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Phosphorylation of Tudor-SN, a novel substrate of JNK, is involved in the efficient recruitment of Tudor-SN into stress granules

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ABSTRACT

Posttranslational modifications of certain stress granule (SG) proteins are closely related to the assembly of SGs, a type of cytoplasmic foci structure. Our previous studies revealed that the Tudor staphylococcal nuclease (Tudor-SN) protein participates in the formation of SGs. However, the functional significance of potential Tudor-SN modifications during stress has not been reported. In this study, we demonstrated that the Tudor-SN protein was phosphorylated at threonine 103 (T103) upon stimulation with arsenite. In addition, c-Jun N-terminal kinase (JNK) was found to be responsible for Tudor-SN phosphorylation at the T103 site. We further illustrated that either a T103A mutation or the suppression of phosphorylation of T103 by the JNK inhibitor SP600125 inhibited the efficient recruitment of Tudor-SN into SGs. In addition, the T103A mutation could affect the physical binding of Tudor-SN with the G3BP (Ras-GAP SH3 domain-binding protein) protein but not with the HuR (Hu antigen R) protein and *AGTR1-3′UTR* (3′-untranslated region of angiotensin II receptor, type 1) mRNA cargo. These data suggested that JNK-enhanced Tudor-SN phosphorylation promotes the interaction between Tudor-SN and G3BP and facilitates the efficient recruitment of Tudor-SN into SGs under conditions of sodium arsenite-induced oxidative stress. This finding provides novel insights into the physiological function of Tudor-SN modification.

1. Introduction

In response to environmental stress (e.g., oxidative stress, heat shock, UV irradiation, viral infection or hyperosmotic stress), eukaryotic cells often shut down translation initiation, leading to the formation of cytoplasmic RNA foci, which are known as stress granules [1]. SGs contain non-translating mRNAs and stalled pre-initiation complexes, as well as some RNA-binding proteins, and are proposed to regulate the translation efficiency and stability of mRNA under stress conditions [1,2]. SG assembly is considered to be a consequence of signaling cascades that are activated by environmental stress [3–5]. A crucial factor among the signaling cascades is posttranslational protein modification (particularly phosphorylation), which efficiently regulates the primary aggregation or dynamic assembly of SGs [3–5]. For example, one or more of the serine/threonine kinases, such as protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2) and heme-regulated inhibitor kinase (HRI), can induce the phosphorylation of eukaryotic translation initiation factor 2 alpha

Abbreviations: SG, stress granule; Tudor-SN, Tudor staphylococcal nuclease; JNK, c-Jun N-terminal kinase; PKR, protein kinase R; PERK, PKR-like endoplasmic reticulum kinase; GCN2, general control nonderepressible 2; HRI, heme-regulated inhibitor kinase; eIF2α, eukaryotic translation initiation factor 2 alpha; eIF4E, eukaryotic translation initiation factor 4E; mTORC1, mammalian target of rapamycin complex 1; SND1, staphylococcal nuclease domain containing 1; *AGTR1-3′UTR*, 3′-untranslated region of angiotensin II receptor, type 1; PP1, protein phosphatase 1; λ-PPase, λ-protein phosphatase; KLH, keyhole limpet hemocyanin; MAPK, mitogen activated protein kinase; CDKs, cyclin-dependent protein kinases; G3BP, Ras-GAP SH3 domain-binding protein; HuR, Hu antigen R; TTP, tristetraprolin; CDC2, cell division control 2; TCLs, Total cell lysates; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IF, Immunofluorescence; RIP, RNA-binding protein immunoprecipitation; SE, standard error; ANOVA, one-way analysis of variance.

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(eIF2α) in distinct types of environmental stress [3,4]. The phosphorylation of eIF2α inhibits protein translation by reducing the concentration of the eIF2-GTP-tRNAi^{Met} ternary complex and then initiates the formation of SGs [6]. In addition, SG formation can also be induced by the inactivation of the eukaryotic translation initiation factor 4E (eIF4E), which is mediated by the phosphorylation of 4EBP1, a key inhibitor known to prevent the formation of mammalian target of rapamycin complex 1 (mTORC1)-dependent eIF4E-eIF4G interactions [7]. Apart from phosphorylation, modifications of proteins including acetylation, ubiquitination and methylation also affect SG formation through different mechanisms [8,9].

