23 were cloned into bacterial vector pET28a, then using the specific primers designed and the high fidelity Pfu DNA polymerase they were amplified. The resulting samples analyzed by running agarose gel electrophoresis are represented in Fig. 4. The specific amplification of all four human TIM23 fragments of different sizes was confirmed by DNA marker used and ethidium bromide staining for visualization of DNA bands under a UV

transilluminator. Later these bands were excised and purified by the gel extraction method. The concentration of DNA as determined by the Nanodrop spectrophotometric method was found to be (~500ng μL^{-1}) which was further used for restriction digestion and ligation into a bacterial plasmid vector.

E. Cloning of human TIM23 fragments into bacterial vector pET28a

The pET28a vector was isolated from bacterial colonies and separated on agarose gel (1%) electrophoresis. The specific band corresponding to pET28a vector was excised from the gel and DNA was extracted by a plasmid isolation kit. Successfully separated and extracted the human TIM23 fragments (after amplification by PCR) by a gel extraction kit (QIA Miniprep columns). The DNA concentration of vector (pET28a) and insert (human TIM23) samples as determined by Nanodrop spectrophotometer were found to be ~500 ng μL^{-1}. The ligation product of incubating the pET28a and human TIM23 samples by T4 DNA ligase was transformed into DH5α bacterial cells by heat shock method. Bacterial Colonies were observed after overnight incubation at 37°C and screened the cells for clones by subjecting the isolated DNA samples to double digestion with *EcoR I* and *Hind III*. These samples were analyzed by agarose gel (1%) electrophoresis as shown in Fig. 5 and 6. The size of the inserts was confirmed by marker DNA as indicated by the arrow marks. No insert band was found in undigested samples while the digested samples had clearly shown the inserts with the required molecular size. These experiments confirm the positive molecular clone of human TIM23 fragments in the bacterial expression vector (i.e., pET28a). Further, these clones were transformed into BL21DE3 bacterial expression cells for overexpression of recombinant human TIM23.

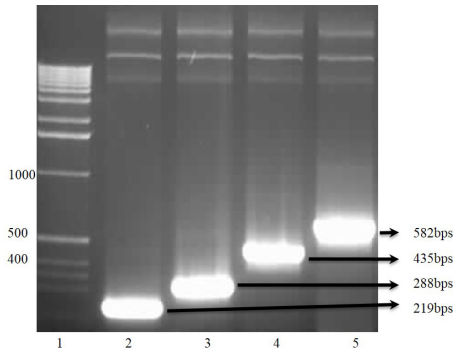

Fig. 4. Amplification analysis of human TIM23 fragments by agarose gel electrophoresis (1%). Lane 1 indicates DNA marker (base pairs), loaded amplified fragments of 219 base pairs, 288 base pairs, 435 base pairs and 582 base pairs on lane number 2, 3, 4, and 5 respectively.

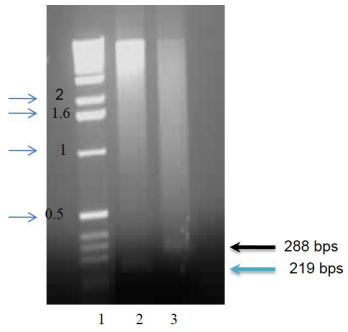

Fig. 5. Restriction digestion analysis of human TIM23 fragments 1 and 2 by agarose gel electrophoresis (1%). Lane 1 indicates DNA marker (kb). Lane 2 and 3 indicates inserts with corresponding sizes of 219 and 288 base pairs, respectively.

