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Research paper

Positive regulatory role of c-Src-mediated TRIM25 tyrosine phosphorylation on RIG-I ubiquitination and RIG-I-mediated antiviral signaling pathway

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ABSTRACT

Retinoic acid-inducible gene I (RIG-I) detects viral RNAs and induces antiviral responses. During viral RNA recognition by RIG-I, tripartite motif protein 25 (TRIM25) plays a critical regulatory role by inducing K63-linked RIG-I polyubiquitination. Previous proteomics analysis revealed several phosphorylation sites on TRIM25, including tyrosine 278 (Y278), yet the roles of these modifications remain elusive. Here, we demonstrated that TRIM25 interacted with c-Src and underwent tyrosine phosphorylation by c-Src kinase upon viral infection and the phosphorylation is required for the complete activation of RIG-I signaling. Analysis using a c-Src inhibitor and TRIM25 mutant, in which tyrosine 278 is substituted by phenylalanine (Y278F), suggested that the phosphorylation positively regulates K63-linked polyubiquitination of RIG-I and subsequent antiviral signaling. The TRIM25 Y278F mutant displayed decreased E3-ubiquitin ligase activity *in vitro*, suggesting that this phosphorylation event affects the E3-ligase activity of TRIM25. Thus, we provide a molecular mechanism of c-Src-mediated positive regulation of RIG-I signaling.

1. Introduction

Several innate immune sensors detect viral infection and trigger antiviral immune responses including type I interferon production. Among these sensors, retinoic acid inducible gene-I (RIG-I) recognizes viral RNA in the cytoplasm and induces type-I interferon production to restrict viral replication [1]. Upon recognition of viral RNA, RIG-I translocates into the mitochondria and interacts with mitochondrial adaptor protein MAVS (VISA/IPS-1/Cardif) [2–4]. Activated MAVS recruits several signaling molecules such as TRAF6, TRAF3, TBK1, and the IKK complex leading to the activation of NF-κB, IRF-3, and AP-1 and subsequent production of interferon [4–6].

Like other signaling pathways, the RIG-I-mediated antiviral signaling pathway is modulated by various post-translational modifications such as the ubiquitination and phosphorylation of key signaling molecules [7,8]. For instance, several E3-ubiquitin ligases such as tripartite motif protein 25 (TRIM25) and Riplet activate RIG-I by conjugating a K63-linked polyubiquitin chain onto RIG-I [9–11]. TRIM25-mediated polyubiquitination of K172 on RIG-I or the non-covalent interaction of a K63-linked polyubiquitin chain with RIG-I promotes oligomerization of RIG-I and its subsequent interaction with MAVS [9,12,13]. Cells also utilize various enzymes such as linear ubiquitin assembly complex (LUBAC), CYLD, and USP3 to negatively regulate ubiquitination by inhibiting the ubiquitination process or eliminating the ubiquitin chain from RIG-I. This suggests that ubiquitination is one of the key regulatory steps in RIG-I signaling [14–16]. Phosphorylation also plays critical regulatory roles in the RIG-I signaling pathway. For instance, the phosphorylation of S8, T170, and T770 residues of RIG-I by protein kinase C α/β (PKC α/β) and casein kinase II (CKII) negatively regulates RIG-I signaling by keeping RIG-I in a repressed state [17–19]. c-Src tyrosine kinase is also reported to be required for efficient RIG-I-mediated antiviral signaling and it interacts with proteins participating in RIG-I signaling such as RIG-I, MAVS, and TRAF3, although the underlying molecular mechanism is not clear [20].

Several phosphorylation sites on TRIM25 were identified from studies using large-scale proteomic approaches, suggesting that the activity of TRIM25 is also regulated by phosphorylation [21,22]. To broaden our understanding of RIG-I-mediated antiviral signaling, this study explored the role of tyrosine phosphorylation on TRIM25 activity.

2. Material and methods

2.1. Cells and viruses

HEK293T and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented

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with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). SYF (ATCC CRL-2459), a murine embryonic fibroblast cell line deficient in c-Src, yes, and fyn, and the SYF-cSrc (ATCC CRL-2489) cell line were purchased from ATCC and maintained in DMEM supplemented with 10% FBS and antibiotics (penicillin and streptomycin). A TRIM25 knockout mouse embryonic fibroblast cell line and vero cells were maintained in DMEM supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Sendai virus (SeV; Cantell strain) was purchased from Charles River. Enhanced green fluorescence protein (eGFP)-fused vesicular stomatitis virus (VSV-eGFP) was described previously [9].

2.2. Plasmids and reagents

pEBG-RIG-I-2Cards (GST-RIG-IN), pIRES-V5-TRIM25, pIRES-HA-TRIM25, HA-Ub, pIRES-V5-TRIM25-ΔRING, V5-TRIM25-ΔCCD, V5-TRIM25-ΔSPRY, and V5-TRIM25-SPRY plasmids were described previously [23,24]. The GFP-c-Src plasmid was kindly provided by Dr. Jihye Seong (KIST, Korea). pcDNA3 c-Src (Addgene, #42202), pCMV6-Myc-fyn (Origene, RC224691), and pCMV6-Myc-Lck (Origene, RC219430) were purchased from the indicated manufacturers.

Protein phosphatase 2 (PP2) was purchased from Sigma-Aldrich and Lipofectamine 2000 was purchased from Thermo Fisher Scientific. PolyI:C LMW and 5′triphosphate double stranded RNA were purchased from Invivogen.

