Differential Phosphorylation of RhoGDI Mediates the Distinct Cycling of Cdc42 and Rac1 to Regulate Second-phase Insulin Secretion*

Received for publication, October 2, 2009, and in revised form, December 18, 2009 Published, JBC Papers in Press, December 22, 2009, DOI 10.1074/jbc.M109.072421

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Cdc42 cycling through GTP/GDP states is critical for its function in the second/granule mobilization phase of insulin granule exocytosis in pancreatic islet beta cells, although the identities of the Cdc42 cycling proteins involved remain incomplete. Using a tandem affinity purification-based mass spectrometry screen for Cdc42 cycling factors in beta cells, RhoGDI was identified. RNA interference-mediated depletion of RhoGDI from isolated islets selectively amplified the second phase of insulin release, consistent with the role of RhoGDI as a Cdc42 cycling factor. Replenishment of RhoGDI to RNA interference-depleted cells normalized secretion, confirming the action of RhoGDI to be that of a negative regulator of Cdc42 activation. Given that RhoGDI also regulates Rac1 activation in beta cells, and that Rac1 activation occurs in a Cdc42-dependent manner, the question as to how the beta cell utilized RhoGDI for differential Cdc42 and Rac1 cycling was explored. Co-immunoprecipitation was used to determine that RhoGDI-Cdc42 complexes dissociated upon stimulation of beta cells with glucose for 3 min, correlating with the timing of glucose-induced Cdc42 activation and the onset of RhoGDI tyrosine phosphorylation. Glucose-induced disruption of RhoGDI-Rac1 complexes occurred subsequent to this, coincident with Rac1 activation, which followed the onset of RhoGDI serine phosphorylation. RhoGDI-Cdc42 complex dissociation was blocked by mutation of RhoGDI residue Tyr-156, whereas RhoGDI-Rac1 dissociation was blocked by RhoGDI mutations Y156F and S101A/S174A. Finally, expression of a triple Y156F/S101A/S174A-RhoGDI mutant specifically inhibited only the second/granule mobilization phase of glucose-stimulated insulin secretion, overall supporting the integration of RhoGDI into the activation cycling mechanism of glucose-responsive small GTPases.

Regulated release of insulin requires two main components as follows: 1) negative regulation to ensure low levels of insulin release under resting conditions, and 2) positive regulation to facilitate robust responsiveness to elevated fuel/glucose conditions. The components of first-phase insulin release are well characterized (1); glucose enters into the beta cell through the constitutively plasma membrane localized GLUT2 transporter and via its metabolism leads to a net increase in intracellular ATP/ADP ratio. This triggers closure of the ATP-sensitive K+ channel and membrane depolarization, which in turn causes the voltage-dependent calcium channel to open, allowing calcium entry (2–4). Calcium entry induces fusion of the insulin granules present in the readily releasable pool at the plasma membrane by means of a soluble NSF attachment protein receptor (SNARE)2-dependent mechanism (5, 6). Immediately following the first phase comes a second and sustained phase of insulin release, which is attributed to the refilling of the readily releasable pool by mobilizing intracellular insulin granule storage pools (7, 8). Although this refilling mechanism is thought to require an amplification signal emanating from glucose, this signal and the amplifying targets have remained elusive.

Refilling of the readily releasable pool involves trafficking of the insulin granules to the cell surface, and this has been shown to involve coordinated re-organization of the cytoskeletal framework (9–12). Regulation of remodeling is known to involve GDP/GTP cycling of the small Rho family GTPases Cdc42 and Rac1, both of which have been implicated in granule mobilization and sustained insulin release (13–15). Earlier studies indicated that both are selectively activated in response to glucose in islets and clonal beta cell lines and that their inactivation by clostridial toxins impairs insulin release (16, 17). Using RNAi depletion, we recently demonstrated that Cdc42 functions as a proximal regulator of second-phase insulin release from mouse islets, triggering Pak1 activation, which in turn activates Rac1 (14). Consistent with the lack of effect of Cdc42 depletion upon first-phase insulin release, Cdc42 is not activated by or required for KCl-stimulated insulin release (14, 16, 18). Furthermore, studies using a cycling-deficient Cdc42 indicated that it is the cycling of Cdc42, and not simply its activation, that is imperative for glucose-stimulated secretion (18).

Small Rho family GTPases cycle between the GDP-bound/inactive and GTP-bound/active states via the actions of gua-

* This work was supported, in whole or in part, by National Institutes of Health Grant DK076614 (to D. C. T.). This work was also supported by American Diabetes Association Career Development Award 1-03-CD-10 and an Indiana University Research Support Fund Grant.

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2 The abbreviations used are: SNARE, soluble NSF attachment protein receptor; RNAi, RNA interference; ERK, extracellular signal-regulated kinase; MKRBB, modified Krebs-Ringer bicarbonate buffer; BIA, radioimmunoassay; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; PVDF, polyvinylidene fluoride; siRNA, small interfering RNA; IP, immunoprecipitation; RT, reverse transcription; GFP, green fluorescent protein; EGFP, enhanced GFP; pV, pervanadate; WT, wild type.
nine nucleotide exchange factors (GEFs), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (GDIs). GEFs catalyze the release of bound GDP, resulting in the formation of the GTP-bound active GTPase, whereas GTPase-activating proteins stimulate GTPase activity leading to inactivation (19). GDIs control the access of GTPases to GEFs, GTPase-activating proteins, and to membranes where effectors reside (20, 21). Interestingly, caveolin-1 functions as a GDI for Cdc42 in beta cells, interacting with the fraction of Cdc42 that localizes to the insulin granule through direct interaction with the v-SNARE VAMP2 (22). However, caveolin-1 does not suffice as the only GDI for Cdc42, given that a large proportion of Cdc42 protein resides in the soluble cytosolic fraction of the beta cell.

In an effort to identify the cytosolic GDI for this pool of Cdc42, we employed a tandem affinity purification approach whereby a dual-tagged form of human Cdc42 was adenovirally introduced into MIN6 beta cells, and Cdc42-associated proteins were identified by mass spectrometry. In this screen, RhoGDI was overwhelmingly found to bind to cytosolic Cdc42 in MIN6 beta cells. This identification was compatible with earlier findings by Regazzi et al. (23) that within cytosolic extracts of clonal beta cells Cdc42 co-sedimented with RhoGDI. Furthermore, RhoGDI has also been shown to function as a cytosolic GDI for Rac1 in islet beta cells, although the specific phase of secretion and their interaction in response to glucose had not been assessed (24).

