Original Article

Rho Guanosine Diphosphate–Dissociation Inhibitor Plays a Negative Modulatory Role in Glucose-Stimulated Insulin Secretion

Anjaneyulu Kowluru and Rajakrishnan Veluthakal

Extant studies have implicated the Rho subfamily of guanosine triphosphate–binding proteins (G-proteins; e.g., Rac1) in physiological insulin secretion from isolated β-cells. However, very little is known with regard to potential regulation by G-protein regulatory factors (e.g., the guanosine diphosphate–dissociation inhibitor [GDI]) of insulin secretion from the islet β-cell. To this end, using Triton X-114 phase partition, co-immunoprecipitation, and sucrose density gradient centrifugation approaches, we report coexistence of GDI with Rac1 in insulin-secreting β-cells (INS cells). Overexpression of wild-type GDI significantly inhibited glucose-induced, but not KCl- or mastoparan-induced, insulin secretion from INS cells. Furthermore, glucose-stimulated insulin secretion (GSIS) was significantly increased in INS cells in which expression of GDI was inhibited via the small interfering RNA–mediated knockdown approach. Together, these data appear to suggest an inhibitory role for GDI in the glucose metabolic signaling cascade, which may be relevant for GSIS.
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The monomeric, small molecular mass guanosine triphosphate (GTP)–binding proteins (G-proteins) have been shown to play significant regulatory roles in cell proliferation, survival, and demise (1,2). At least four major classes of such proteins have been described. The Ras superfamily of G-proteins (e.g., H-Ras) is implicated in gene expression, cellular proliferation, and motility. The Rho subfamily of G-proteins (e.g., Rac) regulates actin cytoskeletal remodeling and reorganization. The Rab (e.g., Rab 3A) and ADP-ribosylation factor 6 (ARF6) subfamilies of G-proteins are known to regulate intracellular vesicle trafficking, and the Ran family participates in the regulation of nuclear transport of macromolecules and plays a crucial role in providing directionality to this trafficking process (1,2). Unlike Rho G-proteins, which undergo prenylation and methylation at their COOH-terminal cysteine residues, ARF6 undergoes myristoylation (1,2). Together, such posttranslational modification steps have been proven to be essential for the targeting and association of these proteins with their membranous sites for optimal effector interaction (1–5).

In a manner akin to the heterotrimeric G-proteins, small G-proteins cycle between their guanosine diphosphate (GDP)-bound (inactive) and GTP-bound (active) conformations, which appear to be tightly regulated by various G-protein regulatory factors (RGFs). At least three types of RGFs have been described for small G-proteins (1,2). The GDP/GTP exchange factors stimulate conversion of the GDP-bound form to the GTP-bound form; the GDP-dissociation inhibitors (GDIs) elicit inhibition of this reaction; and the GTPase-activating proteins facilitate conversion of the GTP-bound to the GDP-bound form by activating the intrinsic GTPase function of candidate G-proteins. Several earlier studies have demonstrated that the GDIs play an important role in spatial determination in the actin cytoskeletal control (1,2). The GDIs are endowed with at least three biochemical properties. The first one involves the ability of the GDI to prevent dissociation of GDP from Rho G-proteins. Second, they appear to inhibit the intrinsic and GTPase-activating protein–catalyzed hydrolysis of GTP. The third biochemical activity involves the ability of GDI to sequester back Cdc42, Rac, and Rho from its putative membranous site, thereby inhibiting its interaction with their respective effector proteins (1,2,6–16).

In the context of the islet β-cell, the stimulus-secretion coupling of glucose-stimulated insulin secretion (GSIS) involves well-regulated trafficking of insulin-laden secretory granules for their docking and fusion with the plasma membrane (17,18). Emerging evidence suggests that such cellular events are delicately controlled by G-proteins, which have been implicated in cytoskeletal remodeling to facilitate granule movement (3–5). Several earlier studies, including our own, suggested critical involvement of small G-proteins, such as Rac1, Cdc42, and ARF6, in GSIS from normal rat islets, human islets, and clonal β-cell preparations (3–5,19–31). Such conclusions were drawn primarily based on data accrued from experiments using *1*) inhibitors of requisite posttranslational modifications (e.g., prenylation, carboxylmethylation, and palmitoylation) of these G-proteins; *2*) clostridial toxins, which irreversibly monoglucosylate and inactivate specific G-proteins; and *3*) gene depletion experiments. Despite this compelling evi-

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ARF6, ADP-ribosylation factor 6; GDI, guanosine diphosphate–dissociation inhibitor; GDP, guanosine diphosphate; G-protein, guanosine triphosphate–binding protein; GSIS, glucose-stimulated insulin secretion; GTP, guanosine triphosphate; Mas, mastoparan; PIP_2, phosphatidylinositol 4,5-bisphosphate; RGF, G-protein regulatory factor; RhoGDI, Rho guanosine diphosphate–dissociation inhibitor; siRNA, small interfering RNA.

dence, very little is understood with regard to identity of and regulation by G-protein RGFs (e.g., GDI) in physiological insulin secretion. With this in mind, the present study was undertaken to determine subcellular localization of GDI in insulin-secreting cells and to examine its roles in GSIS. Data from these studies demonstrate a negative modulatory (i.e., inhibitory) role for GDI in the stimulus secretion coupling of GSIS.

RESEARCH DESIGN AND METHODS

Mastoparan (Mas) was purchased from Biomol (Plymouth Meeting, PA). Antisera directed against Rac1 and Rho GDP-dissociation inhibitor (RhoGDI) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Rac activation assay kit was purchased from Cytoskeleton (Denver, CO). The rat insulin ELISA kit was purchased from American Laboratory Products (Windham, NH).

