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Rab-geranylgeranyl transferase regulates glucose-stimulated insulin secretion from pancreatic β cells

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Abbreviations: FTase, farnesyl transferase; GSIS, glucose-stimulated insulin secretion; GGTase, geranylgeranyl transferase; MVA, mevalonic acid; REP1, Rab escort protein1; RGGT, Rab geranylgeranyl transferase

A growing body of evidence implicates essential roles for small molecular weight G-proteins (e.g., Cdc42, Rac1, Arf6 and Rab3A and Rab27A) in islet β-cell function including glucose-stimulated insulin secretion (GSIS). One of the known mechanisms for optimal activation of small G-proteins involves post-translational prenylation, which is mediated by farnesyltransferase (FTase) and geranylgeranyl transferases (GGTases I and II). The FTase catalyzes incorporation of a 15-carbon farnesyl group while the GGTase mediates incorporation of a 20-carbon geranylgeranyl group into the C-terminal cysteines of G-proteins. The FTase, GGTase I and GGTase II prenylate Ras, Cdc42/Rac1, and Rab G-proteins, respectively. While considerable evidence exists on FTase/GGTase I-mediated regulation of GSIS, very little is known about GGTase II (also referred to as Rab GGTase; RGGT) and its regulatory proteins in the cascade of events leading to GSIS. Herein, we provide the first immunological evidence to suggest expression of α- and β-subunits of RGGT in clonal INS 832/13 β-cells, normal rat islets and human islets. Furthermore, Rab escort protein1 (REP1), which has been shown to be critical for prenylation of Rab G-proteins, is also expressed in these cells. Furthermore, evidence is presented to suggest that siRNA-mediated knockdown of α- or β-subunits of RGGT and REP1 markedly attenuates GSIS in INS 832/13 cells. These findings provide the first evidence in support of key roles for RGGT and its regulatory proteins in GSIS.

Introduction

Rab GTPases represent the largest family of small G-proteins, and it is estimated that > 60 members of these G-proteins are associated with different subcellular compartments in mammalian cells.[1] In a manner akin to all G-proteins, Rab GTPases function as molecular switches that alternate between the GTP-bound (active) and the GDP-bound (inactive) conformations. A growing body of evidence in multiple cell types implicates key regulatory roles for Rab GTPases in several cellular processes including secretory vesicle budding, uncoating, motility and fusion.[1] Among several Rab GTPases identified, Rab3A and Rab27A have been implicated in the islet β-cell function.[2-12] Indeed, data from multiple laboratories have implicated critical regulatory roles for these signaling proteins in the islet β-cell function, including glucose-stimulated insulin secretion (GSIS).[2-12] It is noteworthy in the context of the current study that studies by Yaekura and associates have demonstrated insulin secretory deficiency and glucose intolerance in Rab3A null mice further affirming essential roles of this protein in islet function, including GSIS.[13]

Like the majority of small GTPases (Rac1, Cdc42, Rho, Arf6), the Rab GTPase activation-deactivation cycles are also controlled precisely by regulatory factors such as guanine nucleotide exchange factors (GEFs), GTP-activating proteins (GAPs) and GDP-dissociation inhibitors (GDIs; see ref. 16 for a recent review). In addition, in a manner akin to the members of Ras and Rho subfamily of G-proteins, Rab GTPases are post-translationally modified via geranylgeranylation by a distinct class of prenyl-transferases referred to as Rab geranylgeranyl transferases (RGGT; or geranylgeranyl transferase-II; GGTase-II). The RGGT catalyzes the transfer of geranylgeranyl pyrophosphate, a 20-carbon-derivative of mevalonic acid (MVA) to the C-terminal cysteines of Rab GTPases; such a signaling step has been shown to facilitate their membrane targeting for optimal interaction with the effector proteins and/or fusion of secretory vesicles with the plasma membrane.[14,15] Like the farnesyl transferase (FTase) and geranylgeranyl transferase-I, which prenylate the Ras and Rho subfamilies of GTPases, respectively,[14-17] the RGGT is heterodimeric and requires activation of both α- and β-subunits for the holoenzyme assembly and catalytic activation.[14-17] Lastly, in

