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Coronin-1 is phosphorylated at Thr-412 by protein kinase Cα in human phagocytic cells

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ABSTRACT

Coronin-1, a hematopoietic cell-specific actin-binding protein, is thought to be involved in the phagocytic process through its interaction with actin filaments. The dissociation of coronin-1 from phagosomes after its transient accumulation on the phagosome surface is associated with lysosomal fusion. We previously reported that 1) coronin-1 is phosphorylated by protein kinase C (PKC), 2) coronin-1 has two phosphorylation sites, Ser-2 and Thr-412, and 3) Thr-412 of coronin-1 is phosphorylated during phagocytosis. In this study, we examined which PKC isoform is responsible for the phosphorylation of coronin-1 at Thr-412 by using isotype-specific PKC inhibitors and small interfering RNAs (siRNAs). Thr-412 phosphorylation of coronin-1 was suppressed by Gö6976, an inhibitor of PKCα and PKCβI. This phosphorylation was attenuated by siRNA for PKCα, but not by siRNA for PKCβ. Furthermore, Thr-412 of coronin-1 was phosphorylated by recombinant PKCα *in vitro*, but not by recombinant PKCβ. We next examined the effects of Gö6976 on the intracellular distribution of coronin-1 in HL60 cells during phagocytosis. The confocal fluorescence microscopic observation showed that coronin-1 was not dissociated from phagosomes in Gö6976-treated cells. These results indicate that phosphorylation of coronin-1 at Thr-412 by PKCα regulates intracellular distribution during phagocytosis.

1. Introduction

Professional phagocytes (e.g., neutrophils, monocytes and macrophages) play an important role in initial host-defense responses [1,2]. The phagocytic process is considered to consist of three stages accompanied by drastic cellular transformation: 1) attachment of the particles to the cell; 2) engulfment of the particles; and 3) phagosome-lysosome fusion. The cellular structures are dynamically changed via rapid remodeling of the actin cytoskeleton assisted by actin-binding proteins [3,4]. The cytoskeleton rearrangements associated with morphological changes are modulated by a number of kinases and phosphatases that regulate protein phosphorylation [5]. Protein kinase C (PKC), a serine/threonine kinase family protein, consists of at least ten isoforms, which are subdivided into three groups based on their structure and activator requirements. The conventional PKC isoforms (PKCα, PKCγ, and the alternatively spliced transcript variants PKCβI/βII) require binding of diacylglycerol (DAG) and a phospholipid in a Ca^{2+}-dependent manner for activation. Novel PKC isoforms (PKCδ, PKCε, PKCη and PKCθ) also respond to DAG but in a Ca^{2+}-independent manner. Atypical PKC isoforms (PKCζ, PKCι/λ) require no second messenger binding for activation [6]. Several PKC isoforms are complexly associated with various processes of phagocytosis [7,8].

Coronin-1 is a member of the coronin actin-binding protein family, which contains a so-called WD repeat consisting of tryptophan (W) and aspartic acid (D) and is selectively expressed in immune cells [9]. We previously reported that, during phagocytosis by human neutrophils and neutrophil-like differentiated HL60 cells, coronin-1 and F-actin were transiently accumulated in phagocytic cups and phagosomal membranes, and the phagosomes fused with lysosomes after dissociation of coronin-1 from phagosomes [10,11]. Moreover, we showed that coronin-1 has two phosphorylation sites (Ser-2 and Thr-412 in humans), and Thr-412 of coronin-1 was phosphorylated by PKC during

Abbreviations: PKC, protein kinase C; ATP, adenosine 5'-triphosphate; PS, phosphatidylserine; OpZ, opsonized zymosan; HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

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phagocytosis [12,13]. We also revealed that a pan-PKC inhibitor suppressed phosphorylation of coronin-1 and dissociation of coronin-1 from phagosomes [11]. The relationship between the intracellular parasitism of *Mycobacterium tuberculosis* (Mtb) in murine macrophages and dysfunction of coronin-1 was suggested by the result that the failure in the fusion of lysosome with phagosomes containing mycobacteria was accompanied by prolonged localization of coronin-1 surrounding phagosomes [14]. Taken together, these findings suggested that coronin-1 plays a crucial role in phagocytosis by controlling phagosome-lysosome fusion via phosphorylation at Thr-412 of coronin-1. Thus, the phosphorylation mechanism of coronin-1 seems to be important for innate immunity, including leukocyte phagocytosis. In this study, we attempted to identify the PKC isoforms responsible for the phosphorylation at Thr-412 of coronin-1.