Normal rat islets and insulin-secreting cells. Islets were isolated from normal Sprague-Dawley rats (200–250 g body wt) using the collagenase digestion method as we described previously (20–23). βTC3 cells were provided by Dr. Shimon Efrat (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). INS cells were provided by Dr. Goudong Li (National University of Singapore, Singapore). βTC3 or INS-1cells were cultured as described previously (24,28,29).

Translocation and membrane association of Rac1. This was determined according to the method we described previously (29,30). In brief, after incubation with low (3 mmol/l) or stimulatory (20 mmol/l) concentrations of glucose or Mas (30 μmol/l), cells were homogenized by sonication (3 × 15 s each) and subjected to a single-step centrifugation at 105,000*g* for 60 min. Total membrane (pellet) and soluble (supernatant) fractions were separated and used for the determination of relative abundance of Rac1 in these fractions by Western blotting.

Rac1 activation assay. The degree of glucose- or Mas-induced activation of Rac1 was determined using a Rac1 activation assay kit as we described recently (29,30). In brief, β-cells were exposed to basal (3 mmol/l) or stimulatory (20 mmol/l) concentrations of glucose or Mas (30 μmol/l) for 30 min in Krebs Ringer buffer medium. Lysates (3–5 mg/ml) were clarified by centrifugation for 5 min at 4,800*g*, and p21-binding domain of p21-activated kinase beads (20 μl) were added to the supernatant. The mixture was then rotated for 1 h at 4°C and pelleted by centrifuging at 4,000*g* for 3 min. This pellet was washed once with lysis buffer followed by a rinse in wash buffer (25 mmol/l Tris, pH 7.5, 30 mmol/l $MgCl_2$, 40 mmol/l NaCl, and 150 mmol/l EDTA) and was then reconstituted in Laemmli buffer. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, and the relative abundance of Rac1 was determined by Western blotting and densitometry.

Triton X-114 partition assay. Hydrophilic and hydrophobic phases of the total soluble and particulate fractions from INS cells were isolated using Triton X-114 essentially according to a method described by Regazzi et al. (32) without further modifications. Relative abundance of Rac1 and GDI in each of the individual hydrophilic and hydrophobic phases derived from the cytosolic and membranous fractions was determined by Western blotting.

Sucrose density gradient centrifugation. This was done according to a method described previously (6). In brief, INS cell cytosolic fractions were loaded onto 4-ml gradients of 5–20% sucrose (wt/vol) in 20 mmol/l Tris-HCl, pH 7.5, 5 mmol/l $MgCl_2$, 6 mmol/l β mercaptoethanol, and protease inhibitor cocktail. The gradients were spun in a Beckman MLS-50 rotor for 18–20 h at 180,000*g*. Fractions of 300 μl were collected beginning at the top of the gradient, and the relative abundance of Rac1 and GDI in each fraction was determined by Western blotting. Relative density of the bands was quantitated by densitometry.

Coimmunoprecipitation assays. INS cell lysates (500 μg protein) were incubated overnight with anti-RhoGDI or with rabbit preimmune serum at 4°C with constant rocking. After this, Protein G-Agarose (20 μl) was added, and the mixture was further incubated for an additional 4 h at 4°C with constant mixing. The beads were briefly washed (three times) with 0.5 ml PBS and reconstituted in Laemmli buffer, and the solubilized proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Relative abundance of Rac1 in the immunoprecipitates was determined by Western blotting as described above.

Overexpression of wild-type RhoGDI. Human RhoGDI (wild type) cloned into pcDNA3.1+ at *Eco*RI (5′) and *Xho*I (3′) was purchased from the University of Missouri Rolla cDNA Resource Center (Rolla, MO). INS-1 cells were subcultured at 70–80% confluence and transfected using Effectene (Qiagen, Valencia, CA) with a maximum 0.2-μg plasmid DNA constructs (wild-type RhoGDI) per well of 24-well dishes. Twenty-four hours after transfection, the level of expression of GDI was determined by Western blot analysis.

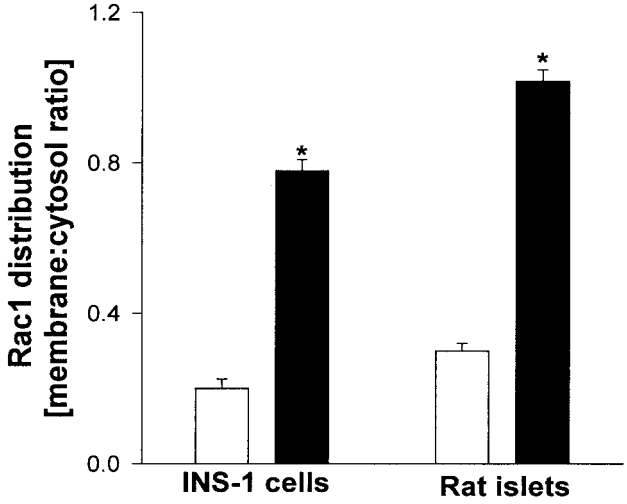

FIG. 1. Glucose stimulates translocation of Rac1 to the membrane fraction in INS cells and islets. INS cells or rat islets were exposed to basal (3 mmol/l) (□) or stimulatory (20 mmol/l) (■) concentrations of glucose for 30 min, as indicated. After incubation, cells were lysed, and total particulate and soluble fractions were isolated (see RESEARCH DESIGN AND METHODS). Relative abundance of Rac1 in those two fractions was determined by Western blotting. Data are expressed as membrane-to-cytosolic ratios and are means ± SE from three experiments. *P < 0.001 for 3 vs. 20 mmol/l glucose-treated conditions.

