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POT1b regulates phagocytosis and NO production by modulating activity of the small GTPase Rab5

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ABSTRACT

The protection of telomeres 1 (POT1) protein is a 75-kDa protein that plays an important role in telomere protection, which is related to telomere elongation. Although POT1 is present in and acts in the nuclei, little is known about the functions of POT1 in the cytosol. We here examined the role of POT1b in phagocytosis in a macrophage-like RAW 264 cell line. We found that POT1 was present in the cytosol, where it was bound to Rab5, which is a protein important for endocytosis. POT1b knockdown in RAW 264 cells increased Rab5 activity and facilitated the phagocytosis of whole cells of *Escherichia coli* and *Staphylococcus aureus*. Furthermore, POT1b knockdown enhanced the expression of inducible nitric oxide synthase (iNOS), followed by the promotion of nitric oxide (NO) generation in response to stimulation by bacterial whole cells. These results suggest that POT1b negatively regulates phagocytosis by controlling Rab5 activity and thereby modulates bacteria-induced NO generation. These findings suggest that POT1b participates in innate immune responses.

1. Introduction

Phagocytosis is an important process in innate immune responses [1–3]. It is mainly performed by specialized cells, including macrophages, monocytes and neutrophils, that function to clear large pathogens, such as bacteria or yeast, or large debris such as the remnants of dead cells and arterial deposits of fat [2,4]. Several steps, including the binding of ligands at the cell surface and the activation of a signaling pathway leading to F-actin polymerization, have been shown to be important in phagocytosis [4,5]. Furthermore, vesicular trafficking by small GTPases, Rab proteins, plays a key role in the controlled maturation of phagosomes [6,7]. Rab5 was initially shown to be localized to early endosomes and the plasma membrane and to participate in endocytosis [8–10]. Rab5 has also been observed on nascent phagosomes [3,11,12]. Rab5 acts as a molecular switch in the regulation of fusion of early endosomes and phagosomes with target membranes through conformational changes between the GTP-bound active and GDP-bound inactive forms [10,13,14]. A large number of proteins have been reported interact with active Rab5 on early endosome, including phagosome membranes, and these include early endosome antigen 1 (EEA1) [15,16], APPL [17,18], Rabaptin-5 [19,20], caveolin-1 [21–23], and plastin [24].

The protection of telomeres 1 (POT1) protein, a 634-amino-acid protein, binds to telomeric single-stranded DNA, protecting chromosome ends from being detected as sites of DNA damage [25]. Hagiwara et al. found candidate Rab5-interacting proteins in the bovine spleen cytosol using a combination of biochemical and proteomic analytic techniques [26]. As a result of the analysis, POT1 was shown to be a possible Rab5-interacting protein. Although many reports have shown that POT1 functions in the nuclei, the possible functions of POT1 in the cytosol have not been studied.

Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to produce L-citrulline and nitric oxide (NO) [27]. Three family members have been characterized: neuronal NOS, which is found primarily in neuronal tissue; inducible NOS (iNOS), which is induced by various cytokines and microbial products in macrophages during inflammation; and endothelial NOS, which is expressed in endothelial cells and which maintains endothelial function [28]. NO is a messenger molecule that has diverse functions throughout the body. In macrophages, NO mediates tumoricidal and bactericidal actions. However, NO overproduction followed by iNOS mRNA induction has been shown to augment inflammatory responses and to contribute to the pathogenesis of various diseases, including inflammatory diseases [29,30].

Abbreviations: POT1b, protection of telomeres 1b; NO, nitric oxide; EEA1, early endosome antigen 1; NOS, nitric oxide synthase; iNOS, inducible NOS; PBS, phosphate-buffered saline; GFP, green fluorescence protein; IgG-HRP, immunoglobulin G-horseradish peroxidase; GAP, GTPase-activating protein.

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In this study, we showed that POT1b, an ortholog of POT1, interacts with Rab5 and regulates bacterial phagocytosis and that POT1b also regulates the stimulation of NO production by bacterial whole cells.

2. Materials and methods

2.1. Cell culture

Raw 264 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin (DMEM–10% FBS–1% P.S.).

2.2. Antibodies and siRNAs

Antibodies were obtained from the following sources: anti-rabbit IgG-Alexa 555 (Life Technologies Corporation, Grand Island, NY, USA), anti-rabbit Rab5 and anti-mouse green fluorescence protein (GFP; Novus Biologicals LLC, Littleton, CO, USA), anti-mouse EEA1 and anti-mouse iNOS (BD Biosciences, San Jose, CA, USA), anti-rabbit POT1 ab21382 (Abcam Plc, Cambridge, UK), anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) and anti-rabbit IgG-HRP (IBL-America Inc., Minneapolis, MN, USA), anti-mouse GAPDH (MBL International, Woburn, MA, USA), and anti-mouse FLAG (Sigma, Tokyo, Japan).

