Glucose regulates the cortical actin network through modulation of Cdc42 cycling to stimulate insulin secretion

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Nevins, Angela K., and Debbie C. Thurmond. Glucose regulates the cortical actin network through modulation of Cdc42 cycling to stimulate insulin secretion. Am J Physiol Cell Physiol 285: C698–C710, 2003. First published May 21, 2003; 10.1152/ajpcell.00093.2003.—Glucose-stimulated insulin granule exocytosis in pancreatic β-cells involves cortical actin remodeling that results in the transient disruption of the interaction between polymerized actin with the plasma membrane t-SNARE (target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex. To examine the mechanism underlying the initiation of cortical actin remodeling, we have used the actin-nucleating/stabilizing agent jasplakinolide to show that remodeling is initiated at a step proximal to the ATP-sensitive K⁺ channels in the stimulus-secretion pathway. Confocal immunofluorescent microscopy revealed that cortical actin remodeling was required for glucose-stimulated insulin secretion. Furthermore, glucose was found to mediate the endogenous activation state of the Rho family GTPase Cdc42, a positive proximal effector of actin polymerization, resulting in a net decrease of Cdc42-GTP within 5 min of stimulation. Intriguingly, glucose stimulation resulted in the rapid and reversible glucosylation of Cdc42, suggesting that glucose inactivated Cdc42 by selective glucosylation to induce cortical actin rearrangement. Moreover, expression of the constitutively active form of Cdc42 (Q61L) inhibited glucose-stimulated insulin secretion, whereas the dominant negative form (T17N) was without effect, suggesting that glucose-stimulated insulin secretion requires Cdc42 cycling to the GDP-bound state. In contrast, KCl-stimulated insulin secretion was unaffected by the expression of dominant negative or constitutively active Cdc42 and ceased to modulate endogenous Cdc42 activation, consistent with glucose-dependent cortical actin remodeling. These findings reveal that glucose regulates the cortical actin network through modulation of Cdc42 cycling to induce insulin secretion in pancreatic β-cells.

jasplakinolide; glucosylation; syntaxin; insulin granule; exocytosis

THE EFFICIENT SECRETION OF INSULIN in response to glucose is critically important to whole body glucose homeostasis. Insulin is secreted from intracellularly localized storage granules, and these granules must undergo a series of regulated steps to finally achieve fusion with the plasma membrane and release insulin into the extracellular milieu (53, 55). When extracellular glucose rises, the glucose transporters (GLUT-2) at the β-cell surface facilitate the uptake of glucose into the cell, resulting in an increase in ATP through metabolism of the glucose (44). This ATP alters the basal ATP/ADP ratio, causing the ATP-sensitive K⁺ (KATP) channels to close, depolarizing the cell and leading to the opening of Ca²⁺ channels, overall increasing cytoplasmic Ca²⁺ concentration ([Ca²⁺]) (1, 13, 51, 57). In response to the rise in intracellular Ca²⁺, some “primed” insulin storage granules fuse and release insulin, while intracellular storage granules mobilize to join granule pools closer to the cell surface (7, 22, 54).

The rate-limiting step in glucose-stimulated insulin secretion is thought to be contained within the steps that include the trafficking/mobilization, priming, docking, and fusion of the insulin granules (35). The priming/docking/fusion steps of insulin granule exocytosis involve the specific pairing of the t-SNARE [target membrane SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein] syntaxin 1 and SNAP-25 with the granule v-SNARE (vesicle membrane SNAP receptor) protein VAMP-2 (vesicle-associated membrane protein) (16, 25, 28, 46, 52, 56, 68, 72). Thus, upon glucose stimulation, insulin granules bind with the syntaxin and SNAP-25 t-SNAREs on the plasma membrane via their VAMP-2 protein to dock/tether and become primed for fusion (66). Primed vesicles are incorporated into the plasma membrane after a fusion reaction, and insulin is secreted into the extracellular space.

The trafficking of the insulin granules to the cell surface requires components of the cytoskeletal framework of microtubules and actin filaments (27, 32, 34, 42). Microtubules have been shown to be required for directional guidance of the granule and for sustained insulin secretion (2, 17, 19, 45). By contrast, the role of actin filaments is less clear. For example, actin filaments have been proposed to function in some of the motive force behind granule transport (49, 64). However, data obtained using F-actin disruption agents showed enhanced secretagogue-induced insulin secretion, suggesting instead that F-actin blocks granule...
movement (37, 43, 49, 62, 69). Recently, we have shown that glucose transiently modulates cortical actin organization and disrupts the interaction of polymerized actin with the plasma membrane t-SNARE complex (62). However, the question of how glucose elicits the reorganization of F-actin still remains.

