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The binding of TBK1 to STING requires exocytic membrane traffic from the ER

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ABSTRACT

Stimulator of interferon genes (STING) is essential for the type I interferon and pro-inflammatory responses against DNA pathogens. In response to the presence of cytosolic DNA, STING translocates from the endoplasmic reticulum (ER) to the Golgi, and activates TANK-binding kinase 1 (TBK1), a cytosolic kinase that is essential for the activation of STING-dependent downstream signalling. The organelles where TBK1 binds to STING remain unknown. Here we show that TBK1 binds to STING at the Golgi, not at the ER. Treatment with brefeldin A, an agent to block ER-to-Golgi traffic, or knockdown of Sar1, a small GTPase that regulates coat protein complex II (COP-II)-mediated ER-to-Golgi traffic, inhibited the binding of TBK1 to STING. Endogenous TBK1 was recruited to the Golgi when STING was transported to the Golgi, as shown by immunofluorescence microscopy. STING variants that constitutively induce the type I interferon response were found in patients with autoinflammatory diseases. Even these disease-causative STING variants could not bind to TBK1 when the STING variants were trapped in the ER. These results demonstrate that the Golgi is an organelle at which STING recruits and activates TBK1 for triggering the STING-dependent type I interferon response.

1. Introduction

The innate immune response is critical for efficient host defense against microbial invasion. Invading pathogens are identified by pattern recognition receptors in the host cell, which initiates a series of signalling events that leads to the production of type I interferons, proinflammatory cytokines, and other downstream antiviral proteins [1,2]. STING, also known as MITA, ERIS, MPYS, or TMEM173, is an ER-localized transmembrane protein that is essential for the innate immune response against cytosolic DNA [3–6]. Cytosolic DNA activates a DNA-binding protein cyclic GMP–AMP (cGAMP) synthase, leading to the generation of cGAMP in the cytosol [7,8]. Other cyclic dinucleotides (CDNs) including cyclic di-GMP and cyclic di-AMP are secreted by intracellular bacteria following infection [9,10]. CDNs bind to STING and activate STING to trigger the type I interferon responses [11].

Mechanistically, STING serves as a scaffold for TBK1 and the transcription factor interferon regulatory factor 3 (IRF3) [12,13]. IRF3 is phosphorylated by TBK1, after which it translocates into the nucleus to stimulate the transcription of type I interferons such as interferon β [14]. Interestingly, after the binding of STING to CDNs, STING translocates from the ER to perinuclear compartments that include the Golgi [15,16]. We have recently found that phosphorylated TBK1, the active form of TBK1, localized at the Golgi not at the ER [17]. The binding of TBK1 to STING is considered to be a critical step prior to phosphorylation of TBK1 [12,13]. However,

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where this occurs after STING binds to CDNs at the ER, remains unknown.

In this study, we found that the binding of TBK1 to STING occurred at the Golgi, not at the ER. We also provide evidence that the biding requires exocytic membrane traffic of STING from the ER. Our results show that the Golgi has a central role in activating STING-dependent type I interferon responses.

2. Methods

2.1. Reagents

The following reagents were purchased from the manufacturers as noted: 2-bromopalmitate (Wako); Doxycycline (Takara); brefeldin A and DMXAA (Sigma-Aldrich). Antibodies used in this study were as follows: mouse anti-GFP (JL-8, dilution 1:1000 for western blotting) (Clontech); mouse anti-GFP (3E6, dilution 1:500 for immunoprecipitation), Alexa 594- or 647- conjugated secondary antibodies (A21203, A21207, A31573, A11016, A21448, dilution 1:2000) (Thermo Fisher Scientific); rabbit anti-TBK1 (ab40676, dilution 1:1000 for western blotting, dilution 1:200 for immunofluorescence) (Abcam); rabbit anti-phospho-TBK1 (D52C2, dilution 1:1000), rabbit anti-phospho-IRF3 (4D4G, dilution 1:1000), rabbit anti-IRF3 (D83B9, dilution 1:1000) and rabbit anti-phospho-STING (#85375, dilution 1:1000) (Cell Signaling Technology); mouse anti-calreticulin (612136, dilution 1:200) (BD Biosciences); rabbit anti-calnexin (ADI-SPA-865, dilution 1:200) (Enzo Life Sciences); mouse anti-α-tubulin (DM1A, dilution 1:5000) (Sigma-Aldrich); sheep anti-mouse IgG antibody-HRP (NA9310V, dilution 1:4000) and donkey anti-rabbit IgG antibody-HRP (NA9340V, dilution 1:4000) (GE Healthcare); sheep anti-TGN38 (AHR499G, dilution 1:200) (Serotec); Mouse TrueBlot ULTRA rat anti-mouse IgG antibody-HRP (eB144, dilution 1:2000) and Rabbit TrueBlot mouse anti-rabbit IgG antibody-HRP (eB182, dilution 1:2000) (Rockland Immunochemicals Inc.).