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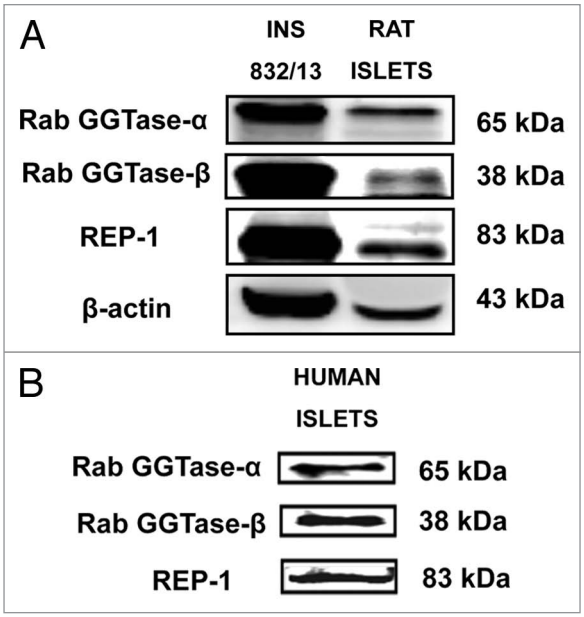

Figure 1. Immunological identification of α and β subunits of RGGT and REP1 in INS 832/13 cells, rat islets and human islets. Protein (40 μg) from cytosolic fraction of INS 832/13 cells and rat islets (see *Materials and Methods)* and total protein lysate (10 μg) from human islets were resolved by 10% SDS-PAGE and probed for α- and β-subunits of RGGT and REP1 as described in the text. Data depicted in **(A)** are representative of three experiments involving INS 832/13 cells and rat islets. Data in **(B)** are accrued from one human islet preparation.

contrast to the Ras and Rho GTPases, the prenylation of Rab GTPases has been shown to be regulated by the Rab escort proteins (REPs), which complex with RGGT (i.e., REP-RGGT complex), and recent evidence from Seabra's laboratory appears to implicate REP-RGGT complex formation as requisite for Rab GTPase prenylation.[14,15]

Despite the accumulating evidence to implicate essential roles for Rab GTPases in vesicular transport and insulin secretion,[2-12] very little is known with regard to their functional regulation by RGGT and REP in the islet β-cell. As a logical extension to our ongoing studies in the area of FTase and GGTase-I,[16-21] we undertook the current investigation to determine the expression and roles of RGGT and REP-1 in GSIS in pancreatic β-cells. Data from these studies provide the first evidence to implicate novel regulatory roles for RGGT and REP1 in GSIS.

Results

Immunological detection of RGGT and REP1 in insulin-secreting cells. Findings depicted in **Figure 1** suggest that the α (~65 kDa) and the β (~38 kDa) subunits of RGGT are expressed abundantly in INS 832/13 cells, normal rat islets and human islets. Further, we also observed a significantly high level of expression of REP1 (~83 kDa) in all the three cell types studied (**Fig. 1**). The relative abundance (i.e., protein expression adjusted to actin levels) of RGGT α-subunit was 30% higher in rat islets as compared with that in INS 832/13 cells. On the other hand, the relative abundance of REP-1 and β-subunit of RGGT was 50% lower in rat islets as compared with that in INS 832/13 cells. Together, these data suggest that RGGT and REP1 are expressed in pancreatic β-cells albeit at varying degrees.

siRNA-mediated knockdown of α- and β-subunits of RGGT inhibits GSIS. We next assessed roles for RGGT in GSIS in INS 832/13 cells. To accomplish this, we quantitated GSIS in these cells following knockdown of α or β subunits of RGGT via siRNA methodology. GSIS from cells transfected with the scrambled siRNA (mock) is taken as control. Data in **Figure 2** suggest a significant reduction on the expression of the RGGT α-subunit (**Fig. 2A**; ~45% inhibition) or β-subunit (**Fig. 2B**; ~48% inhibition) following transfection with respective siRNAs for these subunits. Data in **Figure 2C** indicate a significant increase in GSIS in these cells transfected with the control, scrambled siRNA (bar 2 vs. bar 1). However, the degree of GSIS was markedly attenuated in these cells following inhibition of expression of the α-subunit of RGGT (bar 4 vs. bar 2) or β-subunit (bar 6 vs. bar 2). Interestingly, combined knockdown of both α and β subunits did not exert additional inhibitory effects on GSIS compared with the degree of inhibition seen following knock-down of each of these subunits individually (bar 8 vs. bar 2). The basal secretion was, however, not significantly affected in these cells following depletion of these subunits individually or in combination (bar 3 vs. 1, bar 5 vs. 1 and bar 7 vs. 1, respectively). The incremental response for insulin secretion (from basal to stimulatory glucose) indicated ~53% and ~44% reduction in INS 832/13 following knockdown of the α and β subunits of RGGT, respectively. Taken together, these data suggest that activation of RGGT is necessary for GSIS to occur.