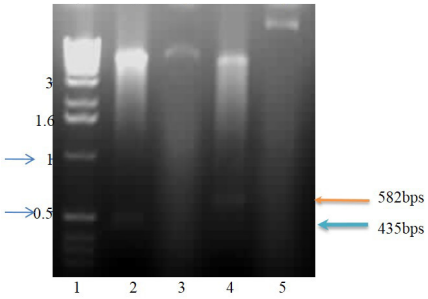

Fig. 6. Restriction digestion analysis of human TIM23 fragments 3 and 4 by agarose gel electrophoresis (1%). Lane 1 indicates DNA marker. Lane 2 and 4 indicates inserts with corresponding sizes of 435 and 582 base pairs, respectively. Lane 3 and 5 indicates undigested samples of plasmid DNA.

F. Overexpression of recombinant human Tim23 fragments

After successful confirmation of clones in a pET28a expression vector, they were transformed into BL21DE3 competent cells. Samples were analyzed on SDS-PAGE (12% and 15%) under reducing conditions using coomassie staining and represented in Fig. 7 and 8. The results revealed that recombinant human Tim23 fragment 1 was not over-expressed with IPTG induction (lane numbers 2 to 4) whereas, overexpression of fragments 2, 3 and 4 was observed as depicted in Fig. 7 and 8. The molecular weight of fragment 2 corresponded to ~12 kDa (lane 7) as confirmed by the protein marker loaded on lane 5 (Fig. 7).

The recombinant human Tim23 fragments 3 and 4 overexpression was also observed (lane number 2 and 4 respectively in Fig. 8) The molecular weight of fragment 3 corresponded to ~18 kDa and that of fragment 4 corresponded to 22 kDa when compared and validated with protein marker loaded in lane 3 under these experimental conditions. As the protein of interest, Tim23 is mitochondrial inner membrane protein the overexpressed cells were sonicated to separate the proteins into their soluble (supernatant) and

insoluble (pellet) fractions and analyzed by gel electrophoresis (SDS-PAGE) to confirm the fractionation of protein in pellet or supernatant.

G. Analysis of soluble and insoluble fractions (Tim23 fragments)

Analysis of fractionation of the Tim23 proteins in these samples (pellet and supernatant) using SDS-PAGE (15%) and coomassie staining is depicted in Fig. 9. The gel images s indicated the over-expression of mutant (fragment 2) at different optical densities (i.e., 0.6, 0.8, and 1) only in pellet fractions, separated on lanes 2, lanes 6, and 8. However, no over-expressed protein band was observed in supernatant fractions.

The recombinant human Tim23 fragments 3 and 4 samples were separated on SDS-PAGE (12%) and the gel pictures obtained after Coomassie staining are represented in Fig. 10 and 11. The samples were over-expressed after growing bacterial cells and reached different optical densities (0.6, 0.8 and 1). Over-expression of these Tim23 fragments was confirmed at different optical densities (i.e., 0.6, 0.8, and 1) based on the bands observed in the gel images (Fig. 10 and 11).

The results indicated the over-expression of fragments 3 and 4 in pellet fractions but not in the supernatant samples. This experiment confirmed that main Tim23 fragments 2, 3, and 4 (mutants) settled as pellet fractions after sonication of bacterial cells similar to the full-length protein which was also detected in the pellet fractions. The SDS-PAGE analysis of the recombinant Tim 23 protein cloned, expressed, and isolated in the pellet fraction authenticated its identity.

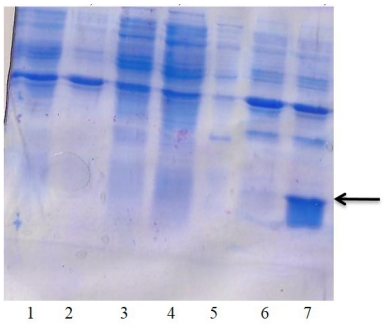

Fig.7. Expression analysis of *r*human Tim23 fragments 1 and 2 by SDS-PAGE (15%). Induced and uninduced samples of human Tim23 (fragments 1 and 2) were separated and analyzed by SDS-PAGE (15%). Samples containing recombinant Tim23 fragments 1 were loaded on to lane numbers 1 to 4. Lane 1: uninduced human Tim23 fragment 1; Lane 2: induced at 0.6 O.D.; Lane 3: induced at 0.8 O.D.; Lane 4: induced at 1.0 O.D.; Lane 5: indicates protein marker. Samples containing recombinant Tim23 fragments 2 were loaded onto lane numbers 6 to 7. Lane 6: uninduced human Tim23 fragment 2; Lane 7: human Tim23 fragment 2 induced at 1 O.D.

