ORIGINAL ARTICLE

Protein Farnesylation–Dependent Raf/Extracellular Signal–Related Kinase Signaling Links to Cytoskeletal Remodeling to Facilitate Glucose-Induced Insulin Secretion in Pancreatic β-Cells

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OBJECTIVE—Posttranslational prenylation (e.g., farnesylation) of small G-proteins is felt to be requisite for cytoskeletal remodeling and fusion of secretory vesicles with the plasma membrane. Here, we investigated roles of protein farnesylation in the signaling steps involved in Raf-1/extracellular signal–related kinase (ERK1/2) signaling pathway in glucose-induced Rac1 activation and insulin secretion in the pancreatic β-cell.

RESEARCH DESIGN AND METHODS—These studies were carried out in INS 832/13 cells and normal rat islets. Molecular biological (e.g., overexpression or small interfering RNA [siRNA]–mediated knockdown) and pharmacologic approaches were used to determine roles for farnesylation in glucose-mediated activation of ERK1/2, Rac1, and insulin secretion. Activation of ERK1/2 was determined by Western blotting. Rac1 activation (i.e., Rac1.GTP) was quantitated by p21-activated kinase pull-down assay. Insulin release was quantitated by enzyme-linked immunosorbent assay.

RESULTS—Coprovision of structure-specific inhibitors of farnesyl transferase (FTase; e.g., FTI-277 or FTI-2628) or siRNA-mediated knockdown of FTase β-subunit resulted in a significant inhibition of glucose-stimulated ERK1/2 and Rac1 activation and insulin secretion. Pharmacologic inhibition of Raf-1 kinase using GW-5074 markedly reduced the stimulatory effects of glucose on ERK1/2 phosphorylation, Rac1 activation, and insulin secretion, suggesting that Raf-1 kinase activation may be upstream to ERK1/2 and Rac1 activation leading to glucose-induced insulin release. Lastly, siRNA-mediated silencing of endogenous expression of ERK1/2 markedly attenuated glucose-induced Rac1 activation and insulin secretion.

CONCLUSIONS—Together, our findings provide the first evidence of a role for protein farnesylation in glucose-mediated regulation of the Raf/ERK signaling pathway culminating in the activation of Rac1, which has been shown to be necessary for cytoskeletal reorganization and exocytotic secretion of insulin. *Diabetes* 59:967–977, 2010

In the majority of cells, transduction of extracellular signals involves ligand binding to a receptor, often followed by the activation of one (or more) G-proteins and their respective effector proteins. The pancreatic islet β-cell is unusual in that regard because glucose, the major physiological insulin secretagogue, lacks an extracellular receptor. Instead, metabolic and cationic events of glucose metabolism are implicated in insulin secretion (1–4). Changes in calcium concentration not only initiate insulin release, but also regulate protein kinases culminating in insulin secretion (1–5). In addition to calcium-dependent protein kinases, several other kinases, including the mitogen-activated protein kinases (e.g., extracellular signal-regulated kinases [ERK1/2]) have been identified and characterized in the pancreatic islet β-cell (5–7). Emerging evidence also suggests G-protein–mediated regulation of protein kinases in multiple cell types, including the islet β-cell. For example, p21-activated kinase (PAK1), and Raf-1 kinase are regulated by Cdc42/ Rac1 (8,9) and H-Ras (10,11), respectively. Furthermore, specific regulatory factors of small G-proteins (e.g., guanosine diphosphate dissociation inhibitor) are modulated by phosphorylation-dephosphorylation (12).

The majority of small G-proteins (e.g., Cdc42, Rac1, and H-Ras) undergo requisite posttranslational prenylation at their COOH-terminal cysteine residues, which control their intracellular trafficking and localization to facilitate optimal interactions with their respective effector proteins (13–15). Protein prenylation involves incorporation of either farnesyl pyrophosphate, a 15-carbon derivative of mevalonic acid, by the farnesyl transferases (FTases), or geranylgeranyl pyrophosphate, a 20-carbon derivative of mevalonic acid, by the geranylgeranyl transferase (GGTase) into the COOH-terminal cysteine (13–15). At least three distinct prenylating enzymes have been described (16–18). The FTase and GGTase-I are often referred to as *CAAX* prenyl transferases because their substrate proteins share a conserved *CAAX* motif at their COOH-terminal region. The GGTase-II prenylates the Rab subfamily of proteins at a different motif and hence is termed a non–*CAAX* prenyl transferases. FTase, GGTase-I, and GGTase-II are heterodimeric (i.e., consisting of α- and β-subunits). Both FTase and GGTase-I share a common α-subunit, but different β-subunits. The α-subunit is the regulatory subunit, whereas the β-subunit confers substrate specificity. Goalstone et al. have demonstrated that the α-subunit of FTase/GGTase undergoes phosphoryla-

tion, leading to functional activation of the enzyme resulting in the prenylation of G-proteins (19). Using pharmacologic inhibitors of prenylation, earlier studies have demonstrated regulatory roles for small G-proteins in insulin secretion in islet β-cells (15,20–23); these observations were further confirmed by molecular biological approaches involving a dominant-negative mutant of FTase/GGTase-I α subunit (23).

Evidence from multiple laboratories implicates the mitogen-activated protein kinase signaling pathway in the regulation of islet β-cell function (6,24–28). Some of these investigations have implicated roles for small G-proteins in glucose- or growth factor–induced activation of ERK1/2 in β-cells leading to insulin gene transcription. The current study is aimed at verifying our recently proposed model implicating protein farnesylation in glucose-mediated activation of ERK1/2 and insulin secretion in the pancreatic β-cell (14). Using pharmacologic and molecular biological approaches, we present the first evidence to suggest that glucose-induced Rac1 activation and insulin secretion require the intermediacy of a H-Ras–independent, but Raf-1/ERK1/2–dependent signaling step.

