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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

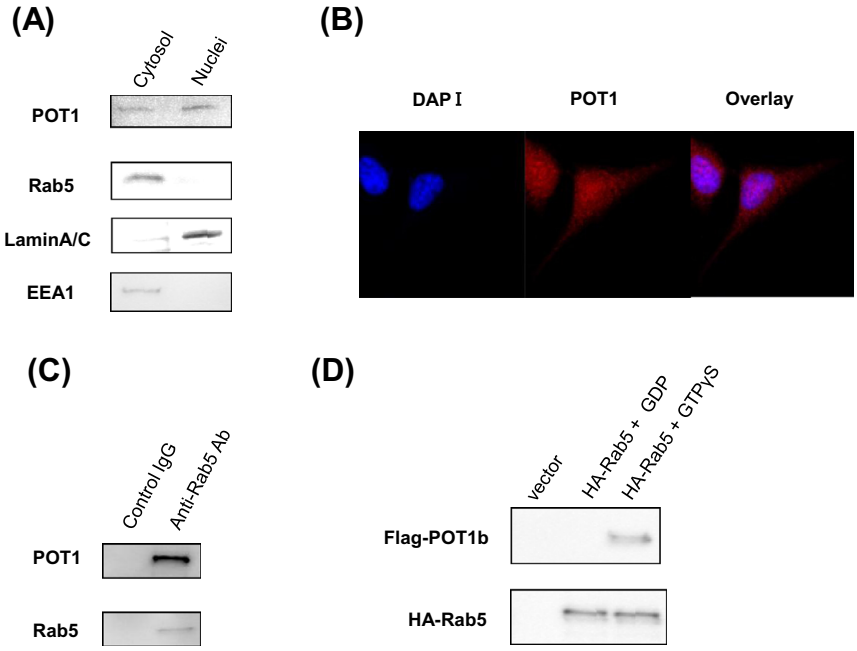

Fig. 1. The protection of telomeres 1 (POT1) protein is present in the cytosol and binds to Rab5. (A) Western blotting of POT1, Rab5, early endosome antigen 1 (EEA1) (an endosome protein), and laminA/C (a nuclear membrane protein) in the cytosolic and nuclear fractions of RAW 264 cells. (B) RAW 264 cells were stained with anti-POT1 antibody (red), and DNA was stained with DAPI (blue). (C) Endogenous POT1 and Rab5 were immunoprecipitated from RAW 264 lysates with anti-Rab5 antibody. POT1 and Rab5 were analyzed by Western blotting with antibodies specific for POT1 and Rab5. (D) Flag-POT1b was co-expressed with HA-Rab5 in RAW 264 cells and subjected to immunoprecipitation with anti-HA antibody in the presence of GTPγS or GDP. The proteins were analyzed by Western blotting.

endogenous Rab5 (Fig. 1C). These findings indicate that POT1 was bound to Rab5.

To further examine the interactions of POT1b with Rab5, plasmids encoding HA-Rab5 and Flag-POT1b were co-transfected into RAW 264 cells, and immunoprecipitation assays were performed with GDP or GTPγS. As shown in Fig. 1D, POT1b was coimmunoprecipitated with GFP-Rab5-GTPγS and not with GFP-Rab5GDP. These findings indicate that POT1b was bound to the active form of Rab5.

3.2. POT1b regulates Rab5 activity

We next investigated whether POT1b affects Rab5 activity using methods described by Liu et al. [31]. The levels of endogenous Rab5-GTP were too low to be detected in this assay. Thus, RAW 264 cells were cotransfected with plasmids encoding GFP-Rab5 and POT1b siRNA to overexpress GFP-Rab5 and knock down POT1b. As shown in Fig. 2A, introducing POT1b siRNA into RAW 264 cells inhibited POT1b expression. Rab5-GTP in the cell lysates was pulled down with GST-tagged R5BD protein, and Western blotting was then performed to determine the relative amounts of Rab5-GTP with anti-GFP antibody. Knockdown of POT1b increased Rab5-GTP levels in the GFP-Rab5-overexpressed cells (Fig. 2B). These results suggest that POT1b regulates Rab5 activity.

