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Glucose-stimulated Cdc42 Signaling Is Essential for the Second Phase of Insulin Secretion*

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The small Rho family GTPases Cdc42 and Rac1 have each been shown to function in insulin exocytosis and are presumed to function in actin remodeling and insulin granule mobilization. However, whether either GTPase is required for the mobilization phase of insulin release (second phase) and are linked in a common signaling pathway has remained unknown. Here we demonstrate that small interfering RNA-mediated depletion of Cdc42 from isolated islets results in the selective loss of second-phase insulin release. Consistent with a role in this nutrient-dependent phase, Cdc42 activation was detected exclusively in response to D-glucose and was unresponsive to KCl or non-metabolizable glucose analogs in MIN6 β-cells. Cdc42 activation occurred early in secretion (3 min), whereas Rac1 activation required ~15–20 min, suggesting Cdc42 as proximal and Rac1 as distal regulators of second-phase secretion. Importantly, Rac1 activation and function was linked in a common pathway downstream of Cdc42; Cdc42 depletion ablated glucose-induced Rac1 activation, and expression of constitutively active Rac1 in Cdc42-depleted cells functionally restored glucose-stimulated insulin secretion. Occurring at a time midway between Cdc42 and Rac1 activations was the phosphorylation of p21-activated-kinase 1 (Pak1), and this phosphorylation event required Cdc42. Moreover, small interfering RNA-mediated Pak1 depletion abolished Rac1 activation and glucose-stimulated insulin release, suggesting that Pak1 may mediate the link between Cdc42 and Rac1 in this pathway. Taken together, these data substantiate the existence of a novel signaling pathway in the islet β-cell whereby Cdc42 functions as a key proximal transmitter of the glucose signal early in stimulus-secretion coupling to support the later stage of insulin release.

Insulin is released from pancreatic islet β-cells in two distinct phases in response to glucose stimulation. The first phase of insulin release can be elicited by elevation of intracellular calcium levels to trigger fusion of granules pre-docked at the plasma membrane, a glucose stimulation. The second phase of insulin release requires the amplifying action of glucose and is presumed to require mobilization of storage pool granules to the cell surface to sustain insulin release for an hour or longer depending upon how long the β-cell detects stimulatory levels of glucose. Thousands of storage granules exist behind a filamentous actin (F-actin) barrier in the pancreatic β-cell, and F-actin remodeling is known to mobilize granules to the target membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)[2] sites at the cell surface, yet the mechanisms involved in remodeling and granule mobilization are largely unknown and untested.

We and others have presented evidence to suggest that the key to actin remodeling in the islet β-cell may lie in the glucose-specific activation of the small Rho family GTPase protein Cdc42 (1–5). We have recently demonstrated that Cdc42 is kept in its GDP-loaded and inactivated state by its interaction with Caveolin-1, whereby Caveolin-1 functions as a novel Cdc42 guanine dissociation inhibitor (GDI) in β-cells. Caveolin-1 binds to Cdc42 present on secretory granules, and the complex rapidly translocates to the plasma membrane and dissociates within 3 min of glucose stimulation (6). The caveolin-1-Cdc42 complex is associated with the vesicle (v)-SNARE protein VAMP2 on the insulin granules and disruption of the Cdc42-VAMP2 interaction attenuates glucose but not KCl-stimulated insulin secretion (2). This glucose-specific Cdc42 activation is also coupled to interactions between target SNARE proteins and F-actin (1). Moreover, expression of the constitutively active form of Cdc42 (Q61L) inhibited glucose-stimulated insulin secretion but was without effect upon K^+-stimulated secretion, suggesting that specifically glucose-stimulated insulin secretion requires Cdc42 cycling to the GDP-bound state. Importantly, blockage of cycling using the Q61L mutant correlated with formation of cortical F-actin that was resistant to reorganization in response to glucose stimulation. This was distinctly different from the response to constitutively active Cdc42 in PC12 neuroendocrine cells (7) and demonstrates the selective responsiveness of Cdc42 to glucose in the β-cell. This fits well with the concept of the β-cell using its cytoskeleton differently from neuronal cells, due to intrinsic differences in the pattern of release of neurotransmitter from

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[2] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; GDI, guanosine diphosphate dissociation inhibitor; G-protein (GTPase), guanosine triphosphate (GTP)-binding protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; MKRBB, modified Krebs-Ringer bicarbonate buffer; Pak1, p21-activated kinase; PM, plasma membrane; PVDF, polyvinylidene fluoride; RIA, radioimmunoassay; siRNA, small interfering RNA; IB, immunoblot; GFP, green fluorescent protein; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary; PBD, protein binding domain.

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that of insulin. However, how Cdc42 signals the remodeling of F-actin in the β-cell remains unknown.

Another Rho family GTPase, Rac1, has also been implicated in insulin secretion from β-cells. Like Cdc42, Rac1 was shown to translocate to the plasma membrane and undergo activation within 15 min of glucose stimulation. However, whether Rac1 activation occurred earlier than 15 min was not tested (5, 8). Based upon this late time of activation in the scheme of stimulus-secretion coupling and also upon indirect evidence showing that a Rac1 mutant disrupted insulin release only in the latter half of the stimulatory period, Rac1 has been implicated as a factor mediating the second phase of secretion (8). However, no direct evidence for Rac1 function or for the function of Cdc42 in biphasic secretion from islets exists to show that either play essential roles in second-phase secretion.

Cdc42 and Rac1 are well studied in other cell types and have been shown to function sequentially in a common pathway or to function in divergent pathways (9–11). When in a common pathway, the order of their activations can vary depending upon the cell type. For example, in the control of cell motility, activation of Cdc42 leads to the sequential activation of Rac1 (12, 13). Alternatively, in other cell types this order of activation is either reversed, or Cdc42 and Rac1 activations function antagonistically (14, 15). In addition, Rac1 has been shown to engage in cross-talk with yet another small G-protein, ARF6, to coordinate the signaling cascade underlying epithelial cell migration (16). Such a cross-talk mechanism has been suggested to explain Rac1 function in pancreatic β-cells (17), although neither this potential mechanism nor its potential participation in a mechanism involving Cdc42 has been examined.

In this report we use multiple siRNA-mediated depletion approaches to provide direct evidence for a role for Cdc42 as a key proximal regulator specifically of second-phase insulin release and that Rac1 activation is downstream of Cdc42 in a common pathway. Activation of this pathway was selectively responsive to D-glucose and not to non-metabolizable analogs of glucose or KCl stimulation, consistent with the role of this pathway in signaling the second and not the first phase of insulin granule exocytosis. The mechanism of Cdc42 signaling to Rac1 was further shown to involve Pak1: 1) Pak1 became activated in response to glucose at ~5 min, midway between the times of activation of Cdc42 (3 min) and Rac1 (20 min), and 2) siRNA-mediated depletion of Cdc42 inhibited Pak1 phosphorylation, and depletion of Pak1 resulted in ablation of Rac1 activation. These data demonstrate that the early activation of Cdc42 during the time of first-phase secretion acts as a conduit for distant and late effects upon insulin release in the second phase.

EXPERIMENTAL PROCEDURES

Materials—The EZ-Detect Cdc42/Rac1 activation kit containing mouse anti-Cdc42 and Rac1 antibodies was purchased from Pierce. Mouse anti-Myc antibody, mouse anti-hemagglutinin antibody, rabbit anti-Pak1 antibody, rabbit anti-actin antibody, rabbit IgG, and protein G Plus-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific anti-Pak1 (Thr-423) antibody was purchased from Chemicon (Temecula, CA). Goat anti-rabbit horseradish peroxidase and anti-mouse horseradish peroxidase secondary antibodies were acquired from Bio-Rad. Lipofectamine2000 was purchased from Invitrogen. Hyperfilm-MP and enhanced chemiluminescence (ECL) reagent were obtained from Amersham Biosciences. The RIA grade bovine serum albumin, D-glucose, 2-deoxy-glucose, L-glucose, 3-orthomethyl glucose, and KCl were obtained from Sigma. The human C-peptide, rat insulin, and sensitive rat insulin RIA kits were purchased from Linco Research Inc. (St. Charles, MO). All siRNA oligonucleotides for targeting endogenous mouse Cdc42 and Pak1 were purchased from Ambion (Austin, TX): Cdc42 si-A, GCAAUGAGUGCUAGUUUUUtt; Cdc42 si-B, CCCAAAUUGAUCUCAGAGAtt; Cdc42 si-C, CCUUUGAAAUACACCAAAUtt; Pak1, CAUCAAAUAUCACAAAGUCtt.