2. Materials and methods

2.1. Reagents

Adenosine 5'-triphosphate (ATP) disodium salt hydrate, 1,4-diazabicyclo-2,2,2-octane, Ficoll PM400, human serum (blood group AB), phosphatidylserine, poly-L-lysine, rhodamine-conjugated phalloidin, Triton X-100 and zymosan A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calphostin C, chelerythrine, Gö6976 and Gö6983 were from Calbiochem (San Diego, CA, USA). Hybond-ECL nitrocellulose membranes and ECL Select were products of GE Healthcare (Piscataway, NJ, USA). Bovine serum albumin fraction V and caluculin A were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). The Alexa Fluor 647 protein labeling kit, Dynabeads protein G and Lipofectamine RNAiMAX were from Invitrogen (Carlsbad, CA, USA). Recombinant PKCα was purchased from Sigma-Aldrich. Recombinant PKCβI and PKCε were from Cyclex (Nagano, Japan). Nonidet P-40 and Opti-MEM medium were supplied by Nacalai Tesque (Kyoto, Japan) and Life Technologies (Gaithersburg, MD, USA), respectively.

2.2. Antibodies

A monoclonal antibody against human coronin-1 (N7) that recognizes the C-terminal region of the molecule was prepared in our laboratory [10]. Monoclonal antibodies against phospho-Thr412 (2B4, IgG1/k) and non-phospho (412pep, IgG1/k) of human coronin-1 were established in our previous study using the Cys-^{407}NRGLDpTGRRRA^{417} phosphopeptide of coronin-1 conjugated with keyhole limpet hemocyanin (KLH) [12]. Anti-PKCα (C-20) and anti-PKCδ (C-17) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-PKCβ (Clone 36/PKCb) was from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat antibody to mouse IgG and HRP-conjugated rabbit antibody to goat IgG were purchased from Kirkegaard & Perry Laboratories Inc. (Guildford, UK). Alexa Fluor 488-conjugated goat anti-mouse IgG was from Invitrogen.

2.3. Cell culture and transfection

HL60 and HEK293T cells were grown in RPMI1640 medium (FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C under a humidified atmosphere with 5% CO_2. HL60 cells were treated with 1.25% DMSO for 4 days and differentiated cells were collected by density gradient centrifugation using Ficoll. HEK293T cells stably expressing human coronin-1 (HEK-hCoro1) were established in our previous study [12]. Synthetic small interfering RNA (siRNA) duplexes against human PKCα (sense strand, CACAUUCAGCAAGUAGGAA), human PKCβ (CAGAGUAAGGGCAUCAUUU) and human PKCδ (GUUGAUGUCUGUUCAGUAU) were purchased from Sigma-Aldrich. The siRNA was introduced into HEK-hCoro1 cells with Lipofectamine RNAiMAX according to the manufacturer's instructions. Briefly, siRNA (25 pmol) in Opti-MEM medium (125 μl) was mixed with RNAiMAX (7.5 μl) in Opti-MEM (125 μl), and incubated for 5 min at room temperature. The mixtures were added to HEK-hCoro1 cells (5 x 10^5 cells in a 6-well plate) and these cells were cultured for 40 h.

2.4. Cell stimulation

HL60 cells (1 x 10^6 cells) were treated with/without PKC inhibitors (chelerythrine, calphostin C, Gö6983 or Gö6976) at 4 °C for 30 min followed by treatment with calyculin A (100 nM) at 37 °C for 20 min. These cells were lysed with TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40), and the supernatants were recovered after centrifugation at 15,000×*g* for 20 min. The recovered supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with anti-phospho-Thr-412 of coronin-1 antibody [clone: 2B4] against phospho-Thr-412 and anti-coronin-1 antibody [clone: 412pep] against total coronin-1 as loading controls.