Toward this end, we show here that RhoGDI is selectively required for control of the second sustained phase of glucose-stimulated insulin secretion in isolated mouse islets. RNAi-mediated depletion of RhoGDI significantly reduced glucose-induced Cdc42 activation in MIN6 cells. Acute glucose stimulation (3 min) resulted in tyrosine phosphorylation of RhoGDI and transient dissociation of endogenous RhoGDI-Cdc42 complexes, whereas RhoGDI-Rac1 complexes dissociated at a later time point (by ∼20 min) and correlated with the serine phosphorylation of RhoGDI. These data pinpoint RhoGDI as a key orchestrator of the differential cycling and signaling events of cytosolic Cdc42 and Rac1 to support the second phase of insulin release.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies recognizing RhoGDI, phospho-RhoGDIα (Ser-101), Cdc42, Rac1, caveolin 1, ERK, phospho-ERK (Thr-202/Tyr-204), actin, and mouse IgG, and protein G Plus-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PY20 antibody was obtained from BD Biosciences. The 4G10 mouse anti-phosphotyrosine monoclonal was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit and mouse anti-EGFP antibodies were from Abcam Inc. (Cambridge, CA) and Clontech, respectively. Mouse anti-VAMP2 was purchased from Synaptic Systems (Gottingen, Germany). Goat anti-rabbit and anti-mouse horseradish peroxidase secondary antibodies were acquired from Bio-Rad. The EZ-Detect Cdc42 activation kit containing mouse anti-Cdc42 antibody and mammalian c-Myc tag IP/co-IP application set were purchased from Pierce. Lipofectamine 2000 and ECL reagents were purchased from Invitrogen and Amersham Biosciences, respectively. The sodium orthovanadate and hydrogen peroxide, RIA-grade bovine serum albumin, diazoxide, and n-glucose were obtained from Sigma. The human C-peptide and sensitive rat insulin RIA kits were purchased from Linco Research Inc. (St. Charles, MO). Three siRNA oligonucleotides targeting endogenous mouse RhoGDI (Ambion, Austin, TX) were used, designated as siA, siB, and siC, respectively; siA is GGAAACUGGAAAGGAC-GA5t; siB is GGUGUGGAGUACCGGAUA5t; and siC is GGUCAAUUGCCAAAUCU5tt. A non-targeting siRNA (siCon) was obtained from Ambion. Purified recombinant His-Cdc42, His-Rac1, and RhoGDI-GST proteins for quantitative immunoblotting analysis were purchased from Cytoskeleton (Denver, CO).

**Plasmids**—The full-length human Cdc42 with N-terminal Myc tag was subcloned into the EcoRI and BamHI sites of pcDNA3. The full-length human RhoGDIα in pcDNA3.1 was a kind gift from Dr. Anjan Kowluru. pGEX4T3-RhoGDI-WT and -Y156F plasmids were kindly provided by Drs. G. M. Bokoch and C. DerMardirossian ( Scripps Research Institute, La Jolla, CA) and used as templates to generate PCR products to subclone into BglIII and HindIII sites of the pEGFP-C1 vector. The pEGFP-RhoGDI-S101A/S174A, pEGFP-RhoGDI-Y156F, and pEGFP-RhoGDI-Y156F/S101A/S174A mutants were made using the QuikChange® Multi Site-directed mutagenesis kit from Stratagene (Cedar Creek, TX). Adenoviral delivery plasmids were constructed by insertion of the annealed complementary oligonucleotide sequence of siA for RhoGDI or of siCon into the 5′-Xhol and 3′-SpeI sites of the pSilencer-Adeno-CMV vector (Ambion) using methods described previously (14). High purity cesium-chloride viral particles were obtained from Viraquest Inc. (North Liberty, IA), and viruses were packaged with GFP for use in identification of transduced islet cells in experiments. All constructs were verified by DNA sequencing analysis.

**Tandem Affinity Purification Screen**—Human Cdc42-WT cDNA was subcloned into the pNTAP shuttle vector for recombination as part of the InterPlay adenoviral tandem affinity purification system kit (Stratagene). MIN6 cells at 60% confluence were transduced with either NTAP-Cdc42WT-Ad or the control vector (TAP-CAT-Ad) CsCl-purified particles for 2 h at 37 °C (multiplicity of infection = 100) as described previously (14). Cells were then washed twice with phosphate-buffered saline and incubated 48 h longer in MIN6 culture medium at 37 °C, 5% CO2. GFP fluorescence was visualized in greater than 95% of cells in all experiments. The TAP-CAT control and our TAP-Cdc42WT bait proteins were subsequently co-purified for identification of associated proteins using the Interplay tandem affinity purification kit (Stratagene) via LHT linear ion-trap liquid chromatography-tandem mass spectrometry protein identification (INCAPS, Indianapolis, IN).

**RNA Isolation and RT-PCR**—Total RNA from MIN6 cells and isolated mouse pancreatic islets was obtained using the RNeasy mini kit (Qiagen, Valencia, CA). RNA (1 μg) was reverse-transcribed with TaqMan (Applied Biosystems, Foster City, CA), and 1% of the product was used for RT-PCR. The primers used for detection of RhoGDI isoforms (α, β, and γ) were as follows: RhoGDIα, forward 5′-cagaacaggacacccgctg
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and reverse 5’-cagtgctgctaccaaggtc; RhoGDIβ, forward 5’-actggagacagcagctaatc and reverse 5’-gagtcgcataggtgcc; RhoGDIγ, forward 5’-gatggctgctgaggaat and reverse 5’-catgtagtacgctgcgtgg. RT-PCR was performed with BioMix Red (Biolane, Taunton, MA) for 30 cycles: 94 °C for 1 min, 56 °C for 1 min, and 71 °C for 1 min with a final 10-min elongation at 71 °C. PCR products were visualized on 2% agarose gels.

Subcellular Fractionation—Subcellular fractions were isolated as described previously (25). Briefly, MIN6 cells were disrupted by 10 strokes through a 27-gauge needle prior to centrifugation at 900 × g for 10 min. Post-nuclear supernatants were centrifuged at 5500 × g for 15 min; supernatant was centrifuged at 25,000 × g for 20 min to pellet the storage granule fraction. The supernatant was further centrifuged at 100,000 × g for 1 h to obtain the cytosolic fraction. Plasma membrane fractions were obtained by mixing the postnuclear pellet with Buffers A and B for centrifugation at 113,000 × g for 1 h to obtain an interface, which was collected for centrifugation at 3000 × g for 10 min to pellet the plasma membrane. All pellets were resuspended in the 1% Nonidet P-40 lysis buffer.