Plasmids—The full-length mouse Cdc42 cDNA was obtained using the following PCR primers to screen a MIN6 cDNA library: forward primer, 5′-AGAGAATTCATGGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCAGACAATTAAGTGA-3′; and reverse primer, 5′-CTCGGATCCTCATAGCAGCACACACCTGCGGCT-3′. The PCR product contained an engineered N-terminal Myc tag and was subcloned into the EcoRI and BamHI sites of pcDNA3 (Invitrogen), and the construct was verified by sequencing. The four plasmid-based siRNA constructs, pSilencer1.0-Cdc42 (siCdc42), were generated as previously described (18). Briefly, annealed complimentary double-stranded oligonucleotides encoding 19 nucleotides of mouse Cdc42 followed by a loop region (TTCAAGAGA) and the antisense of the 19 nucleotides were inserted into ApaI and EcoRI sites of the pSilencer1.0 vector (Ambion). The pSilencer1.0 control construct (siCon) was generated as previously described (19). Sequences of four pSilencer siCdc42 constructs were: siCdc42#1, ATTCCCATCGGAATATGTA; siCdc42#2, GTTATCCACAGACAGATGT; siCdc42#3, CTAACCACTGTCCAAAGAC; siCdc42#4, GCCTATTACTCCAGAGACT. The sequence of siCdc42#4 was used to generate the siCdc42-Ad adenovirus, inserted into the 5′ XhoI and the 3′ SpeI site of the pSilencer-Adeno-CMV vector (Ambion). Adenoviral LacZ backbone purchased from Ambion was used as a control (Con-Ad). Recombination, expression, purification, and titration of cesium chloride viral particles were performed by Viraquest Inc. (North Liberty, IA). Adenoviruses were packaged with green fluorescent protein (GFP) to facilitate identification of transduced cells. The pCMV6-myc-Pak1 and pCGN-HA-Rac1(Q61L) constructs were kindly provided by Dr. Lawrence Quilliam (Indiana University School of Medicine, Indianapolis, IN).

Cell Culture, Transient Transfection, and Secretion Assays—CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml L-glutamine. At 80–90% confluence cells were electroporated with 40 μg of DNA as previously described (20). After 48 h of incubation, cells were harvested in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 10% glycerol, 50 μM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 137 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml leupeptin) and

lysates were cleared by centrifugation at 14,000 × *g* for 10 min at 4 °C. Proteins present in lysates were resolved by 12% SDS-PAGE and depletion detected by immunoblotting.

MIN6 cells (a gift from Dr. John Hutton, University of Colorado Health Sciences Center, Denver, CO) were cultured in DMEM (25 mM glucose) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine, and 50 μM β-mercaptoethanol as described previously (19). At 50–60% confluence cells were co-transfected with 2.5 μg of each plasmid DNA and human proinsulin (pCB6/INS, a gift from Dr. Chris Newgard, Duke University, Durham, NC) as previously described (21). For the insulin secretion rescue assay, DNA quantities were reduced to 1.5 μg of each of the three different plasmids to accommodate the constraints of the DNA:transfectin ratio for optimal transfection efficiency. After 48 h of incubation, cells were washed twice with and incubated for 2 h in freshly prepared modified Krebs-Ringer bicarbonate buffer. Cells were stimulated with 20 mM D-glucose or 50 mM KCl for the times indicated in the figures. MKRBB was then collected and centrifuged at 14,000 × *g* for 5 min for subsequent quantitation of secretion using human C-peptide radioimmunoassay kit (Linco Research). Transfection of siRNA oligonucleotides into MIN6 cells was achieved using Lipofectamine2000 (Invitrogen) with 100 nM oligonucleotides to obtain ~80–90% transfection efficiency. A non-targeting RNA (scrambled siRNA, also obtained from Ambion) was used as a control. Transfected cells were maintained in the supplemented Dulbecco's modified Eagle's medium for 48 h and subsequently incubated in MKRBB and stimulated as described above. Insulin secreted into the MKRBB was quantitated using a rat insulin RIA kit (Linco). Cells were harvested in 1% Nonidet P-40 lysis buffer for detection of Cdc42, Pak1, or Rac1 depletion or activation.

Subcellular Fractionation—Subcellular fractions were isolated as previously described (2). All steps of fractionation procedure were performed at 4 °C unless stated otherwise. Briefly, MIN6 cells at 70–80% confluence were washed with cold phosphate-buffered saline and harvested into 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, and 1 mM dithiothreitol containing the following protease inhibitors: 10 μg/ml leupeptin, 4 μg/ml aprotinin, 2 μg/ml pepstatin, and 100 μM phenylmethylsulfonyl fluoride). Cells were disrupted by 10 strokes through a 27-gauge needle, and homogenates were centrifuged at 900 × *g* for 10 min. Postnuclear supernatants were centrifuged at 5500 × *g* for 15 min, and the subsequent supernatant was centrifuged at 25,000 × *g* for 20 min to obtain the secretory granule fraction in the pellet. The supernatant was further centrifuged at 100,000 × *g* for 1 h to obtain the cytosolic fraction. Plasma membrane fractions (PM) were obtained by mixing the post-nuclear pellet with 1 ml of buffer A (0.25 M sucrose, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4) and 2 volumes of buffer B (2 M sucrose, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4). The mixture was overlaid with buffer A and centrifuged at 113,000 × *g* for 1 h to obtain an interface containing the plasma membrane. The interface was collected and diluted to 2 ml with homogenization buffer for centrifugation at 6000 × *g* for 10 min, and the resulting pellet was collected as the plasma membrane fraction. All pellets were resuspended in the 1% Nonidet P-40 lysis buffer to solubilize membrane proteins.

Cdc42 and Rac1 Activation Assays—To specifically detect the GTP-loaded forms of Cdc42 or Rac1, the EZ-Detect Cdc42/Rac1 activation kit from Pierce was used as described previously (2). Freshly made fractions (100 μg) or whole cell lysate (500 μg) were combined with 20 μg of GST-Pak1-PBD-agarose for 1 h at 4 °C with constant rotation. After 3 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 (PVDF) membranes. Membranes were immunoblotted with mouse anti-Cdc42 or mouse anti-Rac1 antibodies. The relative abundance of eluted Cdc42/Rac1 was determined by Western blotting and densitometry.

Co-immunoprecipitation and Immunoblotting—For Pak1 immunoprecipitation, 2 mg of cleared detergent lysates were combined with 2 μg of rabbit anti-Pak1 antibody and allowed to rotate for 2 h at 4 °C. Protein G Plus-agarose beads were added, and reactions were rotated at 4 °C for an additional 2 h. After three washes with lysis buffer the resulting immunoprecipitates were subjected to 12% SDS-PAGE followed by transfer to PVDF membranes for immunoblotting with a phospho-specific (Thr-423) Pak1 antibody (Chemicon). Membranes were incubated with primary antibody at 4 °C overnight followed by incubation with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature and visualized by enhanced chemiluminescence.

Adenoviral Transduction of MIN6 Cells—MIN6 cells at 60% confluence were transduced with either siCdc42-Ad or siCon-Ad CsCl-purified particles for 2 h at 37 °C (multiplicity of infection of 100). Transduced cells were then washed twice and incubated for 48 h in complete medium at 37 °C, 5% CO_2. GFP fluorescence was visualized in >95% of cells in all experiments. Transduced cells were subsequently preincubated in MKRBB for 2 h and stimulated with 20 mM D-glucose for 20 min. Cleared detergent lysates were prepared for assessment of Rac1 activation as described above.

Mouse Islet Isolation, Adenoviral Transduction, and Perifusion—Pancreatic mouse islets were isolated, adenovirally transduced, and used for perifusion as previously described (18, 22). Briefly, pancreata pooled from five 8–12-week-old male mice (C57BL/6J) were batch-digested with collagenase and purified using a Ficoll density gradient. After isolation, 120 islets were hand-picked into two groups and immediately transduced at an multiplicity of infection of 50 with either siCdc42-Ad or siCon-Ad CsCl-purified particles for 1 h at 37 °C. Transduced islets were then washed twice and incubated overnight in RPMI 1640 at 37 °C, 5% CO_2. GFP fluorescence was visualized in greater than 95% of islets in all experiments. For perifusion analysis, 50 GFP-positive (GFP+) islets were handpicked onto a column between two layers of Cytodex 3 beads (Amersham Biosciences), washed twice with Dulbecco's phosphate-buffered saline (magnesium-free), and preincubated for 30 min at 37 °C in freshly gassed KRBH buffer (10 mM HEPES, pH 7.4, 134 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4) containing 0.1% bovine serum albumin. Islets