2.3. TRIM25 mutagenesis and generation of mutant MEF cell lines

Mutation of TRIM25 Y278F was performed by conventional combination PCR. The PCR product was cloned into an *XbaI/XhoI*-cleaved pIRES-V5 vector as was performed for wildtype (WT) TRIM25. TRIM25 knock-out (KO) MEFs were transfected with vector, WT, or Y278F mutant plasmids and selected by cultivation with puromycin (1 μg/ml) to establish stable cell lines.

2.4. Co-immunoprecipitation (co-IP) and immunoblotting (IB)

HEK293T cells or SYF/SYF-c-Src cells were infected with SeV for the indicated hours or transfected with the indicated plasmids and incubated for 36 h. Cells were then lysed using Triton X-100 lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) containing a protease inhibitor cocktail (Pierce). After centrifugation, supernatants were incubated with the indicated antibodies overnight at 4 °C. Mixtures were further incubated with protein A/G beads for 2–4 h. After washing with lysis buffer, bound proteins were eluted using 1X sample buffer (50 mM Tris pH6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.02% bromophenol blue). Cell lysates or co-IP samples were subjected to SDS-PAGE and immunoblotting using the indicated antibodies. Anti-p-tyrosine, anti-HA, anti-Myc, anti-c-Src, anti-GFP, anti-Ub, anti-K63 Ub, and anti-V5 antibodies were purchased from Cell Signaling Technology. The anti-TRIM25 (Merck Millipore), and anti-GST (Abcam) antibodies were purchased from the indicated manufacturers.

2.5. Luciferase assay

HEK293T cells were transfected with GST-RIG-IN and TRIM25 plasmids as indicated. IFN-β promoter firefly luciferase or NF-κB promoter firefly luciferase were co-transfected with the mentioned plasmids. Thymidine kinase (TK) Renilla luciferase reporter plasmids were also co-transfected to normalize transfection efficiency. After 36 h, cells were lysed and subjected to a dual-luciferase assay (Promega) according to the manufacturer's instructions.

2.6. Virus infection and plaque assay

TRIM25 KO MEFs complemented with pIRES V5 vector, pIRES V5 TRIM25 WT, or pIRES V5 TRIM25 Y278F plasmid were infected with VSV-eGFP virus (multiplicity of infection [m.o.i.] = 0.1) for 12 h and the supernatants were harvested to determine the viral titers. Virus titer was determined by plaque assay. Briefly, vero cells were incubated with serially diluted supernatants containing VSV-eGFP for 2 h, then supernatants were discarded. MEM (2X) and 1.8% methylcellulose semisolid media were added to cells and further incubated for 48 h. Then, media were removed by aspiration followed by staining with crystal violet containing 20% EtOH. Virus plaques were counted and virus titer was calculated.

2.7. Enzyme-linked immunosorbent assay (ELISA)

TRIM25 WT, KO or mutant MEFs were infected with SeV for 12 or 18 h. Levels of IFN-β and interleukin-6 (IL-6) in the supernatants were determined by ELISA using BD OptEIA ELISA kits (BD biosciences) according to the manufacturer's protocol.

2.8. In vitro ubiquitination assay

HEK293T cells were transfected with vector, pIRES-V5-TRIM25-WT, or TRIM25-YF plasmids and incubated for 36 h at 37 °C. Cells were then harvested and lysed using 0.5% Triton X-100 lysis buffer. Cell lysates were incubated with V5 beads for 12 h at 4 °C and then the beads were washed 5 times with the Triton X-100 lysis buffer. The equal amounts of beads were used as E3-ligase for the *in vitro* ubiquitination assay. The *in vitro* ubiquitination assay was conducted using the Human Ubiquitination Conjugation Initiation kit (Boston Biochem). The same amount of beads was incubated with E1, E2 enzyme, ubiquitin, and 10X buffer for 2 h at 37 °C according to the manufacturer's instructions. The reactions were terminated by adding 10 mM EDTA. The synthesis of ubiquitin chains was analyzed by IB.

2.9. RNA isolation and quantitative real-time reverse-transcription-polymerase chain reaction (RT-qPCR)

Expression of genes encoding IFN-β and ISG15 was analyzed by RT-PCR using a CFX Connect (Bio-Rad) real-time PCR system. Total RNAs were isolated from harvested cells using an RNA isolation kit (GeneAll) according to the manufacturer's instructions. After quantitative analysis of total RNA, reverse transcription polymerase chain reaction (RT-PCR) was performed using an RT kit (Enzynomics). Quantitative PCR was performed using 2 μl synthesized cDNA as a template. IFN-β mRNA was amplified using the primer pair 5-AAGAGTTACACTGCCTTTGCCATC-3 (forward) and 5-CACTGTCTGCTGGTGGAGTTCATC-3 (reverse). ISG15 was amplified using the primer pair 5-CCTCTGAGCATCCTGGT-3 (forward) and 5-AGGCCGTACTCCCCCAG-3 (reverse). IFN-β and ISG15 mRNA levels were normalized to those of β-actin, which was amplified with the primer pair 5-TGGAATCCTGTGGCATCCATGAAAC-3 (forward) and 5-TAAAACGCAGCTCAGTAACAGTCCG-3 (reverse).

3. Results

3.1. TRIM25 undergoes tyrosine phosphorylation upon viral infection

Until now, threonine 91, serine 97, serine 100, tyrosine 278 (Y278), and threonine 427 residues of TRIM25 are known to be phosphorylated (Fig. 1A). However, the function of these post-translational modifications remains unknown. The tyrosine 278 residue is conserved across various species, suggesting that it may have a critical role in protein function (Fig. 1A). To examine whether tyrosine phosphorylation of TRIM25 is related to RIG-I-mediated antiviral signaling, the tyrosine phosphorylation status of TRIM25 under viral infection was analyzed.