siRNA-mediated knockdown of RhoGDI expression. Endogenous RhoGDI expression was inhibited by transfection of interfering small interfering RNA (siRNA). A 21-nucleotide siRNA sequence corresponding to the mouse RhoGDI coding region between nucleotides 96 and 117 (13) was obtained from Dharmacon (Lafayette, CO). Cells were transfected with siRNA at a final concentration of 40 nmol/l using Trans-IT-TKO reagent (Mirus Bio, Madison, WI). To assess specificity of RNA interference, control cells were transfected (as above) with nontargeting RNA (i.e., scrambled siRNA) duplexes (also obtained from Dharmacon). The nontargeting RNA includes at least four nucleotide mismatches with all known mouse, rat, and human genes. Transfected cells were maintained in complete growth medium for 48 h. Efficiency of RhoGDI knockdown was determined by Western blots of lysates derived from nontransfected and scrambled siRNA- and siRNA GDI–transfected cells.

Other assays. Protein concentration in β-cell subcellular fractions was quantitated according to Bradford using BSA as a standard. Insulin release was quantitated by ELISA as described previously (24,28,29). The statistical significance of the differences between the experimental conditions was determined by Student's t test. P values <0.05 were considered significant.

RESULTS

Glucose promotes the membrane association and activation of Rac1 in β-cells. It is well established that small G-proteins, including those belonging to the Rho subfamily (e.g., Rac1 and Cdc42), are cytosolic in their distribution and are translocated to their membranous sites after cellular activation (5). Data in Fig. 1, which represent the membrane-to-cytosol ratio of relative abundance of Rac1, demonstrate a significant increase in glucose-induced translocation of Rac1 from the cytosolic to the membrane fraction in INS cells as well as in normal rat islets. Next, we quantitated the ability of glucose to activate Rac1 in insulin-secreting cells. For this, we used the pak21 binding assay (29,30) as a measure of activation of Rac1 in insulin-secreting β-cells (e.g., INS-1 or βTC3) as well as normal rat islets. Data in Fig. 2*A* indicate significant activation, by glucose, of Rac1 in these cells. Mas, a tetradecapeptide from wasp venom, which is a global activator of G-proteins and insulin secretion in β-cells (see below) (25,29), also activated Rac1 in INS cells by nearly

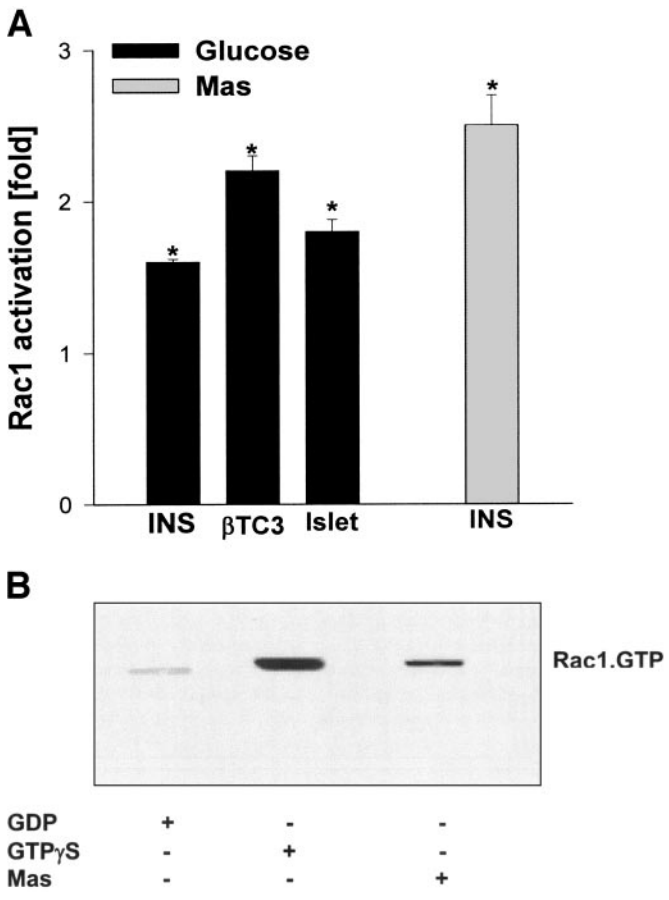

FIG. 2. *A*: Glucose or Mas activates Rac in insulin-secreting cells. Clonal β-cells (INS or βTC3) or normal rat islets were incubated in presence of basal (3 mmol/l) or stimulatory (20 mmol/l) glucose or Mas (30 μmol/l) for 30 min, as indicated. Amounts of activated (GTP-bound) Rac were determined by a pull-down assay (see RESEARCH DESIGN AND METHODS). Data are expressed as fold stimulation over basal glucose and are means ± SE from three experiments. *P = 0.01 for Rac1 activation demonstrable in the presence of 3 vs. 20 mmol/l glucose treatment conditions in individual β-cell preparations, as indicated in the figure. *B*: Incubation of INS cell lysates with guanosine 5′-[γ-thio]triphosphate (GTPγS) or Mas, but not GDP, results in the activation of Rac1. INS cell lysates are incubated with Mas (30 μmol/l) or GTPγS (10 μmol/l) or GDP (10 μmol/l) for 30 min. Amount of activated Rac1 was determined by a pull-down assay (as in *A*). These data indicate significant activation of Rac1 in lysates incubated with either Mas or GTPγS but not GDP. A representative blot from two experiments is shown.