2.5. In vitro kinase assay

HL60 cells (5 x 10^6 cells) were lysed with TNE buffer, the lysate was subjected to immunoprecipitation with Dynabeads protein G (10 μl) and anti-coronin-1 antibody (N7, 1 μg), and the immunoprecipitates were incubated with recombinant PKCα, PKCβI or PKCε (200 ng) in a reaction buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 50 μM ATP, 100 μg/ml phosphatidylserine (PS)) at 30 °C for 3 h. These immunoprecipitates were washed with phosphate-buffered saline (PBS) and analyzed by SDS-PAGE and western blotting.

2.6. Phagocytosis assay

Zymosan was opsonized with human serum (blood group AB) at 37 °C for 30 min and then the opsonized zymosan (OpZ) was fluorescently labeled using an Alexa Fluor 647 labeling kit. The phagocytosis assay was performed as described previously [11]. Briefly, HL60 cells were attached to poly-L-lysine (20 μg/ml)-coated slide glass on ice. Alexa Fluor 647-conjugated OpZ was added to the cells on ice for 30 min. The cells were incubated at 37 °C for 2 min and then immediately on ice and treated with Gö6976 for 30 min. Subsequently, the cells were incubated at 37 °C for 30 min and fixed with 3.8% neutral buffered formaldehyde.

For analysis of the localization of coronin-1 and F-actin (filamentous actin), the cells were permeabilized by treatment with 0.2% Triton X-100 in PBS for 10 min at room temperature, incubated with anti-coronin-1 antibody (N7, 3 μg/ml) for 1 h and washed with PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200) and rhodamine-conjugated phalloidin (15 units/ml) in PBS containing 3% BSA for 30 min. Fluorescently labeled cells were washed well with PBS and then mounted with 2.3% 1,4-diazabicyclo-2,2,2-octane in 20 mM Tris-HCl (pH 8.0) containing 90% glycerol on glass slides. Coronin-1 (green) and F-actin (red) localized around the OpZ (blue) taken up by cells were observed with a confocal laser scanning microscope (Radiance 2100; Bio-Rad, Hercules, CA, USA).

3. Results

3.1. PKCα/β inhibitor blocks phosphorylation of coronin-1 at Thr-412

We previously reported that the phosphorylation status of coronin-1 at Thr-412 is controlled by constitutive turnover of the phosphorylation/dephosphorylation cycle, which involves PKC and phosphatases [12]. First, we analyzed the phosphorylation of coronin-1 of HL60 cells treated with calyculin A (a Ser/Thr phosphatase inhibitor). In western blotting analysis using an antibody specific for phospho-Thr-412 of coronin-1 (anti-phospho-Thr-412), phosphorylation of coronin-1 was

time-dependently increased after calyculin A treatment (Fig. 1A). In our previous study, we found that the phosphorylation of coronin-1 at Thr-412 was suppressed by chelerythrine (a pan-PKC inhibitor) [12]. We therefore examined the effects of three different types of PKC inhibitors, calphostin C (an inhibitor of PKCα, βI/βII, γ, δ, ε, θ, η), Gö6983 (an inhibitor of PKCα, βI/βII, γ, δ, ζ) and Gö6976 (an inhibitor of PKCα, βI), in this study. Phosphorylation of Thr-412 was suppressed by all these PKC inhibitors (Fig. 1B). As shown in Fig. 1C, Gö6976 suppressed the phosphorylation of coronin-1 at Thr-412 in a dose-dependent manner. These results suggest that the constitutive turnover of phosphorylation at Thr-412 of coronin-1 was catalyzed by PKCα and/or PKCβ.

3.2. PKCα is involved in phosphorylation of coronin-1 at Thr-412

We next examined whether PKCα and/or PKCβ were involved in phosphorylation of coronin-1 at Thr-412 by using siRNA. First, we established HEK293T cells that stably expressed coronin-1 and then treated them with the siRNAs for PKCα, PKCβ or PKCδ. Then, the expressions of PKCs in these siRNA-treated cells were analyzed by western blotting using anti-PKCα, anti-PKCβ, anti-PKCδ and anti-coronin-1 antibodies. As shown in Fig. 2A, the results confirmed that each siRNA specifically decreased the corresponding isoform. Next, we treated these cells with calyculin A and detected phosphorylated coronin-1 in the lysates by anti-phospho-Thr-412 antibody. The results indicated that the phosphorylation of coronin-1 at Thr-412 was attenuated by the siRNA for PKCα, but not by the siRNAs for PKCβ or PKCδ (Fig. 2B).

