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Constitutive Activation of Rac1 in Pancreatic β Cells Facilitates F-Actin Depolymerization but Exerts No Influence on the Increase of Pancreatic β Cell Mass and Facilitation of Insulin Secretion

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Insulin secretion from pancreatic β cells has an important role in the onset of type 2 diabetes. Insulin secretion from pancreatic β cells is regulated by pancreatic β cell mass and their insulin secretory function. By using pancreatic β cell-specific Rac1-knockout mice, we recently showed that Rac1 deletion, even with no reduction in pancreatic β cell mass, inhibits F-actin depolymerization, which causes insulin secretion to decline. However, the effect of Rac1 deficiency on the growth and apoptosis of pancreatic β cells was not clarified. Further, the effect of constitutive Rac1 activation on the secretion of insulin from pancreatic β cells has not been studied. Here, we used pancreatic islets isolated from pancreatic β cell-specific Rac1-knockout mice to evaluate the growth and apoptosis of pancreatic β cells. We found that Rac1 deficiency does not influence the growth or apoptosis of pancreatic β cells. Further, when a constitutively activated form of Rac1 (G12V) is expressed, F-actin depolymerization was increased in the pancreatic β cell lines, which had no effect on pancreatic β cell growth or glucose-stimulated insulin secretion. These findings indicate that excessive Rac1 expression or activation in pancreatic β cells facilitates F-actin depolymerization, but has no effect on insulin secretion.

Increased insulin resistance due to obesity and other diseases, and impaired insulin secretion from pancreatic β cells have important roles in the onset of type 2 diabetes. Particularly, among eastern Asians, including the Japanese, impaired insulin secretion has a significant influence because the numbers of type 2 diabetes patients are rapidly increasing, despite a low percentage of obese people in the general population.

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In recent years, the cytoskeleton and cell adhesion of pancreatic β cells have been shown to play important roles in insulin secretion. In addition, the participation of low molecular weight G proteins in insulin secretion is gaining attention. An experiment using a cultured cell line showed that a reduction in the expression of CDC42, a low molecular weight G protein, leads to a reduction in the secretion of insulin (23). Additionally, Rab27a, a small membrane-bound GTPase, was shown to have an important role in the docking of an insulin granule with the plasma membrane (8). We previously showed that insulin signaling in pancreatic β cells influences glucose tolerance by regulating the pancreatic β cell mass (6, 12, 21). Further, it is becoming increasingly evident that the pancreatic β cell mass plays an important role in the onset of type 2 diabetes. On the other hand, recently, we reported that a deficiency of Rac1, a low molecular weight G protein, in pancreatic β cells resulted in impaired insulin secretion, although there was no change in the pancreatic β cell mass (1). Pancreatic β cell-specific Rac1-knockout mice showed hyperglycemia due to the impairment of insulin secretion in a glucose-specific manner. In addition, we showed that stimulation of a rat pancreatic β cell line (INS-1 cells) with glucose, but not KCl, led to the activation of Rac1, thereby facilitating F-actin depolymerization, and that the recruitment of insulin granules is inhibited in Rac1-deficient cells because of impaired actin depolymerization (1).

Thus, we showed that Rac1 is an important molecule for controlling glucose-stimulated insulin secretion in pancreatic β cells; however, no detailed studies have been conducted on the role of Rac1 in the proliferation of these cells. Moreover, it is not known whether the activation of Rac1 facilitates insulin secretion. If activated Rac1 facilitates insulin secretion in pancreatic β cells, then it can be considered a treatment target. Here, we first studied whether Rac1 activation influences the proliferation capacity of pancreatic β cells; further, we evaluated F-actin reorganization and insulin secretion by inducing the expression of activated Rac1 in a pancreatic β cell line.

MATERIALS AND METHODS

Mice

We generated heterozygous pancreatic β -cell-specific *Rac1*-knockout (β Rac1^{-/-}) mice by crossing Rac1 flox/flox mice, which harbor a modified endogenous *Rac1* gene in which the exon is flanked by loxP sites (9), with mice expressing the Cre recombinase gene under the control of the rat insulin-2 gene (7), as described previously. The mice were backcrossed to the C57/BL/6J mouse strain (Clea Japan, Tokyo, Japan) for at least 8 generations. The animals were maintained in a 12-h light/dark cycle and fed normal chow from the time of weaning (3 weeks). All experiments were performed using male mice. This study was performed in accordance with the guidelines of the Animal Ethics Committee of Kobe University Graduate School of Medicine.