Cell Culture, Transient Transfection, and Secretion Assays—MIN6 beta cells were cultured in Dulbecco’s modified Eagle’s medium (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 (26). Transfection of siRNA oligonucleotides into MIN6 cells was achieved as described previously (14). After 48 h of incubation, cells were washed and incubated in glucose-free modified Krebs-Ringer bicarbonate buffer (MKBHB: 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 bovine serum albumin) for 2 h prior to stimulation with 20 mM d-glucose. 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, 5 μg/ml leupeptin) and cleared by centrifugation at 14,000 × g for 10 min at 4 °C. For the secretion rescue assay, MIN6 cells at 50–60% confluence were co-transfected with 60 pmol of siCon or mouse siA (RhoGDI) oligonucleotides plus 0.5 μg of human proinsulin DNA (pcB6/INS, gift from Dr. Chris Newgard, Duke University, Durham, NC) and 0.5 μg of pcDNA3.1-human RhoGDI plasmid (pcDNA3.1 vector as negative control) using Lipofectamine 2000. After 48 h of incubation, cells were washed twice with and incubated for 2 h in freshly prepared MKRBB, followed by stimulation with 20 mM d-glucose for 1 h. MKRBB was then collected and centrifuged at 14,000 × g for 5 min for subsequent quantitation of human C-peptide secretion using a human C-peptide radioimmunoassay kit (Linco Research). MIN6 cells at 50–60% confluence were co-transfected with 1 μg of pEGFP-C1 vector (control), WT, or triple mutant RhoGDI plus 1 μg of human proinsulin cDNA using Transfectin. After 48 h of incubation, cells were washed and incubated in freshly prepared MKRBB for 2 h. Cells were then either stimulated with 40 mM KCl alone or incubated with 250 μM diazoxide for 5 min, followed by addition of 40 mM KCl and then stimulated with 20 mM d-glucose. MKRBB was then collected for subsequent human C-peptide quantitation as above.

Cdc42 Activation Assays—The EZ-Detect Cdc42 activation kit from Pierce was used as described previously (25). Freshly made cleared detergent cell lystate protein (500 μg) was combined with 20 μg of GST-Pak1-PDB-agarose. Following washes, proteins eluted were subjected to 12% SDS-PAGE for immunoblotting with mouse anti-Cdc42 antibody.

Mouse Islet Isolation, Adenoviral Transduction, and Perifusion—Pancreatic mouse islets were isolated, adenovirally transduced, and used for perifusion as described previously (14, 27). Briefly, pancreata from 10- to 14-week-old male mice (C57Bl/6J) were digested with collagenase and purified using a Ficoll gradient density. After isolation, islets were immediately transduced at a multiplicity of infection = 100 with either siRhoGDI-Ad or siCon-Ad CsCl-purified particles for 1 h at 37 °C, washed, and incubated overnight in RPMI 1640 medium at 37 °C, 5% CO2. GFP fluorescence was visualized in greater than 95% of islets in all experiments. 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 KRBH Buffer (10 mM Hepes, pH 7.4, 134 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4 containing 0.5 mg/ml bovine serum albumin) containing 2.8 mM glucose. Islets were perfused 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 16.7 mM glucose stimulation for 35 min. Insulin secreted into eluted fractions was quantitated by a sensitive rat insulin RIA immunoassay kit (Linco Research, Inc.).

Co-immunoprecipitation and Immunoblotting—For each immunoprecipitation, 2 mg of cleared detergent lystate was combined with 2 μg of antibody for 2 h at 4 °C. Co-immunoprecipitation reactions from cytosolic fractions used 1 mg of protein with 2 μg of antibody. Protein G Plus-agarose beads were added and reactions rotated for an additional 2 h. Following three washes with 1% Nonidet P-40 lysis buffer, the resulting immunoprecipitates were subjected to 12% SDS-PAGE followed by transfer to PVDF membrane for immunoblotting and visualization by ECL using a Chemi-Doc system (Bio-Rad). QuantityOne software (Bio-Rad) was used for quantitative analysis of immunoblots.

Quantitation of RhoGDI, Cdc42, and Rac1 Proteins in MIN6 Cell Cytosolic Fraction—Cytosolic fractions of MIN6 cells were subjected to electrophoresis and known quantities of GST-RhoGDI, His-Cdc42, and His-Rac1 recombinant proteins on 12% SDS-PAGE followed by transfer to PVDF membranes and immunoblotting with anti-RhoGDI, Cdc42, or Rac1 antibodies, respectively. Proteins were detected using enhanced chemiluminescence, using exposure well within the linear range of the blot, and quantitated using the Bio-Rad Quantity One software package.

Statistical Analysis—All data are expressed as means ± S.E. Data were evaluated for statistical significance using Student’s t test.
RESULTS

**RhoGDI Associates with the Cytosolic Pool of Cdc42 in a Glucose-sensitive Manner**—To identify Cdc42 cycling proteins functional in insulin-secreting beta cells, a tandem affinity purification screen using a dual-tagged form of Cdc42 was performed in clonal mouse MIN6 beta cells. MIN6 cells were transduced with control adenovirus expressing chloramphenicol acetyltransferase (TAP-CAT) or TAP-Cdc42. The TAP-Cdc42 protein was immunodetected at ~30 kDa, although endogenous Cdc42 was seen at the expected 22-kDa size (Fig. 1A, lanes 1 and 2). An additional ~26-kDa band was present only in crude lysates and is presumed to be a degradation product of TAP-Cdc42; further purification via the dual streptavdin-binding peptide and calmodulin-binding peptide tags resulted in elimination of this product and detection of the single 30-kDa band (Fig. 1A, lane 3). This purified fraction was then subjected to mass spectrometry, resulting in detection of 46 peptides of the guanine dissociation inhibitor protein RhoGDI. RhoGDI was confirmed as a specific endogenous binding partner of Cdc42 in MIN6 beta cells by co-immunoprecipitation (Fig. 1B). Both Cdc42 and RhoGDI proteins were detected in human islets, mouse islets, and MIN6 cells, validating use of the mouse islet and MIN6 cell model systems for subsequent studies (Fig. 1C). Of the three RhoGDI isoforms evaluated by RT-PCR analysis, RhoGDIα was predominant in both MIN6 cells and isolated mouse islets (Fig. 1D). Subcellular fractionation of MIN6 cells localized RhoGDI principally to the cytosol, similar to its localization in rat beta cells (24). Cdc42 localized to each of the three fractions, with ~80% of total present in the cytosolic fraction. Caveolin 1 localized only to plasma membrane and storage granule fractions (Fig. 1E), consistent with its role as the GDI for the granule-localized pool of Cdc42 (22). The presence of VAMP2 in the plasma membrane and storage granule fractions and exclusion from cytosol validated the fraction integrities. Cdc42-RhoGDI complexes could be co-immunoprecipitated from the cytosolic fraction (Fig. 1F), implicating RhoGDI as the GDI for the cytosolic pool of Cdc42.