3.3. POT1b modulates phagocytosis

Rab5 is a critical and rate-limiting component of the docking and fusion processes of the endocytic pathway [10]. To assess the potential role of POT1b in phagocytosis, we examined the effects of POT1b on the phagocytosis of bacterial whole cells by RAW 264 cells. pHrodo Red *E. coli* and *S. aureus* were added to a culture of cells with POT1b knockdown, and uptake of the bacteria in the cells was evaluated on the basis of the fluorescence intensity of the cells. The uptake of pHrodo Red *E. coli* and *S. aureus* increased in a time-dependent manner. However, POT1b knockdown increased the augmentation of uptake of bacteria in the cells (Fig. 3A and B). These findings were consistent with the positive regulation exerted by POT1b knockdown on Rab5 activity

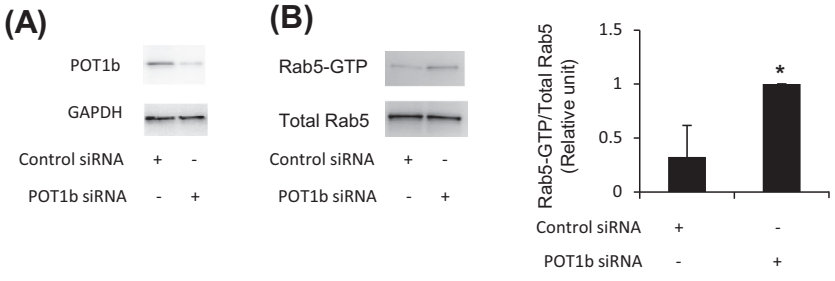

Fig. 2. POT1b regulates Rab5 activity. (A) RAW 264 cells were transfected with POT1b siRNA or control siRNA, and cell lysates were assayed by Western blotting using anti-POT1. (B) Top, amount of Rab5-GTP precipitated by a GST-R5BD pull-down assay examined by immunoblot analysis with anti-GFP antibody. Bottom, total amount of Rab5 in the cell lysates used for the pull-down assay, which was directly determined by immunoblot analyses of the lysate with anti-GFP antibody. Rab5-GTP levels were normalized to the total GFP-Rab5 levels and quantified by ImageJ. Each value ind DNA was stained withn the graph is the mean ± standard deviation (SD) of three independent experiments $^{*}p < 0.05$.

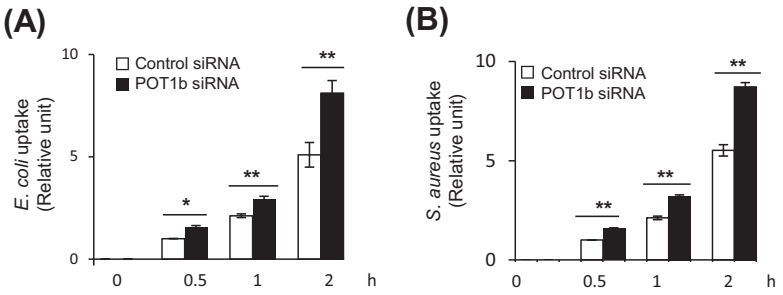

Fig. 3. POT1b regulates phagocytosis. (A and B) POT1b was knocked down in RAW 264 cells and incubated with pHrodo Red *Escherichia coli* (A) and *Staphylococcus aureus* (B) for the indicated *time* periods at 37 °C. Each value in the graph is the mean ± SD of three independent experiments $^{*}p < 0.05$, $^{**}p < 0.01$.

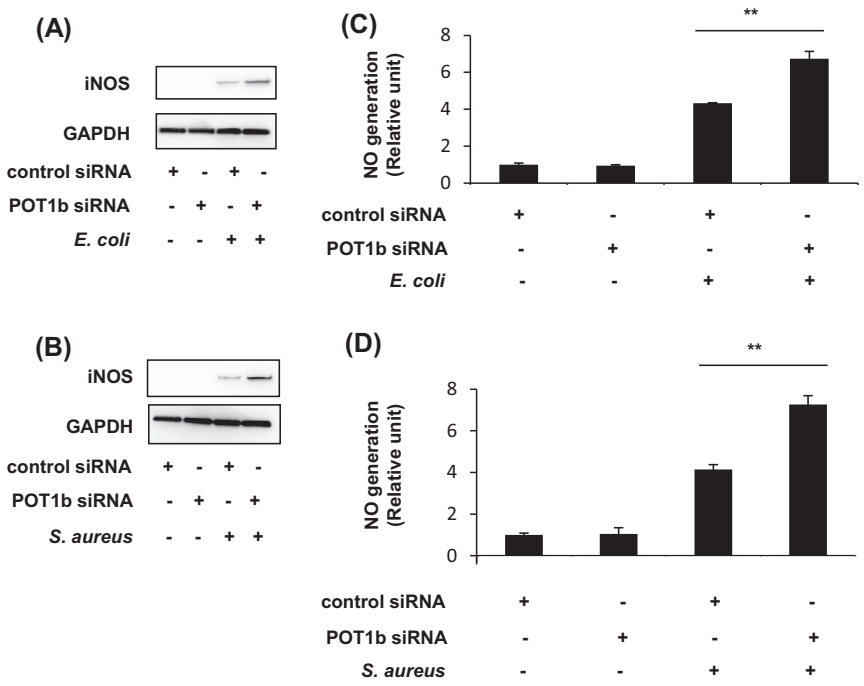

Fig. 4. POT1b regulates bacteria-induced nitric oxide. (A and B) POT1b siRNA or control siRNA was transfected into RAW 264 cells. The cells were incubated with *E. coli* (A) or *S. aureus* (B) for 16 h at 37 °C. The expression levels of iNOS were analyzed by Western blotting with anti-iNOS antibody. (C and D) POT1b siRNA or control siRNA was transfected into RAW 264 cells. The cells were incubated with or without *E. coli* (C) and *S. aureus* (D) for 16 h at 37 °C. NO generation was measured with DAF-2. Each value in the graph is the mean ± SD of three independent experiments $^{**}p < 0.01$.