2.2. Cell culture

Immortalized mouse embryonic fibroblasts (MEFs) were generated from primary MEFs obtained from embryos of *Sting*$^{-/-}$ mice at E13.5 with SV40 large T antigen (addgene #13970). MEFs were cultured in DMEM supplemented with 10% fetal bovine serum/penicillin/streptomycin/glutamine in a 5% CO_2 incubator.

Reconstituted immortalized *Sting*$^{-/-}$ MEFs with mouse STINGs were obtained using retrovirus [18]. Plat-E cells were transfected with pMXs-IRES-puro-EGFP-STING (WT, C205Y, R280Q, R283G)or pRetroX-TetOne-puro-EGFP-STING (WT, V146L, N153S, V154 M, C205Y, R280Q, R283G)and the medium that contained the retrovirus was collected. Immortalized *Sting*$^{-/-}$ MEFs were incubated with the medium and then selected with puromycin.

2.3. RNA interference

siRNA specific to Sar1A (#1: MSS276939, #2: MSS276941) and Sar1B (#1: MSS244655, #2: MSS244656) were purchased from Thermo Fisher Scientific (Cat. No. 10620318). Stealth RNAi™ siRNA Negative Control Hi GC Duplex #3 (Thermo Fisher Scientific) was used as a negative control. A total of 20 nM siRNA was introduced to cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instruction. After 6 h, the medium was replaced by DMEM with 10% fetal bovine serum/penicillin/streptomycin/glutamine and cells were further incubated for 42 h for subsequent experiments.

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 15 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min and quenched with 50 mM NH_4Cl in PBS at room temperature for 10 min. After blocking with 3% BSA in PBS, cells were incubated with primary antibodies, then with secondary antibodies conjugated with Alexa fluorophore. Confocal microscopy was performed using a TCS SP8 (Leica) with a 63 × 1.2 Plan-Apochromat oil immersion lens.

2.5. Immunoprecipitation

Cells were washed with ice-cold PBS and scraped in immunoprecipitation buffer composed of 50 mM HEPES-NaOH (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail (25955, dilution 1:100) (Nacalai Tesque) and phosphatase inhibitors (8 mM NaF, 12 mM β-glycerophosphate, 1 mM Na_3VO_4, 1.2 mM Na_2MoO_4, 5 μM cantharidin, 2 mM imidazole). The cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the resultant supernatants were pre-cleared with Pierce Protein G Plus Agarose (Thermo Fisher Scientific) at 4 °C for 30 min. The lysates were then incubated for 3 h at 4 °C with anti-GFP (3E6) and Pierce Protein G Plus Agarose beads. The beads were washed four times with immunoprecipitation wash buffer (50 mM HEPES-NaOH (pH 7.2), 150 mM NaCl, 0.1% Triton X-100) and eluted with 2 × Laemmli sample buffer. The immunoprecipitated proteins were separated with SDS–PAGE and transferred to PVDF membrane, then analyzed by western blotting.

2.6. Western blotting

Proteins were separated in polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore). These membranes were incubated with primary antibodies, followed by secondary antibodies conjugated to peroxidase. The proteins were visualized by enhanced chemiluminescence using a LAS-4000 (GE Healthcare).

2.7. Total RNA isolation and quantitative real-time PCR

Total RNA from cells was extracted using Isogen II (Nippongene) and reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA). Quantitative real-time PCR was performed using LightCycler 480 SYBR Green I Master (Roche) and LightCycler 480 (Roche Diagnostics). The sequences for the oligonucleotides were as follows. 5′-GCCAAGGTCATCCATGACAACT-3' (GAPDH; sense primer) and 5′-GAGGGGCCATCCACAGTCTT-3' (GAPDH; antisense primer); 5′-GCTGTACGGGCAGACCACAGGAAA-3' (Sar1A; sense primer) and 5′-CGTAGCCTTGCCTCTTGAGCACACT-3' (Sar1A; antisense primer); 5′-CGACCCGGAAGGAGCTGAGAAGAG-3' (Sar1B; sense primer) and 5′-TGCCCGTCCTACACGCTCCAA-3' (Sar1B; antisense primer). Target gene expression was normalized based on GAPDH content.

2.8. Statistical analysis

Error bars displayed throughout this study represent s.d. unless otherwise indicated and were calculated from triplicate samples. Statistical significance was determined with one-way analysis of variance followed by Tukey-Kramer *post hoc* test.; $^{**}P < 0.005$; $^{***}P < 0.001$. Data shown are representative of three independent experiments, including microscopy images and western blotting.

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