RESEARCH DESIGN AND METHODS

Materials. GW-5074 was from Biomol Research Laboratories (Plymouth Meeting, PA). FTI-277 and FTI-2628 were from Calbiochem (San Diego, CA). Mastoparan was purchased from Sigma (St. Louis, MO). Antisera against H-Ras and Raf-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rat insulin ELISA kit was from American Laboratory Products (Windham, NH). Antibodies against phospho-p44/42 ERK kinase (Thr202/Tyr204) and total p44/42 ERK kinase were from Cell Signaling Technology (Danvers, MA). A pool of two target-specific 20- to 25-nucleotide small interfering RNAs (siRNAs) designed to knock down the expression of FTase β-subunit and scrambled siRNA (negative control) were from Applied Biosystems, Ambion (Austin, TX).

Insulin-secreting cells. INS 832/13 cells (provided by Dr. Chris Newgard) were propagated as described in (29). Pancreatic islets from male Sprague-Dawley rats (200–250 g body wt; Harlan Laboratories) were isolated by the collagenase digestion method (29,30).

siRNA-mediated knockdown of H-Ras or the β-subunit of FTase. Endogenous H-Ras or FTase β-subunit expression was depleted by transfecting cells using siRNA at a final concentration of 100 nmol/l using HiPerfect transfection reagent (Qiagen, Valencia, CA). To assess the specificity of RNA interference method, cells were transfected (as above) with nontargeting RNA; this includes at least four nucleotide mismatches with all known mouse, rat, and human gene (i.e., scrambled siRNA) duplexes (Santa Cruz Biotechnology). Efficiency of H-Ras or FTase β-subunit knockdown was determined by Western blot analysis.

Transfection of Raf-1 kinase mutants. INS 832/13 cells were subcultured at 70–80% confluence and transfected using Effectene (Qiagen), with 0.2 μg of plasmid DNA constructs against Raf-1 (mock, vector, wild type, or constitutively active; BXB; provided by Dr. Melanie Cobb) per well of a 24-well plate. The cells were used for insulin secretion studies or for Western blotting studies.

Quantitation of glucose-stimulated insulin secretion. INS 832/13 cells or islets, treated with either FTase inhibitors or Raf-1 inhibitor or transfected with H-Ras siRNAs or wild-type and constitutively active Raf-1 (BXB) mutants were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous absence or presence of inhibitors as indicated in the text. Insulin released into the medium was quantitated by enzyme-linked immunosorbent assay (ELISA) (23,29).

siRNA mediated knockdown of the ERK1 and ERK2. Endogenous expression of either ERK1 or ERK2 was specifically silenced in INS 832/13 cells using siRNA. A pool of three target-specific 19- to 25-nucleotide siRNAs designed to knockdown ERK1 and ERK2 gene was obtained from Santa Cruz Biotechnology. In brief, cells were seeded and grown in 24-well plates in appropriate growth medium to achieve 40–50% confluence. Cells were transfected with ERK1 and/or ERK2 siRNA or scrambled siRNA (as a negative control) using HiPerfect reagent according to the manufacturer's instructions. The efficiency of the siRNA transfection and ERK1/2 knockdown was verified by Western blotting.
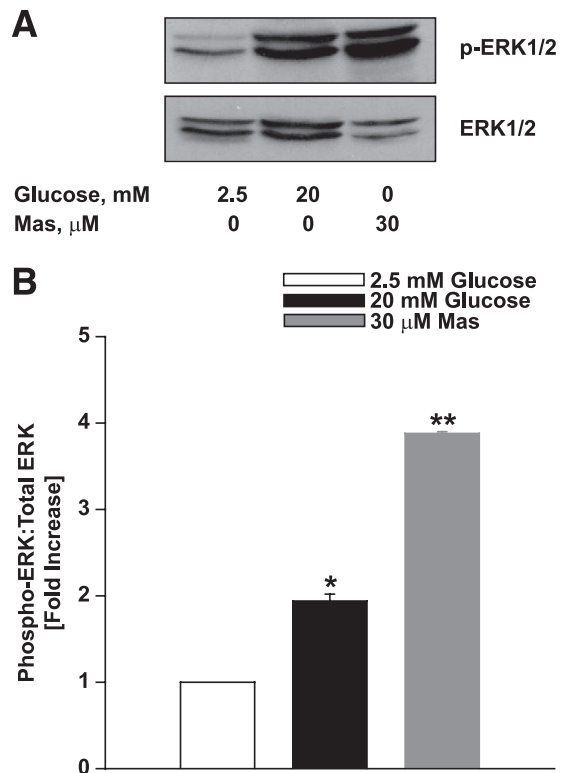

FIG. 1. Stimulatory concentrations of glucose or mastoparan, a global activator of G-proteins, potentiate ERK1/2 phosphorylation in INS 832/13. *A*: INS 832/13 cells were cultured overnight in low-glucose–containing medium and were further incubated in the presence of low (2.5 mmol/l) or high (20 mmol/l) glucose or Mas (30 μmol/l) for 30 min at 37°C. Lysates of cells from above experimental conditions were separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–phosphorylated ERK1/2 (p-ERK1/2) followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody. The same blots were then stripped and reprobed with ERK1/2 antibody. A representative blot from three experiments is shown here. *B*: The relative intensities of p-ERK1/2:total ERK1/2 ratio quantitated by densitometry. Data are expressed as fold increase over basal glucose and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose and **$P < 0.001$ vs. 20 mmol/l glucose.

ERK1/2 phosphorylation assay. INS 832/13 cells or islets, either treated with pharmacologic inhibitors or transfected with specific siRNAs or Raf-1 mutants were incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. After this, the cells were homogenized in a buffer consisting of 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride. Relative abundance of total and phospho-ERK1/2 was determined by Western blotting.

Quantitation of glucose-stimulated Rac1 activation. The degree of Rac1 activation (i.e., GTP-bound form) was determined by the PAK/PBD (p21-activated kinase/p21-binding domain) pull-down assay as described in (23,29,31). Relative abundance of Rac1 in the pull-down samples was quantitated by densitometry of Rac1 bands identified by Western blotting.

Statistical analyses. The statistical significance of the differences between the experimental conditions was determined by Student t test. P values less than 0.05 were considered significant.