2.5-fold. The Rac1 activation assay used in these studies is highly specific, because incubation of INS cell lysates with either guanosine 5′-[γ-thio]triphosphate or Mas, but not GDP (a negative control), resulted in increased binding of Rac1 with pak21 (Fig. 2*B*). Together, data in Figs. 1 and 2, which are compatible with our original observations (30), demonstrate that a stimulatory concentration of glucose facilitates translocation of Rac1 to the membrane fraction, presumably for its interaction with its effector proteins.

As stated above, the functional activation of Rho subfamily of G-proteins is under the fine control of RGFs, such as the GDI (1,2). It is well documented in multiple cell types that inactive, GDP-bound Rac1 remains complexed with GDI in the cytosolic fraction and that dissociation of Rac1 from the GDI/Rac1 complex is necessary for gaining its GTP-bound, active conformation and translocation to the membrane fraction (rev. in 1). Therefore, in the following studies, we examined potential regulatory roles for GDI in GSIS.

Subcellular distribution and potential colocalization of Rac1 and RhoGDI in insulin-secreting cells. To address this, total particulate and soluble fractions were isolated from INS cells by single-step centrifugation (see RESEARCH DESIGN AND METHODS). Western blot analysis suggested that E-cadherin, a known membranous protein, is predominantly localized in the total particulate fraction. This finding suggests that the cytosolic fraction is relatively devoid of membranous protein contamination (Fig. 3*A*, *a*). Further Western blot analysis of these fractions indicated that RhoGDI is predominantly localized in the cytosolic fraction derived from islets (Fig. 3*A*, *b*) and INS cells (Fig. 3*A*, *c*). In a second approach, Triton X-114 phase partition analysis revealed that both Rac1 and GDI are predominantly distributed in the hydrophilic compartment of the soluble phase, further raising a possibility that Rac1 and GDI might coexist in this compartment (Fig. 3*B*). In a third approach, Western blot analysis of individual fractions isolated from β-cell lysates subjected to sucrose density gradient centrifugation further suggested emergence of both Rac1 and GDI in the molecular weight region of 40–45 kDa, which represents the combined molecular weights of each of these proteins (Fig. 3*C*). Lastly, in the fourth approach, data from co-immunoprecipitation (i.e., pull-down assays) demonstrated that an antiserum directed against RhoGDI (but not the preimmune serum) specifically precipitated Rac1 from INS cell lysates (Fig. 3*D*). Together data in Fig. 3*A*–*D* suggest that Rac1 and GDI might coexist and/or remain complexed in the soluble compartment of the islet β-cells.

GDI plays a negative modulatory role in GSIS. We next examined potential contributory roles, if any, of GDI in GSIS in insulin-secreting cells. We addressed this by two independent approaches. In the first, we overexpressed either the empty vector or wild-type GDI mutant in INS cells and then studied GSIS in those cells. In the second, we knocked down GDI expression via the RNA interference approach (using siRNA for GDI or scrambled siRNA as a negative control) and quantitated GSIS in control and GDI–knocked-down cells. Data in Fig. 4 represent expression profiles of GDI in INS cells under these two experimental approaches. We observed significant increase in the expression of GDI in cells transfected with wild-type GDI (Fig. 4, *left panel*). Analysis of data from three individual transfection experiments indicated a 30 ± 5% increase in the expression of GDI under these experimental conditions. In INS cells subjected to siRNA-mediated GDI knockdown, we observed a marked reduction in the expression levels of GDI only when transfected with siRNA for GDI, but not the scrambled siRNA (Fig. 4, *right panel*). Analysis of data from three individual transfection experiments indicated a 65 ± 5% inhibition in the expression of endogenous GDI under these experimental conditions. Relative abundance of α-tubulin remained unchanged under these experimental conditions (not shown). Together, these data verify our experimental system in which to validate potential roles of GDI in GSIS. Data in Fig. 5 demonstrate significant reduction in GSIS in INS cells overexpressing the wild-type form of GDI. In contrast, GSIS was significantly increased in cells in which expression of GDI was inhibited via the RNA interference approach (Fig. 6). However, no significant differences were seen between nontransfected cells and cells transfected with the scrambled siRNA (Fig. 6). Together, these data appear to suggest a negative modulatory (i.e., inhibitory) role for GDI in GSIS.

