Glucose-stimulated insulin secretion is controlled by both exocytosis and endocytosis in pancreatic β-cells. Although endocytosis is a fundamental step to maintain cellular responses to the secretagogue, the molecular mechanism of endocytosis remains poorly defined. We have previously shown that in response to high concentrations of glucose, guanosine 5’-diphosphate (GDP)-bound Rab27a is recruited to the plasma membrane where IQ motif-containing guanosine 5’-triphosphatase (GTPase)-activating protein 1 (IQGAP1) is expressed, and that complex formation promotes endocytosis of secretory membranes after insulin secretion. In the present study, the regulatory mechanisms of dissociation of the complex were investigated. Phosphorylation of IQGAP1 on serine (Ser)-1443, a site recognized by protein kinase Cε (PKCε), inhibited the interaction of GDP-bound Rab27a with IQGAP1 in a Cdc42-independent manner. Glucose stimulation caused a translocation of PKCε from the cytosol to the plasma membrane. In addition, glucose-induced endocytosis was inhibited by the knockdown of IQGAP1 with small interfering RNA (siRNA). However, the expression of the non-phosphorylatable or phosphomimetic form of IQGAP1 could not rescue the inhibition, suggesting that a phosphorylation-dephosphorylation cycle of IQGAP1 is required for endocytosis. These results suggest that IQGAP1 phosphorylated by PKCε promotes the dissociation of the IQGAP1-GDP-bound Rab27a complex in pancreatic β-cells, thereby regulating endocytosis of secretory membranes following insulin secretion.

**Key words** endocytosis; insulin; diabetes

**INTRODUCTION**

Membrane recycling maintains the cellular responses to secretagogues in secretory cells. In pancreatic β-cells, glucose promotes insulin secretion to control glucose homeostasis. These cells are also able to take up insulin secretory membranes from the plasma membrane after insulin secretion. This retrograde transport is known as glucose-induced endocytosis. Although glucose-induced endocytosis is essential for β-cells to realize proper insulin secretion, the molecular mechanism of endocytosis remains poorly defined.

The small guanosine 5’-triphosphatase (GTPase) Rab27a is a Rab family member that acts during the translocation and docking stages of glucose-regulated exocytosis in pancreatic β-cells. These stages of exocytosis are controlled by the GTP-bound state of Rab27a and its effector proteins. We previously demonstrated that GTP-bound Rab27a is converted to the guanosine 5’-diphosphate (GDP)-bound state in response to high glucose concentrations. Moreover, GDP-bound Rab27a interacts with its GDP-dependent effector proteins and controls glucose-induced endocytosis after insulin secretion. These results indicate that Rab27a is a key molecule that regulates membrane recycling in pancreatic β-cells.

Coronin 3 is a GDP-bound Rab27a effector protein in pancreatic β-cells. Coronin 3 promotes F-actin assembly when it binds to GDP-bound Rab27a. Cytoskeletal rearrangement occurs during the late stage of glucose-induced endocytosis.

**MATERIALS AND METHODS**

**Plasmids and Antibodies** pTB701-protein kinase Cε (PKCε)-green fluorescent protein (GFP) was generously provided by Dr. Naoaki Saito (Kobe University). PMA was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other constructs were obtained as reported previously. Point mutations were generated using QuikChange Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA, U.S.A.). The rabbit polyclonal anti-GFP antibody, mouse monoclonal anti-GFP antibody, mouse monoclonal anti-Flag M2 antibody, mouse monoclonal anti-T7 antibody, rabbit polyclonal anti-Cdc42 antibody, and rabbit polyclonal anti-Rab27a antibody were purchased from MBL (Aichi), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), Sigma-Aldrich, Merck Millipore (Burlington, MA, U.S.A.), Thermo Fisher Scientific (Bonn, Germany), and Sigma-Aldrich, respectively.