3.3. Coronin-1 at Thr-412 is phosphorylated directly by PKCα

To evaluate whether PKCα directly phosphorylated Thr-412 of coronin-1, we assessed phosphorylation of coronin-1 by *in vitro* kinase assay. First, we isolated coronin-1 by immunoprecipitation from the HL60 cell lysate using anti-coronin-1 antibody (N7). The immunoprecipitated coronin-1 was then incubated with recombinant PKCα, phosphatidylserine and ATP. Finally, we analyzed the phosphorylation of coronin-1 by western blotting using anti-phospho-Thr-412 antibody. The results indicated that PKCα—but not either PKCβI or PKCε (Fig. 3B)—directly phosphorylated Thr-412 of coronin-1 (Fig. 3A).

3.4. Gö6976 inhibits the dissociation of coronin-1 from phagosomes

Transient accumulation of coronin-1 on phagosomes and successive dissociation from phagosomes seem to be necessary for lysosomal fusion [10,11,15]. Our previous studies suggested that phosphorylation of coronin-1 at Thr-412 was an important process for the dissociation of coronin-1 from phagosomes [12]. We therefore examined the effects of Gö6976 on the intracellular distribution of coronin-1 during phagocytosis in HL60 cells by observation with confocal microscopy. We found that coronin-1 was dissociated from phagosomes in untreated-HL60 cells, whereas coronin-1 remained on phagosomes in Gö6976-treated HL60 cells, as observed in chelerythrine-treated cells in our previous study [11] (Fig. 4). These results strongly suggest that PKCα-catalyzed phosphorylation of coronin-1 at Thr-412 regulates the intracellular distribution of coronin-1 during phagocytosis.

4. Discussion

In this study, we revealed that Thr-412 of human coronin-1, which seems to be important for phagosome-lysosome fusion in phagocytes [11,12], was phosphorylated by PKCα. To clarify which PKC isoforms are involved in the phosphorylation of coronin-1 at Thr-412, we focused on a previous study showing that Thr-412 of coronin-1 was constitutively phosphorylated in leukocytes [12]. We analyzed the phosphorylation of Thr-412 using HL60 cells treated with a serine/threonine phosphatase inhibitor, calyculin A. Phosphorylation of coronin-1 at Thr-412 was time-dependently increased after the treatment of HL60 cells with calyculin A, and the phosphorylation was suppressed by several PKC inhibitors (Fig. 1A and B). Gö6976, a specific inhibitor of PKCα and PKCβ, blocked the Thr-412 phosphorylation in calyculin A-treated cells (Fig. 1B and C). These results strongly suggest that the phosphorylation of Thr-412 was regulated by PKCα and/or PKCβ. To assess the involvement of PKCα and/or PKCβ in the phosphorylation, we established PKCα- or PKCβ-depleted cells by siRNA using HEK293T cells expressing coronin-1 and analyzed the Thr-412 phosphorylation. The results indicated that PKCα is responsible for the Thr-412 phosphorylation (Fig. 2). Moreover, Thr-412 of purified coronin-1 was phosphorylated *in vitro* by PKCα but not by PKCβ (Fig. 3). Finally, we found that inhibition of the Thr-412 phosphorylation by Gö6976 was accompanied by impairment of the dissociation of coronin-1 from phagosomes (Fig. 4). These results suggest that coronin-1 is phosphorylated at