POT1b siRNA MSS231798 and siRNA negative control LO were obtained from Life Technologies Japan (Tokyo, Japan).

2.3. Transfection of cultured cells

RAW 264 cells were transfected using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions.

2.4. Vector constructs

Rab5 in pcDNA3.1 and GFP-Rab5 in pcDNA3 constructs were kindly provided by Dr. Y. Yamamoto (Tokyo University of Agriculture, Tokyo, Japan). For the expression of HA-fused proteins, Rab5 DNA was amplified by PCR and inserted into pCMV-HA (Clontech Laboratories Inc., Mountain View, CA, USA). The GST-R5BD vector was a kind gift from Dr. G. Li (University of Oklahoma Health Science Center, Oklahoma City, USA). The FLAG-POT1b vector was kindly donated by Dr. S. Chang (Yale University, New Haven, USA).

2.5. Preparation of cytosolic and nuclear fractions

Cytosolic and nuclear fractions were prepared using a NucBuster Protein Extraction Kit (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions.

2.6. Immunostaining

RAW 264 cells were fixed with cold methanol for 10 min. Nonspecific binding of the antibodies was blocked by incubation with 5% sheep serum in phosphate-buffered saline (PBS, pH 7.4) for 60 min. The cells were then incubated with anti-POT1 antibody (1:100 dilution) in PBS supplemented with 5% sheep serum for 60 min. The bound primary antibodies were visualized with Alexa Fluor 555-conjugated anti-rabbit IgG polyclonal antibody (1:250 dilution) in PBS supplemented with 1% sheep serum. After washing with PBS, the cells were mounted onto slide glasses and observed by confocal fluorescence microscopy (Carl Zeiss Microscopy GmbH, Gottingen, Germany).

2.7. Immunoprecipitation

RAW 264 cells were lysed for 30 min at 4 °C with a buffer [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, and protease inhibitor]. The clarified lysates were incubated with antibodies for 2 h at 4 °C, with GDP or GTPγS (a nonhydrolyzable GTP analog) or without an additive. The immune complexes were precipitated with protein A-Sepharose beads (Adar Biotech Ltd., Rehovot, Israel) for 2 h at 4 °C and then washed extensively with lysis buffer. The beads were resuspended in SDS sample buffer and analyzed by Western blotting.

2.8. GST-R5BD pull-down assay

The GST-R5BD pull-down assay was based on the method described by Liu et al. [31]. Cells transfected with GFP-Rab5wt were washed twice with PBS and lysed for 5 min in 1 mL of lysis buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 2% glycerol, and protease inhibitor). The cell extracts were clarified by centrifugation at 10,000×*g* for 5 min at 4 °C, and the supernatants were incubated with 20 μL of GST-R5BD bound to the glutathione-Sepharose 4B beads for 20 min at 4 °C under rotation. The beads were subsequently rinsed with lysis buffer, resuspended in SDS sample buffer, and analyzed by Western blotting.

2.9. Uptake assay

To measure bacterial phagocytosis by the cells, monolayers of RAW 264 cells were preincubated with serum-free DMEM without phenol red for 1 h at 37 °C in 96-well plates. The cells were then incubated with 100 μg/mL of pHrodo Red *Escherichia coli* and *Staphylococcus aureus* BioParticles (Life Technologies Japan) for the periods at 37 °C. Fluorescence intensity of the cells was then measured using SpectraMax M3 (Molecular Devices, LLC, Sunnyvale, CA, USA).

2.10. Measurement of NO

Monolayers of RAW 264 cells were preincubated with serum-free DMEM without phenol red for 1 h at 37 °C in 96-well plates. The cells were subsequently incubated with 100 μg/mL of *E. coli* and *S. aureus* BioParticles (Life Technologies Corporation) for 16 h at 37 °C. The cells were then incubated with 10 μM of DAF-2 (Sekisui Medical Co., Ltd., Tokyo, Japan) for 1 h at 37 °C. Fluorescence intensity of the media was measured using SpectraMax M3 (Molecular Devices Corporation).

3. Results

3.1. POT1b interacts with Rab5

We first examined the localization of POT1 in RAW 264 cells. We prepared cytosolic and nuclear fractions of the cells and analyzed the existence of POT1 in the fractions by Western blotting. As shown in Fig. 1A, EEA1 and Rab5 were detected in the cytosolic fraction but not in the nuclear fraction in the cells. LaminA/C, which is a typical nuclear protein, was detected in the nuclear fraction of the cells. POT1 was detected in both the cytosolic and nuclear fractions (Fig. 1A). We also examined the localization of POT1 by confocal fluorescence microscopy. POT1 was localized in both the cytosol and nuclei of the cells (Fig. 1B). Next, we examined whether POT1 bound to Rab5 in the cytosol. We immunoprecipitated the cytosolic fraction of RAW 264 cells with anti-Rab5 antibody and then immunoblotted with anti-POT1 and anti-Rab5 antibodies. Endogenous POT1 was coimmunoprecipitated with