One endogenous islet cell factor that is involved in regulation of the actin cytoskeleton and functions in a glucose-dependent and specific manner is the Rho family GTPase Cdc42. Cdc42 is an upstream positive effector of F-actin (see recent reviews, Refs. 23 and 67). Moreover, Cdc42 is found localized with insulin secretory granules in pancreatic β-cells (30, 31). Cdc42 has been shown to be required for glucose- but not KCl-stimulated insulin secretion in rat islets (29). Cdc42 has also recently been demonstrated to function in mastoparan-stimulated insulin release through an indirect interaction with the exocytosis SNARE protein syntaxin (15). However, the molecular role of Cdc42 in glucose-stimulated insulin secretion remains unclear.

We have recently documented that glucose stimulation for 5 min results in a diminished amount of phalloidin-stained cortical actin in MIN6 β-cells and in isolated rat islets (62). Here we have investigated the mechanism underlying this phenomenon. Specifically, using the actin nucleating/stabilizing agent jasplakinolide, we have shown that cortical actin reorganization induced by glucose occurs at a proximal step in the stimulus-secretion pathway. Importantly, our data show that stimulation with glucose results in alterations in the cycling of endogenous Cdc42 and show correlations between Cdc42 glucosylation and a transient depolymerization of cortical actin, all after 5 min of glucose stimulation. These data support a model whereby cortical actin 1) is signaled to reorganize by a glucose-stimulated regulatory signal, and 2) acts as a dynamic chaperone of granules to maintain granule pool sizes to ensure that insulin release is not rate-limited by depletion of primed granules.

MATERIALS AND METHODS

Materials. Radioimmunoassay (RIA)-grade bovine serum albumin (BSA), d-glucose, mouse anti-insulin antibody, and phalloidin-conjugated FITC were obtained from Sigma (St. Louis, MO). Rabbit polyclonal anti-Cdc42 antibody and mouse monoclonal anti-c-myc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Latrunculin B and jasplakinolide were purchased from Calbiochem (La Jolla, CA) and Molecular Probes (Eugene, OR), respectively. Streptolyxin-O was purchased from Sigma. PAK1-PBD agarose and the mouse anti-RL2 and -syntxin 1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Collagenase and Ficol were purchased from Sigma for the islet isolation. Monoclonal anti-Cdc42 and -syntxin 6 antibodies and Texas red-conjugated secondary antibody were purchased from BD Transduction Laboratories (Lexington, KY) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively. Vectashield was obtained from Vector Laboratories (Burlingame, CA). The MIN6 cells were a gift from Dr. John Hutton (University of Colorado Health Sciences Center). The enhanced chemiluminescence (ECL) kit and Hyperfilm-MP were obtained from Amersham Biosciences (Piscataway, NJ).

Cell culture, insulin secretion, and insulin content assays. MIN6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; 25 mM glucose) equilibrated with 5% CO2 at 37°C. The medium was supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, l-glutamine, and 50 mM β-mercaptoethanol, as described previously (65). MIN6 cells were used between passages 49 and 56. Insulin secretion was determined using cells grown in 35-mm wells to 60–80% confluence. Cells were washed twice with and incubated for 2 h in 1 ml of modified Krebs-Ringer-bicarbonate buffer (MKRBB; 5 mM KCl, 120 mM NaCl, 15 mM HEPES, pH 7.4, 24 mM NaHCO3, 1 mM MgCl2, 2 mM CaCl2, and 1 mg/ml BSA, RIA grade) that was gassed with 95% O2 for 30 min. Latrunculin B (10 μM) and jasplakinolide (5 μM) were solubilized in DMSO (vehicle) and added to the MKRBB for the 2-h preincubation period as indicated. Cells were stimulated with the secretagogue (20 mM glucose or 50 mM KCl) for the time indicated. The Krebs-Ringer-bicarbonate buffers were collected, microcentrifuged for 5 min at 4°C to pellet cell debris, and stored at −20°C. Insulin secreted into the supernatant was quantitated using a rat insulin immunoassay kit (Linco Research, St. Charles MO). Cells were subsequently harvested in NP-40 detergent lysis buffer (25 mM Tris, pH 7.4, 1% NP-40, 10% glycerol, 50 mM NaFl, 10 mM Na4P2O7, 137 mM NaCl, 1 mM NaVO3, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin) and lysed for 10 min at 4°C, and lysates were cleared of insoluble material by microcentrifugation for 10 min at 4°C. Insulin content of the cells was measured by RIA.

Immunofluorescence and confocal microscopy. MIN6 cells at 40% confluence plated on glass coverslips were incubated in MKRBB for 2 h, followed by stimulation with 20 mM glucose or 50 mM KCl, and were then fixed and permeabilized in 4% paraformaldehyde and 0.1% Triton X-100 for 10 min at 4°C. Fixed cells were blocked in 1% BSA and 5% donkey serum at room temperature, followed by incubation with primary antibody (1:100) for 1 h. Cells were then washed with phosphate-buffered saline (PBS) and incubated with phallolidin-conjugated FITC (1:1,000) and/or Texas red or FITC secondary antibody for 1 h. Cells were washed again in PBS and overlaid with Vectashield mounting medium, and coverslips were mounted onto slides for confocal fluorescence microscopy using a Zeiss 510 confocal microscope. Because MIN6 cells proliferate as “pseudo-islets” in cell clusters, cells were randomly selected for imaging analysis based on similarity of cluster size as viewed with a ×100 objective. Images presented were captured using identical settings unless otherwise specified.

