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Research paper

STRAP positively regulates TLR3-triggered signaling pathway

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ABSTRACT

Toll-like receptor (TLR) signaling drives the innate immune response by activating nuclear factor-κB (NF-κB) and interferon regulatory factor (IRF). We have previously shown that STRAP interacts with TAK1 and IKKα along with NF-κB subunit p65, leading to the activation of pro-inflammatory cytokines. However, the roles of STRAP in TRIF/TBK1-mediated TLR3 activation and the subsequent type I interferon (IFN) production are not fully elucidated. Here, we demonstrate that STRAP acts as a scaffold protein in TLR3-triggered signaling. STRAP strongly interacts with TBK1 and IRF3, which enhances IFN-β production. As a consequence, STRAP knockdown reduces the level of both pro-inflammatory cytokine and IFN in TLR3 agonist-stimulated macrophages, whereas its overexpression significantly enhances production of these cytokines. Furthermore, the C-terminus of STRAP is essential for its functional activity in TLR3-mediated IL-6 and IFN-β production. These data suggest that STRAP is a positive regulator of the TLR3-meditated NF-κB and IRF signaling pathway.

1. Introduction

Toll-like receptors (TLRs) are pattern recognition receptors that sense a wide range of pathogen associated molecular patterns (PAMPs), such as microbial nucleic acids and surface glycoprotein, to trigger innate immune system. Upon the detection of PAMPs, TLRs recruit TIR domain containing adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). MyD88- and TRIF-dependent pathways lead to the expression of genes encoding pro-inflammatory cytokine and type I interferon (IFN). In particular, TLR3 exclusively induces the TRIF pathway in response to double-stranded RNA (dsRNA) or its synthetic analog polyriboinosinic:polyribocytidylic acid (polyI:C), and acts on series of downstream signaling molecules including TNF Receptor Associated Factor 3 (TRAF3) and TRAF6. TRAF3 is responsible for the activation of TANK binding kinase 1 (TBK1) and IFN regulatory factor 3/7 (IRF3/7), and TRAF6 promotes the activation of transforming growth factor β-activated kinase 1 (TAK1) and the IκB kinase (IKK) complex-nuclear factor (NF)-κB pathway [1,2].

Serine/threonine kinase receptor associated protein (STRAP) is identified as an inhibitory factor for transforming growth factor-β (TGF-β) signaling via its association with TGF-β receptor I and decapentaplegic homolog 7 (Smad7) [3]. Because TGF-β regulates cell proliferation, the inhibitory effect of STRAP on TGF-β signaling contributes to tumorigenesis, which is supported by STRAP overexpression in colon, lung, and breast cancer [4,5]. Moreover, recent studies have reported that STRAP regulates not only TGF-β signaling but also a variety of signal transduction pathway including the PI3K/PDK, ASK1, and p53 pathway, which are involved in the regulation of cell proliferation and apoptosis [6–8]. STRAP contains a WD40 domain, which is a short structural motif of approximately 40 amino acids that usually ends with a tryptophan-aspartic acid (WD) dipeptide. The WD40 repeat is involved in diverse signaling pathway via its mediation of various protein-protein interactions. In particular, our previous study shows that STRAP is involved in activating TLR2/4-mediated cytokine production via the facilitation of TAK1-IKKα-p65 interactions [9]. However, the involvement of STRAP in TRIF-TBK1-mediated TLR3 activation and the subsequent IFN-β production has not been fully understood.

In this study, we report that STRAP positively regulates TLR3 ligand-induced NF-κB and IRF3 signaling as a scaffold protein. The interaction of STRAP with TBK1 and IRF3 enhances IFN-β production under polyI:C stimulation. We also find that TLR3-mediated IFN-β production is significantly affected by STRAP expression levels. In addition, the C-terminus of STRAP is essential for its role in the TLR3-mediated signaling pathway. Collectively, these findings provide

Abbreviations: STRAP, serine/threonine kinase receptor associated protein; TLR, Toll-like receptor; IFN, interferon; IRF, IFN regulatory factor; TBK1, TANK binding kinase 1; TRAF, TNF receptor associated factor; TAK1, transforming growth factor β-activated kinase 1

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evidence that STRAP functions as a scaffold protein involved in the regulation of both the NF-κB and IRF3 signaling pathways under TLR3 activation.

2. Materials and methods

2.1. DNA constructs

Mouse STRAP and STRAPΔC mutants were tagged with GFP and subcloned into retroviral pLHCX vector (Clontech, Mountain View, CA) (GFP-STRAP and GFP STRAPΔC). The shRNA oligonucleotides against STRAP or GFP (control) were annealed and subcloned into the pSUPER retroviral vector (Oligoengine, Seattle, WA). All constructs were verified by sequencing.

2.2. Cell culture

Murine RAW 264.7 macrophages (ATCC TIB-71) and human embryonic kidney (HEK) 293T cells (ATCC CRL-11268) were grown in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, UT) containing 7.5% fetal bovine serum (FBS; Hyclone) and penicillin/streptomycin (Hyclone). Cells were maintained in an atmosphere of 5% CO2 in a 37 °C humidified incubator.