**Cell Culture and Transfection** The insulin-secreting pancreatic β-cell line MIN6 was a gift from Dr. Jun-Ichi...
Miyazaki (Osaka University). COS-7 cells were obtained from the Cell Resource Center for Biomedical Research (Tohoku University). MIN6 and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15 and 10% fetal bovine serum (FBS), respectively. Negative control (NegaConNaito1) and IQGAP1 (MSS219911) stealth small interfering RNAs (siRNAs) were purchased from RNA interference (RNAi) Co., Ltd. (Tokyo) and Thermo Fisher Scientific. For transient expression, cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s instructions.

Co-immunoprecipitation COS-7 cells were transfected with the indicated plasmids. The cell lysate was subjected to immunoprecipitation with a rabbit polyclonal anti-GFP antibody as described. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed using a mouse monoclonal anti-GFP, anti-Flag M2, or anti-T7 antibody. The binding assay using MIN6 cells was performed as described previously. In brief, MIN6 cells expressing either GFP-IQGAP1-WT or GFP-IQGAP1-S1443A were incubated with 20mM glucose solution supplemented with 1µM PMA for 10min. The cell extracts were subjected to immunoprecipitation with an anti-GFP antibody and immunoblotting with an anti-GFP, anti-Rab27a, or anti-Cdc42 antibody.

Immunofluorescence MIN6 cells expressing GFP-tagged proteins were stimulated with 20mM glucose for the times indicated in the figures. The cells were fixed and stained as previously described. For uptake of Alexa-568-labeled transferrin (Thermo Fisher Scientific), MIN6 cells were incubated with 5µg/mL Alexa-568-transferrin for 5 min in 20mM glucose. The number of cells with a cytoplasmic staining pattern (more than 8 cells/dish, four independent dishes for each plasmid) was counted. Images were taken using an LSM710 confocal system (Carl Zeiss, Oberkochen, Germany) attached to an Axio Observer Z1 (Carl Zeiss). TIRF microscopy was performed as described previously.

Statistical Methods Data are presented as means ± standard deviation (S.D.) and statistically analyzed using Tukey-Kramer’s multiple comparison test. p values less than 5% were regarded as significant.

RESULTS

IQGAP1 C-Terminal Region Inhibits the Binding of GDP-Bound Rab27a to the GTPase-Related Domain of IQGAP1

Our previous studies showed that GDP-bound Cdc42 promotes the formation of a complex between GDP-bound Rab27a and IQGAP1, and that the GTPase-related domain (GRD) of IQGAP1 is a binding site for GDP-bound Rab27a. Since the C-terminal (CT) region of IQGAP1 regulates its activity, we first investigated the effect of the CT region on the binding of GDP-bound Rab27a to IQGAP1-GRD using COS-7 cells. GFP fusions of both IQGAP1-GRD/CT and full-length (WT) IQGAP1 were immunoprecipitated with anti-GFP antibody (Fig. 1A). Although IQGAP1-GRD/CT and IQGAP1-WT were mainly immunoprecipitated (Supplementary Fig. S1), only a few degradation products of IQGAP1 were also immunoprecipitated, in agreement with our previous report. Moreover, both Cdc42-G12V, a mutant that maintains Cdc42 in the GTP-bound state, and Rab27a-T23N, which

Fig. 1. Phosphorylation of IQGAP1 on Ser-1443 Inhibits the Binding of IQGAP1 to GDP-Bound Rab27a