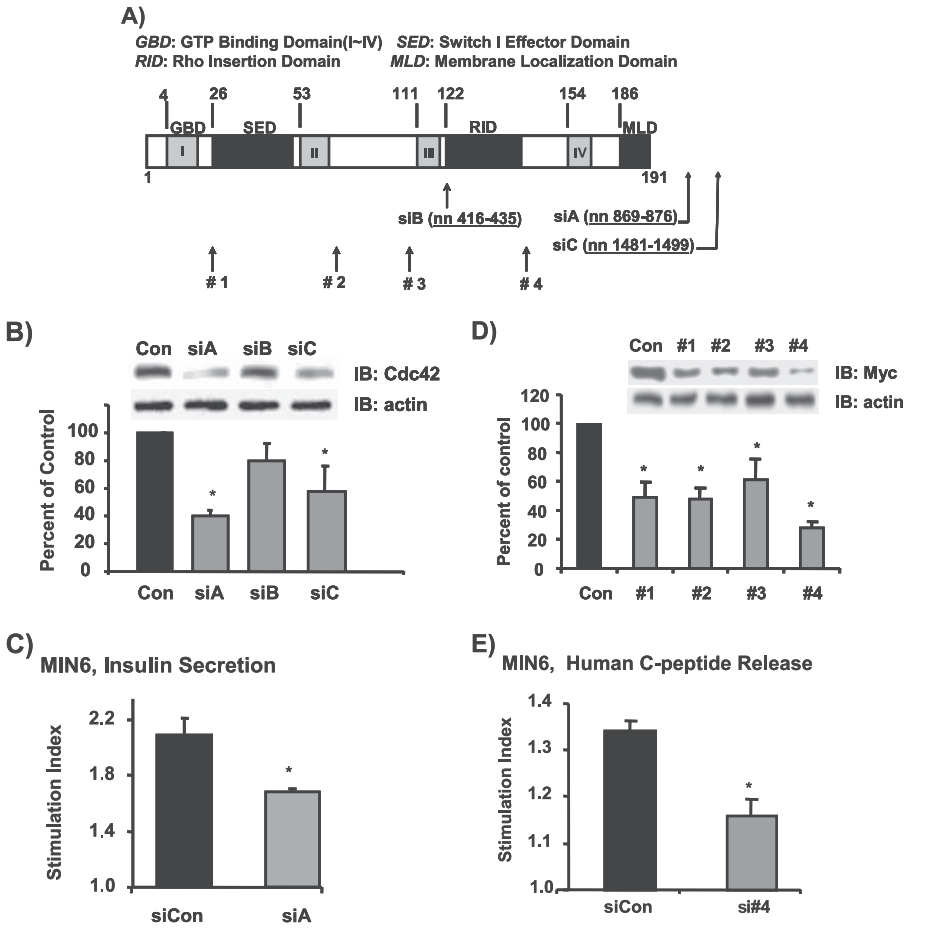

FIGURE 1. **Depletion of Cdc42 by siRNA impairs glucose-stimulated insulin secretion in MIN6 cells.** *A*, locus of siRNA oligonucleotides within the mouse Cdc42 mRNA (NM_009861). siA-siC sites were targeted by commercially available siRNA oligonucleotides. #1-#4 sites were used in a plasmid-based siRNA delivery system. *B*, MIN6 cells were transfected with three different commercially available Cdc42 siRNA and control oligonucleotides using Lipofectamine2000 as described under "Experimental Procedures." After 48 h of incubation, whole cell detergent lysates were prepared and subjected to 12% SDS-PAGE for immunoblotting with anti-Cdc42 and anti-actin (loading control) antibodies. Data represent the average ± S.E. for four independent experiments. *, $p < 0.05$ *versus* siCon. *Con*, control. *C*, MIN6 cells were transfected using Lipofectamine2000 with siA and control oligonucleotides, and after 48 h incubation cells were preincubated in glucose-free MKRBB for 2 h followed by stimulation with 20 mM glucose for 20 min. Insulin secreted into the MKRBB was measured by RIA. Data are shown as the ratio (average ± S.E.) of insulin released after stimulation relative to basal level from three independent experiments; *, $p < 0.05$ *versus* basal. *D*, four double-stranded Cdc42 siRNAs in the pSilencer1.0 vector (Ambion). CHO-K1 cells were co-electroporated with each siRNA construct and mouse pcDNA3-myc-Cdc42 as a reporter of depletion efficiency. After 48 h of incubation, whole cell detergent lysates were prepared and subjected to 12% SDS-PAGE for immunoblotting with anti-Myc and anti-actin (loading control) antibodies. Data represent the average ± S.E. for four independent electroporations using at least two independent batches of cesium chloride-purified DNA. *, $p < 0.05$ *versus* siCon. *E*, MIN6 cells were transfected with either control or Cdc42 siRNA (*si#4*) plasmid DNAs plus human pro-insulin DNA. After 48 h of incubation, cells were preincubated in MKRBB for 2 h followed by a 1-h stimulation with 20 mM glucose. Human C-peptide secreted into the media from the transfected cells was measured by RIA. Data represent the average ± S.E. of at least four independent experiments. *, $p < 0.05$ *versus* siCon.

were then perifused at a flow rate of 0.3 ml/min for 10 min in KRBH buffer containing 2.8 mM glucose with eluted fractions captured at 1-min intervals followed by D-glucose stimulation (20 mM) for 35 min. Insulin secreted into eluted fractions was quantitated by a sensitive rat insulin RIA immunoassay kit (Linco Research, Inc.). This entire procedure was repeated for a total of five independent perifusion experiments. For validation of Cdc42 depletion in transduced islets, 200 GFP+ islets were handpicked and lysed in SDS-PAGE sample buffer, and proteins were resolved on 12% SDS-PAGE for immunoblotting.

Statistical Analysis—All data are evaluated for statistical significance using Student's *t* test. Data are expressed as the average ± S.E.

RESULTS

Depletion of Cdc42 by siRNA Impairs Glucose-induced Insulin Secretion in β-Cells—We and others have demonstrated that impairment of Cdc42 activation or cycling leads to inhibition of glucose-stimulated insulin secretion from pancreatic β-cells (1–3, 23). Cdc42 knock-out mice are not available for study because they die early in embryogenesis (24), so we used siRNA-mediated depletion of Cdc42. We used seven different siRNA sequences to target multiple domains and functional regions of Cdc42 (Fig. 1*A*), three commercially available oligonucleotides and four that we generated and expressed by a plasmid-based system. The three commercially available siRNA oligonucleotides were transiently transfected into MIN6 cells (designated as siA, siB, and siC) using the reagent Lipofectamine 2000 to obtain ~80–90% of cells transfected, as gauged using fluorescently labeled oligonucleotides. Two of the three oligonucleotides successfully depleted endogenous Cdc42 protein from MIN6 cells, with siA depleting to the greatest extent at 60% (Fig. 1*B*). Depletion of Cdc42 by siA also resulted in a 30% inhibition of glucose-induced insulin release compared with the control oligonucleotide (Fig. 1*C*). In an independent approach we also tested the effects of plasmid-based siRNA delivery using four 19-mer sequences (designated as #1–#4) in the pSilencer vector as we have described previously (18). Due to the low transfection efficiency of plasmid into MIN6 cells, we chose to first evaluate the efficiency of each siRNA at depleting Cdc42 using the easily transfectable CHO-K1 cells. CHO-K1 cells were co-transfected with an Myc-tagged mouse Cdc42 plasmid to evaluate the effectiveness of each mouse-specific siRNA on depletion of mouse Cdc42 (CHO-K1 cells express endogenous hamster Cdc42). All four Cdc42 siRNA constructs effectively depleted Myc-tagged mouse Cdc42 expression (Fig. 1*D*), with si#4 being the most effective (~70% knockdown). To determine the functional impact of Cdc42 depletion upon glucose-stimulated insulin

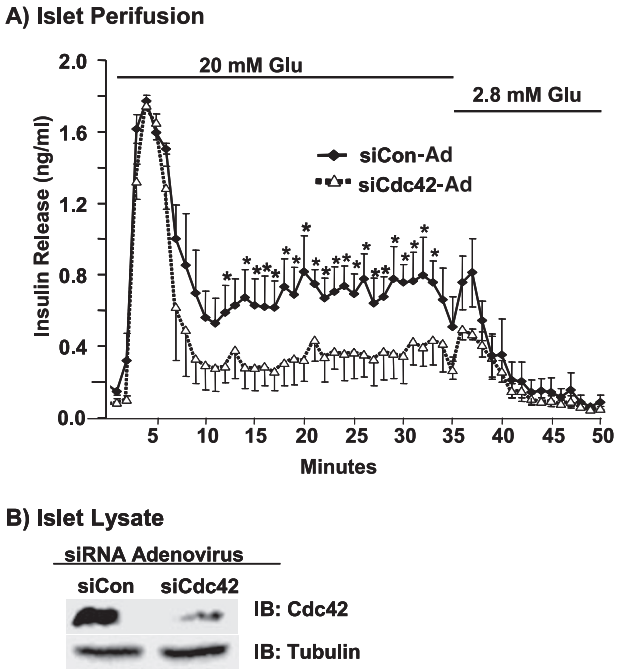

FIGURE 2. **Cdc42 depletion in isolated mouse islets results in the selective inhibition of second-phase insulin secretion.** *A*, mouse islets were isolated and immediately transduced with control (*siCon-Ad*) or siCdc42 (*siCdc42-Ad*) adenoviral particles for 1 h at 37 °C (multiplicity of infection of 50). Islets were then cultured overnight, after which GFP+ islets were handpicked into groups of 50 and placed onto columns for perifusion analysis at 1-min interval with 2.8 or 20 mM glucose. Data are the average ± S.E. of five independently isolated sets of islets. *, $p < 0.05$ *versus* siCon. *B*, 200 islets transduced with siCon-Ad or siCdc42-Ad were solubilized in SDS sample buffer, and protein was resolved on 12% SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted for the presence of Cdc42 or tubulin (loading control).