RESULTS

Glucose and mastoparan augment ERK1/2 phosphorylation in pancreatic β-cells.

Data in Fig. 1 indicate that incubation of INS 832/13 cells with a stimulatory concentration of glucose (20 mmol/l) led to nearly two-fold increase in the phosphorylation of ERK1/2. Moreover, incubation of these cells with mastoparan (Mas), a global activator of G-proteins (32) and insulin secretion (7,21,33–
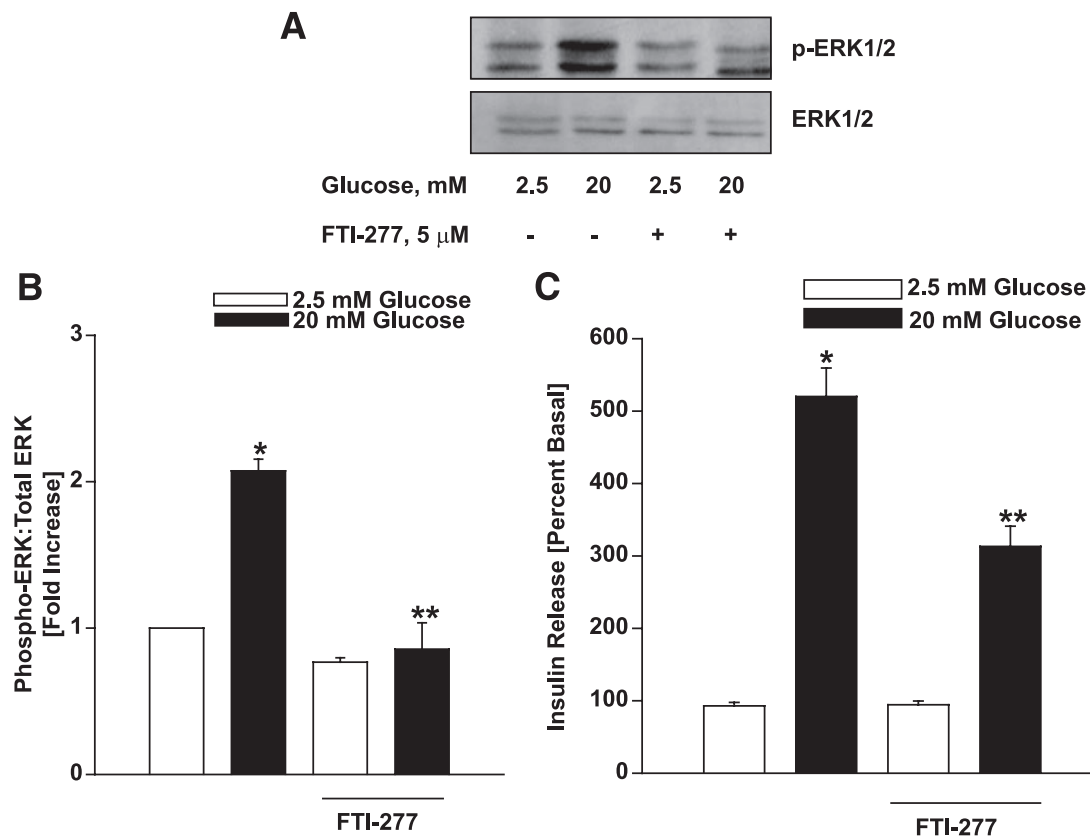
FIG. 2. FTI-277, a specific inhibitor of protein farnesylation, markedly attenuates ERK1/2 phosphorylation and insulin secretion in INS 832/13 β-cells. *A*: INS 832/13 cells were treated with either diluent alone or FTI-277 (5 μmol/l) as indicated in the figure and cultured overnight in low-glucose media. Cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of either diluent alone or FTI-277. Cell lysates were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The same blots were then stripped and reprobed with ERK1/2 antibody. A representative blot from three experiments is shown here. *B*: The relative intensities of p-ERK1/2:total ERK1/2 ratio quantitated by densitometry. Data are expressed as fold increase over basal glucose and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose and **$P < 0.001$ vs. 20 mmol/l glucose in the absence of FTI-277. *C*: INS 832/13 cells were treated with either diluent alone or FTI-277 (5 μmol/l) and cultured overnight in low-glucose media. Cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of either diluent alone or FTI-277. Insulin released into the medium was quantitated by ELISA. The data are expressed as percentage of basal and are means ± SEM from three independent experiments. *$P < 0.05$ vs. 2.5 mmol/l glucose and **$P < 0.05$ vs. 20 mmol/l glucose in the absence of FTI-277.

36), markedly stimulated ERK1/2 phosphorylation. The pronounced effects of Mas on ERK1/2 phosphorylation suggested that one (or more) G-proteins may be involved in this signaling cascade. To further assess roles of G-proteins in the sequence of events leading to glucose-induced ERK1/2 phosphorylation and insulin secretion, we undertook a pharmacologic approach to specifically inhibit the G-protein activation via attenuating the β-cell FTases through the use of site-specific inhibitors of farnesylation.

FTase inhibition markedly reduces glucose-induced activation of ERK1/2 and insulin release in pancreatic β-cells. Data described in Fig. 2*A* and *B* demonstrate that incubation of INS 832/13 cells with a stimulatory concentration of glucose (20 mmol/l) leads to a significant increase in ERK1/2 phosphorylation, data compatible with those provided in Fig. 1. FTI-277, a known inhibitor of protein farnesylation, totally abrogated the effect of glucose to stimulate ERK1/2 activation without significantly affecting the basal phosphorylation of ERK1/2 seen in the presence of 2.5 mmol/l glucose. Under these conditions, FTI-277 also inhibited glucose-stimulated insulin secretion (GSIS) significantly without exerting any effects on basal insulin secretion (Fig. 2*C*). In a manner akin to INS 832/13 cells (Fig. 2), glucose significantly stimulated the phos-

phorylation of ERK1/2 in isolated rat islets (Fig. 3*A*) and such a signaling step was sensitive to FTase inhibition. FTI-277 completely inhibited glucose-induced activation of ERK1/2 without significantly affecting the basal phosphorylation (Fig. 3*A*). Data in Fig. 3*B* further confirm the inhibitory effects of FTI-277 on GSIS in normal rat islets. Moreover, FTI-2628, another structure-specific FTase inhibitor, completely inhibited GSIS in normal rat islets. Together, data in Figs. 2 and 3 implicate a role for protein farnesylation in glucose-induced activation of ERK1/2 phosphorylation and insulin secretion in INS 832/13 cells and isolated rat islets.