The Tudor staphylococcal nuclease (Tudor-SN) protein, also known as SND1 (staphylococcal nuclease domain containing 1) or p100, is a kind of multifunctional protein that is implicated in a variety of cellular processes, such as gene transcription, adipogenesis, pre-mRNA splicing, breast cancer metastasis, cell cycle and stress granule assembly [10–15]. Our previous studies revealed that Tudor-SN, a crucial component of SGs, efficiently associates with G3BP in SGs via the SN domain under different stress conditions [15]. Tudor-SN is also important for the aggregation of *AGTR1-3′UTR* (*3′-untranslated region of angiotensin II receptor, type 1*) mRNA granules [16]. Moreover, Tudor-SN was identified as a novel poly(A)$^+$ mRNA binding protein, which regulates the dynamic nature of G3BP-positive SGs and the stabilization of SG-associated mRNAs during cellular stress [17]. Here, we further investigate the molecular mechanism of Tudor-SN-containing SG assembly in terms of Tudor-SN protein phosphorylation.

In the present study, Tudor-SN was identified as a novel c-Jun N-terminal kinase (JNK) target. Tudor-SN can be phosphorylated by JNK at residue T103 in response to sodium arsenite-induced oxidative stress. In addition, the phosphorylation of T103 facilitates the efficient aggregation of Tudor-SN into SGs by promoting the binding of Tudor-SN with G3BP.

2. Materials and methods

2.1. Cell culture, plasmids and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The sodium arsenite (Sigma Aldrich, Aldrich, St Louis, MO, USA), JNK inhibitor SP600125 (S146001, Selleck Chemicals), protein phosphatase 1 (PP1, P0754, New England Biolabs) or λ-protein phosphatase (λ-PPase, P0753, New England Bio-labs) were used to treat the cells or the total cell lysates (TCLs). A HeLa stable cell line with Tudor-SN-Knockout (Tudor-SN$^{-/-}$ HeLa) was generated via a modified CRISPR/Cas9 double-nicking gene editing system [18,19]. Briefly, a pair of A and B sgRNAs (A: 5′-CACCGAG GTTGATCTGCCGCTCAGG-3′; 3′-CTCCAAC TAGACGGCGAGTCCCAAA-5′; B: 5′-CACC GTGCTGGAAATCTT GCTCGC-3′; 3′-CACGA CCTTTAGAACGAGCGCAAA-5′) that could specifically identify the upstream and downstream sequence within exon 2 of the *Tudor-SN* gene were designed and synthesized. Two recombinant eukaryotic expressional plasmids (pX462-Tudor-SN-sgRNA-A, pX462-Tudor-SN-sgRNA-B) were constructed from the pX462 carrier vector (pX462-Tudor-SN-sgRNA-A, pX462- Tudor-SN-sgRNA-B). After enzyme digestion with BbsI (FD1014, Fermentas) and gene sequencing, the two recombinant plasmids were co-transfected into HeLa cells. Puromycin was then used to screen positive cells and prepare the monoclonal Tudor-SN$^{-/-}$ HeLa cell line. The plasmids encoding RFP-tagged wild-type Tudor-SN (RFP-Tudor-SN^{wt}) or FLAG-tagged wild type Tudor-SN (FLAG-Tudor-SN^{wt}) were generated as described previously [15]. The FLAG-Tudor-SN^{T103A} and RFP-Tudor-SN^{T103A} mutant plasmids were constructed by the GENEWIZ Company (China). The GFP-G3BP plasmid was kindly provided by Dr. Jamal Tazi (Montpellier, France). Plasmids were transfected into HeLa cells using Lipofectamine 2000 transfection reagent (11668-019, Invitrogen), according to the manufacturer's protocols.