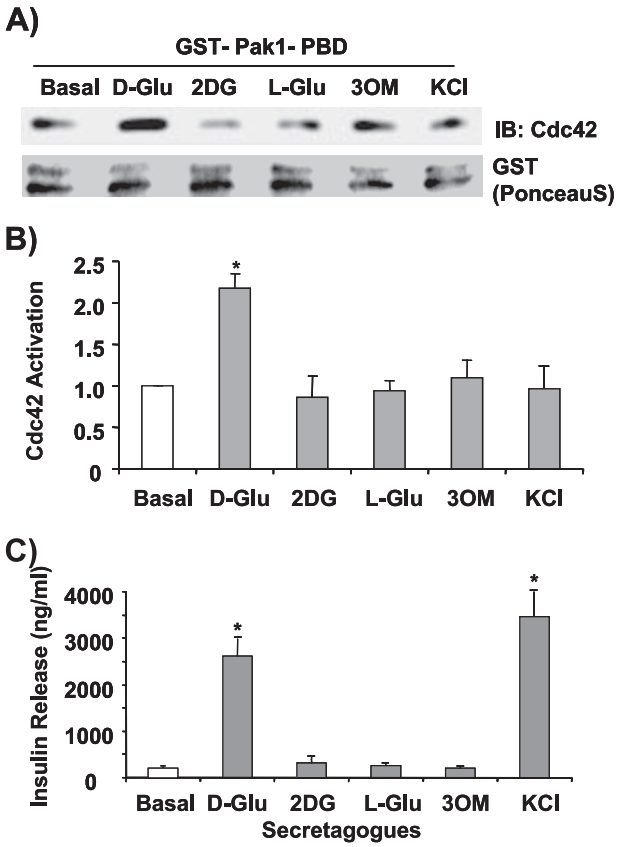

FIGURE 3. **Cdc42 activation in MIN6 cells is specific to D-glucose.** *A*, after 2 h of incubation in glucose-free MKRBB buffer, MIN6 cells were stimulated with 20 mM D-glucose (*D-Glu*), 2-deoxyglucose (*2DG*), L-glucose (*L-Glu*), 3-orthomethylglucose (*3OM*), or 50 mM KCl for 3 min. Cleared detergent cell lysates were prepared and immediately subjected to GST-Pak1-PBD interaction assays. Equal loading of lanes was verified by Ponceau S staining of the GST-Pak1-PBD. *B*, optical density quantitation of Cdc42 activation for three independent experiments; *, $p < 0.05$ *versus basal*. *C*, insulin secretion in response to stimulation with the various secretagogues for 30 min was determined by insulin RIA; *, $p < 0.05$ *versus* basal.

secretion, a human proinsulin plasmid was co-transfected with si#4 into MIN6 cells to serve as a reporter of secretion from transfectable cells. Human proinsulin is packaged and processed to human C-peptide and insulin in secretory granules in a manner similar to that of the mouse proinsulin present in the MIN6 cells, but the human C-peptide is immunologically distinct from that of the mouse C-peptide. Consistent with the effect of the commercial siRNA oligonucleotide siA, our plasmid-based si#4 inhibited ~50% of the glucose-induced human C-peptide release (Fig. 1*E*). These results suggested that Cdc42 is required for glucose-stimulated insulin secretion.

Cdc42 Is Essential for Second-phase Insulin Release from Islets—To address whether Cdc42 is required in first, second, or both phases of glucose-stimulated insulin secretion, we used isolated islets transduced with siCdc42 adenovirus. Freshly isolated mouse islets were transduced with adenoviruses encoding either siCdc42#4 (siCdc42-Ad) or the matched control (siCon-Ad) for 24 h. Adenoviruses were packaged with GFP, enabling selection of 50 transduced islets for each perifusion chamber. GFP+ islets were used for perifusion study in parallel chambers, and insulin secretion was measured every minute over a 60-min time period as described previously (19). After an equilibration period of 30 min, islets were perifused for 10 min in KRBH buffer containing 2.8 mM glucose (basal) followed by stimulation for 35 min at 20 mM glucose and, finally, returned to basal conditions for an additional 15 min. Insulin release kinetics showed biphasic responsiveness from islets isolated from wild-type mice that mimicked those previously reported (25). Control islets exhibited a peak within 5 min of glucose stimulation, consistent with the occurrence of first-phase insulin secretion. The first-phase peak was followed by a drop to the sustained second phase that persisted at a level ~2–3-fold higher than basal level until glucose levels were reduced back to basal (Fig. 2*A*). In islets transduced with siCdc42, first-phase secretion was similar to control islets. Quantitation of the area under the curve for first phase was 97 ± 5% of siControl-treated islets ($p = 0.3$). However, siCdc42-Ad islets did show a significant reduction in second-phase secretion in each of the five independent islet perifusion experiments. Efficiency of Cdc42 depletion from islets was validated by immunoblotting (Fig. 2*B*). Thus, these data indicated that Cdc42 was functionally essential for facilitating second phase but not first-phase insulin secretion in mouse islets.

Metabolism of Glucose Is Required for Cdc42 Activation—Second-phase insulin secretion is elicited in response to glucose and not in response to KCl. Previous studies had suggested that Cdc42 functioned only in response to glucose (1). To determine whether glucose transport and/or glucose metabolism was essential to trigger Cdc42 activation, MIN6 cells were stimu-

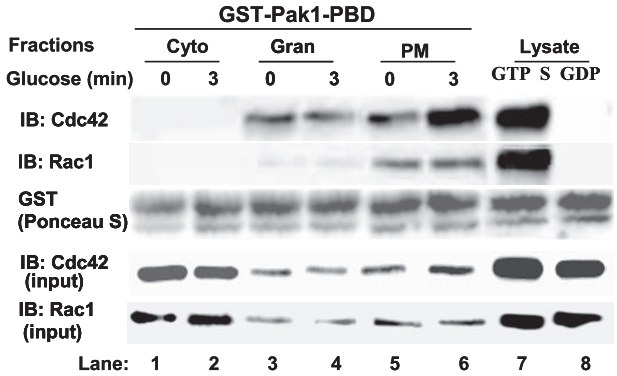

FIGURE 4. **Glucose-stimulated Cdc42 activation occurs at the plasma membrane and precedes Rac1 activation in MIN6 cells.** MIN6 cells were incubated in glucose-free MKRBB for 2 h and either left unstimulated or were stimulated with 20 mM glucose for 3 min. Plasma membrane (*PM*), cytosol (*Cyto*), and granule (*Gran*) fractions were prepared as described under "Experimental Procedures." Each fraction (100 μg protein) was subjected to the GST-Pak1-PBD interaction assay, and eluted protein was resolved on 12% SDS-PAGE for immunoblotting for the presence of Cdc42 or Rac1 as indicated. The localization of Cdc42 and Rac1 proteins in each fraction was also assessed (*IB*, *input*). Ponceau S staining used as loading control. GTPγS and GDP were used as experimental controls.

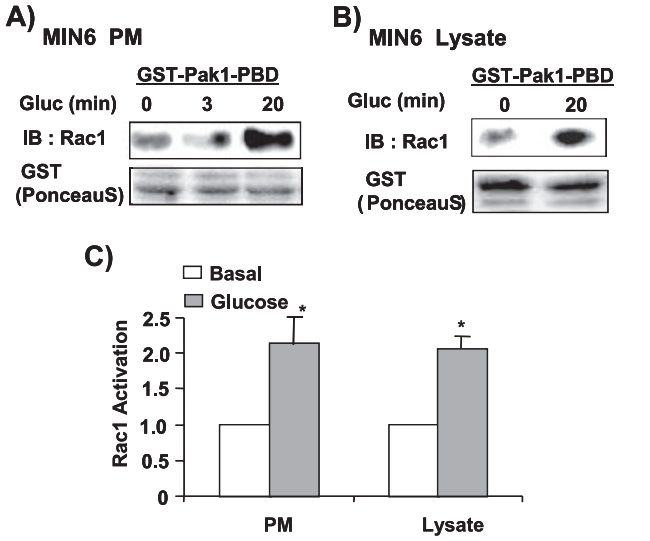

FIGURE 5. **Glucose-induced Rac1 activation requires 20 min and occurs at the plasma membrane in MIN6 cells.** MIN6 cells were incubated in MKRBB for 2 h before stimulation with 20 mM glucose for 3 or 20 min. PM fractions (*A*) or whole cell detergent lysates (*B*) were prepared and immediately used in the GST-Pak1-PBD interaction assay. Eluted proteins were resolved on 12% SDS-PAGE, transferred to PVDFs, and immunoblotted with mouse anti-Rac1 antibody. Ponceau S staining served as an indicator of loading. *C*, optical density quantitation of Rac1 activation at PM and in lysate from three independent experiments; *, $p < 0.05$ *versus* basal.