Islet isolation

Pancreatic islets were isolated by collagenase digestion of exocrine pancreas and Histopaque density-gradient centrifugation, as described previously (4, 11).

Plasmids

Rac1 (G12) was provided by Dr. Shuji Ueda (Kobe University Graduate School of Agricultural Science). cDNA encoding HA $\times$ 3-tagged Rac1 (G12V) was subcloned into pEF-BOS (17) for expression in mammalian cells.

Cell culture and plasmid transfection

At 24 h before transfection, INS-1 cells were re-plated in 12-well plates (60-mm dishes) and transfected with the Rac1 (G12V) vector or empty vector using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). After further incubation for 72 h, the cells were harvested for the evaluation of insulin secretion and protein expression.

Immunoblot analysis

We prepared lysates of isolated islets or INS-1 cells as described previously (10, 16). The lysates were probed with antibodies to Rac1 (BD Biosciences Pharmingen, San Diego, CA, USA), PAK1, a phospho-Thr423 form of PAK1, Akt, a phospho-Thr308 form of Akt, a phospho-Ser473 form of Akt, S6, phospho-Ser235 and -Ser236 forms of S6, or the cleaved form of caspase 3 (all from Cell Signaling, Denver, MA, USA) and β -actin (Sigma-Aldrich, St. Louis, MO, USA).

Assay of insulin secretion from INS-1 cells

Insulin secretion from INS-1 cells was measured as described previously (1, 12). After a 30-min incubation in Krebs-Ringer-bicarbonate-4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 2 mM NaHCO_3 , 10 mM HEPES, and 0.1% bovine serum albumin) containing the indicated stimulators, and the supernatant was assayed for insulin secretion by using an enzyme-linked immunosorbent assay kit with a mouse insulin standard (Morinaga Institute of Biological Science, Yokohama, Japan). The insulin content was determined after extraction by using acid ethanol. A number of studies have reported significant data in insulin assays on INS-1 cells transfected with plasmids using Lipofectamine 2000 (2,4)

F-actin staining analysis

F-actin was stained using an F-actin Visualization Biochem Kit (Cytoskeleton, Denver, CO, USA). INS-1 cells cultured on a 12 $\times$ 12-mm coverslip were fixed using a fixative solution and incubated for 10 min. After washing the coverslip, the cells were treated with a permeabilization buffer (pH 7.0) and incubated for 5 min. After washing, F-actin was stained using rhodamine phalloidin and incubated for 30 min. For Rac1 staining, an anti-Rac1 antibody (BD Biosciences) was used.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM) and compared using analysis of the variance. A P-value less than 0.05 was considered significant.

RESULTS

Because Rac1-deficient mice are reportedly embryonic lethal (22), we created pancreatic β cell-specific Rac1-knockout mice to conduct the analyses. We showed that Rac1-knockout mice exhibit abnormal glucose tolerance because insulin secretion is inhibited due to impaired F-actin reorganization in Rac1-deficient pancreatic β cells (1). We reported that there was no significant difference in the pancreatic β cell mass between these mice and control mice. Further, we also showed that insulin signaling is important for the regulation of pancreatic β cell mass (6, 12, 21). In the present study, we investigated insulin signaling using pancreatic islets isolated from a pancreatic β cell-specific Rac1-knockout

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mouse. There was no significant difference in insulin signaling in the pancreatic islets of the knockout mouse when compared with the control mouse (Fig. 1a). In addition, no significant difference was observed in the expression levels of proliferating cell nuclear antigen (PCNA), a proliferation marker, and cleaved caspase 3, an apoptosis marker (Fig. 1b). These results showed that Rac1 deficiency in pancreatic β cells does not influence insulin signaling, proliferation, or apoptosis, revealing that the impairment of insulin secretion seen in pancreatic β cell-specific Rac1-knockout mice is not dependent on pancreatic β cell mass.