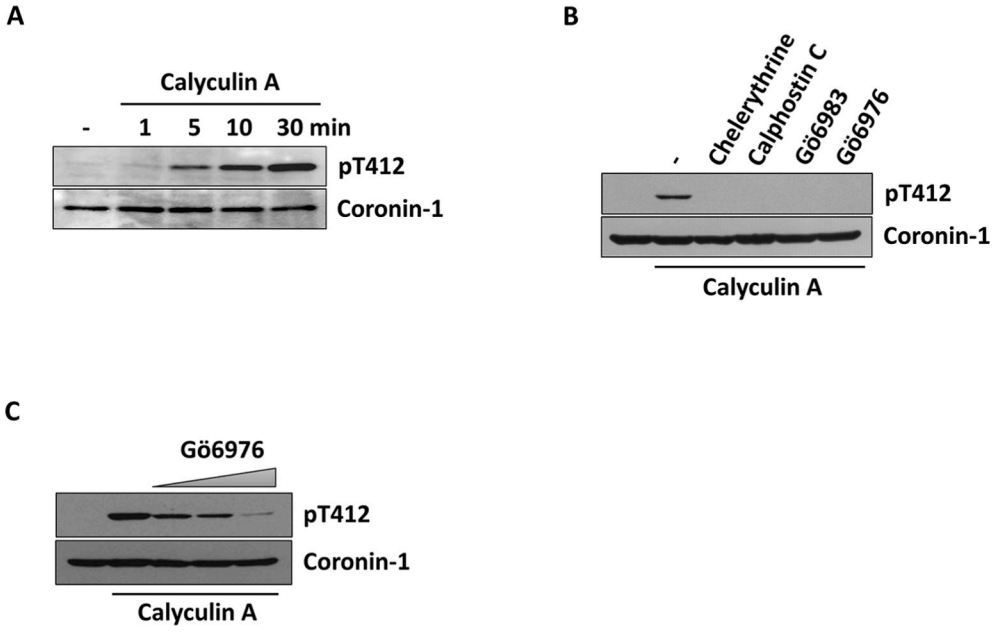

Fig. 1. Effect of PKC inhibitors on the phosphorylation at Thr-412 of coronin-1. (A) HL60 cells were treated with calyculin A (100 nM) for 0, 1, 5, 10 or 30 min. (B) HL60 cells were pre-treated with chelerythrine (30 μM), calphostin C (15 μM), Gö6983 (15 μM) or Gö6976 (1 μM), followed by treatment with calyculin A (100 nM). (C) HL60 cells were pre-treated with Gö6976 (0, 0.1, 0.3, 1 μM) and then treated with calyculin A (100 nM). The cells were lysed with TNE buffer and the lysates were analyzed by SDS-PAGE and western blotting with anti-phospho-Thr-412 of coronin-1 antibody. Total coronin-1 was detected using anti-coronin-1 (412pep) antibody as an internal standard. The experiments were repeated five times, and representative results are shown.

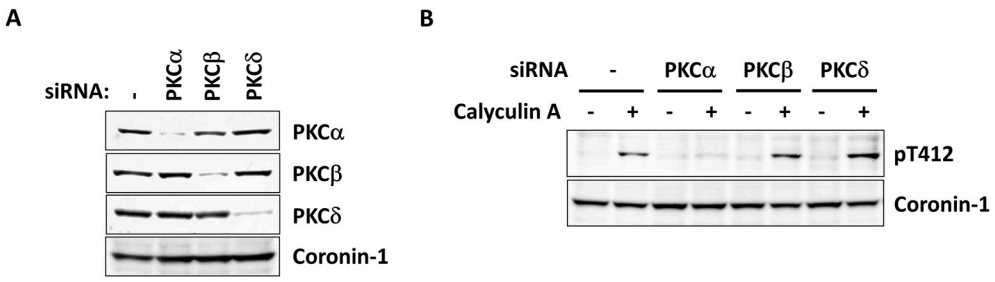

Fig. 2. Efficacy of siRNA for PKCs on phosphorylation at Thr-412 of coronin-1. HEK293T cells stably expressing coronin-1 were transfected with siRNAs against PKCα, PKCβ or PKCδ. (A) These cells were lysed with TNE buffer and the lysates were analyzed by SDS-PAGE and western blotting with anti-PKCα (1:400), anti-PKCβ (1 μg/ml), anti-PKCδ (1:400) and anti-coronin-1 (1 μg/ml) antibodies. (B) The cells were treated with calyculin A (50 nM) and lysed with TNE buffer. The lysates were analyzed by SDS-PAGE and western blotting with anti-phospho-Thr-412 of coronin-1 antibody. Total coronin-1 was detected using anti-coronin-1 (412pep) antibody as an internal standard. The experiments were repeated five times, and representative results are shown.