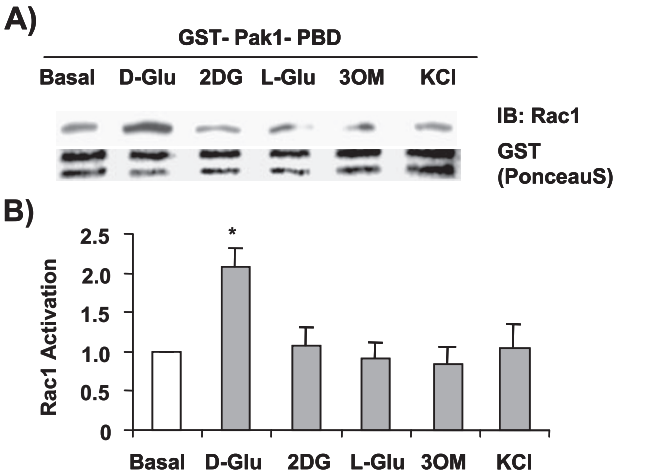

FIGURE 6. **Activation of Rac1 in MIN6 cells is specific to D-glucose.** *A*, after 2 h of incubation in glucose-free MKRBB buffer, MIN6 cells were stimulated with either 20 mM D-glucose (*D-Glu*), 2-deoxyglucose (*2DG*), L-glucose (*L-Glu*), 3-orthomethylglucose (*3OM*), or 50 mM KCl for 20 min. Cleared detergent cell lysates were prepared and immediately subjected to GST-Pak1-PBD interaction assays, and eluted protein was subjected to 12% SDS-PAGE for immunoblotting with anti-Rac1 antibodies. Equal loading of lanes was verified by Ponceau S staining of the GST-Pak1-PBD. *B*, optical density quantitation of Rac1 activation for three independent experiments; *, $p < 0.05$ *versus* basal.

lated with either 20 mM D-glucose, L-glucose (non transportable), 2-deoxyglucose (taken up by glucose transporters and phosphorylated similar to glucose but cannot be metabolized further), 3-orthomethylglucose (taken up by glucose transporters but could not be phosphorylated), or 50 mM KCl for 3 min for assessment of activation using the GST-Pak1-PBD interaction assay. Of these compounds, only D-glucose was found to induce Cdc42 activation (Fig. 3*A*). Quantitation of three independent experiments showed that D-glucose rapidly increased levels of activated Cdc42 by greater than 2-fold (Fig. 3*B*). Although KCl failed to activate Cdc42, it did effectively trigger insulin secretion as did D-glucose (Fig. 3*C*), showing that the MIN6 cells were responding appropriately by secreting insulin in response to the known secretagogues. Because KCl tends to elicit a faster secretory response, we also assessed Cdc42 activation levels in cells stimulated with KCl for 30 s, 1 min, or 2 min, and all failed to elicit Cdc42 activation (data not shown). These data indicated that Cdc42 activation in glucose-stimulated insulin release is selective for glucose and not KCl and that both glucose transport and metabolism beyond glucose 6-phosphate is required.

Cdc42 Activation Occurs at the Plasma Membrane and Precedes Glucose-induced Rac1 Activation—We have previously shown that Cdc42 becomes activated within 3 min of glucose stimulation in whole cell lysate in MIN6 cells (1), and Cdc42 translocated from the granule pool to the PM during that time. To determine whether Cdc42 became activated in the granule pool or upon arrival at the PM, we examined the activation status of Cdc42 in these different fractions. Although the Cdc42 present in the cytosol was not activated under either condition (Fig. 4 *lanes 1–2*), there was activated Cdc42 present in the granule pool, although this level was not increased in response to glucose (Fig. 4, *lanes 3–4*). In fact, the glucose-stimulated increase in activated Cdc42 was exclusively localized to the PM pool of Cdc42 (Fig. 4, *lanes 5–6*). Consistent with our previously results in MIN6 cell whole lysate, the abundance of activated Cdc42 was found to increase 2-fold within 3 min after glucose stimulation (Fig. 4, *Cdc42 (input)*, *lanes 5–6*). These data suggested that glucose triggers translocation of Cdc42-bound granules to the PM, and at the PM Cdc42 becomes activated.

In the βTC3 β-cell line Rac1 has been shown to become activated within 15–20 min of glucose stimulation (8). To determine whether this late time of activation held true in the MIN6 line of β-cells, we assessed the Rac1 activation using the same fraction preparations used to assess Cdc42 activation. Similar to Cdc42, in the cytosol no detectable activated Rac1

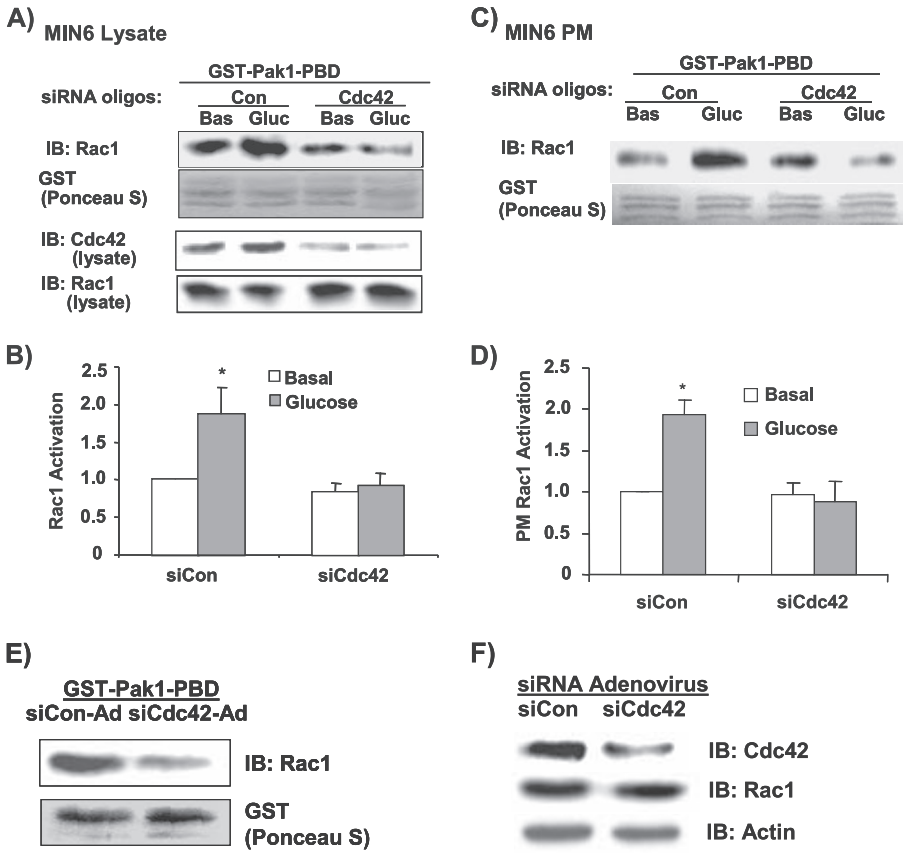

FIGURE 7. **Cdc42 expression is required for glucose-induced Rac1 activation in MIN6 cells.** *A*, MIN6 cells were transduced with siCdc42 (siA) or siControl siRNA oligonucleotides. After 48 h of incubation, MIN6 cells were preincubated in MKRBB for 2 h and then either left unstimulated or were stimulated with glucose (20 mM) for 20 min. Whole cell detergent lysates were prepared and immediately subjected to GST-Pak1-PBD interaction assays. Eluted proteins were resolved on 12% SDS-PAGE and transferred to PVDF for immunoblotting to detect activated Rac1. Cdc42 and Rac1 abundance in lysates was confirmed by immunoblotting. *B*, quantitation by optical density scanning and calculation of the ratio of Rac1 activated upon glucose stimulation relative to basal level for three independent experiments; *, $p < 0.05$ *versus* si*Con* under basal conditions. *C*, MIN6 cells were transfected with siCon or siCdc42 oligonucleotides, and after 48 h of incubation and subsequent glucose stimulation for 20 min, PM fractions were prepared and immediately subjected to GST-Pak1-PBD interaction assays. *D*, optical density quantitation of ratio of Rac1 activation at PM upon glucose stimulation compared with basal levels. Data are the average ± S.E. of three independent experiments. *, $p < 0.05$ *versus* siCon under basal conditions. *E*, MIN6 cells transduced with either siCon-Ad or siCdc42-Ad for 48 h were preincubated in MKRBB buffer for 2 h and stimulated with glucose for 20 min. Lysates were prepared and immediately subjected to GST-Pak1-PBD interaction assays. Eluted protein was separated on 12% SDS-PAGE and transferred to PVDF for immunoblotting with anti-Rac1 antibody. Equal loading of lanes was verified by Ponceau S staining of the GST-Pak1-PBD. Data are representative of two independent experiments with identical results. *F*, MIN6 cells were transduced with siCon-Ad or siCdc42-Ad as in *E* above, and detergent lysates were prepared. Proteins (40 μg) were resolved on 12% SDS-PAGE and transferred to PVDF for immunoblotting with anti-Rac1, Cdc42, and anti-actin (loading control) antibodies.