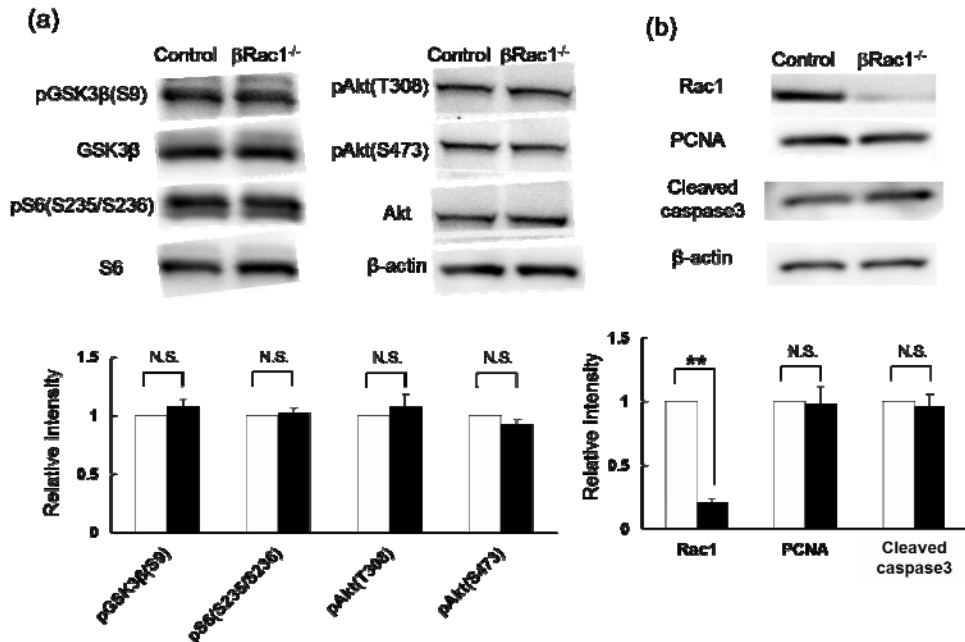


Fig. 1. Effects of β -cell Rac1 ablation on insulin signaling and proliferation in pancreatic islets. (a, b) Islets isolated from 10-week-old control and β Rac1^{-/-} mice were subjected to immunoblot analysis with antibodies to the indicated proteins. The bar graph shows quantification of the expression levels of the indicated proteins. The white bars indicate control mice; the black bars indicate β Rac1^{-/-} mice. Data are means $\pm$ SEM of 3 independent experiments. **P < 0.01, N.S.: not significant.

We confirmed Rac1 expression by using a rat pancreatic β cell line, INS-1 cells (1). In addition, we showed that Rac1 is not activated by either low-glucose or KCl stimulation, but is activated when the cells are stimulated with a high level of glucose. In this study, we prepared INS-1 cells transfected with an activated Rac1 (G12V) vector. Evaluation of Rac1 activation in these cells by using immunoblotting showed the enhanced expression of activated Rac1 (Fig. 2a). In addition, we observed the enhanced phosphorylation of PAK1, which is located downstream of Rac1 (Fig. 2b). However, there was no significant difference in insulin signaling between the 2 groups (Fig. 2c), and there was no significant change in the expression levels of PCNA or cleaved caspase 3 (Fig. 2d).