Cdc42 activation assay and immunoblotting. A glutathione S-transferase (GST) fusion-protein, corresponding to the p21-binding domain of human p21-activated kinase (PAK1-PBD agarose) was used to specifically detect and interact with the GTP form of Cdc42 in MIN6 cell lysates as described previously (5). Briefly, cleared detergent lysates were prepared from cells incubated in MKRBB buffer for 2 h at 37°C and stimulated with glucose for 5 min. Freshly made cleared cell lysate (1 mg) was combined with 10 μg of PAK1-PBD agarose for 1 h at 4°C. After three washes with lysis buffer, proteins were eluted from the agarose beads and subjected to electrophoresis on 12% SDS-PAGE, followed by transfer to polyvinylidene difluoride membrane. Membranes were immunoblotted with rabbit anti-Cdc42 or mouse anti-Myc antibodies, and proteins were visualized by ECL.
Isolation, culture, and stimulation of insulin secretion of mouse islets. Pancreatic mouse islets of Langerhans were isolated using a modification of the previously described method (14, 33). Briefly, pancreata from 8- to 12-wk-old male C57Bl/6J mice were digested with collagenase and purified using a Ficoll density gradient. After isolation, islets were cultured overnight in complete CMRL-1066 culture medium (CMRL-1066 medium supplemented with 2 mM glutamine, 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin). Fresh islets were hand picked and cultured in complete CMRL-1066 overnight. Islets were washed twice with Krebs-Ringer bicarbonate buffer (10 mM HEPES, pH 7.4, 134 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, and 1.2 mM KH2PO4) containing 2 mM glucose and 0.1% BSA. Groups of 10 islets were preincubated in Krebs-Ringer bicarbonate buffer in the presence or absence of 5 µM jasplakinolide for 1 h, followed by stimulation with 20 mM glucose in the continued absence or presence of jasplakinolide for 1 h. Media were collected to measure insulin secretion, and islets were harvested in NP-40 lysis buffer to determine cellular insulin content by RIA.

Adenoviral infection of MIN6 cells. The NH2-terminal Myctagged Cdc42 adenoviral particles were generated in collaboration with Dr. Jeffrey Pessin (SUNY Stony Brook, Stony Brook, NY) by using cDNA constructs previously described (4, 11) and purchased from The University of Iowa Gene Transfer Vector Core Facility (Iowa City, IA). MIN6 cells at 50–60% confluence were infected in complete medium at a multiplicity of interest (MOI) of 100 for 2 h at 37°C, followed by two washes with PBS and 48 h of incubation at 37°C. Infection resulted in >90% of cells infected as determined by immunofluorescence using anti-Myc antibody to visually detect infected cells.

RESULTS

Actin rearrangement potentiates glucose-stimulated insulin secretion. We have recently reported (62) that glucose transiently induces rearrangement of cortical actin. To investigate the importance and role of cortical actin rearrangement in the process of insulin secretion, we treated MIN6 β-cells with the cell-permeant agent jasplakinolide, which rearranges F-actin by specifically binding to F-actin and inducing nucleation of F-actin oligomers (9, 60). Initially, we determined the time required for jasplakinolide to visually achieve steady state after rearranging cortical actin and nucleating visible microfilaments to be ~1 h, concurrent with the time by which the G:F-actin ratio was converted from ~85:15% to 1:99% (data not shown) in an actin solubility assay (50). Jasplakinolide treatment had no significant stimulatory effects in the absence of secretagogues, indicating that actin rearrangement alone was not sufficient to initiate insulin secretion (Fig. 1A). Surprisingly, jasplakinolide treatment consistently potentiated insulin secretion initiated by glucose, with insulin levels exceeding that of glucose-stimulated untreated or vehicle (DMSO)-treated MIN6 cells (Fig. 1A). Glucose stimulation of the vehicle-treated MIN6 cells resulted in a characteristic time-dependent increase in insulin secretion within 10 min (Fig. 1A). After glucose stimulation, both the rate and extent of insulin secretion, after 5 min of stimulation with glucose, were increased 3.7-fold in jasplakinolide-treated cells compared with control (vehicle treated) cells (Fig. 1A). Vehicle-treated cells behaved indistinguishably from untreated cells (data not shown). Although potentiated insulin secretion by jasplakinolide was no longer increased at a linear rate by 10 min, even after 60 min the secretion rate was considerably higher than that evoked by glucose alone (data not shown). This increase in secretion was not accompanied by any significant change in the total cellular content of insulin (Fig. 1B). Identical results were obtained using the β-HC9 β-cell line, indicating that this effect is not peculiar to the MIN6 cells (data not shown). These data indicated that jasplakinolide-induced rearrangement of F-actin facilitates glucose-stimulated insulin secretion in cultured β-cells.