(Fig. 2B). These results suggest that POT1b regulates bacterial phagocytosis by controlling Rab5 activity.

3.4. POT1b regulates NO induction by bacteria

Macrophages are the main proinflammatory cells involved in the response to invading pathogens and the release of many proinflammatory molecules, including NO. Heat-inactivated bacteria increase with NF-κB activation, and this induces iNOS expression in RAW 264 cells through a Toll-like receptor (TLR)-regulated pathway [32]. Thus, we investigated whether POT1b influences NO generation by bacterial phagocytosis in RAW 264 cells. RAW 264 cells with POT1b knockdown were incubated with heat-killed *E. coli* and *S. aureus*, and the expression levels of iNOS in the cells were analyzed by Western blotting with anti-iNOS antibody. As shown in Fig. 4A and B, treatment of whole cells of *E. coli* and *S. aureus* induced iNOS expression in RAW 264 cells. However, iNOS induction was upregulated by POT1b knockdown in the cells (Fig. 4A and B). Next, we measured NO generation in the cell cultures with DAF-2, NO indicator (Fig. 4C and D). NO was generated slightly in RAW 264 cells that were not treated with bacterial cells. NO was significantly induced in bacteria-treated RAW 264 cells (Fig. 4C and D). However, NO induction in bacteria-treated RAW 264 cells was increased with POT1b knockdown in the cells (Fig. 4C and D). These results suggest that POT1b regulates bacteria-induced NO production.

4. Discussion

Many reports have shown that POT1 has a role in nuclei. However, the significance of the role of POT1 in the cytosol has not been studied. In this study, we first revealed that POT1b, an ortholog of POT1, interacts with Rab5 and then showed that POT1b negatively regulates Rab5 activity and modulates phagocytosis. We also showed that POT1b controls NO induction induced by bacteria.

Chen et al. reported that among the six main telomeric proteins, TRF1, RAP1, TIN2, and TRF2 mainly localize to the nucleus, whereas TPP1 and POT1 are found in both the cytoplasm and nucleus [33]. They suggested that cytoplasmic telomeric proteins may perform

additional functions that are distinct from their nuclear counterparts. However, the significance of the role of POT1 in the cytosol has not been studied. We here showed that POT1 localized in both the cytosol and nucleus (Fig. 1A and B). Interestingly, POT1b interacted with active Rab5 in the cytosol, and POT1b knockdown increased phagocytosis by regulating Rab5 activity. These lines of evidence suggest that POT1b may be a crucial regulator of endocytosis.

One of the most intriguing observations during this experiment was that POT1b knockdown increased Rab5 activity (Fig. 2). It has been reported that GAP negatively regulates Rab5 activity [34-37]. The GAPs identified to date are known to contain a Tre-2, Bub2, and Cdc16 (TBC) domain, which directly catalyzes Rab5 GTPase [13]. However, the amino acid sequence of POT1b does not contain a TBC domain. Therefore, POT1b may recruit GAP or act as a cofactor for GAP.

In the present study, we showed that loss of POT1b increased iNOS expression in RAW 264 cells. Heat-inactivated *S. aureus* induces the activation of GSK-3 beta, resulting in an increase in NF-κB activation and iNOS expression in RAW 264 cells through a TLR-regulated pathway [32]. In addition, Rab5 is an upstream protein of GSK-3 beta [17]. Thus, POT1b and Rab5 may contribute to the TLR/GSK-3 beta/NF-κB pathway and NF-κB-regulated NO production. The excessive synthesis of NO by iNOS acts as a major macrophage-derived inflammatory mediator and is also involved in the development of inflammatory disease [38]. In the present study, POT1b knockdown increased iNOS protein expression and NO production by phagocytosis of *E. coli* and *S. aureus* in RAW 264 cells. POT1b may act as a potent regulator of inflammatory mediators.

Collectively, we found that POT1b is a new regulator of bacterial phagocytosis that acted by regulating Rab5 activity. Moreover, POT1b controlled phagocytosis-induced NO production. Further studies will provide new insights into the mechanisms of membrane trafficking and NO generation through POT1b and Rab5.

Acknowledgments

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