2.2. Antibody preparation

Rabbit polyclonal antibodies against pT73 or pT103 were produced by BEIJING B&M BIOTECH CO., LTD (China). Briefly, an MBL antigen retrieval system was utilized to predict the secondary structure features of the Tudor-SN region around the T73 and T103 site, including accessibility, flexibility, surface probability, antigenicity, hydrophilicity and dipole moment. Then, the unphosphorylated and phosphorylated polypeptides, including "PDAKDpTPDEPC" for T73 and "TIENKpTPQGRC" for T103, were designed and synthesized. The C-terminal C (cysteine) was used to efficiently bind the carrier protein, keyhole limpet hemocyanin (KLH). Antiserums were collected from New Zealand White rabbits immunized with these polypeptides four times and purified through the AKTA protein purification system (GE Healthcare Life Sciences).

2.3. LI-COR odyssey infrared imaging system

HeLa cells were untreated or treated with 0.5 mM sodium arsenite for 1 h. Total cell lysates (TCLs) of HeLa cells were collected and separated by SDS-PAGE. The PVDF membranes (Roche) were incubated with the rabbit polyclonal anti-Tudor-SN antibody (1:500 dilution, ab65078, Abcam) and mouse monoclonal anti-β-actin antibody (1:3000, A1978, Sigma Aldrich) in 10 ml LI-COR blocking buffer with gentle agitation overnight at 4 °C. The next day, the membranes were washed three times for 10 min each with 15 ml Tri-buffered saline containing 0.1% Tween (TBS-T) before addition of the secondary antibodies conjugated to a fluorescent entity, IRDye® 800CW donkey anti-Mouse IgG (H + L) (1:15000, 926-32212) and IRDye® 680 donkey anti-rabbit IgG (H + L) (1:15000, 926-32223), for 1 h at room temperature. After three washes with TBS-T, the membrane was visualized and analyzed by Odyssey IR imaging system (LI-COR Biosciences).

For the total phosphorylation of Tudor-SN, TCLs were incubated with the mouse monoclonal anti-Tudor-SN antibody bound to protein G/A agarose (20421, Pierce) overnight at 4 °C with head-over-tail rotation. The immunoprecipitated Tudor-SN proteins were separated by SDS-PAGE and blotted with the rabbit anti-Tudor-SN antibody (1:500, ab65078, Abcam) and mouse monoclonal anti-p-Thr (phospho-threonine) (1:1000, #9386, Cell Signaling Technology), mouse monoclonal anti-p-Tyr (phospho-tyrosine) (1:1000, #9411, Cell Signaling Technology), or mouse monoclonal anti-p-Ser (phospho-serine) antibody (1:1000, ab17465, Abcam). The mouse monoclonal anti-Tudor-SN antibody was used as described previously [15].

For eIF2α phosphorylation, membranes were incubated simultaneously with rabbit monoclonal anti-phosphor-eIF2α (Ser51) (1:1000, #3597, Cell Signaling Technology) and mouse monoclonal anti-eIF2α antibody (1:1000, #2103, Cell Signaling Technology). For T73 or T103 phosphorylation of Tudor-SN, membranes were incubated simultaneously with the mouse anti-Tudor-SN antibody (1:100, sc-271590, Santa Cruz Biotechnology) and rabbit anti-pT73 or anti-pT103 antibody (1:1000).

2.4. Western blotting and antibodies

A western blotting assay was performed as previously described [14]. The following antibodies were used: rabbit polyclonal anti-pT73 or anti-pT103 (1:1000), mouse monoclonal anti-Tudor-SN (1:5), anti-p-JNK (1:1000, #9251, Cell Signaling Technology), anti-JNK (1:1000, #9252, Cell Signaling Technology), anti-p-p38 (1:1000, #4511, Cell Signaling Technology), anti-p38 (1:1000, #8690, Cell Signaling Technology), anti-ERK (1:1000, #9102, Cell Signaling Technology), anti-p-ERK (1:1000, #9101, Cell Signaling Technology), anti-mTOR (1:1000, #2972, Cell Signaling Technology), anti-p-mTOR (1:1000, #2971, Cell Signaling Technology), and mouse monoclonal anti-β-actin antibody (1:3000). The gray scale value of the band was measured using Image J 2X software (NIMH, Bethesda, MD, USA).