To determine whether F-actin also functions in the control of insulin secretion from primary cells, we preincubated isolated mouse islet cells without or with jasplakinolide for 1 h before a 1-h incubation with or
Glucose alters cortical actin at a step proximal to the K\textsubscript{ATP} channels. To determine whether actin rearrangement was important for non-nutrient-induced insulin secretion, we examined the effect of depolarizing levels of KCl (50 mM) on jasplakinolide-treated cells (Fig. 2A). Stimulation by KCl was expected to result in a faster rate of secretion in the initial phase than that induced by glucose, and the vehicle-treated MIN6 cells showed a 3.9-fold increase in insulin secretion after 10 min. However, cells pretreated with jasplakinolide failed to exhibit potentiated secretion, secreting slightly lower than vehicle-treated levels of insulin throughout the time period examined. Moreover, stimulation with secretagogues downstream of KCl similarly failed to show potentiated secretion (data not shown). Thus our data using KCl as the secretagogue agreed with earlier findings in RINm5F cells (69), indicating that glucose but not KCl was capable of mediating F-actin to initiate insulin secretion. However, this difference was not the result of differential insulin granule localization in jasplakinolide-treated cells stimulated with glucose or KCl, because all cells pretreated with jasplakinolide exhibited a marked increase in insulin granules localized to the periphery regardless of secretagogue (Fig. 3B). This increase in peripherally localized granules may be the result of jasplakinolide-induced nucleation of microfilaments or clearance of the microfilament “cell web,” as has been described (9). Nevertheless, despite the increased availability of granules at the cell perimeter, KCl was incapable of eliciting an enhanced response in jasplakinolide-treated cells, which indicated that glucose and KCl had differential access to these granules.

To investigate how jasplakinolide might confer differential responsiveness of cells to stimulation with glucose compared with KCl, we treated cells with vehicle or jasplakinolide and visually assessed cortical actin by phalloidin-FITC labeling in the MIN6 cells (Fig. 4A). Syntaxin 6 was colabeled using Texas red to demarcate the localization of the trans-Golgi network of individual cells within the cortical actin network. Visualization of the midplane view of cell clusters revealed the strong distribution of F-actin as a ring beneath the plasma membrane indicative of cortical actin, which was dramatically diminished after 5 min of stimulation with glucose in >80% of similarly sized cell clusters visualized (Fig. 4A, images 1 and 2). Stimulation with KCl was without effect on cortical actin (Fig. 4A, image 3). Because jasplakinolide competes with phalloidin for binding to F-actin and results in a dramatically reduced fluorescence emission, phalloidin-FITC was used at 1:10 to achieve visualization of F-actin in jasplakinolide-treated cells. Cells pretreated with jasplakinolide exhibited discernible cortical actin rings under basal conditions (Fig. 4A, image 4). By contrast, even with jasplakinolide pretreatment, glucose stimulation for 5 min resulted in markedly reduced cortical actin (Fig. 4A, image 5). However, jasplakinolide-treated cells stimulated with KCl exhibited cortical actin rings similar to those of the basal state (Fig. 4A, image 6). Even when assessed at much earlier time points, stimulation with KCl failed to result in

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**Fig. 2.** Jasplakinolide potentiates glucose-stimulated insulin release from isolated mouse islet cells. A: islets cells were preincubated for 1 h with or without jasplakinolide (5 \( \mu \)M), followed by an incubation under basal (2 mM glucose) or stimulated conditions (20 mM glucose; Gluc) for 1 h. Insulin secretion was measured by RIA. Insulin levels have been normalized (basal = 1) for vehicle or jasplakinolide-treated islets, and data are shown as means ± SE of 3 independent sets of experiments. B: whole cell detergent lysates were prepared from unstimulated cells, and insulin content was quantitated by RIA. Data are shown as means ± SE of 3 independent sets of experiments.
alterations in cortical actin (data not shown). The effect of glucose is specific to d-glucose and is independent of general osmotic effects, because treatment of cells with l-glucose failed to induce alterations in cortical actin in these analyses (data not shown). In addition, green fluorescent protein (GFP)-actin-transfected cells treated with jasplakinolide showed similar alterations in cortical actin in response to glucose (data not shown). Thus both endogenous cortical actin and jasplakinolide-induced cortical actin was markedly reduced in response to stimulation by glucose but not by KCl. Moreover, this responsiveness of cortical actin to glucose correlated with the ability of glucose to initiate insulin secretion.