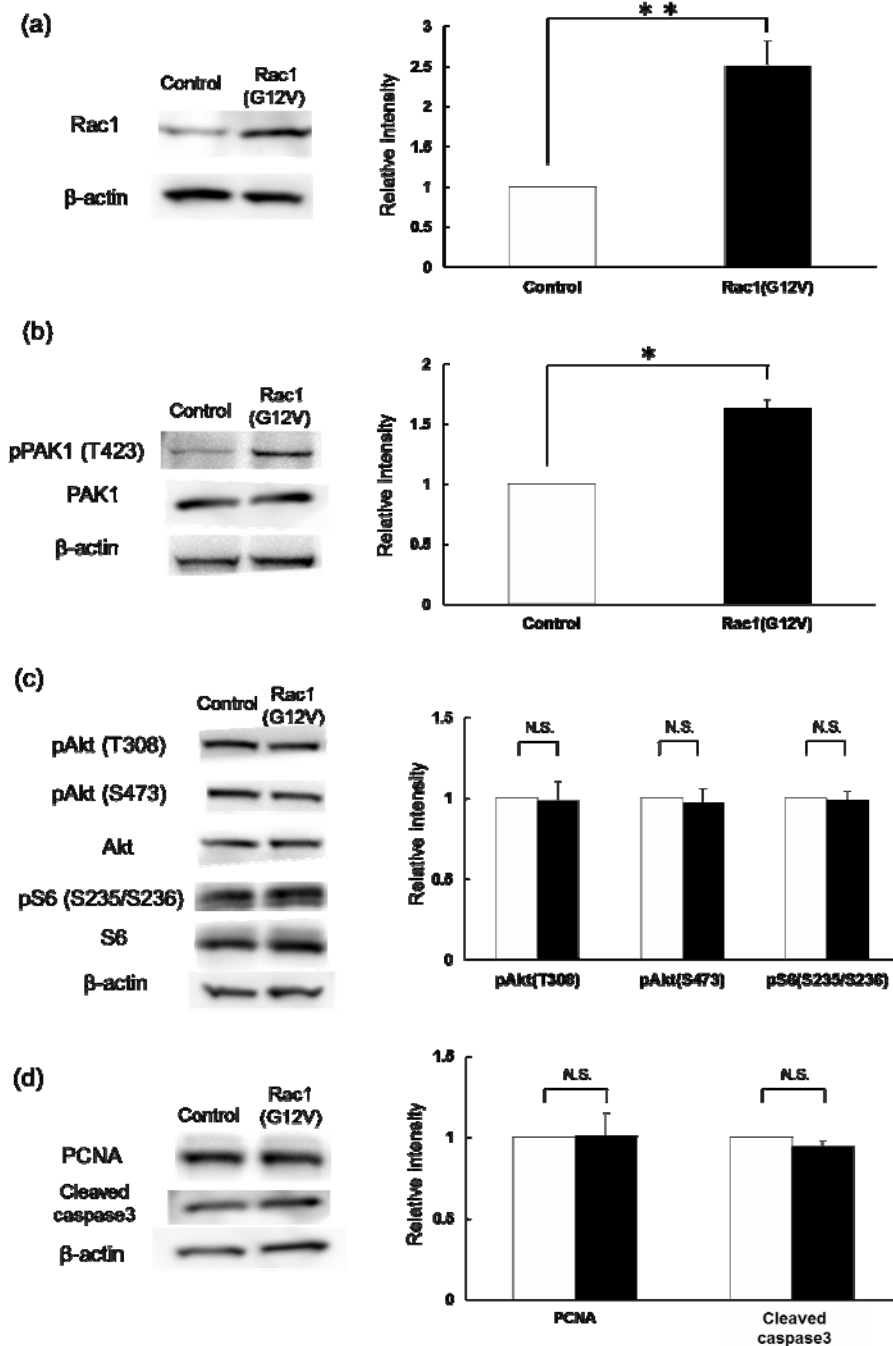


Fig. 2. Effects of constitutively activated Rac1 expression in the pancreatic β -cell INS-1 cell line. (a) GTP-Rac1, the active form of Rac1, was measured in INS-1 cells treated with the empty vector (Control) and activated mutant of Rac1 (G12V). (b–d) INS-1 cells treated with the empty vector (Control) and Rac1 (G12V) were subjected to immunoblot analysis with antibodies to phosphorylated or total forms of PAK1 (b), the indicated proteins for insulin signaling (c), PCNA and cleaved caspase 3 (d), and β -actin as a loading control. The bar graph shows

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quantification of the expression levels of GTP-Rac1 (a), phosphorylated forms of PAK1 (b), and the indicated proteins (c, d). The white bars indicate control; the black bars indicate Rac1 (G12V). Data are means $\pm$ SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, N.S.: not significant.

Previously, we showed that reduced expression of Rac1 in pancreatic β cells inhibits the depolymerization of F-actin, causing impaired F-actin depolymerization that then leads to impaired insulin secretion (1). In the present study, immunostaining for F-actin in the INS-1 cells expressing activated Rac1 revealed a reduction in the expression level of F-actin compared to the control group, even when the glucose level was as low as 2.8 mM (Fig. 3).

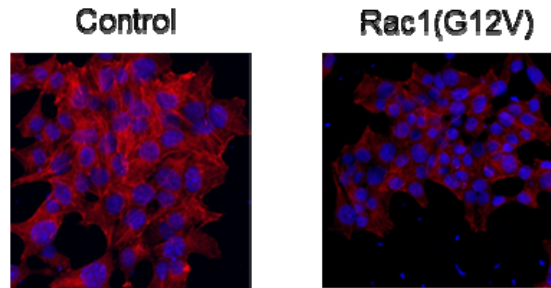


Fig. 3. Effects of constitutively activated Rac1 expression on F-actin depolymerization in the pancreatic β -cell INS-1 cell line. Staining of F-actin in response to treatment with 2.8 mM glucose was assessed in INS-1 cells treated with empty vector (Control) and Rac1 (G12V) with rhodamine phalloidin (red) and DAPI (blue).