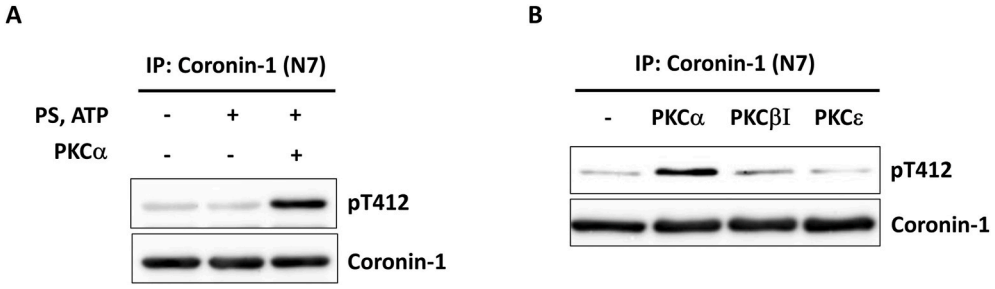

Fig. 3. *In vitro* kinase assay of coronin-1 at Thr-412 with PKC isoforms. The lysate of HL60 cells was subjected to immunoprecipitation with Dynabeads protein G and anti-coronin-1 antibody (N7), and the immunoprecipitates were incubated with PKCα (A) in a reaction buffer with/without PS and ATP (A), and with PKCα, PKCβI or PKCε (B). These immunoprecipitates were analyzed by SDS-PAGE and western blotting with anti-phospho-Thr-412 of coronin-1 antibody. Total coronin-1 was detected using anti-coronin-1 (412pep) antibody as an internal standard. The experiments were repeated five times, and representative results are shown.

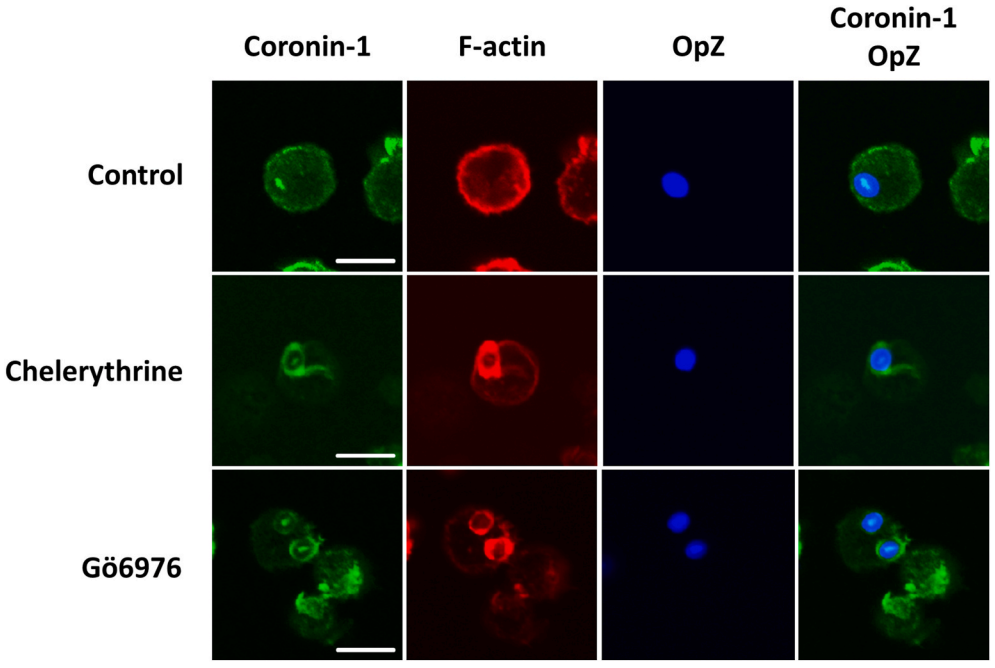

Fig. 4. Effect of Gö6976 on the dissociation of coronin-1 from phagosomes. HL60 cells were incubated with Alexa Fluor 647-labeled opsonized zymosan (OpZ) at 37 °C. After 2 min, phagocytosis was interrupted by cooling on ice, and Gö6976, a PKCα/βI inhibitor, was added for 20 min. Subsequently, phagocytosis was restarted by incubation at 37 °C for 30 min. Finally, the cells were fixed, permeabilized and stained with anti-coronin-1 and rhodamine-labeled phalloidin. Scale bars = 10 μm. The experiments were repeated three times, and representative results are shown.