To quantitate these visible alterations in cortical actin with glucose, we used a coimmunoprecipitation assay. We recently reported (62) that F-actin and not monomeric actin specifically coimmunoprecipitates with the plasma membrane t-SNARE protein syntaxin 1 in MIN6 cells. Jasplakinolide treatment increased the amount of actin coimmunoprecipitated, whereas treatment with the F-actin-depolymerizing agent latrunculin B (61) abolished the interaction, indicating that syntaxin 1 coimmunoprecipitated specifically with F-actin. This interaction was used to evaluate and compare the effects of glucose with KCl on cytoskeletal rearrangement. Glucose stimulation for 5 min resulted in a twofold decrease in the amount of actin coimmunoprecipitated from vehicle-treated cells (Fig. 4B, lanes 1 and 2). Similarly, jasplakinolide-treated cell lysates showed a twofold decrease in actin coimmunoprecipitated with anti-syntaxin 1 (Fig. 4B, lanes 4 and 5). By contrast, stimulation with KCl for 5 min had no effect on the quantity of actin coimmunoprecipitated from cells treated with or without jasplakinolide (Fig. 4B, lanes 3 and 6). Thus these data indicated that glucose had the capacity to rearrange cytoskeletal actin, even in cells treated with jasplakinolide. Taken together, these data suggested that glucose signals alterations in the cytoskeleton early in the secretory pathway, possibly in parallel with or upstream of the KATP channels.

**Glucose-stimulated insulin secretion requires Cdc42 cycling.** Cdc42 is a Rho family small GTPase that associates with specific downstream targets to affect changes in the actin cytoskeleton and has been shown to be preferentially important in glucose-stimulated insulin secretion (29). Because glucose induces alterations of cortical actin by 5 min, we sought to determine whether the activation state of Cdc42 was differentially affected by glucose or KCl stimulation within the initial 5-min period. MIN6 cells were infected with CsCl-purified adenoviral particles (MOI = 100 to achieve infection of >90% of cell population) encoding wild-type (WT), dominant negative GDP-bound (T17N), and constitutively active GTP-bound (Q61L) forms of Cdc42. The PAK1-PBD interaction assay was used to confirm the GTP activation state of the recombinant proteins, utilizing the NH2-terminal myc epitope tag on the recombinant proteins to distinguish them from endogenous Cdc42 (Fig. 5A). All three forms of Cdc42 were present in cleared cell lysates made from infected MIN6 cells (Fig. 5A, lanes 1–3), although the Q61L form of Cdc42 expressed poorly compared with WT and T17N. As expected, the WT and Q61L proteins that bind GTP were able to interact with PAK1-PBD, whereas the GDP-bound T17N form failed to interact with PAK1 (Fig. 5A, lanes 4–6).

To determine whether expression of these various forms of Cdc42 would impact the ability of glucose to affect cortical actin in β-cells, we visualized F-actin by using phalloidin-FITC to compare the T17N- or Q61L-infected cells with control cells (Fig. 5B). The level of infection was assessed to be 90% or greater, as shown by immunofluorescent labeling of the Myc-tagged recombinant Cdc42 proteins (Fig. 5B, images 1, 3, 5, and 7). As expected, the Myc-tagged T17N protein localized cytosolically and the cells showed strong cortical actin
staining (Fig. 5B, images 1 and 2). The Myc-tagged Q61L protein was correctly localized to the plasma membrane and also showed strong cortical actin staining (Fig. 5B, images 3 and 4). Like noninfected cells or cells infected with the WT construct (data not shown), T17N-infected cells were responsive to glucose as visualized by disruption of cortical actin after 5 min of stimulation (Fig. 5B, image 6). In contrast, the cortical actin of Q61L-infected cells was unaffected by glucose (Fig. 5B, image 8). In contrast to either mutant form of Cdc42, endogenous Cdc42 is reportedly punctate and present in both the cytosol and periphery in isolated islets (31). Cdc42 was localized similarly in MIN6 cells (Fig. 5C, image 1). This distribution did not markedly change in response to glucose or KCl (Fig. 5C, images 2 and 3). Because Cdc42 localizes to the plasma mem-
brane upon activation and only a small percentage of Cdc42 is activated in a given cellular pool in response to stimuli (59), this finding was not unexpected. Moreover, colabeling of endogenous Cdc42 and F-actin (using phalloidin-FITC) showed that although there is some colocalization of Cdc42 with cortical actin, much of the Cdc42 was in the cytosol (Fig. 5C, images 4–6).

To correlate the qualitative changes in cortical actin in response to glucose in the T17N-infected cells with quantitative alterations in F-actin, we compared the amount of actin that coimmunoprecipitated with endogenous Cdc42 vs. the T17N form of Cdc42 in the absence and presence of glucose stimulation (Fig. 5D). Cdc42 is known to interact with F-actin via binding with N-WASp (neuronal Wiskott-Aldrich syndrome protein) and the Arp2/3 (actin-related protein) complex (see recent review, Ref. 18). In MIN6 cell lysates, anti-Cdc42 antibody coimmunoprecipitated actin, and this association was markedly reduced in cells stimulated with glucose for 5 min (Fig. 5D, lanes 1 and 2) and was fully ablated by latrunculin B pretreatment of the cells (data not shown). In the same manner, Myc–T17N Cdc42 coimmunoprecipitated actin from unstimulated cells, and this association was completely absent in glucose-stimulated cells (Fig. 5D, lanes 3 and 4). However, the relative G:F-actin ratio was relatively unchanged after 5 min of glucose stimulation such that the cells contained ~83–86% G-actin and 14–17% F-actin (data not shown), consistent with the ratio derived from rat islets (24). In addition, expression of the T17N or Q61L forms of Cdc42 had no statistically significant effect on the G:F-actin ratio, consistent with another study using Cdc42 mutants (36). Thus the decreased cortical actin visualized in glucose-stimulated T17N-expressing cells correlated with decreased interaction between Cdc42 and F-actin.