siRNA-mediated knockdown of FTase β-subunit inhibits glucose-induced ERK1/2 activation and insulin secretion. As stated above, FTase and GGTase-I share a common α-subunit. The β-subunits, which confer substrate specificity, are different however. To further determine whether glucose-induced activation of ERK1/2 and insulin secretion require farnesylation, we depleted the expression of endogenous FTase β-subunit using siRNA methodology and then quantitated glucose-mediated activation of ERK1/2 and GSIS in INS 832/13 cells. Data described in Fig. 4*A* indicate >60% inhibition in the expression of FTase β under our current experimental conditions. Under these conditions, the ability of glucose
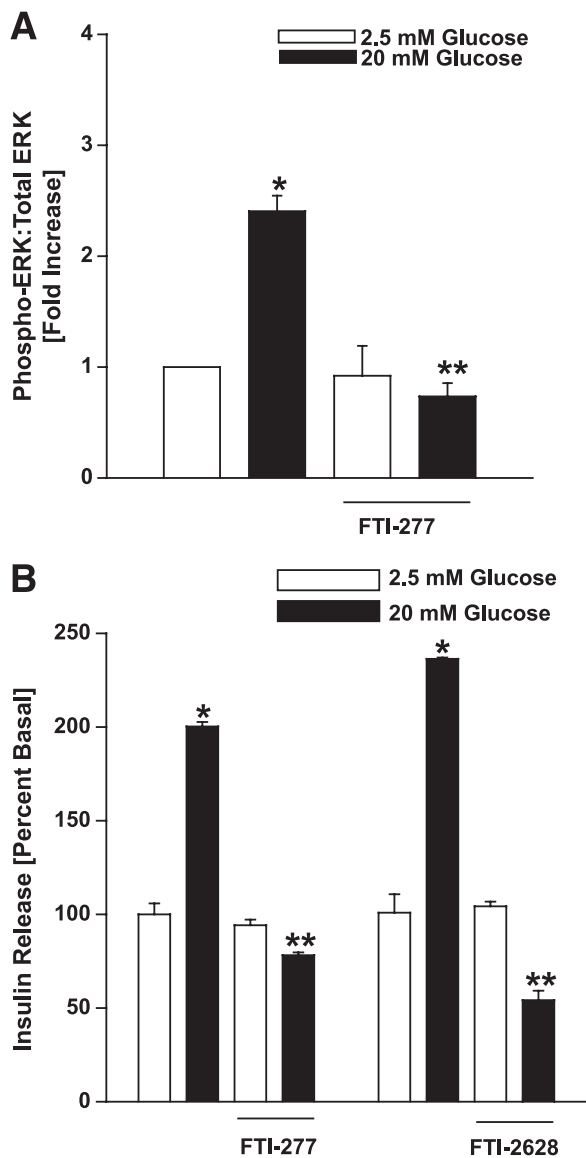
A

B

FIG. 3. Inhibition of protein farnesylation leads to reduction of glucose-stimulated ERK1/2 phosphorylation and insulin secretion in normal rat islets. *A*: Normal rat islets were cultured overnight in low-glucose–containing medium in the presence of diluent alone or FTI-277 (5 μmol/l). The next morning, they were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of FTI-277 or diluent. Islet lysates were separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The relative intensities of p-ERK1/2:total ERK1/2 ratio were plotted based on the densitometric scan. Data are expressed as fold increase and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose and **$P < 0.001$ vs. 20 mmol/l glucose in the absence of FTI-277. *B*: Normal rat islets were cultured overnight in low-glucose–containing medium in the presence of diluent alone. FTI-277 (5 μmol/l), or FTI-2628 (10 μmol/l) as indicated in the figure. Islet lysates were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of either FTI-277, FTI-2628, or diluent. Insulin released into the medium was quantitated by ELISA. The data are expressed as percentage of basal and are means ± SEM from three independent experiments. *$P < 0.05$ vs. 2.5 mmol/l glucose, **$P < 0.05$ vs. 20 mmol/l glucose alone.

to augment ERK1/2 phosphorylation was completely abolished (Fig. 4*B*), findings in line with our pharmacologic data (Figs. 2 and 3). Moreover, we noticed a marked inhibition in GSIS in FTase β-siRNA–transfected cells (Fig.

4*C*). Based on these data, we conclude that a protein farnesylation–sensitive step is necessary for glucose to promote the activation of ERK1/2 phosphorylation and insulin secretion.

siRNA-mediated knockdown of H-Ras expression elicits no significant effects on glucose-induced activation of ERK1/2 and insulin secretion in pancreatic β-cells. It is widely felt that regulation of ERK1/2 involves the sequential activation of Ras/Raf-1 kinase signaling cascade. Because our data indicated that glucose-mediated effects on ERK1/2 phosphorylation require a farnesylation step, and because Ras is a farnesylated protein, we investigated roles of H-Ras in this signaling cascade. To address this, we quantitated glucose-induced ERK1/2 activation and insulin secretion in INS 832/13 cells in which H-Ras expression was knocked down by the siRNA approach. Data in Fig. 5*A* and *B* suggest nearly 50% knockdown of H-Ras expression in INS 832/13 cells under our current experimental conditions. Data in Fig. 5*C* and *D* indicated no significant effects of H-Ras depletion on glucose-induced ERK1/2 phosphorylation in these cells. In addition, we observed that GSIS in INS 832/13 cells was resistant to H-Ras depletion (Fig. 5*E*). These findings indicate that H-Ras plays a minimal regulatory role(s), if any, in signaling events leading to glucose-induced activation of ERK1/2 phosphorylation and insulin secretion.