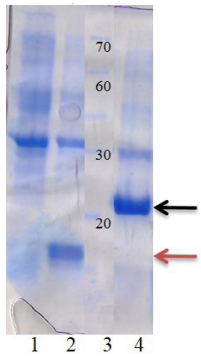

Fig. 8. Expression analysis of *r*human Tim23 fragments 3 and 4 by SDS-PAGE (12%). Recombinant Tim23 fragments 3 containing samples were loaded into lanes 1 and 2. Human Tim23 fragment 3, uninduced in Lane 1, induced (at 1.0 O.D.) in Lane 2, and protein marker in Lane 3. Recombinant Tim23 fragment 4 sample (induced at 1.0 O.D.) was loaded into lane 4.

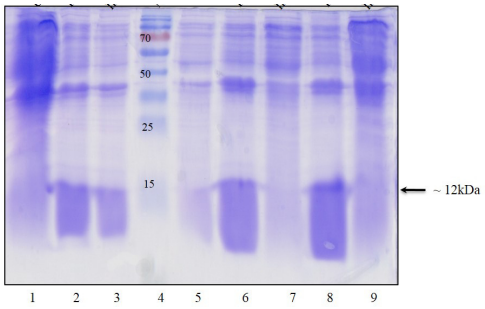

Fig. 9. Expression profile of pellet and supernatant fractions of *r*Human Tim23 fragment 2 by SDS-PAGE (15%). Lane 1: uninduced, Lane 2: Pellet (induced at 0.6 O.D); Lane 3: Supernatant (induced at 0.6 O.D); Lane 4: marker; Lane 5: uninduced; Lane 6: Pellet (induced at 0.8 O.D); Lane 7: Supernatant (induced at 0.8 O.D); Lane 8: Pellet (induced at 1 O.D); Lane 9: Supernatant (induced at 1 O.D).

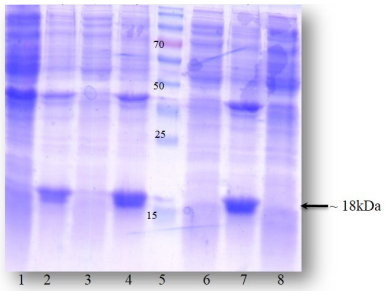

Fig. 10. Expression profile of pellet and supernatant fractions of *r*human Tim23 fragment 3 by SDS-PAGE (12%). Lane 1: uninduced; Lane 2: Pellet (induced at 0.6 O.D); Lane 3: Supernatant (induced at 0.6 O.D); Lane 4: Pellet (induced at 0.8 O.D); Lane 5: Marker; Lane 6: Supernatant (induced at 0.8 O.D); Lane 7: Pellet (induced at 1 O.D), Lane 8: Supernatant (induced at 1 O.D).

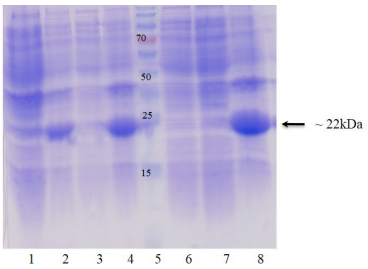

Fig. 11. Expression profile of pellet and supernatant fractions of *r*humanTim23 fragment 4 by SDS-PAGE (12%)**.** Lane 1: uninduced; Lane 2: Pellet (induced at 0.6 O.D); Lane 3: Supernatant (induced at 0.6 O.D); Lane 4: Pellet (induced at 0.8 O.D), Lane 5: Marker; Lane 6: Supernatant (induced at 0.8 O.D); Lane 7: Supernatant (induced at 1 O.D); Lane 8: Pellet (induced at 1 O.D).