was found (Fig. 4, *lanes 1–2*). Unlike Cdc42, however, no significant increase of activated Rac1 was found within the 3 min of glucose stimulation in the PM fractions (Fig. 4, *lanes 5–6*). This lack of detection of Rac1 activation early in stimulus-secretion coupling is consistent with response in βTC3 cells.

Although Cdc42 activation was found to be increased by more than 2-fold in the PM within 3 min of glucose stimulation, glucose-induced Rac1 activation at the PM was not detected until 20 min of glucose stimulation (Fig. 5, *A* and *C*). Similarly, Rac1 activation in MIN6 whole cell lysate level was not apparent until 20 min with glucose (Fig. 5, *B* and *C*) and was consistent with that observed in other β-cell lines and islets (5). Taken together, these results suggest that Cdc42 activation preceded Rac1 activation during glucose-stimulated insulin release from pancreatic β-cells. Furthermore, the glucose-induced increase in Rac1 activation mimicked that of Cdc42 in that it occurred exclusively in response to D-glucose and not in response to KCl or non-metabolizable analogs of glucose (Fig. 6, *A* and *B*). Such a selective response to glucose is consistent with previous reports suggesting that Rac1 functions in second-phase insulin release.

Rac1 Activation Requires Cdc42—Because of the lateness of glucose-induced Rac1 activation, Rac1 has been suggested to function in a later step(s) in the stimulus-secretion coupling cascade (3, 8). However, our perifusion data indicates that Cdc42 is functionally important for second-phase secretion, and yet glucose-induces Cdc42 activation within 3 min of stimulation. This prompted us to question whether Cdc42 and Rac1 activations are linked together in a common signaling pathway. To determine whether Rac1 activation was dependent upon Cdc42, MIN6 cells were transfected with control or Cdc42 siRNA oligonucleotides (using siCon or siA as in Fig. 1), and whole cell lysates were prepared for assessment of Rac1 activation. In siCon-transfected MIN6 cells, Rac1 activation was increased 2-fold within 20 min of glucose stimulation as we routinely observed in non-transfected cells (Fig. 7, *A* and *B*). However, cells depleted of Cdc42 failed to show glucose-induced Rac1 activation. This inhibition of Rac1 activation was not the result of decreased Rac1 expression, as total Rac1 expression was similar in siCon and siCdc42-transfected cell lysates (Fig. 7, *A* and *B*). This inhibition was due to failure of Rac1 to become activated at the PM, as determined by assessment of Rac1 activation in fractions prepared from cells transfected with siCon or siCdc42 oligonucleotides (Fig. 7, *C* and *D*). The failure of glucose to induce activation of Rac1 was also not due to a unique effect of the siA Cdc42 oligonucleotide, since our siCdc42-adenovirus (siCdc42-Ad) also blunted glucose-induced Rac1 activation (Fig. 7*E*), and this was independent of any effects upon Rac1 expression (Fig. 7*F*). Overall these results strongly suggested that Cdc42 was required for the glucose-induced activation of Rac1 in β-cells.

Pak1 Functions in Mediating the Cdc42-dependent Activation of Rac1—Because Cdc42 is required for Rac1 activation during glucose-stimulated insulin secretion, we questioned how Cdc42 activation might lead to Rac1 activation. Because

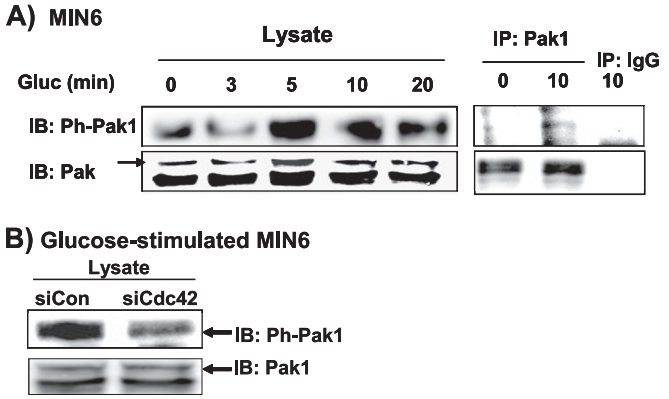

FIGURE 8. **Depletion of Cdc42 impairs glucose-induced Pak1 phosphorylation (Thr-423).** *A*, MIN6 cells were preincubated in MKRBB for 2 h and subsequently stimulated with 20 mM glucose for the times indicated. Cell lysates were used for detection of glucose-induced Pak1 phosphorylation by immunoblotting with a phospho-specific Pak1 (*Ph-Pak1*) antibody relative to Pak antibody (*left panel*). The Pak antibody detects both Pak1 and Pak2, and the position of the Pak1 band is denoted by an *arrow*. Specificity for Pak1 was validated by Pak1 immunoprecipitation compared with an IgG control (*right panel*). Data are representative of three independent experiments. *B*, MIN6 cells were transfected with siCon or siCdc42 oligonucleotides for 48 h and subsequently stimulated with 20 mM glucose for 5 min. Cell lysates were prepared, and proteins were resolved on 12% SDS-PAGE for detection of Pak1 phosphorylation by immunoblotting for Pak1-phosphorylation at Thr-423. Data are representative of three independent experiments.

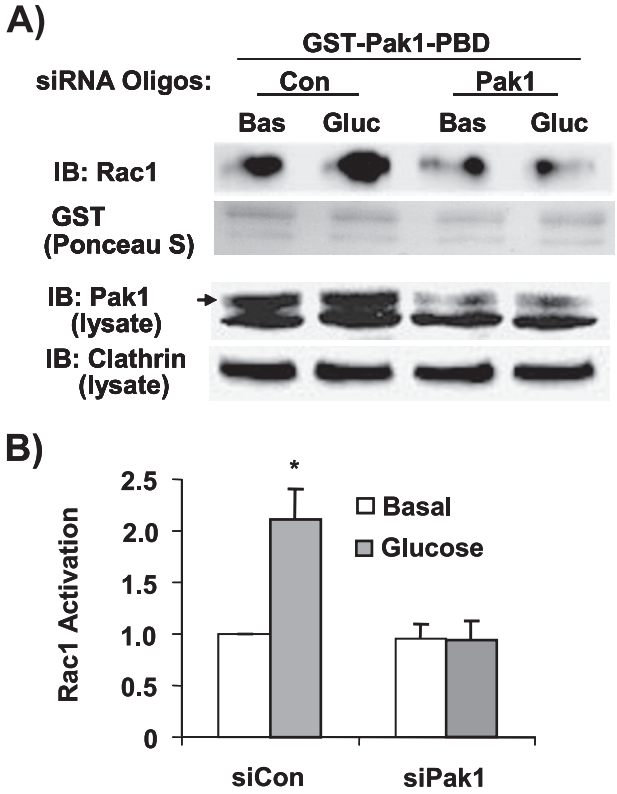

FIGURE 9. **Depletion of Pak1 abolishes glucose-induced Rac1 activation.** *A*, MIN6 cells were transiently transfected with Pak1 or control siRNA oligonucleotides for 48 h. After preincubation in MKRBB buffer for 2h, cells were either left unstimulated or were stimulated with 20 mM glucose for 20 min. Detergent lysates were prepared and immediately subjected to GST-Pak1-PBD interaction assays. Data are representative of three independent experiments. Ponceau S staining was used as the loading control. Lysates (30 μg) were subjected to immunoblotting with Pak1 antibody to validate Pak1 depletion, and clathrin was used as a loading control. *B*, optical density quantitation of Rac1 activation for three independent experiments (average ± S.E.); *, $p < 0.01$ *versus* siCon under basal conditions.