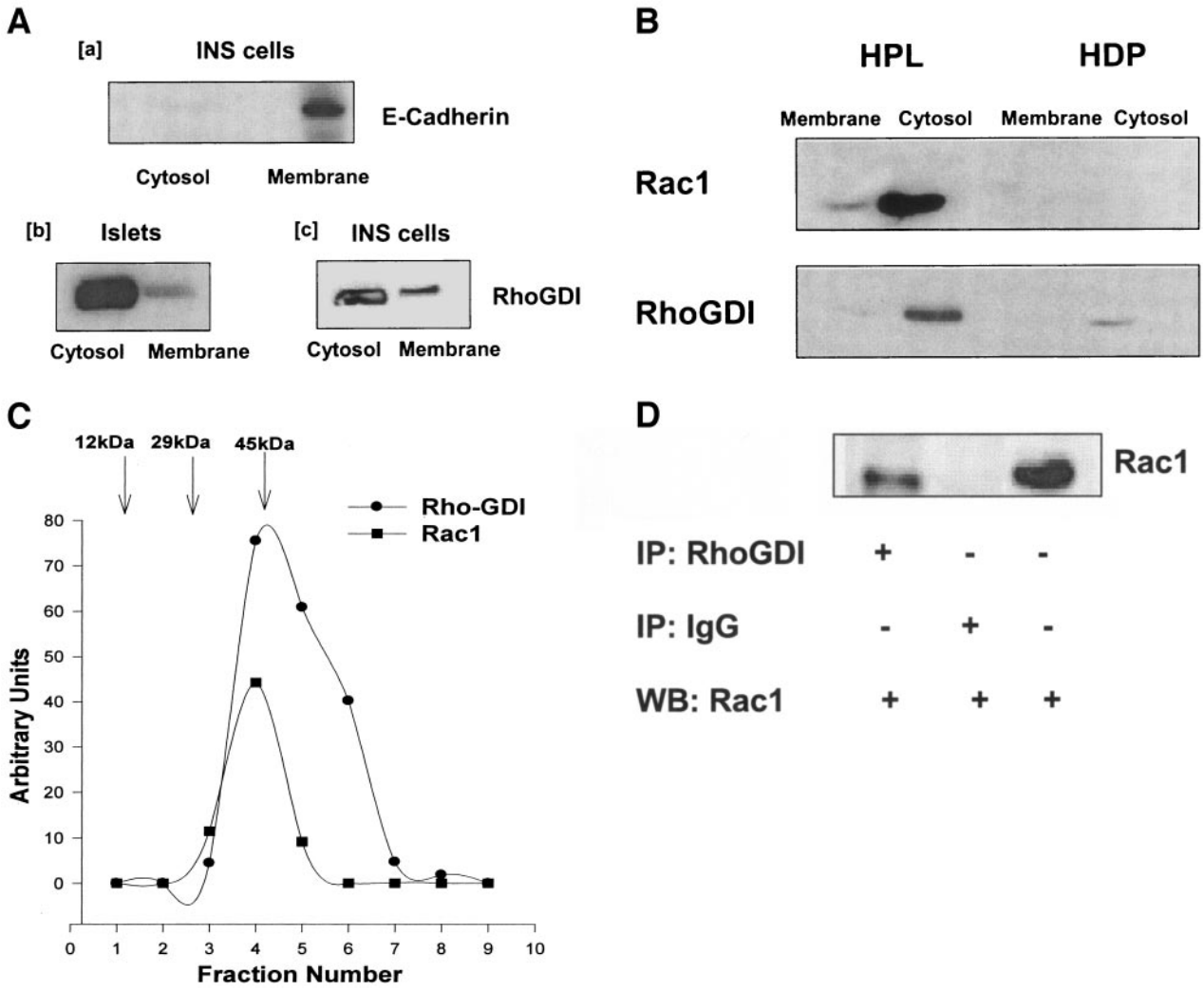

FIG. 3. *A*: **Subcellular distribution of GDI and Rac1 in insulin-secreting cells.** *A*: **Subcellular distribution of RhoGDI in normal rat islets and INS cells. Total membrane and cytosolic fractions from INS cells were isolated (see RESEARCH DESIGN AND METHODS), and relative abundance of E-cadherin, a membrane marker protein, was determined by Western blotting. Data in *a* indicate that cadherin is predominantly localized in the membrane fraction. Relative abundance of GDI in the total membrane and soluble fractions derived from normal rat islets and INS cells is shown in *b* and *c*, respectively.** *B*: **Rac1 and GDI predominantly partition in the hydrophilic compartment of the β-cell. Hydrophilic and hydrophobic phases of the cytosol and membrane fractions from INS cells were isolated using Triton X-114 partition technique (see RESEARCH DESIGN AND METHODS). Relative abundance of Rac1 and GDI in each of these fractions was determined by Western blotting. Data are representative of two experiments with comparable results. Cyto, cytosol; HDP, hydrophobic; HPL, hydrophilic.** *C*: **Sucrose density gradient analysis of β-cell cytosolic Rac and GDI complexes. INS cell cytosolic fraction was subjected to sucrose (5–20%) density gradient centrifugation (see RESEARCH DESIGN AND METHODS). Relative abundance of Rac1 and GDI in each fraction was determined by Western blotting. Density of the bands was quantitated by densitometry and plotted as arbitrary units. These data suggest that both Rac1 and GDI emerge in fractions, corresponding to a molecular mass of ~45 kDa, that represent the combined size of these proteins. Data are representative of at least two individual experiments with comparable data.** *D*: **Coimmunoprecipitation of Rac1 with RhoGDI. INS cell lysates were immunoprecipitated with anti-RhoGDI (*lane 1*) or preimmune serum (*lane 2*). Relative abundance of Rac1 in the immunoprecipitates was determined by Western blotting. Total lysate proteins were probed for Rac1 as a positive control (*lane 3*). A representative blot from two experiments is shown.**

GDI is not required for calcium- or Mas-induced insulin secretion. We next determined regulatory roles for GDI in insulin secretion elicited by a membrane depolarizing concentration of KCl (40 mmol/l) or Mas (30 μmol/l). In these experiments, in contrast to its regulatory roles in GSIS (Figs. 5 and 6), we observed no clear effects of GDI overexpression on either KCl- or Mas-induced insulin secretion (Fig. 7). These data suggest significant regulation by GDI specifically of glucose-stimulated insulin secretion but not calcium- or Mas-stimulated insulin secretion (see below).