The effects of expressing T17N and Q61L forms of Cdc42 upon glucose-stimulated insulin secretion were determined. Cells overexpressing the T17N form of Cdc42 showed an approximately twofold stimulation within 5 min of glucose addition (Fig. 6A), similar to noninfected cells (Fig. 1A). In contrast, cells overexpressing the Q61L form of Cdc42 showed an ablated response to glucose (Fig. 6A), correlating with a lack of responsiveness of cortical actin in Q61L-infected cells to glucose (Fig. 5B, image 8). Consistent with this finding, Q61L-infected cells treated with latrunculin B for 2 h before stimulation showed a significant stimulation by glucose without affecting basal secretion (Fig. 6B). Combined with data demonstrating that Q61L- and WT-expressing cells are equally responsive to KCl (Fig. 6C), these data indicated that the expression of Q61L protein does not inhibit general secretory function of the cells. Taken together, these data suggested that cycling of Cdc42 to the GDP-bound state was required for glucose-stimulated insulin secretion.

To correlate changes in the Cdc42 activation state with the changes observed in cortical actin and insulin secretion over time, we next determined the endogenous level of GTP-bound Cdc42 in cleared cell lysates prepared from MIN6 cells stimulated with glucose over a 5-min time period (Fig. 7). The amount of Cdc42 interacting with the PAK1-PBD (indicating GTP bound) was ~7% of total cellular Cdc42, similar to the level of Cdc42-GTP reported for other cell types (5, 38). The cellular content of Cdc42-GTP rose to ~14% after 3 min of glucose stimulation, falling to levels below that of the basal state after 5 min of stimulation (Fig. 7A). This peak in Cdc42-GTP level correlated with the peak rate of glucose-stimulated insulin secretion between 1 and 3 min (Fig. 7B). The amount of detergent-soluble Cdc42 protein was unchanged in glucose-stimulated compared with unstimulated lysates, and there was no alteration in Cdc42 activation in cells stimulated with KCl over the same time period (data not shown). These data suggested that stimulation with glucose induces changes in the ability of Cdc42 to bind PAK1-PBD or effector molecules. Importantly, the lowest level of Cdc42-GTP was detected after 5 min, the same time that glucose was found to stimulate depolymerization of cortical actin.

The activation state of Cdc42 has been reported to be altered by several posttranslational modifications, including carboxyl methylation and glucosylation (29, 31). However, whereas carboxyl methylation requires GTP and results in Cdc42 activation, glucosylation is not known to be GTP dependent and results in Cdc42 inactivation. To determine whether changes in the...
activation state were correlated with changes in the glucosylation state of Cdc42, we immunoprecipitated Cdc42 from detergent cell lysates made from cells stimulated with or without glucose for 5 min (Fig. 8A, lanes 1 and 2). Although equivalent levels of Cdc42 were immunoprecipitated, 2.3-fold more glucosylated (O-linked) Cdc42 protein was immunoprecipitated from glucose-stimulated cells than from unstimulated cells as determined by immunoblotting using the O-linked N-acetylglucosamine (O-GlcNAc)-specific antibody RL2 (Fig. 8A, inset). This finding represented an increase in glucosylated Cdc42 from approximately ~2% in the basal state to 4% after glucose stimulation for 5 min. The reciprocal immunoprecipitation using the RL2 antibody confirmed this finding, as shown by an increase in glucosylation of Cdc42 after glucose stimulation for 5 min (Fig. 8A, lanes 3 and 4). To determine whether Cdc42 was a specific target of glucosylation, we examined the glucosylation state of syntaxin 1, an indirect binding partner of Cdc42 (15). Although syntaxin 1 was present in the cell lysates (Fig. 8B, lanes 1 and 2), it was not immunoprecipitated with the RL2 antibody (Fig. 8B, lanes 3 and 4). In addition, the insulin granule v-SNARE protein VAMP2 was present in cell lysates but was not glucosylated (data not shown), consistent with studies of glucosylated SNARE proteins in 3T3-L1 adipocytes (10). A more thorough time course of Cdc42 glucosylation revealed that the small but significant increase in glucosylated Cdc42 protein was evident only after 3 min (Fig. 8C) and was reversibly reduced back to basal levels by 30 min of static glucose incubation (Fig. 8D). This rate of reversibility could be increased by removal of glucose at the 5-min time point, followed by incubation in glucose-free MKRBB for 10 min. Taken together, these data suggested a correlation between the timing of inactivation of Cdc42 by selective glucosylation and the timing of cortical actin rearrangement in the initiation of insulin secretion stimulated by glucose.