H. Purification of recombinant human Tim23 fragments

The recombinant human Tim23 fragments were purified by affinity chromatography as the proteins containing His tags bind to the Ni-NTA column. Different fractions along with wash and flow through were analyzed by running polyacrylamide gel electrophoresis (12% and 15%) under reducing conditions. The gels were subjected to coomassie staining after purification and the results obtained are represented in following Fig. 12 and 13. An intense band was detected confirming the purified *r*human Tim23 fragment 2 with corresponding M.Wt ~12 kDa. This experiment confirmed the presence of pure proteins based on their molecular weights (Fig. 12 and 13). These pure fragments (mutant) proteins were used for further analysis along with full-length recombinant human Tim23 protein. The corresponding molecular weight protein bands with ~18 and ~22 kDa indicated and confirmed the presence of purified recombinant human Tim23 fragments 3 and 4.

I. In-vitro phosphorylation assay

Mutants (fragments 2, 3, and 4) of *r*human Tim23 were subjected to phosphorylation under *in-vitro* conditions using radio labelled ^{32}P ATP followed by SDS-PAGE (15%), and transferred to a western blot. Autoradiography was used to investigate the blot as represented in Fig. 14. This study demonstrated the phosphorylation of proteins in the samples including mitochondrial extract and radiolabelled ^{32}P ATP demonstrated. No bands were seen in lane 1 with mitochondrial extract alone (without labeled ATP) and radiolabelled ^{32}P ATP phosphorylated proteins in lane 2 (mitochondrial extract), and phosphorylated fragments 2, 3 and 4 (mutants) recombinant human Tim23 proteins were visualized in lanes 3, 5 and 6, respectively. Corresponding molecular weights of the mutants ~12 kDa (fragments 2), ~18 kDa (fragments 3) and ~22 kDa (fragments 4) were confirmed when compared with the protein markers. Phosphorylated recombinant human Tim23 full-length protein (i.e., ~23 kDa band) was seen in lane 8. Phosphorylation of full-length Tim23 and its mutants (fragments 2, 3 and 4) under *in-vitro* conditions were thus confirmed by this investigation. Non-specific protein (Recombinant purified GRIM-19, M.Wt ~17 kDa) was not phosphorylated under the conditions used for Tim23 and its fragments.

J. Pull-down assay after in-vitro phosphorylation

After the western blot the phosphorylated recombinant proteins on the membrane were analyzed by autoradiography and the image recorded is shown in Fig. 15. This experiment showed phosphorylation of proteins was not observed without labeled ATP (Lane 1). No proteins were observed in lane 2 where recombinant proteins were absent. Lanes 3, 5, 6, and 8 were shown with specific protein bands of fragments 2, 3, 4 and full length of recombinant human Tim23 respectively. The molecular weight of these proteins was compared with the protein marker shown in lane number 4. Single bands containing ~12, ~18, ~22, and ~23 kDa protein bands after autoradiography with pull-down Ni-NTA affinity beads confirmed the specific proteins with phosphorylation of recombinant human Tim23 fragments 2, 3, 4, and full length were identified. A comparative analysis of phosphorylated human Tim23 and its fragments was done and represented in Fig. 16. These analyses clearly showed that *r*human Tim23 fragment 3 phosphorylation was more when compared to other fragments (2 and 4) and *r*human Tim23 (FL).

K. Immunoblotting assay

Following autoradiography, the nitrocellulose membrane was treated with a polyclonal antibody (anti-*r*human Tim23) to specifically identify recombinant full-length human Tim23 and its mutants. The resulting blot developed is represented in Fig. 17. After probing with a polyclonal antibody raised against *r* human Tim23 the specificity of fragments and full-length human Tim23 were identified. No proteins were identified in lanes 1 and 2 as expected after the pull-down assay. Specific bands on lanes 3, 5, 6, and 8 after immunoblotting confirmed the presence of phosphorylated recombinant human Tim23 fragments 2, 3, 4, and full-length Tim23.