Thr-412 by PKCα and that the phosphorylation is a key event for the phagosome-lysosome fusion following dissociation of coronin-1 from phagosomes. PKCα is known as a multifunctional enzyme that plays crucial roles in various cellular signal transduction cascades. In phagocytes, the depletion of PKCα was found to lead to decreasing uptake of bacteria or exogenous materials during phagocytosis [16–18]. The intracellular distribution of coronin-1 cannot be monitored in PKCα-knockout phagocytes due to the decreasing phagocytic activity; we therefore treated the cells with PKC inhibitors after the cells had taken up the particles. In future studies, it will be necessary to clarify this phenomenon by using Thr-412 mutant-expressing coronin-1-deficient cells.

We previously identified two phosphorylation sites (Ser-2 and Thr-412) in human coronin-1 by MALDI-TOF-MS analysis. We then developed monoclonal antibodies specific for phospho-Ser-2 (Oku et al., unpublished data) and phospho-Thr-412 [12] of coronin-1 to evaluate the

roles of the phosphorylation, and found that Thr-412 of coronin-1 was phosphorylated during phagocytosis. These results prompted us to further characterize Thr-412 phosphorylation in the present study. It was also reported that Thr-418 of murine coronin-1 was phosphorylated by cyclin-dependent kinase (CDK) 5 [19]. However, human coronin-1 has no amino acid residue corresponding to Thr-418. The amino acid sequence around Thr-412 of human coronin-1 shows quite high homology with that of murine coronin-1. Our recent study indicated that Ser-412 of mouse coronin-1 was also phosphorylated by PKC, as assessed by a newly established monoclonal antibody specific for phospho-Ser-412 of murine coronin-1 (Oku et al., unpublished data). The phosphorylation of Thr-412 (human) or Ser-412 (mouse) appears to be interesting in relation with the possible regulation of the interaction of coronin-1 with phagosomal membranes.

As mentioned in the Introduction, a relationship between impaired translocation of coronin-1 and intracellular parasitism of Mtb has been suggested. Our present results suggest that the failure in the dissociation of coronin-1 from Mtb-containing phagosomes is due to the suppression of phosphorylation of coronin-1 at Thr-412 by PKCα. Mycobacteria secrete a variety of exoproteins, some of which are known to have relevance with the localization of coronin-1 to phagosomes [14,20,21]. We suggest several hypothetical mechanisms as follows. 1) Protein kinase G (PknG) from mycobacteria is known to be a virulence factor that inhibits phagosome-lysosome fusion [21,22]. Since PknG reduces the expression and activation of PKCα [16,23], it may indirectly suppress phosphorylation of coronin-1 at Thr-412 and inhibit subsequent phagosome-lysosome fusion. 2) Lipoamide dehydrogenase C (LpdC) released from mycobacteria is physically associated with coronin-1 and promotes the accumulation of coronin-1 on phagosomes [24]. Then, the binding of LpdC to coronin-1 may suppress phosphorylation at Thr-412 by steric hindrance. 3) Mycobacteria may secrete protein phosphatases acting on coronin-1 and/or activators of host phosphatase. It was reported that pathogenic mycobacteria produced several protein tyrosine phosphatases and phosphoinositide phosphatases such as protein-tyrosine phosphatase A (PtpA), PtpB and secreted acid phosphatase M (SapM) [25–28]. In addition, it was recently reported that PtpA secreted from *Staphylococcus aureus* was involved in the intracellular survival of macrophages through dephosphorylation of coronin-1 [29]. Thus, phosphorylation at Thr-412 of coronin-1 by PKCα probably manipulates phagosome maturation, and mycobacteria disturb the function of coronin-1 by multiple mechanisms in order to promote their own survival in leukocytes. Finally, elucidation of the regulatory mechanisms of coronin-1 is a potential therapeutic target for tuberculosis.

Authors' roles

TO conceived the project and designed the experiments. TO, YK and RI performed the experiments. TO, YK and TT wrote and edited the manuscript. MT, ST and TT supervised the research.

Ethical approval and consent to participate

We did not use samples collected from patients or animals in any of the experiments.

Consent for publication

All the authors have approved the manuscript and agree with its submission.

Declaration of competing interest

There are no conflicts of interest to declare.

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