DISCUSSION

To the best of our knowledge, this study demonstrates, for the first time, that GDI plays a significant regulatory role in physiological insulin secretion. Several previous studies, including our own, implicated Rho subfamily G-proteins (e.g., Cdc42 and Rac) in physiological insulin secretion (3–5,19–31). Using specific inhibitors, we reported that posttranslational modifications of these G-proteins (e.g., isoprenylation and carboxylmethylation) are critical for physiological insulin secretion (19,22,24). We also demonstrated that such modification steps increase the hydrophobicity of the candidate G-proteins, resulting in their translocation to specific membranous sites for optimal interaction with their effector proteins (22,29,30). Using *Clostridial* toxins, which specifically monoglucosylate and inactivate Rho subfamily of GTPases (e.g., Cdc42 and Rac1), we have been able to further substantiate the involvement of these G-proteins in physiological insulin

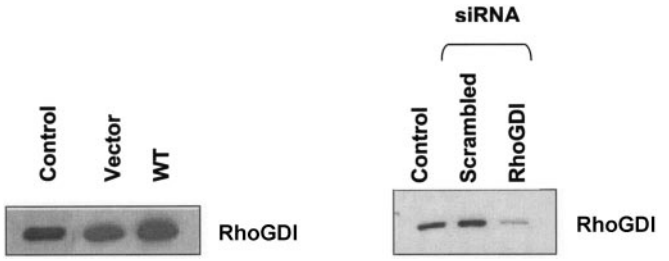

FIG. 4. *A*: Expression profiles of GDI in INS cells used in GSIS experiments. *Left panel* represents expression profile (determined by Western blotting) of GDI in control INS cells or in INS cells overexpressing either vector alone or the wild-type GDI. *Right panel* represents expression profiles of GDI in INS cells in which GDI expression is controlled via the siRNA method. These data indicate a marked reduction in the expression of GDI in cells transfected with siRNA corresponding to the GDI but not the scrambled siRNA.

secretion (23,28,29). Additional studies from our laboratory, including the current study, demonstrated direct activation, by glucose, of Cdc42 and Rac1 in isolated islets and clonal β-cell preparations (22,23,29,30). Lastly, using gene transfection approaches, we have confirmed our original hypothesis that activation of specific G-proteins is critical for physiological insulin secretion (29,30). Along these lines, recent studies from the laboratories of Thurmond (27,31) and Sharp (25) also provided additional insights into contributory roles for Rho subfamily of G-proteins in insulin secretion. Nevins and Thurmond (27) proposed a novel mechanism for regulation, by glucose, of cortical actin network and insulin secretion through modulation of Cdc42 cycling (via the GTP-hydrolytic cycle) in the β-cell. More recently, they described potential interaction between Cdc42 and VAMP-2, one of the SNARE proteins, as a requisite for proper targeting of insulin granules for docking and fusion with the plasma membrane (31). Together, these data (3–5,19–31) support the hypothesis that Rho G-proteins control physiological insulin secretion.

Despite such compelling evidence, very little has been studied with regard to potential regulatory roles for RGFs, specifically the GDI in islet function. Along these lines,

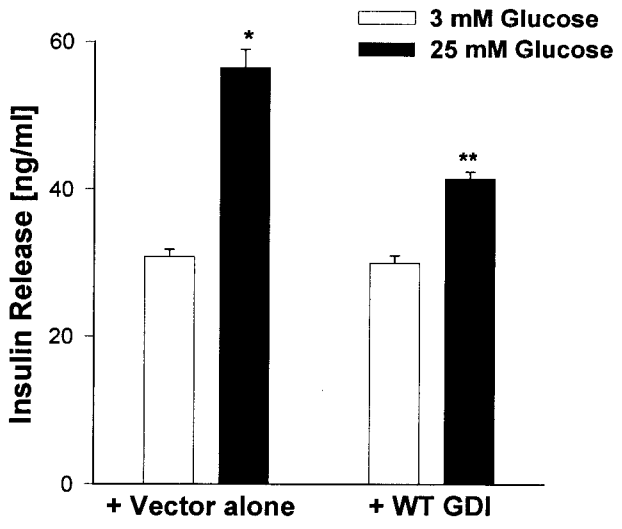

FIG. 5. Overexpression of wild-type GDI inhibits GSIS in INS cells. GSIS was quantitated in INS cells overexpressing wild-type GDI, as indicated in the figure. Data are means ± SE from three experiments. *P = 0.005 for 3 vs. 25 mmol/l glucose. **P = 0.005 for GSIS demonstrable in vector vs. GDI-expressing cells.

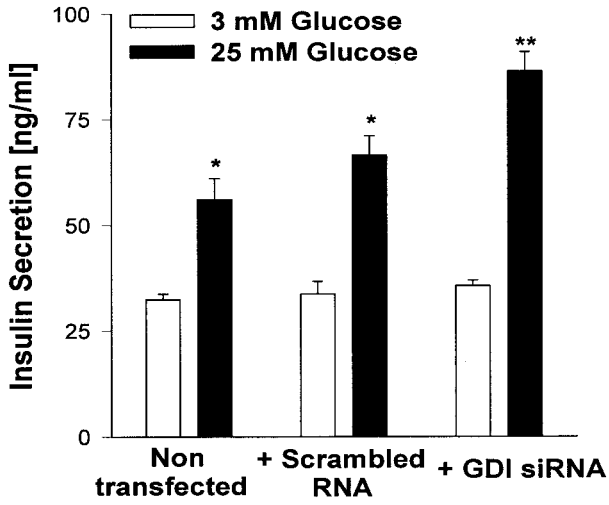

FIG. 6. siRNA-mediated knockdown of GDI increases GSIS in INS cells. GSIS was quantitated in nontransfected INS cells and INS cells transfected with either scrambled siRNA or the GDI siRNA. Data are means ± SE from three experiments. *P = 0.005 for 3 vs. 25 mmol/l glucose. **P = 0.05 for nontransfected or scrambled siRNA-transfected cells vs. GDI siRNA-transfected cells.