Overall, the present findings encourage us to hypothesize that Tim23, an integral membrane protein, plays role in the functioning complex channel for the sorting of preproteins across the inner membrane through chemical modification i.e., phosphorylation. It is also possible that the finding of the study may contribute to developing new approaches for treating mitochondrial diseases. Earlier, Sarma and Mattaparthi (2017) through their molecular dynamic simulation studies showed the phosphorylation mediated conformational changes in Lemur Tyrosine Kinase (LMTK3) an oncogenic kinase and suggested its

possible effects on the substrates leading to breast cancer. The present research study reveals the first comprehensive analysis and evidence for the phosphorylation of a recombinant human Tim23 and paves the path for further in-depth studies to be conducted through site-directed mutagenesis. Understanding the *in-vitro* phosphorylation of human Tim23 will require further studies (*in vivo)* to confirm the kinases and their targeting sites.

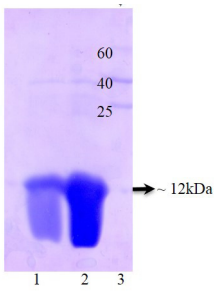

Fig. 12. Analysis of purified *r*human Tim23 fragment 2 by SDS-PAGE (15%). Protein Lane 1 and 2 indicates fragment 2 purified fractions 1 and 2 respectively and lane 3: protein marker.

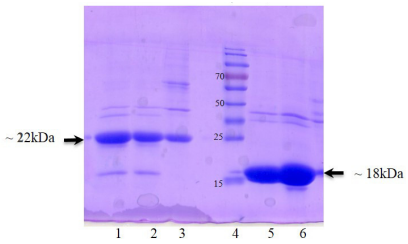

Fig. 13. Analysis of purified *r*human Tim23 fragment 3 and 4 by SDS-PAGE (12%)**.** Lane 1, 2 and 3: indicates purified fractions of fragment 4; Lane 4: protein marker; Lane 5 and 6: purified fractions of fragment 3.

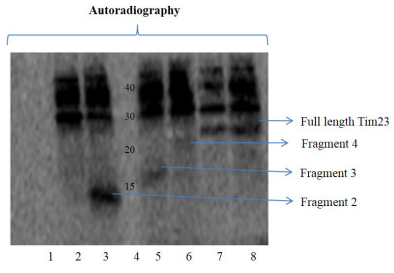

Fig. 14. Autoradiography profile after *in-vitro* phosphorylation of *r*human Tim23 (FL) and fragments. Lane 1: Mitochondrial extract alone; Lane 2: mitochondrial extract (5 µg) with ^{32}P ATP; Lane 3: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 2; Lane 4: marker; Lane 5: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 3; Lane 6: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 4; Lane 7: GRIM-19 with mitochondrial extract and labeled ^{32}P ATP; Lane 8: mitochondrial extract (5 µg) with ^{32}P ATP and *r* human Tim23 (FL).

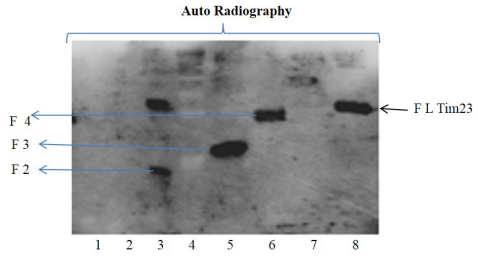

Fig. 15. Autoradiography profile of full length *r*human Tim23 and its fragments after pulled down assay. Lane 1: mitochondrial extract alone; Lane2: mitochondrial extract (5 µg) with radio labeled ^{32}P ATP; Lane 3: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 2: Lane 4: marker; Lane 5: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 3; Lane 6: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 4; Lane 7: GRIM-19 with mitochondrial extract and labeled ^{32}P ATP; Lane 8: mitochondrial extract (5 µg) with ^{32}P ATP and full length *r*human Tim23.