Activation of Raf-1 kinase is necessary for glucose-mediated activation of ERK1/2 and insulin secretion in pancreatic β-cells. We next examined whether Raf-1 kinase activation represents a regulatory step in glucose-induced activation of ERK1/2 phosphorylation and insulin secretion because it has been implicated in the regulation of members of mitogen-activated protein kinase family in multiple cell types including the pancreatic β-cell (6,24–28,37–41). We also examined regulation by Raf-1 kinase pathway of GSIS under the conditions in which it regulates ERK1/2 phosphorylation. In the first series of studies, we determined the effects of GW-5074, a known inhibitor of Raf-1 kinase, on glucose-mediated activation of ERK1/2 phosphorylation and insulin secretion. Data in Fig. 6*A* and *B* demonstrated a near complete inhibition of glucose-induced ERK1/2 activation by GW-5074. We also observed that GW-5074 inhibitor abolished GSIS under the same conditions that it inhibited ERK1/2 phosphorylation (Fig. 6*C*). It should be noted that, unlike GSIS, KCl-stimulated insulin secretion was completely resistant to the inhibitory effects of GW-5074 (Fig. 6*D*); these data rule out potential nonspecific effects of the inhibitor, and afford further support to potential involvement of the Raf-1/ERK1/2 pathway in cytoskeletal reorganization leading to GSIS.

In the second approach, we investigated whether overexpression of a constitutively active mutant of Raf-1 potentiates glucose-induced ERK1/2 phosphorylation and insulin secretion. To address this, INS 832/13 cells were transfected with either wild-type Raf-1 or a constitutively active mutant of Raf-1 (BXB/Raf-1). Data in Fig. 6*E* represent a Western blot, illustrating a significant glucose-mediated activation of ERK1/2 in INS 832/13 cells transfected with wild-type or the BXB/Raf-1–transfected cells. Albeit variable, we noticed a modest increase in ERK1/2 phosphorylation under basal glucose conditions in cells expressing either the wild-type or constitutively active Raf-1 mutants (Fig. 6*E* and *F*). No significant potentiation of glucose-induced ERK1/2 phosphorylation was seen in cells expressing either the wild-type or the constitutively active Raf-1 kinase mutant (Fig. 6*E* and *F*).
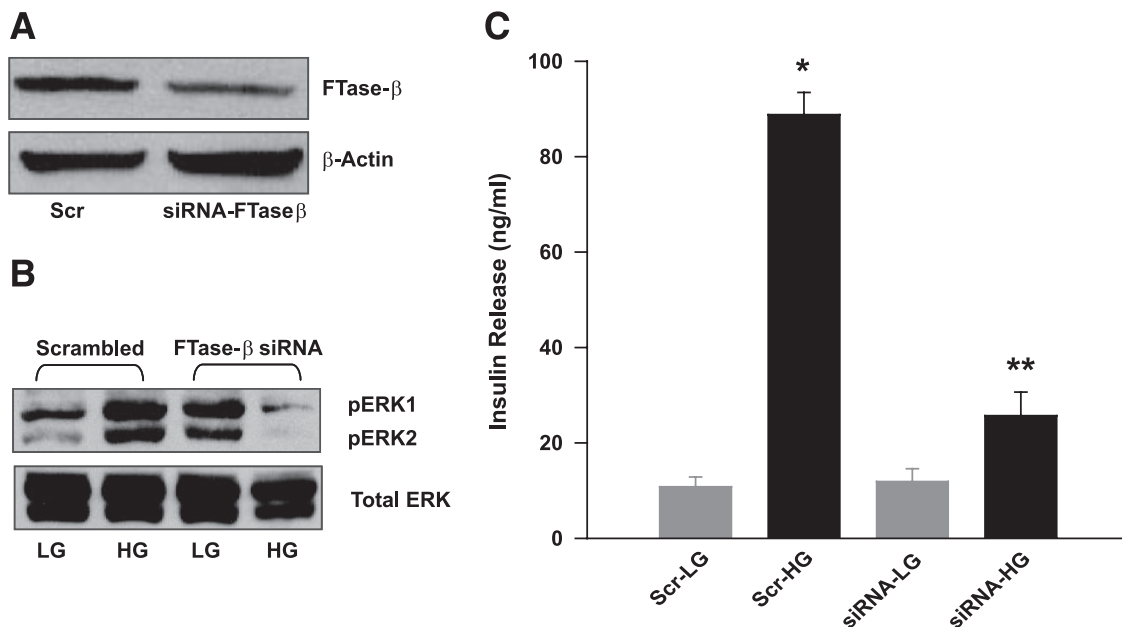
FIG. 4. siRNA-mediated depletion of the β-subunit of FTase leads to inhibition of glucose-stimulated ERK1/2 phosphorylation and insulin secretion in INS 832/13 cells. *A*: INS 832/13 cells were transfected with either scrambled siRNA or siRNA specific for FTase β-subunit as described in the RESEARCH DESIGN AND METHODS section. Expression of the FTase β-subunit was verified by Western blotting. A representative blot from three independent studies yielding similar results is provided here. *B*: Transfected cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Lysates from these cells were separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. A representative blot from three experiments yielding comparable data is shown here. *C*: Transfected cells were incubated with either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Insulin released into the medium was quantitated by ELISA. The data are expressed as percentage of basal and are means ± SEM from eight independent determinations. *$P < 0.05$ for scrambled low vs. high glucose, **$P < 0.05$ for siRNA-FTase β-subunit low vs. high glucose.

However, we observed a significant potentiation of GSIS in cells transfected with the BXB/Raf-1 mutant compared with vector or wild-type Raf-1–transfected cells (Fig. 6*G*). Together, data presented in Fig. 6*A*–*G* support our hypothesis that a farnesylation-requiring, Ras-independent, but Raf-1–dependent signaling event is essential for glucose-mediated regulation of ERK1/2 activation and insulin release in isolated β-cells (see below).