(A) Lysates of COS-7 cells expressing GFP-IQGAP1-WT or GFP-IQGAP1-GRD/CT, Flag-Rab27a-T23N, and either T7-Cdc42-G12V, or T7-Cdc42-T17N were subjected to immunoprecipitation with an anti-GFP antibody and immunoblotting with an anti-GFP, anti-Flag, or anti-T7 antibody. Input indicates the cell lysates before immunoprecipitation. (B) Schematic representation of IQGAP1 and its mutants. GRD, GTPase-related domain; CT, carboxy terminus. (C) Lysates of COS-7 cells expressing T7-Cdc42-G12V, Flag-Rab27a-T23N, and either GFP-IQGAP1-S1443A or GFP-IQGAP1-S1443D were subjected to immunoprecipitation with an anti-GFP antibody and immunoblotting with an anti-GFP, anti-Flag or anti-T7 antibody. Input indicates the cell lysates before immunoprecipitation. (D) MIN6 cells expressing either GFP-IQGAP1-WT or GFP-IQGAP1-S1443A were incubated with 20mM glucose solution supplemented with 1µM PMA for 10min. Cell extracts were subjected to immunoprecipitation with an anti-GFP antibody and immunoblotting with an anti-GFP, anti-Rab27a, or anti-Cdc42 antibody. Input indicates the cell lysates before immunoprecipitation.
mimics the GDP-bound state of Rab27a, co-immunoprecipitated with IQGAP1-WT in COS-7 cells (Fig. 1A), which are also consistent with our previous study. In contrast, neither Cdc42-G12V nor Cdc42-T17N, which mimics the GDP-bound state of Cdc42, co-immunoprecipitated with the IQGAP1-GRD/CT, suggesting that GRD/CT does not contain the complete Cdc42-binding site (Figs. 1A, B). Moreover, Rab27a-T23N did not co-immunoprecipitate with IQGAP-GRD/CT in the presence or absence of Cdc42-G12V. GRD/CT contains a binding site for GDP-bound Rab27a (Fig. 1B). Since IQGAP1-GRD binds GDP-bound Rab27a in a Cdc42-independent manner, these findings suggest that the C-terminal region of IQGAP1 inhibits the binding of GDP-bound Rab27a to IQGAP1.

Inhibition of the Interaction between GDP-Bound Rab27a and IQGAP1 by IQGAP1 Phosphorylation

Ser-1443 in the CT region of IQGAP1 is a major phosphorylation site for protein kinase Cε (PKCε; Fig. 1B) and participates in the intramolecular conformational changes in IQGAP1. We therefore examined whether the phosphorylation of IQGAP1 on Ser-1443 is involved in the interaction of GDP-bound Rab27a with IQGAP1, and that GTP-bound Cdc42 is not involved in the interaction. We next performed an immunoprecipitation assay in the presence or absence of PMA in glucose-stimulated MIN6 cells to examine the interactions with phosphorylated IQGAP1. MIN6 cells are the insulin-secreting pancreatic β-cell line. When GFP-IQGAP1 was immunoprecipitated with anti-GFP-antibody, co-immunoprecipitation of Rab27a was inhibited by PMA treatment (Fig. 1D). In contrast, PMA did not affect the amount of co-immunoprecipitated Rab27a when GFP-IQGAP1-S1443A was immunoprecipitated (Fig. 1D). Since Ser-1443 is a phosphorylation site for PKCε, these results suggest that PKCε phosphorylation of IQGAP1 promotes the dissociation of GDP-bound Rab27a from the IQGAP1 complex.

Activation of PKCε at the Late Stage of Glucose-Induced Endocytosis

Peripheral PKCε catalyzes phosphorylation on Ser-1443 in IQGAP1. We therefore examined the effect of glucose on the intracellular localization of PKCε in pancreatic β-cells. Stimulation of MIN6 cells with 20 mM glucose resulted in the accumulation of PKCε-GFP in the vicinity of the plasma membrane (Figs. 2A, B). We next performed time-lapse imaging analysis using total internal reflection fluorescence (TIRF) microscopy, which specifically images cell surface PKCε-GFP (Fig. 2B). Consistent with our previous study, stimulation with 20 mM glucose caused an
apparent translocation of mCherry-dynamin 2 to the plasma membrane within 40 s (Fig. 2B). In contrast, PKCε-GFP was recruited to the same area after 215 s. We previously showed that glucose stimulation resulted in a redistribution of both EPI64, a GTPase-activating protein (GAP) for Rab27a, and coronin 3, another GDP-dependent effector for Rab27a, to the plasma membrane within 40 s.19) PKCε was recruited to the plasma membrane later than the endocytic machinery. Since PKCε localization to the plasma membrane represents an active state,22) these results suggest that PKCε is activated at the late stage of glucose-induced endocytosis.