We next determined whether the RhoGDI-Cdc42 interaction was sensitive to glucose stimulation, as would be characteristic for a GDI-GTPase interaction in beta cells. Cdc42 activation in MIN6 cells has been shown previously to reach peak activation within 3 min of glucose stimulation (14, 18). As shown in Fig. 2A, anti-Cdc42 co-immunoprecipitated 45% less RhoGDI from cells stimulated with glucose for 3 min than from unstimulated cleared detergent MIN6 lysates (0.55 ± 0.09 of basal level of RhoGDI/Cdc42 ratio, p < 0.05). Reciprocal immunoprecipitation using anti-RhoGDI showed a similar response (Fig. 2B; 0.41 ± 0.06 of basal level of Cdc42/RhoGDI interaction).
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A) MIN6 Lysate

B) Cdc42 Activation

FIGURE 3. Depletion of RhoGDI enhances glucose-induced Cdc42 activation. A, MIN6 cells were transfected with three different commercially available RhoGDIα siRNA (siA, siB, and siC) or negative control (siCon) oligonucleotides using Lipofectamine 2000 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-RhoGDI and anti-actin (loading control) antibodies. The bar graph shows the quantitation by optical density scanning of RhoGDI depletion compared with siCon in three independent experiments; *, p < 0.05. B, detergent cell lysates were prepared from MIN6 cells transfected with siCon or the siA oligonucleotides (siRhoGDI), left unstimulated or glucose (Gluc)-stimulated for 3 min, for immediate use in the GST-Pak1-PBD interaction assay. Eluted proteins were resolved on 12% SDS-PAGE for subsequent immunoblotting with mouse anti-Cdc42 antibody. Ponceau S staining served as an indicator of GST-Pak1-PBD loading, and immunoblotting of input lysate shows equal Cdc42 expression under all conditions. Data are representative of three independent activation assays; activation levels were normalized to siCon = 1.0 in each assay and expressed as fold activation.

ratio, p < 0.05). This decrease was fully recapitulated using only the cytosolic fraction for co-immunoprecipitation (data not shown). These data suggested a role for RhoGDI as the glucose-sensitive cytosolic Cdc42 guanine nucleotide dissociation inhibitor.

RhoGDI Depletion Potentiates Glucose-induced Cdc42 Activation—Guanine nucleotide dissociation inhibitor proteins bind to inactive GTPases. To determine whether RhoGDI was required to maintain inactivation of cytosolic Cdc42, RNAi-mediated depletion of endogenous RhoGDIα was employed. Three different siRNA oligonucleotides (siA, siB, and siC) were capable of reducing endogenous RhoGDI levels, with siA exerting the most efficient protein knockdown (Fig. 3A); siA was thereafter utilized in all subsequent assays. In Cdc42 activation assays, RhoGDI depletion was without effect relative to that from nontargeted siCon-transfected cell lysates under basal, unstimulated conditions (Fig. 3B; 1.2 ± 0.1 versus 1.0, respectively). Conversely, activated Cdc42 levels in glucose-stimulated (3 min) RhoGDI-depleted cells were significantly elevated (Fig. 3B; 2.5 ± 0.2 versus 1.9 ± 0.1-fold increase of siCon-depleted cells, p < 0.05, n = 3). This is in contrast to caveolin-1 depletion, which induced a 2 ± 0.2-fold increase in activated Cdc42 under basal conditions compared with control (p < 0.05) but failed to exert a change with glucose stimulation (2 ± 0.3-fold, n = 3). These data indicated that RhoGDI functioned as a guanine nucleotide inhibitor of Cdc42 activation in MIN6 cells, in a manner distinct from that served by the Cdc42-unique-localized GDI protein, caveolin-1. Rac1 activation in either control- or RhoGDI-depleted cells was unaffected at either of these time points (data not shown).

RhoGDI Depletion Selectively Enhances Second-phase Insulin Release—To determine the requirement for RhoGDI in biphasic insulin secretion, islet perifusion was employed. RNAi-mediated RhoGDI depletion was used rather than islets isolated from RhoGDIα<sup>−/−</sup> knock-out mice because these mice are reported to exhibit severe metabolic abnormalities and infertility (28). As such, freshly isolated C57Bl/6J mouse islets were transduced with either RhoGDIi siRNA (siRhoGDI-Ad) or control siRNA (siCon-Ad) and 40 h later were perfused, and insulin secretion was monitored. GFP expression from islets transduced with siRhoGDI was visualized within the majority of islet cells with high penetration throughout the islet sphere (Fig. 4A). Knockdown efficiency and selectivity for RhoGDI in islets by adenoviral siRhoGDI was validated by immunoblotting (Fig. 4B). For analysis of biphasic insulin release, GFP-expressing islets were handpicked onto columns and perfused for 10 min in KR BH buffer containing 2.8 mM glucose (basal), followed by stimulation for 35 min at 16.7 mM glucose; islets were returned to basal for an additional 15 min to validate reversibility of response (Fig. 4C). Insulin release kinetics of siCon-Ad-transduced islets were similar to those of wild-type mice as reported previously (29). Similarly, siRhoGDI-Ad transduced islets exhibited a peak within 5 min of glucose stimulation, consistent with the occurrence of first-phase insulin secretion. However, islets transduced with siRhoGDI-Ad showed a significant augmentation of second-phase secretion compared with siCon-Ad islets, as quantified by the area under the curve between 18 and 45 min (area under the curve = 45 ± 7 versus siCon-Ad at 25 ± 3, p < 0.05). These data indicated RhoGDI selectively regulates the second phase of glucose-stimulated insulin secretion.

To verify that the enhancement of second-phase insulin release resulted from specific reduction in the RhoGDI protein, we replenished RhoGDI-depleted MIN6 cells with the human form of RhoGDI, because the human sequence is not targeted by this siRNA. A human C-peptide reporter assay was utilized to monitor secretion from transfectable MIN6 cells, wherein the human proinsulin plasmid was co-transfected with siRNA oligonucleotides and pcDNA3.1-RhoGDI. 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. Addition of human RhoGDI protein restored the stimulation index to that of siCon-transfected cells (Fig. 4D). Expression of the human RhoGDI protein was validated by immunoblotting, and its resistance to the siRNA was confirmed (data not shown). Thus, these data revealed that RhoGDI was required as a negative regulator of the second phase of insulin secretion, the same phase known to be regulated by its binding partner Cdc42.
Differential Phosphorylation of RhoGDI Promotes Its Dissociation from Either Cdc42 or Rac1

Kowluru and Veluthakal (24) have previously shown RhoGDI to interact with another small GTPase, Rac1, in rat beta cells. Rac1 activation is dependent upon Cdc42 and peaks 12–17 min after that of Cdc42 (14). Given this, RhoGDI depletion could have impacted activation of Cdc42 or Rac1, or both, to elicit the amplification of second-phase insulin release. Because GDI proteins bind to GTPases in a 1:1 stoichiometry (20), we tested the notion that in the beta cell cytosol, RhoGDI-Cdc42 and RhoGDI-Rac1 complexes exist and are differentially responsive to glucose. Anti-RhoGDI antibody co-immunoprecipitated both Cdc42 and Rac1 from unstimulated MIN6 cell lysates (Fig. 5A). Within 3 min of glucose stimulation, Cdc42 co-immunoprecipitation was reduced by ~50% but recovered fully after 17 min more (Fig. 5, A and B). In contrast, Rac1 remained bound to RhoGDI at 3 min of glucose stimulation but dissociated by nearly 40% after 20 min of glucose stimulation (Fig. 5, A and C). These data suggested that glucose differentially impacted RhoGDI interactions with Cdc42 versus Rac1.

