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Tudor-SN interacts with and co-localizes with G3BP in stress granules under stress conditions

Xingjie Gao [a,b,c,d,1], Lin Ge [a,b,c,1], Jie Shao [a,b,c,d,e], Chao Su [a,b,c], Hong Zhao [a,b,c,d], Juha Saarikettu [e], Xuyang Yao [a,b,c], Zhi Yao [a,b,c,*], Olli Silvennoinen [e,f], Jie Yang [a,b,c,d,*]

[a] Department of Immunology, Basic Medical College, Tianjin Medical University, PR China
[b] Tianjin Key Laboratory of Cellular and Molecular Immunology, PR China
[c] Key Laboratory of Educational Ministry of China, Heping District Qixiangtai Road No. 22, Tianjin 300070, PR China
[d] Laboratory of Molecular Immunology, Research Center of Basic Medical Science, Tianjin Medical University, PR China
[e] Institute of Medical Technology, University of Tampere, Biokatu 8, FI-33014 Tampere, Finland
[f] Department of Clinical Microbiology, Tampere University Hospital, FI-33520 Tampere, Finland

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ABSTRACT

SGs are mRNA containing cytoplasmic structures that are assembled in response to stress. Tudor-SN protein is a ubiquitously expressed protein. Here, Tudor-SN protein was found to physiologically interact with G3BP, which is the marker and effector of SG. The kinetics of the assembly of SGs in the living cells demonstrated that Tudor-SN co-localizes with G3BP and is recruited to the same SGs in response to different stress stimuli. Knockdown of endogenous Tudor-SN did not inhibit the formation of SGs, but retarded the aggregation of small SGs into large SGs. Thus Tudor-SN may not be an initiator as essential as G3BP for the formation of SGs, but affects the aggregation of SGs. These findings identify Tudor-SN as a novel component of SGs.

Structured summary:
MINT-7968768, MINT-7968779: *Tudor-SN* (uniprotkb:Q7KZF4) *physically interacts* (MI:0915) *with G3BP* (uniprotkb:Q13283) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7968800: *Tudor-SN* (uniprotkb:Q7KZF4) and *TIA-1* (uniprotkb:P31483) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7968789: *Tudor-SN* (uniprotkb:Q7KZF4) and *G3BP* (uniprotkb:Q13283) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

Stress granules (SGs) are dynamic dense structures that are rapidly formed in the cytosol in response to a variety of environmental stress stimuli. Stress conditions induce extensive reprogramming in mRNA metabolism including induction of transcription and translation of specific genes to repair stress-induced damage and adapt to changed conditions. As a consequence, many other genes are silenced via the recruitment of mRNA into SG that stalled with translation pre-initiation complexes [1]. Once the stress condition is released, the SGs are disassembled, and mRNAs are repacked into translationally competent mRNPs and proteins are synthesized.

Several components of SGs have been identified, but their composition is still only partially known. SGs are composed of mRNAs in conjunction with a subset of translation initiation factors, including eIF2, eIF2B, eIF4E, the 40S ribosomal subunit, as well as RNA binding proteins. Notable RNA-binding proteins in SGs include TIA-1 [2], and G3BP [3], all of which have self-interaction domains that can contribute to SGs formation. In addition to these core components, SGs contain an eclectic number of proteins, for example deacetylase [4], RNA helicases [5], hnRNP [6], and vary depending on the cell type [7] or duration of the stress signal [8].

Tudor-SN protein was first identified as a coactivator of EBNA2 (Epstein-Barr virus nuclear protein 2) [9], and subsequently discovered as coregulator of pim-1 [10], STAT6 transcription factor in IL-4 mediated gene regulation [11,12], and STAT5 in prolactin (PRL) signaling [13]. It was also copurified with U5 snRNP complex and promote spliceosome assembly in vitro [14]. These studies suggest that Tudor-SN protein participates in several biological responses and may play distinct roles in various cellular events. Interestingly,

* Corresponding authors at: Department of Immunology, Basic Medical College, Tianjin Medical University, PR China. Fax: +86 22 23542581.
E-mail addresses: yaozhi@tmu.cn (Z. Yao), yangj@tijmu.edu.cn (J. Yang).
[1] These authors contribute equally to the paper.

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Tudor-SN is an integral part of RISC (RNA-induced silencing complex) [15], and could recognize hyper-edited double-stranded RNAs (I-dsRNAs) [16], while I-dsRNA molecules specifically binds a complex which comprises many SG components, including G3BP, TIA-1 [17]. Very recently, Tudor-SN was identified as an essential protein for RNA stability and stress tolerance in plants [18]. In our previous study, we identified G3BP as an interaction protein of Tudor-SN in the GST-pull down assay and MOLDI-TOF analysis, which encouraged us to investigate whether Tudor-SN is directly involved in SGs.

2. Materials and methods

2.1. Cells and plasmids

COS-7 cells and HeLa cells were cultured as described previously [12]. COS-7 cells were transfected by electroporation at 220 V/950 mF with a Bio-Rad gene pulser. The transfection of HeLa cells were performed using FuGENE transfection reagent (Roche, Indianapolis) according to the manufacturers' procedures.

Plasmids encoding GFP epitope-tagged G3BP (GFP-G3BP) was kindly provided by Dr. Jamal Tazi. The pSG5 expression plasmids containing full-length Tudor-SN tagged with Flag epitope (pSG5-Tudor-SN), the pGEXT-4T-1 plasmids containing SN domain (GST-SN, 1-639aa), TSN domain (GST-TSN, 640–885aa) or Tudor domain (678–769aa, GST-TD) of Tudor-SN protein were generated as previously described [11,12]. The full-length Tudor-SN (pRFP-Tudor-SN), SN (pRFP-SN, 1–639aa) or TSN (pRFP-TSN, 640–885aa) fragment was cloned and inserted into the EcoRI/XhoI sites of the vector pCherry-C1which was kindly provided by Dr. Johan Peranen. All PCR products were sequenced.