FTase and Raf-1 kinase activation steps are necessary for glucose-induced Rac1 activation in β-cells. Published evidence (8,14,23,39,42) implicates activation of Rho subfamily small G-proteins (e.g., Rac1) in GSIS. Here, we investigated glucose-induced activation of Rac1 (i.e., Rac1-GTP) in control cells and in cells in which Raf-1 kinase activation is negated via pharmacologic inhibition using GW-5074. Stimulatory concentrations of glucose significantly increased the relative abundance of GTP-bound Rac1, which was decreased significantly in cells incubated with GW-5074 (Fig. 7*A* and *B*). It should also be noted that a modest, but significant increase in the activation of Rac1 was seen in GW-5074–treated cells under low-glucose treatment conditions compared with cognate cellular preparations exposed to diluent alone. Total Rac1 did not change under these conditions (Fig. 7*A*). In a preliminary study, we also observed similar inhibitory effects by GW-5074 on glucose-induced activation of Rac1 in rodent islets (159% of control in high-glucose–treated cells in the absence of GW-5074 vs. 72% of control in the presence of GW-5074 and high glucose). However, as in the case of INS 832/13 cells, GW-5074 increased Rac1 activation under basal conditions (data not shown).

To further examine a role for FTase in this signaling cascade, we quantitated glucose-induced activation of Rac1 in INS 832/13 cells in the absence or presence of FTI-277. Data in Fig. 7*C* suggested a significant reduction in glucose-induced Rac1 activation after FTase inhibition. In a manner akin to GW-5074 (Fig. 7*B*), a consistent increase in Rac1 activation was noticed in FTI-277–treated cells under low-glucose conditions. Together, these data implicate that a FTase/Raf-1 kinase–sensitive signaling mechanism(s) may underlie glucose-induced activation of Rac1. It is important to note that the inhibitory effects of GW-5074 on glucose signaling pathway (i.e., ERK1/2 activation, Rac1 activation, and insulin secretion) were not due to its cytotoxicity because no significant effects of this compound on the metabolic cell viability of INS 832/13 cells were noticed. The cell viability rates represented 103.70 ± 2.12% of control in GW-5074 (10 μmol/l)–treated cells (not significant vs. diluent-treated cells; $n = 3$).

ERK1/2 activation is necessary for Rac1 activation and GSIS. To conclusively determine regulatory role for ERK1/2 in glucose-induced Rac1 activation and associated insulin secretion, we quantitated these two events in INS 832/13 cells in which ERK1/2 expression was knocked down using siRNA-ERK1 or siRNA-ERK2, singly or in combination. Data depicted in Fig. 8*A* and *B* suggested a marked reduction in the expression of these proteins after transfection with specific siRNA, but not scrambled siRNA. Transfection of either siRNA-ERK1 or siRNA-ERK2 (100 nmol/l each) or a combination of both (at 50 nmol/l each) markedly attenuated GSIS compared with the scrambled siRNA–transfected cells (Fig. 8*C*). In support of our original hypothesis and compatible with data described above, we also noticed complete inhibition of glucose-induced activation of Rac1 in ERK1/2-depleted cells.
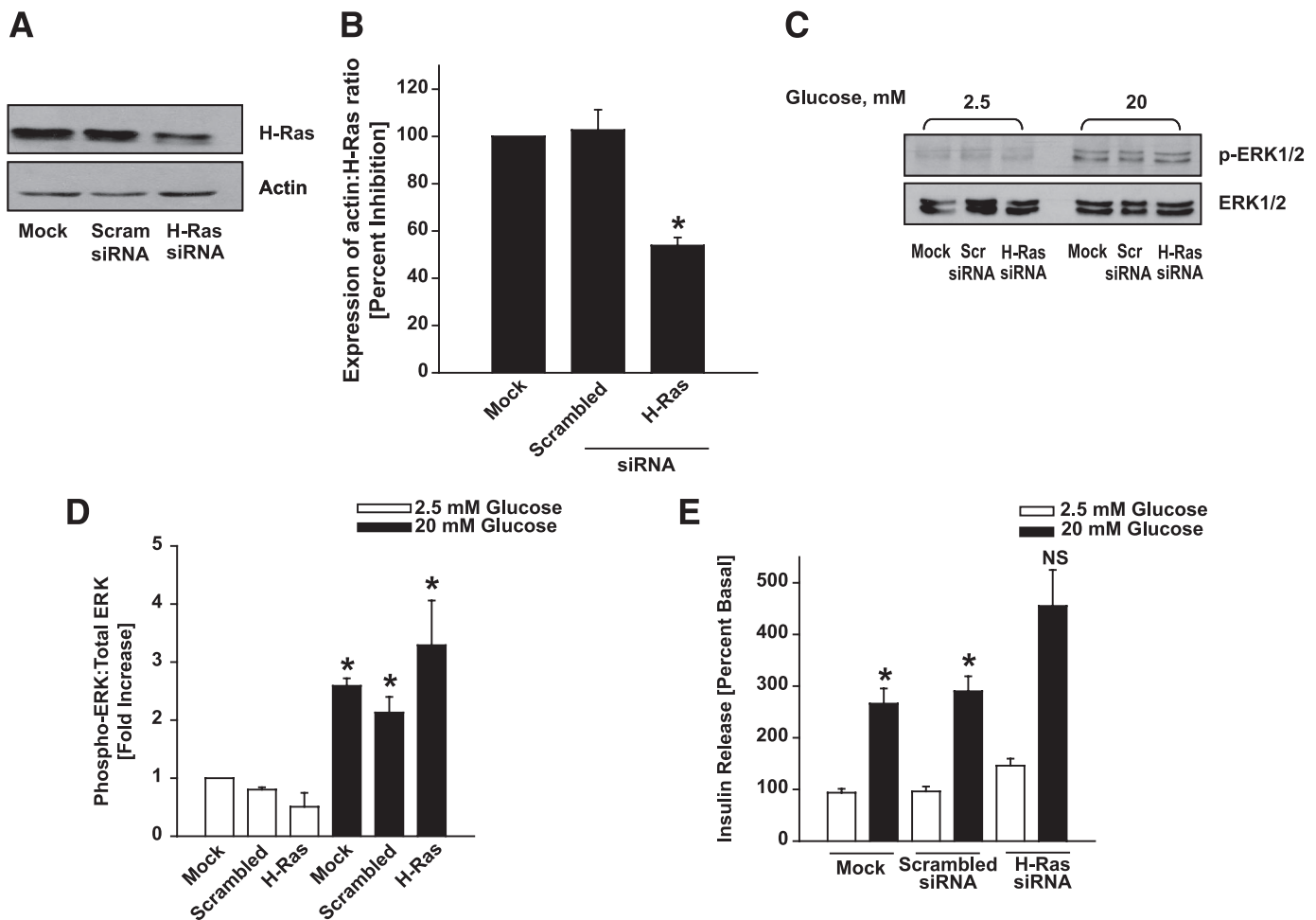
FIG. 5. Glucose continues to stimulate ERK1/2 phosphorylation and insulin secretion in cells in which H-Ras expression was negated using H-Ras siRNA. A: INS 832/13 cells were transfected with mock, scrambled siRNA, or H-Ras siRNA at a final concentration of 100 nmol/l using HiPerfect transfection reagent. Lysates from the above cellular preparations were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with an antibody raised against H-Ras followed by incubation with HRP-conjugated secondary antibody. The same blots were then stripped and reprobed with actin antibody. A representative blot from three experiments is shown here. B: The relative intensities of actin:H-Ras ratio quantitated by densitometry. Data are expressed as means ± SEM from three experiments. *$P < 0.001$ vs. mock- or scrambled siRNA–transfected cells. C: INS-1 832/13 cells were transfected with mock, scrambled siRNA, or H-Ras siRNA at a final concentration of 100 nmol/l using HiPerfect transfection reagent and cultured overnight in low-glucose media. Cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Cell lysates were separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The same blots were then stripped and reprobed with ERK1/2 antibody. A representative blot from three experiments is shown here. D: The relative intensities of p-ERK1/2:total ERK1/2 ratio quantitated by densitometry. Data are expressed as fold increase and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose-treated cells transfected with either mock, scrambled siRNA, or H-Ras siRNA. E: INS 832/13 cells were transfected with mock, scrambled siRNA, or H-Ras siRNA at a final concentration of 100 nmol/l using HiPerfect transfection reagent and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Insulin released into the medium was quantitated by ELISA. Data, which are expressed as percentage of basal, are means ± SEM from three experiments. *$P < 0.01$ vs. 2.5 mmol/l glucose-treated cells. NS, not significant vs. mock- or scrambled siRNA–transfected cells treated with stimulatory glucose concentrations.