To address whether RhoGDI expressed in cytosol is sufficient to function as GDI for both Cdc42 and Rac1, two approaches were utilized. First, sequential IP from MIN6 cytosol was conducted. As shown in Fig. 5D, after three sequential immunoprecipitation reactions, co-immunoprecipitation of Cdc42 and Rac1 was undetectable, although RhoGDI could still be immunoprecipitated, suggesting that the amount of RhoGDI was not limiting. Second, quantitative immunoblotting was employed to evaluate the stoichiometric ratio of these proteins in the cytosolic fraction of MIN6 cells. RhoGDI was found to be present at 0.39 ± 0.02 μmol/liter, whereas Cdc42 and Rac1 were present at 0.15 and 0.11 ± 0.02 μmol/liter, respectively. These quantitative results are consistent with a previous report stating that GDI:GTPase associations occur at a 1:1 stoichiometric ratio in multiple cell types (20).

In other cell types, the GDI-GTPase complex dissociation can occur with stimulus-induced phosphorylation of the GDI protein (20, 30). Given that glucose stimulation of MIN6 beta cells triggers tyrosine phosphorylation of the exocytotic machinery required for second-phase insulin release (31, 32), we examined whether RhoGDI underwent tyrosine phosphor-
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Glucose rapidly induces Cdc42-RhoGDI dissociation in MIN6 beta cells. A, MIN6 cells were incubated in MKRBB for 2 h and left unstimulated or stimulated with 20 mM glucose (Gluc) for 3 or 20 min. Detergent cell lysates were prepared for IP with control IgG. Immunoprecipitates were subjected to 12% SDS-PAGE for immunoblotting (IB). Data are representative of three independent experiments. B, ratio of Cdc42/RhoGDI binding; C, ratio of Rac1/RhoGDI binding under basal conditions was set equal to 1 for normalization of glucose-stimulated ratio in each of three independent experiments. *, p < 0.05 compared with basal. D, cytosolic fractions prepared from MIN6 cells were subjected to three sequential immunoprecipitation reactions (IP1–3) with anti-RhoGDI antibody. Immunoprecipitated proteins were resolved on 12% SDS-PAGE for immunoblotting with the antibodies indicated. Data are representative of three independent experiments.

Pervanadate treatment ablates Cdc42-RhoGDI association in MIN6 beta cells. MIN6 beta cells were incubated in MKRBB for 2 h prior to incubation with either vehicle or freshly made 0.5 mM pervanadate (pV) for 5 min prior to preparation of cleared detergent cell lysates. Lysates were immunoblotted (IB) for the presence of p-ERK and total ERK to validate action of pV treatment (A) and for immunoprecipitation with anti-RhoGDI antibody (B). Immunoprecipitated proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with the antibody indicated. Data are representative of three independent experiments.

Phosphorylation of ERK was used to validate pV-inducible tyrosine phosphorylation in MIN6 beta cells. Glucose rapidly induces Cdc42-RhoGDI dissociation in MIN6 beta cells. However, Rac1 dissociation from EGFP-RhoGDI-Y156F was blocked following a 20-min glucose stimulation (Rac1/RhoGDI-Y156F ratio = 1.1 ± 0.04, compared with control = 1.0, n = 3), consistent with the effect of pV. These results suggested that RhoGDI tyrosine phosphorylation was involved in interactions with both Cdc42 and Rac1.

Because the Y156F mutation in RhoGDI altered its interaction with Rac1 with only longer glucose stimulation periods, we examined the level of RhoGDI tyrosine phosphorylation at longer time points. Indeed, the level of RhoGDI tyrosine phosphorylation had peaked but did remain elevated for the 20-min time period.
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period assessed (Fig. 8, A and B). Unlike pV, glucose stimulation did not induce a visible doublet.

RhoGDI has also been demonstrated to undergo phosphorylation on serine residues 101 and 174 in non-beta cell types (33). To determine whether in beta cells this occurred in response to glucose stimulation, a phosphospecific S101-RhoGDI antibody was used to immunoblot co-immunoprecipitated RhoGDI. Indeed, pS101-RhoGDI detection was increased by 1.6-fold after 10 min of glucose stimulation (Fig. 8, B, ratio of Cdc42 binding to EGFP-RhoGDI proteins under basal conditions was set equal to 1 to normalization of glucose-stimulated ratio in each of three independent experiments; *, p < 0.05 versus basal.

Similar to the approach used to delineate Tyr-156 as the site of action in RhoGDI-Cdc42 dissociation, an EGFP-RhoGDI-S101A/S174A double mutant was compared for its ability to co-immunoprecipitate endogenous Rac1 with that of EGFP-RhoGDI-WT. In the absence of stimulus, the double mutant and WT forms of RhoGDI bound equivalent levels of Rac1 (Fig. 9, A and B). However, although glucose induced the expected 50% loss of Rac1 association with EGFP-RhoGDI-WT, Rac1 failed to dissociate from the EGFP-RhoGDI-S101A/S174A mutant. Cdc42 binding to RhoGDI was unaffected by either mutation of the serine residues or glucose stimulation at this 20-min time point, and it did not alter the complex dynamic upon acute glucose stimulation for just 3 min (ratio of 0.7 ± 0.1 of basal WT for EGFP-RhoGDI-WT compared with 0.7 ± 0.2 for EGFP-RhoGDI-S101A/S174A, n = 3). These data indicated that phosphorylation of serine residues 101 and/or 174 of RhoGDI is required for its dissociation from Rac1 but not from Cdc42.

RhGDI-Rac1 Disassociation Is Selectively Involved in Glucose-induced Amplification—Another method used to assess the second phase of glucose stimulation is by the diazoxide paradigm. This pharmacological paradigm is based upon the premise that the first-phase release is due to ATP-sensitive potassium channel (K_ATP)-dependent triggering, although the second phase/amplifying phase of insulin secretion is mainly regulated by K_ATP channel-independent effects (although a K_ATP channel-dependent signal is still required) (34–38). By subjecting beta cells to depolarizing KCl concentrations in the presence of the K_ATP channel opener diazoxide, low glucose stimulates triggering, while high glucose stimulates amplification. We used this paradigm as a second approach to ascertain the incidence of RhoGDI/Rac1 and RhoGDI/Cdc42 dissociation events within the amplification phase of glucose-stimulated insulin release. MIN6 cells stimulated with glucose for 20 min in the presence of diazoxide plus KCl secreted ~2-fold more insulin compared with non-glucose-stimulated cells (Fig. 10A), which recapitulated the glucose-amplification effect seen otherwise in primary islets, indicating their utility as the model system for our protein-protein interaction studies, and consistent with other reports of the MIN6 line as possessing characteristics of glucose-induced amplification (39–41). Under this paradigm, glucose stimulation for 20 min decreased the amount of Rac1 that co-immunoprecipitated with RhoGDI by 40 ± 7% (Fig. 10B). Although the MIN6 cells maintained the expected triggering response to KCl in terms of insulin secretion (Fig. 10C), the ratio of Rac1/RhoGDI remained unchanged (Fig. 10D). These data indicated that RhoGDI-Rac1 dissociation occurs in response to a glucose-induced amplification signal but not the triggering signal.