earlier studies by Regazzi et al. (32) reported potential complexation of small G-proteins with GDI in HIT-T15 and RINm5F cells. However, no follow-up studies were carried out by these researchers on potential contributory roles of this protein in GSIS. Therefore, our current studies form a logical extension to our ongoing work to determine potential roles of Rho GTPases (e.g., Rac1) and RGFs in GSIS. As indicated above, the GDP-bound, inactive form of Rac1 remains associated with GDI in the cytosolic compartment. After receipt of an appropriate signal(s), Rac1 dissociates from the Rac1/GDI complex to attain the

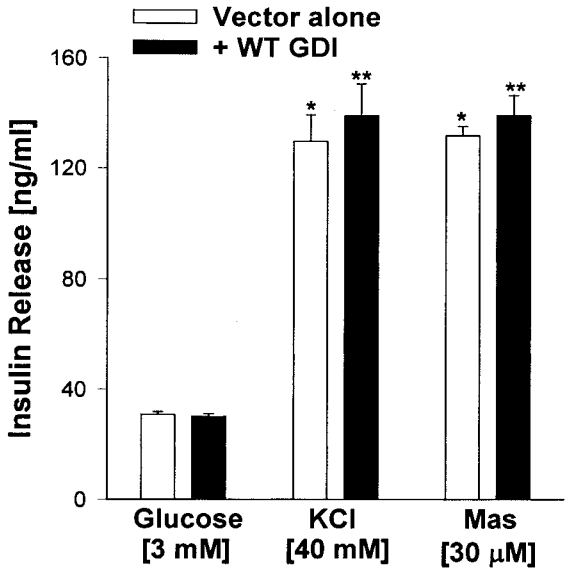

FIG. 7. Lack of effects of GDI on KCl- or Mas-induced insulin secretion in INS cells. Insulin secretion was quantitated in INS cells transfected with either vector alone (□) or wild-type GDI (■) in the presence of basal (3 mmol/l) glucose or KCl (40 mmol/l) or Mas (30 μmol/l), as indicated in the figure. Data are means ± SE from at least three to four independent measurements. *P = 0.005 for stimulation of insulin secretion by KCl or Mas in cells expressing vector alone or wild-type GDI compared with basal insulin secretion seen in the presence of 3 mmol/l glucose. **No significant difference in the insulin secretion from INS cells expressing either vector alone or wild-type GDI.

GTP-bound conformation (i.e., activation) and translocation to the membrane for optimal interaction with its effector proteins. Our current observations lend support for a potential negative modulatory role for GDI in glucose-stimulated, but not calcium- or Mas-stimulated, insulin secretion. From these data, it appears that one or more messengers in glucose metabolic pathway might contribute to the dissociation of Rac1 from Rac1/GDI complex for subsequent activation and translocation of Rac1 to the membrane fraction (Figs. 1 and 2).

Which, then, are those factors that could facilitate the dissociation of Rac1 from Rac1/GDI complex, culminating in the activation of Rac1 function? Extant data from multiple laboratories suggest Rho GTPase functional regulation by biologically active lipids. For example, using cytosolic fractions from human neutrophils, Chuang et al. (6) demonstrated dissociation of RhoGDI/Rac complex by several classes of lipids, including arachidonic acid, phosphatidic acid, and phosphoinositides. Studies from Dagher's laboratory (8,10,11) indicated that phosphoinositides and phosphatidic acid induce the formation of an open complex between RhoA and RhoGDI, presumably via disruption of protein-lipid interactions. This, in turn, promotes the exchange of GTP for GDP and subsequent translocation of activated G-protein to the complex. Using purified Rho G-proteins, Zheng et al. (7) demonstrated significant dissociation by phosphoinositides, specifically phosphatidylinositol 4,5-bisphosphate (PIP_2), of GDP from Cdc42, Rho, and Rac. These data provide an alternative mechanism for the dissociation of GDP from Rho G-proteins. More recently, using 1321N1 astrocytoma cells, Fleming et al. (14) reported regulation of GDP/GTP exchange and subsequent activation of Rac1 by inositol lipids via promoting the binding of Tiam1, a known GDP/GTP exchange factor for Rac1, to the plasma membrane. Together, these findings raise an interesting possibility that glucose-mediated activation of islet endogenous phospholipases (33) and subsequent generation of phospholipid second messengers (e.g., phosphatidic acid; PIP_2) could contribute toward dissociation of Rac1 from Rac1/GDI, culminating in its activation in the islet β-cell. Last, it may be germane to point out that recent observations of Santy and Casanova (34) suggested potential cross-talk between multiple small G-proteins (e.g., ARF6 and Rac1) in the signaling cascade underlying epithelial cell migration. They reported that activation of ARF6 is critical for the activation of downstream signaling steps involving Rac1. Recent observations by Lawrence and Birnbaum (26) in isolated β-cells (Min 6) provide further support to such a potential cross-talk between ARF6-Rac1 signaling pathway in GSIS. Based on the observations of Lawrence and Birnbaum and our current findings, we propose (Fig. 8) that GSIS might constitute initial activation of ARF6, which leads to stimulation of phospholipid-metabolizing enzymes, culminating in the biogenesis of biologically active lipids, such as PIP_2 and phosphatidic acid. These, in turn, might facilitate dissociation of GDI/Rac1 complex to enable Rac1 to gain GTP-bound active conformation and translocation to the plasma membrane fraction for its interaction with its putative effector proteins. Additional studies are needed to examine potential cross-talk between ARF6 and Rac1 in GSIS.