siRNA-mediated knockdown of REP1 inhibits GSIS. We next assessed roles of REP1 in GSIS. To address this, endogenous expression of REP1 in INS 832/13 cells followed by quantitation of GSIS in cells transfected with either the scrambled or REP-1-siRNA was performed. As shown in **Figure 3**, endogenous levels of REP1 were significantly attenuated (~32% inhibition) in these cells following transfection with siRNA-REP1. Furthermore, GSIS was significantly reduced in these cells following depletion of REP1 further affirming a role for REP1 in GSIS. The incremental response for insulin secretion (from basal to stimulatory glucose) indicated ~39% reduction in INS 832/13 following knockdown of the REP1 expression. Together, data reported herein are suggestive of key regulatory roles for RGGT-REP1 signaling axis in GSIS.

Discussion

The overall objective of this brief study is to assess the roles of RGGT and REP-1 in GSIS in pancreatic β-cells. Our data provides the first evidence not only to support expression of these proteins in β-cells, but also their regulatory roles in GSIS. As reviewed recently in,[16,17,22] considerable evidence exists to indicate regulatory roles for small G-proteins (Cdc42, Rac1, Arf6 and Ras) in the cascade of events leading to GSIS. In addition, published evidence from multiple laboratories implicates Rab GTPases in the transport of insulin laden secretory granule toward plasma membrane for fusion and release of insulin.[2-12,16,17,22] Along these

lines, recent evidence also supports localization of, and regulation by, GEFs, GAPS and GDIs in insulin-secreting cells for precise regulation and functional activation of small G-proteins leading to GSIS.[16,17,22]

Another mechanism for the activation of small G-proteins (e.g., Ras, Cdc42, Rac1 and Rab) is posttranslational prenylation of these proteins at their C-terminal cysteine residues, which, in turn, increases their hydrophobicity and their optimal membrane anchoring of these proteins.[16,17] In this context, at least three classes of prenylating enzymes have been described in the literature. The first one is FTase, which catalyzes the incorporation of a 15-carbon derivative of mevalonic acid (MVA) into the C-terminal cysteine residues of candidate substrate proteins. Examples of farnesylated proteins include Ras GTPases, nuclear lamins (lamin A and lamin B) and certain γ-subunits of heterotrimeric G-proteins.[16,17] The GGTase-1,which represents the second class of prenylating enzymes, transfers geranylgeranyl pyrophosphate, a 20-carbon derivative of MVA into the C-terminal cysteines of G-proteins belonging to the Rho G-protein subfamily (e.g., Cdc42 and Rac1). Both FTase and GGTase-I are heterodimeric in composition consisting of α and β subunits. In terms of subunit composition and holoenzyme assembly, both FTase and GGTase-1 share the same α-subunit, but distinct β-subunits. Numerous recent studies from our laboratory, involving pharmacological and molecular biological approaches, have demonstrated essential roles for FTase/GGTase-I in various aspects of islet β-cell function, including G-protein activation (Ras, Cdc42, Rac1) and trafficking, cytoskeletal rearrangements, cell proliferation and insulin secretion.[16,17] The RGGT, which represents the third category of prenylating enzymes, is also heterodimeric in nature, but the composition of α and β subunits are entirely different from FTase and GGTase-I.[14,15] It catalyzes the incorporation of geranylgeranyl pyrophosphate into the C-terminal cysteine residues of Rab GTPases (e.g., Rab3A, Rab27A). Using siRNAs for α- or β-subunits of RGGT, the current study provided additional evidence for key regulatory roles for RGGT in the cascade of events leading to insulin secretion.