KCl stimulation alone failed to separate Cdc42 from RhoGDI, consistent with our previous observation that KCl failed to activate Cdc42 in MIN6 cells. However, we did not observe RhoGDI-Cdc42 dissociation after 3 min of glucose stimulation in this pharmacological paradigm (diazoxide plus KCl ± glucose, data not shown).

A triple RhoGDI phosphorylation mutant (EGFP-RhoGDI-Y156S/S101A/S174A) was expressed in MIN6 cells and assessed for functional impact upon the amplifying phase of glucose-stimulated insulin secretion using the diazoxide paradigm. Cells were transiently transfected to co-express human C-peptide to serve as a reporter of insulin release. In cells with glucose-induced triggering phase blocked by diazoxide plus KCl, overexpression of the WT form of EGFP-RhoGDI significantly inhibited glucose-induced human C-peptide release, as expected given the known inhibitory action of RhoGDI (Fig. 11A). Remarkably, the triple RhoGDI phosphorylation mutant inhibited the human C-peptide release even further. Neither the EGFP-RhoGDI-WT nor the triple phosphorylation mutant altered KCl-induced human C-peptide release from MIN6
cells, indicating specificity of RhoGDI action and importance of its phosphorylation status to the amplifying phase of glucose-stimulated insulin secretion (Fig. 11B).

**DISCUSSION**

In this study we demonstrate that RhoGDI is a key regulator of the second phase of insulin secretion from pancreatic islets, functioning as a master coordinator of the differential temporal activation events of the two critical GTPases involved in the process, Cdc42 and Rac1. RhoGDI was found to undergo sequential phosphorylation events in response to glucose stimulation in beta cells, with tyrosine phosphorylation emerging first followed by serine phosphorylation. The timing of these sequential events coincided with the times at which dissociation of RhoGDI-Cdc42 and RhoGDI-Rac1 complexes was detectable. Furthermore, RhoGDI residues Tyr-156 and Ser-101/Ser-174 were implicated in mediating these sequential phosphorylation events; RhoGDI-Cdc42 dissociation was sensitive only to mutation of Tyr-156, whereas the RhoGDI-Rac1 dissociation was sensitive to mutations of either Tyr-156 or Ser-101/Ser-174. These data support the integration of RhoGDI into the glucose-responsive Cdc42-Rac1 cycling mechanism and expand upon the current model by revealing a possible crucial role for RhoGDI in orchestrating the sustained pattern of insulin granule exocytosis.

The identification of RhoGDI and not a different GDI protein as the cytosolic Cdc42 GDI was initially surprising, given that RhoGDI was previously identified as a Rac1 GDI in the cytosol of beta cells (24). Although we have since demonstrated that Cdc42 is required for both Rac1 activation and for second-phase secretion (14), the question as to how the beta cell uses the same RhoGDI for both Cdc42 and Rac1 reservoirs remained unanswered. This was particularly intriguing given the significantly different activation kinetics of Cdc42 and Rac1 in response to the same glucose stimulus. However, the concept that RhoGDI could feasibly interact with both small GTPases was supported by quantitative evidence showing sufficient RhoGDI protein levels exist to roughly equal the cumulative cellular levels of Rac1 and Cdc42 in multiple cell types (20), accommodating the 1:1 stoichiometric ratio reported for GDI:GTPase association. Combined with the RhoGDI phosphorylation events in other cell systems and the plethora of phosphorylation events in beta cells, we tested the idea that RhoGDI might undergo sequential phosphorylation events to coordinate the timing of Cdc42 and Rac1 activation cycles. Glucose-induced tyrosine phosphorylation of RhoGDI was detectable within 3 min, whereas serine phosphorylation was not detected until our next time point at 10 min, with both detected still at the 20-min time point. Our mutational analyses of RhoGDI-Cdc42 were complemented by the use of pV, indicating that this complex is regulated by RhoGDI tyrosine phosphorylation. However, pV was much more effective at fully abolishing binding than was the Y156F mutation. One reason for this could be that pV, as a global and nonspecific tyrosine phosphatase inhibitor, also altered phosphorylation of other factors involved. Current studies are underway to investigate the potential for Cdc42 tyrosine phosphorylation in this mechanism because it has been reported to occur elsewhere (42).

Distinct from RhoGDI-Cdc42 complexes, RhoGDI-Rac1 dissociation was ablated by both the RhoGDI Y156F mutation as well as the S101A/S174A mutations, but only with the longer 20 min of glucose stimulation. The participation of tyrosine phosphorylation in modulation of the RhoGDI-Rac1 complex was supported by the use of pV studies, although pV treatment merely reduced dissociation in part. Structural analyses show Tyr-156 to be in the binding interface (43), and thus this mutation may have been more potent than the pV data indicated because it induced a significant conformational change in RhoGDI. Alter-
natively, pV may have caused phosphorylation at multiple sites to induce this and other changes. Based upon the structure of RhoGDI, eight of the nine tyrosine residues within RhoGDI are solvent-exposed (43). The structure also reveals both Ser-101 and Ser-174 as solvent-exposed, and in our studies the S101A/S174A mutations fully blunted dissociation of RhoGDI-Rac1 complexes following the 20-min glucose stimulation, consistent with the timing of the occurrence of Rac1 activation as well as detection of RhoGDI serine phosphorylation. The lack of effect upon RhoGDI-Cdc42 complexes by the Ser-101/174 mutations at either 3 or 20 min of glucose stimulation argues against the serine mutations simply inducing major conformational changes. Importantly, Pak1 is a known kinase for Ser-101/Ser-174-RhoGDI (33), and we have shown that in islet beta cells, Pak1 activation occurs prior to and is required for Rac1 activation (14). Because Cdc42 activation occurs prior to that of Pak1, this concurs with the lack of effect of Ser-101/174-RhoGDI mutation upon RhoGDI-Cdc42 association.

In addition to these discrete RhoGDI phosphorylation events, a recent proteomic study identified five dimethylation sites (Lys-50, Lys-52, Arg-111, Arg-152, and Arg-180) within RhoGDI from stimulated NRK49F rat kidney fibroblast cells (44). Given that glucose-induced methylation of proteins has been reported in insulin-secreting cells (45), it may be that juxtaposed dimethylation/phosphorylation modules within RhoGDI constitute functional switches between the Rho family GTPases. However, in addition to the impact of temporally induced RhoGDI modifications to the GDI-GTPase interaction, spatial location/membrane lipid composition can affect these interactions (46). Because no particular lipid domains have been identified as exocytosis active zones in beta cell membranes, this facet of GDI-GTPase interaction awaits further investigation.