Cdc42 and Rac1 do not bind directly (10) and there is a considerable lag between the times of Cdc42 activation (3 min) and Rac1 activation (20 min), it seemed that there must be another factor involved to transmit the signal from Cdc42 to Rac1. We have previously shown that the known Cdc42 effector protein Pak1 exists in MIN6 cells (2). Pak1 is a Ser/Thr kinase that is known to undergo stimulus-dependent phosphorylation on Thr-423, a critical phosphorylation site in the Pak1 activation process after release from autoinhibition (26–28). Activated Cdc42 binding to Pak1 disrupts its dimerization and leads to a series of conformational changes that promotes Thr-423 phosphorylation (29). To investigate this possibility, we first examined Pak1 for Thr-423 phosphorylation in response to glucose stimulation. Small amounts of phosphorylated Pak1 (*Ph-Pak1*) were detected under basal conditions; however, within 5 min of glucose stimulation, the abundance of Ph-Pak1 significantly increased (Fig. 8*A*). The specificity of this increase was verified by comparing Pak1 immunoprecipitates from glucose-stimulated cells with those from unstimulated cells or an IgG control (Fig. 8*A*, *last three lanes*).

To determine whetherCdc42 was required for the glucose-induced phosphorylation of Pak1, phosphorylated Pak1 (Thr-423) levels were assessed in lysates prepared from control or Cdc42-depleted MIN6 cells (Fig. 8*B*). Indeed, the glucose-induced increase in Pak1 phosphorylation was decreased in siCdc42 but not siCon-transfected cells. These data indicate that induction of Pak1 phosphorylation in response to glucose is at least partially, if not exclusively, Cdc42-dependent.

Because the timing of glucose-induced Pak1 phosphorylation precedes that of Rac1 activation, we questioned whether Pak1 served upstream of Rac1 linking Cdc42 and Rac1 activation in a common pathway. Transient transfection of custom Pak1 siRNA oligonucleotides successfully depleted Pak1 protein by more than 50% after 48 h of transfection (Fig. 9*A*, *IB*, *Pak1* in *Lysate*, *upper band* is Pak1). The oligonucleotides were very specific to Pak1, since Pak2 expression was unaffected (*IB*, *Pak1 Lysate*, *lower band* in the doublet). As a result of the Pak1 depletion, glucose-induced Rac1 activation was abolished in MIN6 cells (Fig. 9, *A* and *B*), similar to the effect observed with depletion of Cdc42. Overall, these results strongly suggest Pak1 is involved in mediating the Cdc42-Rac1 activation signaling pathway during glucose-stimulated insulin secretion in pancreatic β-cells.

Pak1 and Rac1 Function in Cdc42-dependent Insulin Secretion—We next sought to determine whether this glucose-mediated sequence of Cdc42 downstream signaling events was coupled to function in insulin secretion. Although it has already been established that Rac1 is functionally important in glucose-stimulated insulin secretion (8), the functional requirement for Pak1 remained unknown. To address this we assessed the effect of Pak1 depletion upon glucose-stimulated insulin secretion. Consistent with the negative effect of Pak1 depletion upon Rac1 activation, Pak1 depletion correspondingly inhibited glucose-induced insulin secretion by >50% (Fig. 10*A*).

To elucidate whether Rac1 indeed functioned downstream of Cdc42 in a common pathway, constitutively active Rac1 was "added-back" to Cdc42-depleted cells to compen-

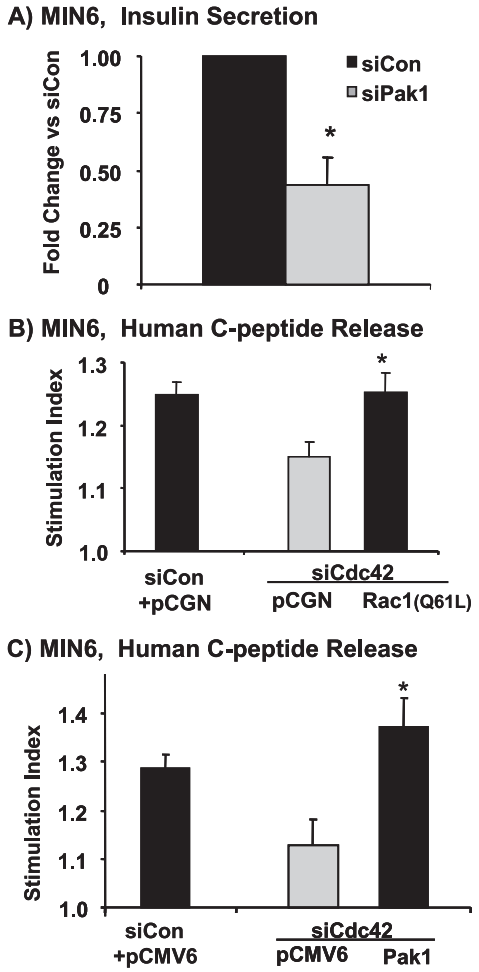

FIGURE 10. **Pak1 and Rac1 function in Cdc42-dependent glucose-stimulated insulin secretion.** *A*, MIN6 cells were transfected using Lipofectamine2000 with siPak1 or control oligonucleotides, and after 48 h of incubation cells were preincubated in glucose-free MKRBB for 2 h followed by stimulation with 20 mM glucose for 20 min. Insulin secreted into the MKRBB was measured by RIA. Data are shown as the ratio of insulin released from siPak1 cells relative to control cells after stimulation from three independent experiments; *, $p < 0.05$ *versus control*. *B*, human proinsulin DNA and control or Cdc42 siRNA plasmid DNAs (siCon and siCdc42, respectively) were co-transfected into MIN6 cells with pCGN-Rac1(Q61L) or pCGN vector alone as described under "Experimental Procedures." After 48 h of incubation, cells were preincubated in MKRBB for 2 h followed by a 1-h stimulation with 20 mM glucose. Human C-peptide secreted into the media from the transfected cells was measured by RIA. Data represent the average ± S.E. of at least five independent experiments. *, $p < 0.05$ *versus* siCdc42 with pCGN vector. *C*, human pro-insulin DNA and control or Cdc42 siRNA plasmids DNAs (siCon and siCdc42, respectively) were co-transfected into MIN6 cells with pCMV6-Pak1 or pCMV6 vector alone. After 48 h of incubation, cells were preincubated in MKRBB for 2 h followed by 1 h of stimulation with 20 mM glucose. Human C-peptide secreted into the media from the transfected cells was measured by RIA. Data represent the average ± S.E. of at least four independent experiments. *, $p < 0.05$ *versus* siCdc42 with pCMV6 vector.

sate for the lack of endogenous activated Rac1. Using the human C-peptide assay system, glucose-stimulated insulin release was significantly reduced in Cdc42-depleted cells co-transfected with the vector control (pCGN) compared with control cells (Fig. 10*B*), and this inhibition was reversed by co-transfection with constitutively active Rac1 (pCGN-Rac1Q61L). Co-expression of wild-type Pak1 in Cdc42-depleted cells also restored glucose-stimulated insulin secretion levels to normal (Fig. 10*C*). Because wild-type Pak1 was capable and constitutively active Pak1 was not required for restoration of function, this might indicate that residual Cdc42 remaining after depletion was sufficient to activate under conditions where Pak1 abundance was not limiting, or that Pak1 functions in multiple pathways impacting insulin release. Expression of recombinant Myc-tagged Pak1 and hemagglutinin-tagged Rac1Q61L proteins in the MIN6 cells was verified by immunoblotting (data not shown). Taken together, these functional data support the biochemical data, suggesting that Pak1 and Rac1 function downstream of Cdc42 in a common pathway mediating glucose-induced insulin secretion in β-cells.

DISCUSSION

In this report we demonstrate for the first time that Cdc42 plays an essential role in insulin granule exocytosis. Finding that Cdc42 was required exclusively for the second phase of insulin release represents an important step in elucidating the proteins and mechanisms responsible for eliciting second-phase secretion, which have remained otherwise elusive. Our data show that the regulation of second-phase secretion begins early during the first phase, with Cdc42 activation occurring within 3 min of glucose stimulation. Also consistent with a selective role in second-phase secretion, Cdc42 activation occurred exclusively in response to D-glucose and was unresponsive to secretagogues such as KCl, which cannot elicit second-phase release. Furthermore, Rac1 activation occurs later at ~20 min of glucose stimulation and as such has been suggested to be important for second-phase secretion (8), although this had not been explicitly tested or linked to Cdc42 until now. Here we show that Rac1 activation is dependent upon glucose-induced activation of Cdc42 via the Cdc42 effector protein Pak1. The sequential cascade of Cdc42 signaling to Rac1 was further supported by functional data demonstrating rescue of insulin secretion by constitutively active Rac1 in Cdc42-depleted cells. This signaling from Cdc42 to Pak1 and on downstream to Rac1 represents the first reported Cdc42 signaling cascade in pancreatic β-cells and supports the notion that Cdc42 functions as the key proximal transmitter of the glucose-specific signal to the actin cytoskeleton to enact remodeling and facilitate second-phase insulin release.