DISCUSSION

The overall objective of this study was to investigate the regulatory roles of protein farnesylation in the signaling events leading to glucose-induced ERK1/2 activation and insulin secretion in pancreatic β-cells. Salient findings of this study are *1*) coprovision of structure-specific inhibitors or siRNA-mediated knockdown of FTase β-subunit endogenous to the β-cell resulted in a significant inhibition of glucose-stimulated ERK1/2 activation and insulin secretion; *2*) siRNA-mediated silencing of ERK1/2 resulted in a significant reduction in glucose-induced Rac1 activation and GSIS, suggesting a pivotal role for ERK1/2 in this signaling event; and *3*) pharmacologic inhibition of Raf-1 kinase markedly reduced the stimulatory effects of glucose on ERK1/2 phosphorylation, Rac1 activation, and insulin

secretion, suggesting that Raf-1 kinase activation may be upstream to ERK1/2 and Rac1 activation resulting in GSIS. Taken together, our data provide the first evidence for the involvement of Raf-1/ERK1/2/Rac1 signaling pathway in GSIS.

A growing body of recent evidence suggests that the stimulatory effects of glucose and other secretagogues on ERK1/2 activation may involve G-protein activation. For example, glucose-dependent insulinotropic peptide was shown to activate ERK via a cAMP-dependent mechanism(s) involving monomeric (e.g., Rap1) but not trimeric (24) G-proteins. In MIN6 cells, glucagon-like peptide 1 (GLP-1) stimulated ERK activity by a mechanism involving protein kinase A– and calmodulin kinase–dependent, but Rap1-, Ras-, or Raf-1–independent, signaling steps (27).
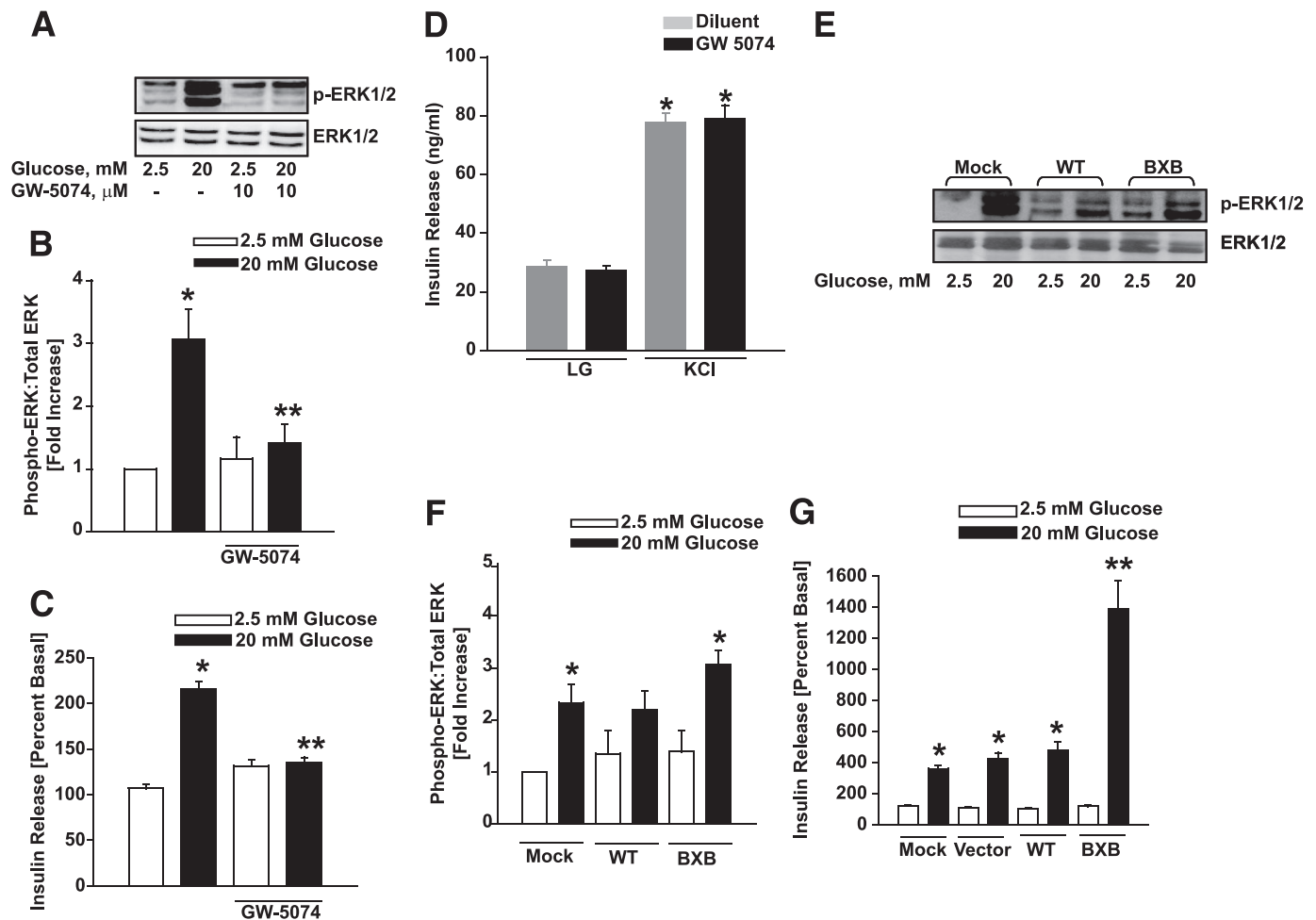
FIG. 6. Raf-1 kinase activation is necessary for glucose-induced ERK1/2 phosphorylation and insulin secretion in INS 832/13 β-cells. *A*: INS 832/13 cells were treated with either diluent alone or GW-5074 (10 μmol/l) as indicated in the figure, and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of either GW-5074 or diluent. Cell lysates were then separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The same blots were then stripped and reprobed with ERK1/2 antibody. A representative blot from three experiments is shown here. *B*: The relative intensities of p-ERK1/2:total ERK1/2 ratio quantitated by densitometry. Data are expressed as fold increase and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose in the absence of GW-5074, **$P < 0.001$ vs. 20 mmol/l glucose in the absence of GW-5074. *C*: INS 832/13 cells were treated with either diluent alone or GW-5074 (10 μmol/l) and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C in the continuous presence of either diluent alone or GW-5074. Insulin released into the medium was quantitated by ELISA. The data are expressed as percentage of basal and are means ± SEM from three independent experiments. *$P < 0.05$ vs. 2.5 mmol/l