F-actin is considered a barrier against insulin granules in low glucose conditions (19). Because F-actin levels are low in INS-1 cells expressing activated Rac1, insulin secretion is likely to be facilitated. Therefore, we investigated insulin secretion from these cells in the presence of 2.8 mM and 16.8 mM glucose. Despite the reduction of F-actin expression in these cells, no significant difference was observed in insulin secretion when compared to the control group (Fig. 4a). In addition, we investigated insulin secretion after stimulation with 30 mM KCl, but no significant difference was observed between the 2 groups (Fig. 4b).

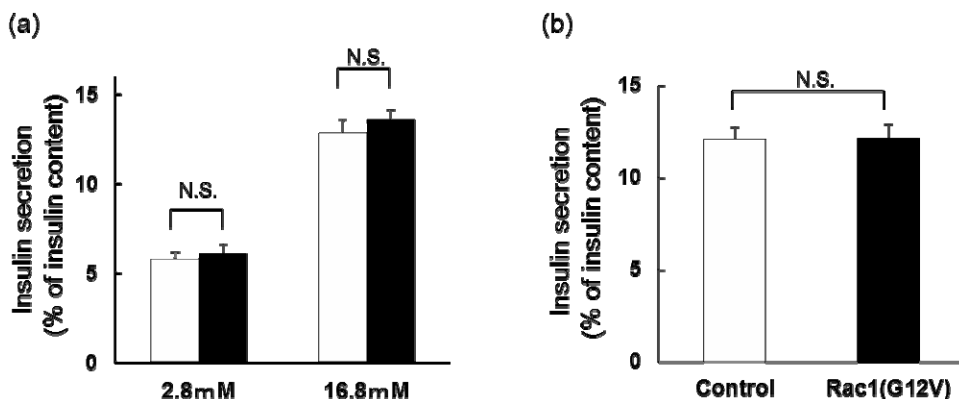


Fig. 4. Effects of constitutively activated Rac1 expression on insulin secretion in the pancreatic β -cell INS-1 cell line. Insulin secretion in response to the indicated concentrations of glucose (a) or 30 mM KCl (b) for 30 min was assessed in INS-1 cells and expressed as a percent of insulin content. Data were obtained from 6 independent experiments. The white bars indicate control cells; the black bars indicate Rac1 (G12V)-expressing cells.

These results showed that the activation of Rac1 facilitates the depolymerization of F-actin, but further, does not enhance insulin secretion.

DISCUSSION

Recently, we showed that although F-actin is depolymerized in pancreatic β cells stimulated by high glucose levels, the depolymerization of F-actin is inhibited in Rac1-deficient pancreatic β cells and Rac1-knockdown INS-1 cells, resulting in F-actin remaining in the cells. Further, we showed that insulin secretion is impaired due to the action of this F-actin as a barrier against insulin granules (1). Considering that the deficiency and inactivation of Rac1 impair insulin secretion in pancreatic β cells, it is interesting to determine whether the constitutive activation of Rac1 facilitates insulin secretion. If activated Rac1 (G12V) facilitates insulin secretion, then the protein could be a potential antidiabetic drug. In our present study, the overexpression of activated Rac1 (G12V) in INS-1 cells led to a reduction in the expression levels of F-actin; however, no significant difference was observed in insulin secretion after overexpressing activated Rac1 (G12V). The cytoskeleton is a known factor in insulin secretion (13, 18, 24); however, it alone is not sufficient to induce insulin secretion because calcium signaling has an essential role in this process (3, 20). The activation of Rac1 appears not to influence calcium signaling, which we consider is the reason why it causes no change in insulin secretion.

However, the inactivation and reduced expression of Rac1 are likely to be involved in impaired insulin secretion in patients with diabetes. In that case, insulin secretion is expected to recover through the activation of Rac1. In addition, Rac1 has been shown to be involved in secretion in cells other than pancreatic β cells (15) and participates in incretin secretion (14). At present, the cytoskeleton is an unlikely target in the diagnosis and treatment of type 2 diabetes; however, advances in research could lead to the clinical targeting of the cytoskeleton in the future.

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