2.3. Reagents & antibodies

PolyI:C and Omicsfect (CP2101) were purchased from Sigma-Aldrich (St. Louis, MO) and Omics Bio (Taipei City, Taiwan), respectively. The following antibodies were used: anti-STRAP (611346, BD Transduction Laboratories, San Jose, CA), anti-TBK1 (ab40676, Abcam, UK), anti-TAK1 (sc-7162, Santa Cruz Biotechnology, Santa Cruz, CA), anti-IKKα (sc-7218, Santa Cruz Biotechnology), anti-p65 (sc-109, Santa Cruz Biotechnology), anti-p-p65 (3033, Cell Signaling Technology, Danvers, MA), anti-IRF3 (sc-9082, Santa Cruz Biotechnology), anti-GFP (sc-9996, Santa Cruz Biotechnology), anti-Tubulin (G094, ABM Inc, Richmond, Canada), anti-CD14 (150101, Biolegend, San Diego, CA), and anti-Lamin A/C (2032, Cell Signaling Technology).

2.4. Retroviral transduction

HEK 293T cells were transfected with plasmids encoding VSV-G and Gag-Pol, along with constructs cloned into retroviral vector (GFP-STRAP, GFP-STRAPΔC, shRNA-GFP, and shRNA-STRAP). The media containing viral particles were harvested at 48 h post-transfection and filtered through a 0.45-μm membrane. Cells were transduced with virus by centrifugation at 2200 rpm for 45 min, and then incubated for 4 h. Transduced cells were incubated with fresh media for 24 h and then selected with hygromycin or puromycin.

2.5. RT-PCR

Total RNA from cultured cells were isolated using an RNA prep kit (GeneAll, Seoul, South Korea), and 1 μg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Enzynomics, Daejeon, South Korea) for 1 h at 42 °C. PCR was performed with appropriate primers and cDNA, and PCR products were analyzed on an agarose gel containing ethidium bromide. Data were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.6. Western blot

Cell lysates were prepared in 1% digitonin with the protease inhibitor cocktail (Roche, Basel, Switzerland) and then re-suspended in denaturing protein loading buffer. Lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 10 min, and incubated with the appropriate antibodies at 4 °C overnight. Membranes were washed three times with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Bands were visualized using an ECL detection reagent (Advansta, Menlo Park, CA).

2.7. Co-immunoprecipitation assay

RAW macrophages were stimulated with polyI:C for the indicated time points and washed twice with PBS. Cells were lysed with 1% digitonin lysis buffer containing the protease inhibitor cocktail (Roche) for 2 h at 4 °C. Cell lysates were incubated with primary antibodies at 4 °C overnight, and protein G-Sepharose beads (Sigma-Aldrich) were added to the samples for 1 h at 4 °C. The beads were washed twice with 0.1% digitonin buffer. Proteins were eluted by boiling in 2% denaturing buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol) for 5 min.

2.8. Cell fractionation

Cells were lysed in extraction buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5% NP-40) on ice for 15 min, centrifuged at 4000 rpm for 10 min. The pellet was lysed in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) on ice for 20 min, centrifuged at 13,000 rpm for 20 min, and the supernatant was collected as the nuclear fraction.

2.9. Statistical analysis

All experiments were repeated at least three times with consistent results. Data are presented as means and standard deviation (s.d.) as noted in the figure legends. Statistical differences between two means were evaluated with the two-tailed, unpaired Student's *t*-test. Differences with *P* values below 0.05 were considered significant. No samples were excluded from the analysis. The data had a normal distribution, and the variance was similar between the groups being compared. No statistical method was used to predetermine sample sizes. Sample size was based on previous experience with experimental variability. The experiments were not randomized. The investigators were not blinded to allocation during experiments or outcome assessment.

3. Results

3.1. STRAP promotes TLR3-mediated cytokine production

The previous observation of STRAP-mediated regulation in the TLR2/4-dependent signaling pathway [9] led us to investigate its potential role in the TLR3-induced NF-κB and IRF3 signaling pathways. To determine whether STRAP is involved in the TLR3 signaling pathway, we first examined mRNA levels of pro-inflammatory cytokines in RAW264.7 macrophages stably expressing STRAP short hairpin RNA (shRNA) or green fluorescence protein (GFP)-tagged STRAP following polyI:C stimulation. Remarkably, STRAP depletion reduced IL-6 mRNA levels under polyI:C stimulation when compared to control cells stably expressing shRNA-GFP (Fig. 1A, left panel). Conversely, GFP-STRAP overexpression increased IL-6 mRNA levels after polyI:C stimulation, compared with control cells stably transfected with the empty (Mock) vector (Fig. 1A, right panel). These patterns were not due to STRAP-mediated altered TLR3 expression because we observed similar expression levels of TLR3 in control and STRAP-overexpressing cells (Supplementary Fig. 1). We further examined mRNA levels of two other pro-inflammatory cytokines, TNF-α and IL-1β, in STRAP-depleted or -overexpressing cells in response to polyI:C stimulation. TNF-α and IL-