**DISCUSSION**

In this report we have demonstrated that cortical actin plays an important role specifically in glucose-stimulated insulin secretion in MIN6 β-cells and mouse islet cells. Cortical actin rearrangement induced by jasplakinolide resulted in an increased number of insulin granules to the cell perimeter, which correlated with increased glucose-stimulated insulin secretion. By contrast, cells expressing the constitutively active form of Cdc42 (Q61L) displayed cortical actin that was resistant to glucose-induced changes, correlating with an inhibition of glucose-stimulated insulin secretion. Induction of changes in cortical actin was specific to glucose, because stimulation with 50 mM KCl or other downstream secretagogues failed to result in potenti- ated insulin secretion in jasplakinolide-treated cells or visible alterations of cortical actin. Previous work (29, 31) suggested that the small Rho-family GTPase Cdc42 was specifically modified by glucose in islet cells. Our data extend this by suggesting that glucose stimulation of β-cells for 5 min results in an increase in glucosylated Cdc42, correlating with a decreased ability of Cdc42 to interact with effector proteins, coordinate with the time at which cortical actin is first visibly altered in response to glucose. The dynamic alterations in cortical actin induced by glucose during static incu-
bation conditions are consistent with the concept that β-cell cortical actin undergoes glucose-dependent remodeling through posttranslational modification of Cdc42 to modulate the level of Cdc42 activation.

The rate of replenishment of granule pools was recently demonstrated to be a critical factor in nutrient-stimulated insulin secretion as opposed to the transient secretion elicited by KCl (3, 48). Because Cdc42 is a key upstream regulator of F-actin that colocalized with insulin secretory granules (30, 31), it appeared to be a perfect target for modification by glucose to exert a large effect in a short time frame. Consistent with the initial transient depolymerization of F-actin 5 min after glucose addition to the media, expression of constitutively active Cdc42 abolished glucose- but not KCl-stimulated insulin secretion within 5 min, suggesting that Cdc42 cycling to the GDP-bound form leads to actin depolymerization. Expression of dominant negative Cdc42 had a small stimulatory but statistically insignificant effect on insulin secretion, suggesting that interruption of Cdc42 cycling overall does not inhibit insulin secretion. To investigate the importance of cycling further studies using Cdc42Hs (F28L), which can undergo constitutive GTP/GDP exchange without interfering with GTP hydrolysis (39), will be needed. Importantly, the inhibition of secretion by the consti-

Fig. 7. Glucose induces changes in the activation state of Cdc42. MIN6 cells were incubated in MKRBB for 2 h before stimulation with 20 mM glucose for 0.5, 1, 3, or 5 min. A: detergent-solubilized cell lysates were prepared and immediately used in a PAK1-PBD interaction assay as described in MATERIALS AND METHODS. Lysate (50 µg) prepared at each time point was loaded alongside the PAK1-PBD eluates of the interaction assay, and proteins were separated on 12% SDS-PAGE, transferred to PVDF, and immunoblotted with mouse anti-Cdc42 to calculate the relative percentage of Cdc42-GTP. Data shown and percentages calculated are representative of at least 4 independent experiments, and data are shown as means ± SE.

Fig. 8. Cdc42 is glucosylated in response to glucose stimulation. MIN6 cells were incubated in MKRBB for 2 h before stimulation with 20 mM glucose for 5 min. A: detergent-solubilized cell lysates were prepared for immunoprecipitation using rabbit anti-Cdc42 (top, lanes 1 and 2) or mouse RL2 (top, lanes 3 and 4) antibodies, and proteins were separated on 12% SDS-PAGE for immunoblotting with Cdc42 antibody. The membrane was subsequently stripped and immunoblotted for the presence of glucosylated Cdc42 protein using the RL2 antibody (bottom, lanes 1 and 2). Inset: glucosylation was quantitated using optical density scanning for 4 independent experiments, and data are means ± SE (normalized to basal = 1 for each experiment). B: lysates containing endogenous syntaxin 1 protein (lanes 1 and 2) were immunoprecipitated using mouse RL2 and immunoblotted for syntaxin 1 (lanes 3 and 4). C: time course of Cdc42 glucosylation. Detergent lysates were prepared from cells stimulated with glucose for 0, 2, 3, 4, and 5 min and immunoprecipitated using anti-Cdc42, and proteins were separated on 12% SDS-PAGE for immunoblotting with RL2 antibody. The membrane was subsequently stripped and immunoblotted with Cdc42 antibody. D: Cdc42 glucosylation is reversible. Detergent lysates were prepared from cells stimulated with glucose for 5 or 30 min and immunoprecipitated using anti-Cdc42, and proteins were separated on 12% SDS-PAGE for immunoblotting with RL2 antibody. In the same experiment, cells were stimulated with glucose for 5 min, washed twice with glucose-free MKRBB, and incubated for 10 min in glucose-free MKRBB, and detergent lysates were prepared and used in the same immunoprecipitation experiment. Data are representative of 3–7 independent experiments.
ness of Cdc42 to glucose and not KCl places Cdc42 in the proximal signaling pathway, perhaps concurrent at the level of K_{ATP} channel closure. Therefore, we propose that glucose triggers insulin secretion while simultaneously initiating the cascade of events necessary to transiently induce actin depolymerization. Because neither cortical actin depolymerization nor granule mobilization is an instantaneous process, it seems advantageous for the β-cell to initiate this process in advance of the need to elicit second-phase insulin secretion.