2.2. GST-pull down assay

GST (glutathione S-transferase) pull down experiments were performed as previously described [12]. The beads-bound GST fusion proteins were incubated with the total cell lysate of transfected COS7 cells or in vitro translated ^{35}S-labeled G3BP protein. After washing, the bound proteins were separated by SDS–PAGE and analyzed by immunoblotting with mouse monoclonal anti-GFP antibody (Sigma, St. Louis, MO, USA) or autoradiography.

The cell-free in vitro translation of full-length G3BP was carried out in a nuclease-treated rabbit reticulocyte lysate (RRL) system (Promega BioSciences, CA, USA) according to the manufacturer's recommendations. The proteins were labeled with L-[^{35}S]-methionine (Amersham Biosciences, USA).

2.3. Co-immunoprecipitation

The total cell lysates of HeLa cells without stress stimuli were collected with Nonidet P-40 lysis buffer (50 mM Tris–HCl, pH 7.6, 300 mM NaCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 20% glycerol, 0.1 mM sodium orthovanadate, 1 mM sodium butyrate), and then incubated with mouse monoclonal anti-Tudor-SN or anti-G3BP (Abcam, Cambridge, UK), as well as rabbit polyclonal IgG (Santa Cruz biotechnology) as control, followed by incubation with protein-G/A-Sepharose (Amersham Pharmacia Biotech). The bound proteins were analyzed by SDS–PAGE and blotted with anti-Tudor-SN or anti-G3BP antibody. The mouse monoclonal anti-Tudor-SN antibody was generated against SN4 domain (amino acids 507–674) of Tudor-SN in Dr. Silvennoinen's lab. The rabbit polyclonal anti-Tudor-SN antibody was generated against TSN domain (amino acids 640–885) of Tudor-SN in our lab.

2.4. Immunofluorescence and confocal microscopy

Cells were grown on glass cover slips. Cellular stress was induced either by treatment with 0.5 mM sodium arsenite (Sigma–Aldrich, St. Louis, MO, USA), or by incubation at 45 °C for different time point (Heat shock). Control cells and treated cells were fixed and permeabilized, and then incubated with mouse monoclonal anti-G3BP and rabbit polyclonal anti-Tudor-SN antibodies, or mouse monoclonal anti-Tudor-SN and rabbit polyclonal anti-TIA-1 (Santa Cruz biotechnology). After washing, cells were incubated with anti-mouse Alexa fluor 488 (Invitrogen) and anti-rabbit Texas-red (Molecular probes, Eugene, Oregan USA) conjugated secondary antibodies. Confocal images were collected using LSM5 program and Zeiss confocal microscope, equipped with an Argon laser (488 nm) and HeNe laser (543 nm) and a ×63 objective. Green emission was detected using a 505-nm low pass filter and red emission using a 630-nm low pass filter [11]. Approximately 200 cells were scored per experiment independently by two different individuals.

For living cell imaging, HeLa cells were transfected with GFP-tagged G3BP, and RFP-Tudor-SN, RFP-SN or RFP-TSN, respectively by using FuGENE transfection reagent according to the manufacturers' procedures. As a control, HeLa cells were transfected with empty vector pEGFP-C1 and p Cherry-C1. After 24 h, the cells were seeded onto glass-bottom dishes (Mat-Tek, Ashland, MA) and cultured overnight. Before observation, the cells with 2 ml culture medium were maintained in a chamber system at 37 °C and 5% CO_2. The images of timed series were acquired as described above.

HeLa cells were transfected with Tudor-SN siRNA or scramble siRNA according to previously described [11]. After 72 h, the cells were seeded onto glass-bottom dishes and cultured overnight. After heat shock at 45 °C for 60 min or treated with 0.5 mM sodium arsenite for 45 min, confocal images were obtained as described above.

2.5. Cell proliferation assay

Cell proliferation was measured with MTS assay. Briefly, cells were plated in 96-well plates at a density of 2×10^3 per well and incubated for 24 h or 48 h, and then the cells were incubated with 20 μl of MTS solution (Promega) for 4 h at 37 °C. The absorbance was measured at 490 nm using ELISA microplate reader Multiskan (Thermo Labsystems).

3. Results

3.1. Tudor-SN interacts with G3BP in vivo and in vitro

Tudor-SN is a multi functional protein composed of four repeats of SN and a Tudor domain followed by a SN5 domain (Fig. 1A). We initially performed GST-pull down assay to verify the interaction of Tudor-SN and G3BP. GST and different GST fusion proteins were bound to glutathione-coupled beads (Fig. 1B) and incubated with total cell lysates of COS7 cells transfected with GFP-G3BP. As shown in Fig. 1C, neither Tudor nor TSN domain associated with G3BP, but the SN domain readily precipitated the GFP-G3BP protein. The beads-bound different GST fusion proteins were also incubated with in vitro translated ^{35}S-labelled full-length G3BP protein. As shown in Fig. 1D, in vitro translated G3BP was found to interact with the GST-SN fusion protein, but not the others. These results indicate that the SN domain of Tudor-SN interacts with G3BP.

To substantiate the in vivo interaction of Tudor-SN and G3BP, the co-immunoprecipitation experiment was performed with endogenous proteins of HeLa cells. As shown in Fig. 2A, G3BP only