In addition to caveolin-1, here we identify another GDI for Cdc42 in beta cells. Why does the beta cell need two different Cdc42 GDI proteins? In the beta cell, Cdc42 resides in both granule membrane compartments as well as cytosolic compartments, whereas in chromaffin cells, Cdc42 is absent from secretory granules (47). Unlike other cell types, the beta cell must be equipped to sustain regulated exocytosis over minutes to hours of time, and it may be that through dynamic compartmentalization and orchestration of Cdc42 cycling that this is achieved. Cdc42 seems to "multitask" in
RhoGDI Regulates Cdc42-mediated Insulin Exocytosis

FIGURE 11. Overexpression of RhoGDI specifically inhibited glucose-induced KATP channel-independent insulin secretion. MIN6 cells at 50–60% confluence were co-transfected with 1 μg of pEGFP-vector, pEGFP-RhoGDI-WT, or triple mutant (Y156F/S101A/S174A) plus 1 μg of human preinsulin cDNA. After 48 h of incubation, cells were incubated in glucose-free MKRBB for 2 h and subsequently subjected to diazoxide (250 μM) for 5 min and then stimulated with 20 mM d-glucose in the presence of 40 mM KCl for 1 h (A) or stimulated with 40 mM KCl alone (B). MKRBB was collected for subsequent human C-peptide quantitation by RIA, and each sample was normalized for protein content. Data in each of three independent experiments were normalized to EGFP = 100% and represent the average ± S.E.; *, p < 0.05 versus EGFP; #, p < 0.05 versus WT.

insulin exocytosis, as Cdc42 activation is required for the targeting of insulin granules to the plasma membrane (22, 25). Such multitasking would likely require multiple GDI proteins. Intriguingly, pilot studies suggested that the depletion of RhoGDI induced mobilization of Cdc42 from the cytosolic compartment to the secretory granule fraction, where it remained in the inactive (GDP) form, which could explain the lack of increase in basal Cdc42 activation in RhoGDI-depleted cells. If Cdc42 activation occurs primarily upon subsequent mobilization of the granules to the plasma membrane, then this could explain the dramatic increase in Cdc42 activation in RhoGDI-depleted cells. If Cdc42 activation occurs primarily upon subsequent mobilization of the granules to the plasma membrane, then this could explain the dramatic increase in Cdc42 activation in RhoGDI-depleted cells upon glucose stimulation. In this model, glucose stimulation would simultaneously free Cdc42 from complexation with RhoGDI while inducing Cdc42-bound insulin granule mobilization to the plasma membrane, whereupon Cdc42 becomes activated. Further studies will be required to determine whether Cdc42 that has dissociated from RhoGDI subsequently binds to the granule GDI caveolin-1 prior to/during granule mobilization to the plasma membrane.

In this study, we show for the first time a definitive role for the RhoGDI in second-phase insulin release using islet perfusion, and for the RhoGDI-Rac1 complex in the amplification phase of glucose-stimulated insulin secretion, revealed by use of the dioxide paradigm. This finding fits well with prior reports that G proteins and GTP levels are intimately involved in the amplification and sustainment of insulin secretion (36, 48). However, although the role of Cdc42 activation as a key proximal signaling event leading to the onset of second-phase insulin secretion is established using islet perfusion, RhoGDI-Cdc42 complexes were not responsive to the 3-min glucose stimulation in the dioxide paradigm, as was expected based upon the ability of glucose alone (without diazoxide) to induce complex dissociation. However, Cdc42-RhoGDI complexes were also unresponsive to KCl stimulation, consistent with previous reports arguing against Cdc42 function in first phase or triggering of insulin secretion. Because the Cdc42 activation cycle occurs so rapidly upon glucose stimulation under non-diazoxide treatment conditions (evident within 30 s, peaks by 3 min, and deactivated by 5 min), pretreatment with diazoxide/KCl may have altered the kinetics of the upstream and yet unknown signaling events leading to Cdc42 activation, thwarting efforts to capture the dissociation event. Pertinent to this, the sulfonylurea drugs that target KATP channel function were recently shown to activate the small GTPase Rap1, through the upstream GEF protein Epac2 (49). Alternatively, it has been argued that high KCl-induced depolarization may invoke artificial effects upon insulin release that are not normally produced by nutrient stimuli (50), overall suggesting that the KATP-independent diazoxide paradigm may not be fully equivalent to the second phase of insulin secretion as characterized by the temporal biphasic release pattern observed during islet perfusion. Regardless, the early action of Cdc42 is consistent with a role as a potential target of an amplification factor or time-dependent potentiation factor that is suggested to be responsible for increased second-phase insulin secretion and believed to initiate prior to the appearance of second-phase insulin secretion (51, 52).

In conclusion, efforts to improve upon diabetes treatments will require new drug targets to achieve more physiological delivery of insulin and improve overall beta cell function. In addition, efforts to increase beta cell mass will be crucial because the residual beta cell mass retained by some diabetic patients is also dysfunctional (53–55). Toward this goal, we demonstrate here the efficacy of investigating the Cdc42 signaling pathway in beta cells as a potential source of novel drug targets. Although not a beta cell-specific protein, RhoGDI displays characteristics of an attractive target in that it appears to be a target of the amplifying factor(s), and although its depletion amplified second phase, it did so without de-regulating secretion. Furthermore, these studies elucidated that targeting the Cdc42 cytosolic reservoir will be beneficial for enhancing glucose-stimulated insulin release in a regulated and metered way that more closely mimics the pattern of secretion from healthy human islets.

Acknowledgments—We are grateful to Dr. Anjan Kowluru and Drs. Bokoch and DerMardrossian for sharing their human RhoGDI and pGEX-RhoGDI-WT constructs, respectively. We thank Dr. Eunjin Oh and Jenna Jewell for confocal microscopy assistance, Michael Kalwat for careful reading of this manuscript, and Shari Uphchurch for administrative assistance. Human islets were obtained through the National Institutes of Health/ICR/ABCC distribution program.

REFERENCES
1. Rhodes, C. J. (2000) in Diabetes Mellitus: A Fundamental and Clinical Text (LeRoith, T., and Olefsky, J. M., eds) pp. 20–38, Lippincott Williams & Wilkins, Philadelphia
2. Cook, D. L., and Hales, C. N. (1984) Nature 311, 271–273
3. Meglasson, M. D., and Matschinsky, F. M. (1986) Diabetes Metab. Rev. 2, 163–214
4. Satin, L. S., and Cook, D. L. (1985) Pflügers Arch. 404, 385–387
5. Kiraly-Borri, C. E., Morgan, A., Burgoyne, R. D., Weller, U., Wollheim, C. B., and Lang, J. (1996) Biochem. J. 314, 199–203

3 Z. Wang and D. C. Thurmond, unpublished data.
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6. Rorsman, P., Eliasson, L., Renström, E., Gromada, J., Barg, S., and Göpel, S. (2000) *News Physiol. Sci.* **15**, 72–77

7. Henquin, J. C., Ishiyama, N., Nenquin, M., Ravier, M. A., and Jonas, J. C. (2002) *Diabetes* **51**, S60–S67

8. Straub, S. G., Shammugam, G., and Sharp, G. W. (2004) *Diabetes* **53**, 3179–3183

9. Li, G., Rungger-Brandl, E., Just, I., Jonas, J. C., Aktories, K., and Wollheim, C. B. (1994) *Mol. Biol. Cell* **5**, 1199–1213