Our data support a molecular mechanism wherein Cdc42 becomes glucosylated upon stimulation with glucose, which correlates with the observed transient loss of cortical actin induced by glucose. The endogenous modification of Cdc42 by glucosylation in response to stimulation of β-cells with glucose for only 5 min is a novel finding. O-linked glucosylation is highly dynamic, with rapid cycling in response to cellular signals analogous to phosphorylation. Previous studies have shown that Cdc42 is glucosylated on Thr35, which lies in the effector domain of the GTPase and is involved in coordinating the γ-phosphate of bound GTP (26). Glucosylation by Clostridium difficile toxin targets this residue and decreases the GTPase activity by 85% and also inhibits the GTPase-activating protein (GAP) stimulation of Cdc42 GTPase activity (58). In islets and β-cells, glucosylation by this toxin specifically inhibited glucose- but not KCl-induced insulin secretion within 1 h (29). The abundance of O-GlcNAc in islet β-cells markedly and transiently increases in response to 20 mM glucose (40). O-GlcNAc transferase is highly enriched in β-cells relative to other known cell types and is thought to be of vital importance for β-cell response to glucose loads (21). In fact, hyperglycemia leads to the rapid and reversible accumulation of O-GlcNAc specifically in β-cells in vivo (40). Moreover, in a recent study glucosylation was blocked by incubation of mouse islets with the glutamine:fructose-6-phosphate amidotransferase (GFAT) inhibitor azaserine, resulting in inhibition of glucose-stimulated insulin secretion (73), and inhibition was reversed by the addition of GlcNac. Intriguingly, many regulatory proteins that interact with actin contain O-GlcNAc, as well as proteins thought to bridge the cytoskeleton to the plasma membrane such as vinculin, talin, and the synapsins (12, 20, 41). In addition, we have recently reported the specific glucosylation of Munc18c, a SNARE accessory protein that regulates GLUT-4 vesicle fusion, suggesting that glucosylation may also exert distal effects on the exocytosis machinery (10).

Further studies are required to investigate the potential regulation of insulin granule exocytosis by O-GlcNAc at these various steps in the stimulus-secretion pathway.

Recently, it was reported (15) that Cdc42 indirectly interacts with syntaxin 1, and this interaction is promoted by the secretagogue mastoparan or by constitutively activating Cdc42 using the Q61L mutation. Mastoparan, a hormone receptor-mimetic peptide isolated from wasp venom, stimulates insulin release from pancreatic β-cells independently of a requirement for increased [Ca^{2+}]. In support of this, we report that jasplakinolide cannot potentiate secretion elicited by secretagogues downstream of glucose or in Streptolysin-O-permeabilized cells stimulated under high-Ca^{2+} buffer conditions (data not shown). Thus our data are consistent with the notion that Cdc42 is involved in glucose-stimulated insulin secretion and further suggest that Cdc42 may be part of the protein complex that bridges to the SNARE protein complexes at the plasma membrane.

Syntaxin 1A has also been demonstrated to interact with L-type Ca^{2+} channels, and it may be that this localization is sufficient to expedite granule fusion after stimulation by secretagogue (6, 70, 71). Although it has also been shown that actin can tether to channels, latrunculin B depolymerization of F-actin resulted in potentiation rather than inhibition of Ca^{2+} induced secretion (62). However, it is possible that Ca^{2+} entry activates F-actin-severing proteins such as scinderin, which clears F-actin. In fact, in mouse pancreatic β-cells, scinderin-derived actin-binding peptides reduced Ca^{2+}-dependent exocytosis of insulin granules (8). Furthermore, immunofluorescence studies in chromaffin cells show a stimulus-initiated spatial and temporal correlation between scinderin relocalization and actin depolymerization (63). Because it has been shown that exocytosis in these cells occurs in cortical areas devoid of F-actin (47), it seems possible that redistribution of scinderin to locations near K_{ATP} and/or Ca^{2+} channels could facilitate granule movement to channel-localized syntaxin 1A SNARE complexes.

Nearly 30 years ago it was discovered that F-actin-depolymerizing agents potentiates the secretion of insulin stimulated by glucose. Our results demonstrate that glucose can induce transient actin cytoskeletal rearrangements in isolated mouse islets and in MIN6 β-cells. We propose that glucose signals the transient reduction of filamentous cortical actin to coordinate granule mobilization and pool refilling that will ultimately promote granule exocytosis.

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DISCLOSURES

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REFERENCES


42. Malaisse WJ, Malaisse-Lagae F, Van Obberghen E, Somers G, Devis G, Ravazza M, and Orci L. Role of microtubules in...