Ras-related proteins are usually found in the inactive GDP-bound state in the cytosolic fraction. They cycle between two conformational states; one bound to GTP ("active" state) and localized to the plasma membrane, and the other bound to GDP ("inactive" state) and kept in the cytosol (30). Unlike other cell types, in β-cells the pool of Cdc42 that translocates to the PM to become activated is principally the granule pool, and this granule pool of Cdc42 translocates in parallel with VAMP2 and the Cdc42 GDI protein caveolin-1 to the PM in response to glucose (2). Once at the PM, Cdc42 dissociates from caveolin-1 (6). Consistent with our previous observation of this translocation mechanism, our new data expand upon this by showing that once at the PM Cdc42 becomes activated. Thus, these data suggest that glucose triggers first the translocation of Cdc42-bound granules to the PM, and once there the GDI caveolin-1 dissociates and Cdc42 becomes activated. Determination of how D-glucose triggers first the translocation of Cdc42 and then its activation awaits further investigation.

These studies showed that glucose metabolism is required to trigger Cdc42 activation, which is consistent with a selective functional role for Cdc42 in second-phase insulin release. Cdc42 failed to undergo activation, translocation, or dissociation from its GDI in response to KCl, and depletion of Cdc42 from isolated islets failed to impair the first phase of insulin secretion. This lack of response to KCl is reminiscent of our finding that KCl stimulation fails to elicit visual changes in cortical F-actin remodeling, whereas glucose has a profound impact upon remodeling (1). This differs considerably from Cdc42 response and function in PC12 cells, since in PC12 cells Cdc42 is KCl-activated (7). Also different is the enhanced secretory response of PC12 cells expressing the constitutively active form of Cdc42 (7), since in β-cells the expression of the same Cdc42 mutant results in ablation of glucose-stimulated secretion (1). However, expression of the constitutively active Cdc42 in β-cells did not impair KCl-stimulated secretion, again demonstrating the high degree of selectivity of Cdc42 function in response to glucose.

The selectivity of response to glucose carried through the cascade downstream to Rac1 activation as well, since Rac1 activation occurred exclusively in response to D-glucose in our study. Rac1 activation was found to occur significantly later, requiring 20 min as opposed to the 3 min required for maximal Cdc42 activation. However we were surprised to find that Rac1 activation was dependent upon Cdc42. Although both Cdc42 and Rac1 have been shown to function as positive effectors in the β-cell, it has been speculated that the two GTPases might function in parallel but not necessarily common pathways. The reasoning behind this speculation was based upon overexpression studies of Cdc42 and Rac1 mutants. Although expression of constitutively active Cdc42 impaired glucose-stimulated insulin release, expression of the active mutant of Rac1 had no effect (8). Moreover, expression of the dominant-negative Rac1 mutant inhibited glucose-induced insulin release; dominant-negative Cdc42 was without effect (1). However, now that we know Cdc42 signals downstream to Rac1, it is possible that these different responses are rooted in differences of temporal cycling of these GTPases. For example, Cdc42 needs to cycle to the GDP-bound form to induce F-actin disruption in time to facilitate granule mobilization for the second phase of secretion (which begins within 10 min of glucose stimulation), and subsequent Rac1 activation at 15–20 min serves to restore cortical F-actin. This model would be supported by our data showing that constitutively active Cdc42 blocked F-actin disruption and with the data showing that dominant-negative Rac1 blocked a late event in exocytosis. However, it is important to note that the mutant forms of Cdc42 and Rac1 have been examined in cultured β-cell lines and not in isolated islets such that extrapolation of these data to biphasic secretion is limited and that future studies with these mutants will need to be performed in islets.

These differences in Cdc42 signaling and actin remodeling in β-cells compared with other cell types may exist because β-cells release insulin in discrete phases over a long period of time. The pattern of biphasic release in the perifusion protocol is highly reproducible between laboratories and among many different batches of islets with using different islet isolation procedures and different perifusion apparatus designs. This shows just how carefully metered biphasic insulin release is and strongly suggests that the readily releasable pool of granules must be refilled from the storage pool in a carefully timed and metered manner. F-actin is well suited to the task of pool refilling, since release and refill events must occur across the expanses of the cell, yet be coordinated to occur simultaneously to achieve the precisely timed biphasic release of insulin. Because biphasic release is elicited by glucose, glucose must be able to transmit signals to coordinate these simultaneous events. Cdc42 fits the profile expected of such a proximal factor receiving the signal transmitted from glucose, and this is further substantiated by evidence showing that it participates directly in granule docking with SNARE proteins (2) and is required for second-phase insulin release.

Our previous studies using overexpression of Cdc42 mutants demonstrated that Cdc42 was important for actin remodeling and exocytosis from β-cells but fell short of proving that Cdc42 was essential for these processes. Because Cdc42 null mice die early in embryogenesis (24), we used multiple types of siRNA-mediated depletion regimens that allowed us to successfully order the first signaling cascade to emanate from Cdc42 in β-cells: Cdc42 → Pak1 → Rac1. Although Cdc42 signaling to Rac1 and Pak1 is not unique, this particular order of activation differs from other cells, where this order is either reversed (15) or Cdc42 and Rac1 activations are antagonistic (14). Pak1 is a known downstream effector of Cdc42, binding exclusively to the GTP-loaded form of Cdc42. Although we did demonstrate that siRNA-mediated depletion of Pak1 by ~50% resulted in total ablation of Rac1 activation, the mechanism by which phosphorylation and activation of Pak1 leads to induction of Rac1 activation remains unclear. Recently, Pak1 was suggested to activate Rac1 by inducing the dissociation of Rac1 from RhoGDI, mediated by phosphorylation of RhoGDI in HeLa cells (31). In β-cells the same signaling pathway might exist, since RhoGDI·Rac1 complexes were found in β-cells (5). Dissociation of this complex by factors such as arachidonic acid, phosphatidic acid, and phosphoinositides have been suggested to participate in the activation of Rac1 (32–34). Alternatively, there may be additional factors other than Pak1 that participate in dissociating the Rac1·RhoGDI complex. For example, the Pak exchange factor Cool-2/αPIX has been suggested to function in a Cdc42-to-Rac1 signaling pathway (10, 35). Recently, Cool-1/βPIX has been shown to mediate the localized activation of Rac1 in non-β-cells (11). In addition, cross-talk among multiple small G-proteins may also exist (ARF6 and Rac1) (16). One disconnect in the importance of Rac1 in supporting second-phase insulin release is that Rac1 activation does not occur until the second phase of secretion is well under way. As mentioned before, one possible role for Rac1 may be in F-actin formation and granule corralling as part of the metering mechanism of granule pool refilling. Determination of this mechanism awaits further investigation.

In addition to activation of Rac1, Pak1 in the β-cell may also participate in actin remodeling by signaling to myosin light chain kinase. Calcium-calmodulin-dependent phosphorylation of myosin and myosin light chain kinase (MLCK) activity has previously been demonstrated in pancreatic islets (36, 37), and treatment of islet cells with MLCK inhibitors or specific anti-MLCK antibodies alters insulin secretion (38–40). Pak1 can

signal to myosin light chain kinase in more than one way, however. Pak1 can either directly deactivate myosin light chain kinase (MLCK) to induce actin depolymerization (41, 42), or it can indirectly activate MLCK through Raf and extracellular signal-regulated kinase signaling (42). In support of this possibility, a recent study using human islets showed that Raf became activated in response to glucose to mediate extracellular signal-regulated kinase activation (43). Cdc42 activation has also been linked to downstream activation of extracellular signal-regulated kinase (44). Alternatively, Pak1 can also signal to LIM kinase to evoke changes in the cytoskeleton, although this has not been examined in islet β-cells to date. Thus, future studies of Pak1 will be required to discern its role in transmitting the Cdc42-specific signal to induce F-actin remodeling in β-cells and biphasic insulin release.

In sum, these data demonstrate that Cdc42 activation occurring during first-phase secretion serves to trigger downstream events for second phase. This identification of Cdc42 as essential for second-phase secretion is the first evidence for a selective signaling pathway that is exclusively responsive to glucose to elicit the second phase of insulin release. Because Cdc42 is a known F-actin effector protein, our data showing an essential role for Cdc42 in just second-phase secretion supports the concept that second-phase secretion requires cytoskeletal remodeling to mobilize granules. In this report we also provide evidence for an underlying mechanism for this by demonstrating that Cdc42 signals downstream to Pak1 and on to Rac1. Although these signaling molecules are widely expressed in many cell types, in the islet β-cell this cascade is very specific to glucose. To address how this can be so glucose-specific, future investigations into the events leading to the activation of Cdc42 in insulin exocytosis will be required.

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