Our current studies also identified roles for REP1 in GSIS. At least two mechanisms have been proposed for geranylgeranylation of Rab GTPases by RGGT. The first model suggests that formation of Rab-REP complex is requisite for geranylgeranylation of Rab by RGGT.[14,15] More recent studies by Baron and Seabra[14] have provided evidence in support of an alternate model in which these investigators demonstrated that REP-RGGT complex formation is necessary prior to prenylation of Rab GTPases. These signaling steps remain to be verified in the β-cell in relation to GSIS.

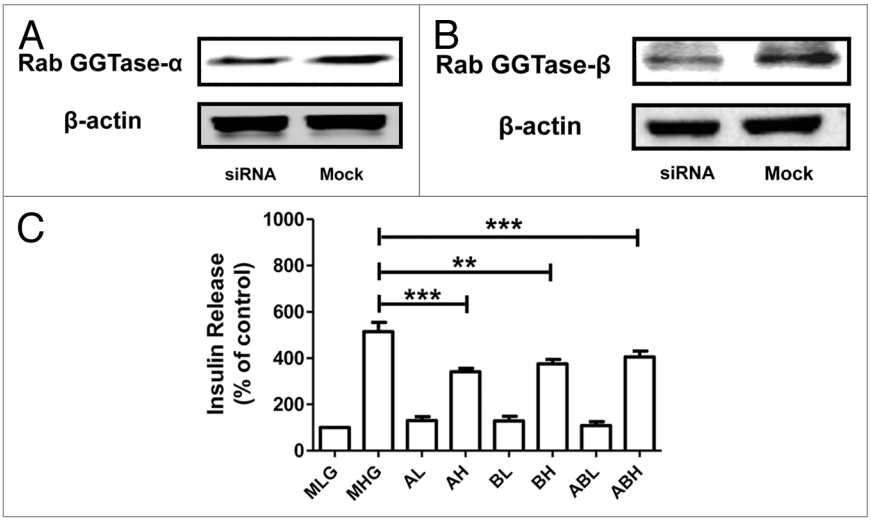

Figure 2. siRNA-mediated depletion of α- and β-subunits of RGGT individually or in combination significantly reduces glucose-induced insulin secretion in INS 832/13 cells. INS 832/13 cells were transfected with siRNA for α and β-subunits of RGGT (singly or in combination) or the scrambled siRNA, following which they were stimulated with either low (2.5 mM; LG) or high glucose (20 mM; HG) for 45 min. The efficiency of transfection was determined by immunoblotting and representative blot has been provided (**A and B**), respectively. Protein loading was determined by actin content in individual lanes. Insulin release was assayed with an insulin ELISA kit (see Materials and Methods). Data are representative of four independent experiments and results are shown as means ± SEM. The mean complimentary value for insulin secretion in the medium under basal conditions (i.e., control) represented 6.7 ng/ml (n = 4). **p < 0.01, ***p < 0.001.

In conclusion, we provide the first evidence to implicate novel regulatory roles for RGGT and REP1 in the cascade of events leading to physiological insulin secretion. Additional studies are needed to identify the candidate Rab GTPases whose prenylation is under the control of glucose-derived signals to facilitate insulin secretion. These aspects of RGGT biology in islet function are being investigated in our laboratories currently.

Materials and Methods

Materials. Small interfering RNA (siRNA) to Rabggta (s133067), Rabggtb (s130052) and scrambled siRNA (negative control; 4390843) were purchased from Ambion. siRNA to REP-1(SASI_Rn01_00095751) was from Sigma Aldrich Co. The rat insulin ELISA kit was from American Laboratory Products. Antisera directed against Rab GGTase β-subunit (ARP49163_P050) were from Aviva System Biology. Antisera to Rab GGTase α-subunit (sc-365901) and REP-1 (sc-14763) were purchased from Santa Cruz Biotechnology. Anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugates and Enhanced Chemiluminescence (ECL) kits were from Amersham Biosciences. All other reagents used in these studies were from Sigma Aldrich Co. unless stated otherwise.