10. Thurmond, D. C., Gonelle-Gispert, C., Furukawa, M., Halban, P. A., and Pessin, J. E. (2003) *Mol. Endocrinol.* **17**, 732–742

11. Tomas, A., Yermen, B., Min, L., Pessin, J. E., and Halban, P. A. (2006) *J. Cell. Mol. Med.* **11**, 52042–52051

12. Tsuboi, T., da Silva Xavier, G., Leclerc, I., and Rutter, G. A. (2003) *J. Biol. Chem.* **278**, 52042–52051

13. Li, J., Luo, R., Kowluru, A., and Li, G. (2004) *Am. J. Physiol. Endocrinol. Metab.* **286**, E818–E827

14. Wang, Z., Oh, E., and Thurmond, D. C. (2007) *J. Biol. Chem.* **282**, 9536–9546

15. Kowluru, A. (2008) *J. Clin. Invest.* **11**, 981–986

16. Kowluru, A., Li, G., Rabaglia, M. E., Segu, V. B., Hofmann, F., Aktories, K., and Metz, S. A. (1997) *Biochem. Pharmacol.* **54**, 1097–1108

17. Kowluru, A., Seavey, S. E., Li, G., Sorenson, R. L., Weinhaus, A. J., Nesher, R., Rabaglia, M. E., Vadakekalam, J., and Metz, S. A. (1996) *J. Clin. Invest.* **98**, 540–555

18. Nevins, A. K., and Thurmond, D. C. (2003) *Am. J. Physiol. Cell Physiol.* **285**, C698–C710

19. Bishop, A. L., and Hall, A. (2000) *Biochem. J.* **348**, 241–255

20. DerMardirossian, C., and Bokoch, G. M. (2005) *Trends Cell Biol.* **15**, 356–363

21. Olofsson, B. (1999) *Cell. Signal.* **11**, 545–554

22. Nevins, A. K., and Thurmond, D. C. (2006) *J. Biol. Chem.* **281**, 18961–18972

23. Regazzi, R., Ikikuchi, A., Takai, Y., and Wollheim, C. B. (1992) *J. Biol. Chem.* **267**, 17512–17519

24. Kowluru, A., and Veluthakal, R. (2005) *Diabetes* **54**, 3523–3529

25. Nevins, A. K., and Thurmond, D. C. (2005) *J. Biol. Chem.* **280**, 1944–1952

26. Spurlin, B. A., and Thurmond, D. C. (2006) *Mol. Endocrinol.* **20**, 183–193

27. Lacy, P. E., and Kostianovsky, M. (1967) *Diabetes* **16**, 35–39

28. Togawa, A., Miyoshi, I., Ishizaki, H., Tanaka, M., Takakura, A., Nishioka, H., Yoshida, H., Doi, T., Mizoguchi, A., Matsura, N., Niho, Y., Nishimune, Y., Nishikawa, S., and Takai, Y. (1999) *Oncogene* **18**, 5373–5380

29. Preitner, F., Ibberson, M., Franklin, L., Binnert, C., Pende, M., Gjinovci, A., Hansotia, T., Drucker, D. J., Wollheim, C., Curberlin, R., and Thorens, B. (2004) *J. Clin. Invest.* **113**, 635–645

30. DerMardirossian, C., Rocklin, G., Seo, J. Y., and Bokoch, G. M. (2006) *Mol. Biol. Cell* **17**, 4760–4768

31. Oh, E., and Thurmond, D. C. (2006) *J. Biol. Chem.* **281**, 17624–17634

32. Oh, E., and Thurmond, D. C. (2009) *Diabetes* **58**, 1165–1174

33. DerMardirossian, C., Schnelzer, A., and Bokoch, G. M. (2004) *Mol. Cell* **15**, 117–127

34. Henquin, J. C. (2000) *Diabetes* **49**, 1751–1760

35. Bratanova-Tochkova, T. K., Cheng, H., Daniel, S., Gunawardana, S., Liu, Y. J., Mulvaney-Musa, J., Schermerhorn, T., Straub, S. G., Yajima, H., and Sharp, G. W. (2002) *Diabetes* **51**, S83–S90

36. Straub, S. G., and Sharp, G. W. (2002) *Diabetes Metab. Res. Rev.* **18**, 451–463

37. Sugawara, K., Shibasaki, T., Mizoguchi, A., Saito, T., and Seino, S. (2009) *Genes Cells* **14**, 445–456

38. Ohara-Imaizumi, M., Nakamichi, Y., Tanaka, T., Ishowa, H., and Nagamatsu, S. (2002) *J. Biol. Chem.* **277**, 3805–3808

39. Horie, M., Mizuno, N., Tsuji, K., Haruna, T., Ninomiya, T., Ishida, H., Seino, Y., and Sasayama, S. (2001) *Cardiovasc. Drugs Ther.* **15**, 31–39

40. Park, K., Goto, M., Takei, R., Maruyama, A., Kobayashi, K., Miyazaki, J., Cho, C., and Akaie, T. (2000) *J. Biomater. Sci. Polym. Ed.* **11**, 903–913

41. Shigeto, M., Katsura, M., Matsuda, M., Ohkuma, S., and Kaku, K. (2006) *J. Pharmacoal. Sci.* **101**, 293–302

42. Tu, S., Wu, W. J., Wang, J., and Cerione, R. A. (2003) *J. Biol. Chem.* **278**, 49293–49300

43. Hoffman, G. R., Nassar, N., and Cerione, R. A. (2000) *Cell* **100**, 345–356

44. Guerrera, I. C., Keep, N. H., and Godovac-Zimmermann, J. (2007) *J. Proteome Res.* **6**, 2623–2630

45. Lim, Y., Shin, J. S., Paik, W. K., and Kim, S. (2003) *Biochem. Biophys. Res. Commun.* **305**, 292–298

46. Johnson, J. L., Erickson, J. W., and Cerione, R. A. (2009) *J. Biol. Chem.* **284**, 23860–23871

47. Gasman, S., Chasserot-Golaz, S., Bader, M. F., and Vitale, N. (2003) *Cell. Signal.* **15**, 893–899

48. Zhao, Y., Fang, Q., Straub, S. G., and Sharp, G. W. (2008) *J. Biol. Chem.* **283**, 5306–5316

49. Zhang, C. L., Katoh, M., Shibasaki, T., Minami, K., Sunaga, Y., Takahashi, H., Yokoi, N., Iwasaki, M., Miki, T., and Seino, S. (2009) *Science* **325**, 607–610

50. Hlatapatka, K., Willenborg, M., and Rustenbeck, I. (2009) *Am. J. Physiol. Endocrinol. Metab.* **297**, E315–E322

51. Nekher, S., and Cerasi, E. (1987) *Endocrinology* **121**, 1017–1024

52. Nekher, S., and Cerasi, E. (2002) *Diabetes* **51**, S53–S59

53. Gerich, J. E. (2002) *Diabetes* **51**, S117–S121

54. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) *Diabetes* **52**, 102–110

55. Rogers, S., and Silink, M. (1985) *Arch. Dis. Child.* **60**, 200–203