We also observed that unlike GSIS, overexpression of wild-type GDI had no significant effects on high KCl– or Mas-induced insulin secretion. The current findings are compatible with our earlier observations. First, we recently reported that overexpression of dominant-negative mutant of Rac1 (N17Rac1) inhibited only glucose but not high KCl–stimulated insulin secretion, suggesting that Rac1 activation may not be necessary for insulin secretion elicited by a membrane-depolarizing concentration of potassium (30). Second, based on extensive biochemical, microscopic, and physiological data, we proposed recently (29) that Mas-induced insulin secretion requires Rac1 activation, presumably via its direct effects of GTP/GDP exchange. These data, thus, appear to argue against a putative requirement for GDI in Mas-induced insulin secretion; data are in agreement with current findings.

Last, it may be also be germane to point out that the current studies do not rule out the possibility that similar mechanism might underlie glucose-stimulated activation of other Rho G-proteins, such as Cdc42, because GDI has also been shown to complex with GDP-bound form of Cdc42 (1,2,7,9). Recent evidence from the laboratory of Bokoch (15,35) has identified pak 21-kinase, an effector protein for Cdc42, as the RhoGDI kinase, which phosphor-

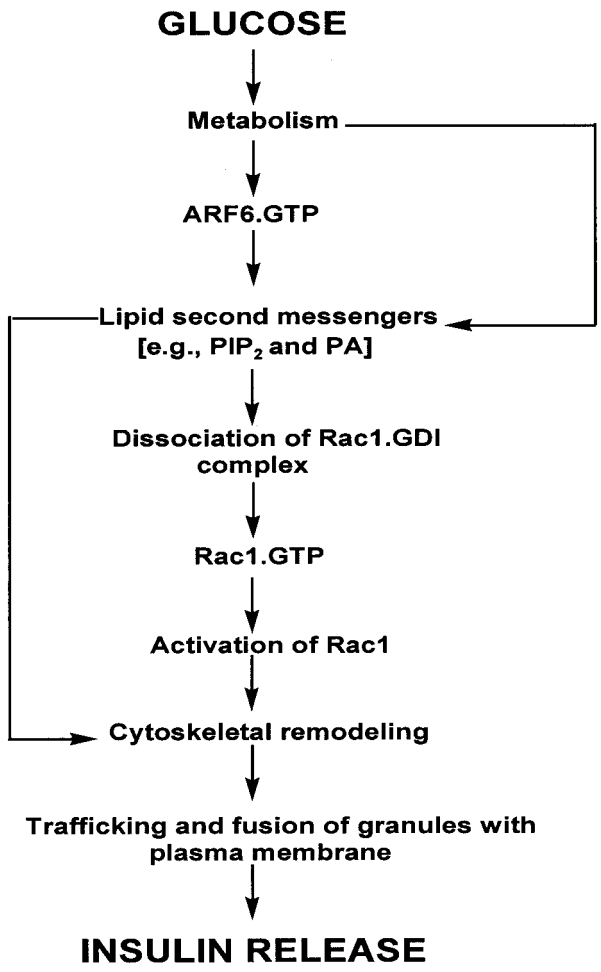

FIG. 8. Proposed model to implicate GDI in the signaling steps leading to GSIS. We propose that GSIS from the β-cell involves activation and potential cross-talk between ARF6 and Rac1. Our model states that glucose, via its metabolism, facilitates the conversion of the inactive ARF6.GDP to active ARF6.GTP, which, in turn, activates the β-cell endogenous phospholipase D to generate phosphatidic acid. This, in turn, activates PIP-4-phosphate-5 kinase to yield PIP_2. Phosphatidic acid and/or PIP_2 promotes the dissociation of Rac1 from GDI, after which Rac1 attains its GTP-bound active conformation via GTP/GDP exchange. ARF6.GTP singly, or in concert with Rac1.GTP, facilitates cytoskeletal remodeling, leading to fusion of insulin-laden secretory granules with the plasma membrane and the exocytosis of insulin.

ylates RhoGDI and facilitates the dissociation of Rac1 from Rac1/GDI complex. Therefore, it is likely that GSIS involves potential cross-talk between multiple Rho GTPases (e.g., Cdc42 and Rac1) as we originally proposed previously (23).

Together, based on the available body of evidence, we propose the following model: *1*) GSIS involves one or more Rho subfamily G-proteins (e.g., Rac1 and Cdc42), *2*) these G-proteins are bound to GDI in their GDP-bound inactive conformation and remain complexed in the cytosolic fraction, *3*) posttranslational modifications (e.g., isoprenylation) of these G-proteins are critical for their association with GDI because it prefers the prenylated form of G-proteins over their unprenylated counterparts, and inhibitors of prenylation markedly reduce GSIS (3–5, 24), and *4*) one or more metabolites of glucose (e.g., biologically active lipids) are essential for dissociation of Rac1 from the Rac1/GDI complex before its activation and translocation to the membrane fraction. Future studies will need to determine potential abnormalities, if any, in glucose-mediated activation of this signaling cascade in models of impaired insulin secretion (5,36). These studies are currently under way in our laboratory.

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