Insulin-secreting β-cells. INS 832/13 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 10 mM HEPES (pH 7.4). The medium was

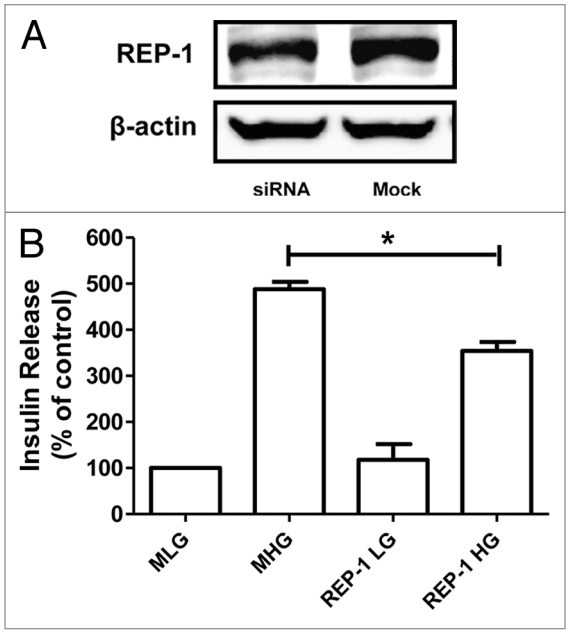

Figure 3. siRNA-mediated depletion of REP1 significantly reduces glucose-induced insulin secretion in INS 832/13 cells. INS 832/13 cells were transfected with siRNA to REP1 or scrambled siRNA, following which they were stimulated with either low (2.5 mM; LG) or high glucose (20 mM; HG) for 45 min. Transfections efficiency of REP1 was determined by immunoblotting and representative blot has been provided (**A**). Protein loading was determined by actin content in individual lanes. Insulin release was assayed with an insulin ELISA kit (see *Materials and Methods*). Data are representative of three independent experiments and results are shown as means ± SEM. The mean complimentary value for insulin secretion in the medium under basal conditions (i.e., control) represented 2.2 ng/ml (n = 3). *p < 0.05.

changed twice, and cells were subcloned weekly. Rat islets were isolated from normal Sprague-Dawley rats by collagenase digestion.[18,21] Human islets were obtained from Prodo Laboratories.

siRNA-mediated knockdown of REP1, α or β subunit of RGGT. Endogenous expression of REP1, RGGT α or β-subunits was knocked down by transfecting INS 832/13 cells using siRNA. In brief, INS 832/13 cells were seeded in 12-well plates and transfected with gene specific siRNA were performed at 50–60% confluence at a final concentration of 100 nM using HiPerFect transfection reagent (Qiagen). Further, to assess specificity of siRNA, cells were transfected in parallel with nontargeting siRNA (scrambled siRNA; 100 nM). Transfected cells were cultured in complete growth medium for 48 h and efficiency of knockdown was determined by western blot analysis.

Insulin release studies. Following transfections with specific siRNA for 48 h the INS 832/13 cells were incubated with Krebs ringer bicarbonate buffer for 1 h, prior to stimulation with low glucose (2.5 mM) or high glucose (20 mM) for 45 min at 37°C. Insulin released into the medium was quantified by ELISA as described earlier.[18,20,21,23]

Subcellular fractionation and western blotting. INS 832/13 cells, rat or human islets were sonicated in a homogenization buffer (HB, 20 mM TRIS-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 250 mM Sucrose, 10 μg/ml leupeptin and 2 μg/ml aprotinin) and centrifuged for 30 min at 100,000 g. The supernatant from this centrifugation step is used for western blotting. In brief, proteins were separated by SDS-PAGE on 10% (w/v) polyacrylamide mini gels and electrotransferred to nitrocellulose membrane. The membranes were blocked with 5% non fat dry milk in TBS-T (10 mM TRIS-HCl; pH 7.4), 8.8 g/liter NaCl and 0.1% Tween 20 for 2 h at room temperature. The membranes were then incubated overnight at 4°C with antisera rose against the RGGT α- (1:100) or β-subunits (1:400) or REP1 (1:400) in TBS-T containing 5% BSA. The membranes were washed five times for 5 min each with TBS-T and probed with appropriate horseradish peroxidase-conjugated secondary antibodies in 5% non-fat dry milk in TBS-T at room temperature for 1 h. After washing, the immune complexes comprising of the target proteins were detected using the ECL kit. The membranes were stripped and reprobed for β-actin. The band intensity was quantified and protein expression levels were calculated relative to β-actin in the same sample.

Statistical analysis. Results are expressed as means with their standard errors as indicated. Data are analyzed using one way ANOVA followed by Bonferroni posthoc test (GraphPad Prism 5; GraphPad Software, Inc.). Differences between control and treatment groups were considered significant if p is < 0·05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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