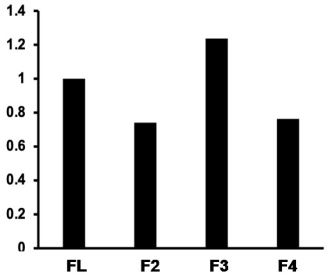

Fig. 16. Comparative analysis of full-length (FL) *r*human Tim23 phosphorylation with its fragments (F2, F3 and F4).

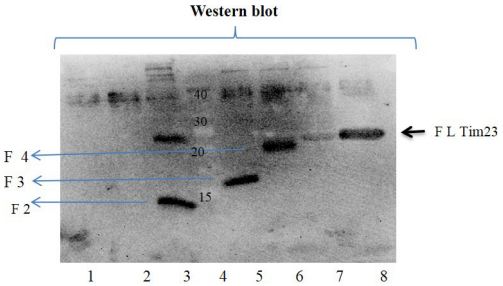

Fig. 17. Immunoblotting after *in-vitro* phosphorylation of full-length human Tim23 and its fragments. Lane 1: mitochondrial extract alone; Lane 2: Mitochondrial extract (5 µg) with radio labeled ^{32}P ATP; Lane 3: mitochondrial extract (5 µg) with labeled ^{32}P ATP and *r*human Tim23 fragment 2; Lane 4: marker; Lane 5: mitochondrial extract (5µg) with labeled ^{32}P ATP and rHuman Tim23 fragment 3. Lane 6; mitochondrial extract (5µg) with labeled ^{32}P ATP and rHuman Tim23 fragment 4. Lane 7; Recombinant GRIM-19 with mitochondrial extract and labeled ^{32}P ATP. Lane 8; mitochondrial extract (5µg) with ^{32}P ATP and full length rHuman Tim23.

CONCLUSIONS

The Autoradiography studies using radiolabelled ($\gamma^{32}P$) ATP showed the phosphorylation of full-length human Tim23 and confirmed by western blotting. The amplification of these fragments was evaluated on electrophoresis containing agarose gel (1%). Following digestion (double) with restriction enzymes and agarose gel (1%) electrophoresis revealed the particular insertions of the TIM23 fragments that had been cloned and inserted into the bacterial pET28a vector. Tim23 fragments (f2, f3, and f4) were induced and over expressed in BL21 (DE3) cells by IPTG. The over-expression of proteins was evaluated on SDS-PAGE. The presence of a single distinct band indicated that the *r* human Tim23 fragments have been purified using affinity chromatography (Ni-NTA), and this has been validated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. The *in-vitro* phosphorylation of recombinant human Tim23 fragments, as well as the full length of the protein, was shown by autoradiography and verified by immunoblotting using an antibody specific to Tim23. Overall, the present study reports the *in-vitro* phosphorylation of full-length human Tim23 and its fragments.

FUTURE SCOPE

The mitochondrial Tim23 is an important protein channel. The present study on *in-vitro* phosphorylation of Tim23 could give tremendous scope to study its mechanism in mitochondrial biogenesis. It also provides directions to target mitochondria in disease-related conditions. Further, the outcome of the study is encouraging and can be extended to investigate the *in vivo* phosphorylation of Tim23 and the detailed mechanism in the mitochondria of human cells. The specific site and residues involved in Tim23 phosphorylation are also needed to be identified.

Author's contribution. CSA carried out all the experimentation, acquisition, and analysis of data and drafting of the manuscript. MZG assisted with the analysis and drafting of the manuscript. KR conceived, designed, and supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

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Conflict of Interest. None.

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