Involvement of IQGAP1 Phosphorylation in Glucose-Induced Endocytosis in Pancreatic β-Cells Finally, we investigated the effect of phosphorylation of IQGAP1 on glucose-induced endocytosis in pancreatic β-cells. In accordance with our previous study,18,19) Alexa-568-labeled transferrin (Tfn) was internalized in glucose-stimulated MIN6 cells (Figs. 3A, B). Moreover, knockdown of IQGAP1 inhibited internalization, which was rescued by RNAi-resistant (RNAiR)-IQGAP1 but not by IQGAP1 (Figs. 3A, B), in agreement with our previous report.18) Unlike wild-type RNAiR-IQGAP1, neither non-phosphorylatable RNAiR-IQGAP1-S1443A nor phosphomimetic RNAiR-IQGAP1-S1443D was able to rescue the inhibition of Tfn internalization induced by IQGAP1 siRNA (p < 0.01; Figs. 3A, B). These results suggest that a phosphorylation and dephosphorylation cycle of IQGAP1 is required for glucose-induced endocytosis in pancreatic β-cells.

DISCUSSION

Our previous study has shown that high concentrations of glucose recruit GDP-bound Rab27a to the plasma membrane, which in turn forms a complex with IQGAP1, and that the IQGAP1-GDP-bound Rab27a complex promotes endocytosis of secretory membranes after insulin secretion.18) The present study further showed that phosphorylation of IQGAP1 on Ser-1443 promotes dissociation of the IQGAP1-GDP-bound Rab27a complex. This results in the promotion of endocytosis after insulin secretion. High glucose also activates PKCε. The kinase phosphorylates IQGAP1 and promotes the dissociation of the complex. (Color figure can be accessed in the online version.)
to inhibit endocytosis after insulin secretion. Considering these findings, the following mechanism is proposed: PKCe, which is activated by glucose stimulation, phosphorylates IQGAP1 on Ser-1443, thereby promoting the dissociation of the IQGAP1-GDP-bound Rab27a complex in pancreatic β-cells and leading to the inhibition of endocytosis. Interestingly, the overexpression of either the non-phosphorylatable or phosphomimetic form of IQGAP1 could not rescue the inhibition of endocytosis induced by IQGAP1 downregulation (Fig. 3), suggesting that a phosphorylation-dephosphorylation cycle of IQGAP1 is required for the regulation of endocytosis.

In the present study, we showed that the phosphorylation state of the CT domain of IQGAP1 is essential for the binding of GDP-bound Rab27a to IQGAP1. Diacylglycerol generated by glucose stimulation via the activation of Ca2+-sensitive phospholipase C is expected to activate PKCe in pancreatic β-cells. PKCe localized to the plasma membrane represents an active state and would be able to phosphorylate IQGAP1. PKCe is very likely to contribute to the dissociation of the IQGAP1-GDP-bound Rab27a complex via phosphorylating IQGAP1. Further studies are required to elucidate these relationships.

IQGAP1 has been shown to bind the exocytosis, a protein complex involved in vesicle trafficking, and regulate the docking state of insulin granules in un-stimulated pancreatic β-cells. On the other hand, the amount of GTP-bound Cdc42, which promotes the dissociation of the exocytosis from IQGAP1, has been shown to be increased by glucose stimulation. Therefore, IQGAP1 is likely to participate in the regulation of exocytosis in pancreatic β-cells. We previously showed that GTP-bound Cdc42 also promotes formation of the IQGAP1-GDP-bound Rab27a complex and that the interaction regulates endocytosis after insulin secretion. This complex is likely to be dissociated by PKCe phosphorylation of IQGAP1, which might advance the endocytic process to the next step. Insulin secretion is thus suggested to be controlled by stage-specific complex formation and the dissociation of IQGAP1 from its specific binding partners.

Endocytosis after glucose-stimulated insulin secretion is essential to maintain constant β-cell volume before and after insulin secretion. Since insulin secretion comprises two phases, bi-phasic endocytosis may also occur. PKCe is translocated to the plasma membrane later than other proteins involved in GDP-bound Rab27a signaling (Fig. 2). This raises the possibility that the phosphorylation of IQGAP1 by PKCe converts endocytosis from the first to the second phase.

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Conflict of Interest The authors declare no conflict of interest.

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