glucose alone, **$P < 0.05$ vs. 20 mmol/l glucose alone. *D*: INS 832/13 cells were treated with either diluent alone or GW-5074 (10 μmol/l) and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low glucose (2.5 mmol/l) or a membrane-depolarizing concentration of KCl (60 mmol/l; osmolality adjusted by reducing NaCl in the incubation medium) for 45 min at 37°C in the continuous presence of either GW-5074 or diluent. Insulin released into the medium was quantified using ELISA. Data are expressed as nanograms per milliliter of insulin released and are mean ± SEM from eight independent determinations. *$P < 0.001$ vs. respective low-glucose controls. *E*: INS-1 832/13 cells were transfected with mock, wild type, and constitutively active Raf-1 (BXB) mutants at a final concentration of 0.2 μg of DNA and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Protein lysates were then separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were then blocked and incubated with anti–p-ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The same blots were then stripped and reprobed with ERK1/2 antibody. A representative blot from three experiments is shown here. *F*: The relative intensities of p-ERK1/2:total ERK1/2 ratio quantitated by densitometry. Data are expressed as fold increase over basal glucose and are means ± SEM from three experiments. *$P < 0.001$ vs. 2.5 mmol/l glucose-treated cells. *G*: INS-1 832/13 cells were transfected with mock, wild type, and constitutively active Raf-1 (BXB) mutants at a final concentration of 0.2 μg of DNA and cultured overnight in low-glucose media. The cells were further incubated in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose for 30 min at 37°C. Insulin released into the medium was quantitated by ELISA. Data are expressed as percentage of basal and are means ± SEM from three independent experiments. *$P < 0.05$ vs. 2.5 mmol/l glucose alone, **$P < 0.05$ vs. mock-, vector-, or wild-type mutant–transfected cells treated with 20 mmol/l glucose.

Arnette et al. (39) demonstrated ERK1/2 activation by glucose and GLP-1 in INS-1 cells. Furthermore, inactive mutants of Ras or Raf-1 markedly reduced glucose-induced activation of ERK, suggesting important regulatory roles for the Ras/Raf-1 signaling steps in this signaling cascade. In contrast, studies by Briaud et al. demonstrated inhibition of IGF-1–mediated, but not glucose-induced, activation of ERK in INS-1 cells after overexpression of a dominant-negative Ras mutant (25). The underlying reasons for these differences between the two studies on the regulatory roles of Ras in glucose-mediated effects on ERK1/2 activation remain to be determined. Lastly, potential regulation of a Rap-1/B-Raf–, but not Ras/Raf-1–, sensitive mechanism(s) in GLP-1 and glucose-mediated activation of ERK was reported in human islets (26).

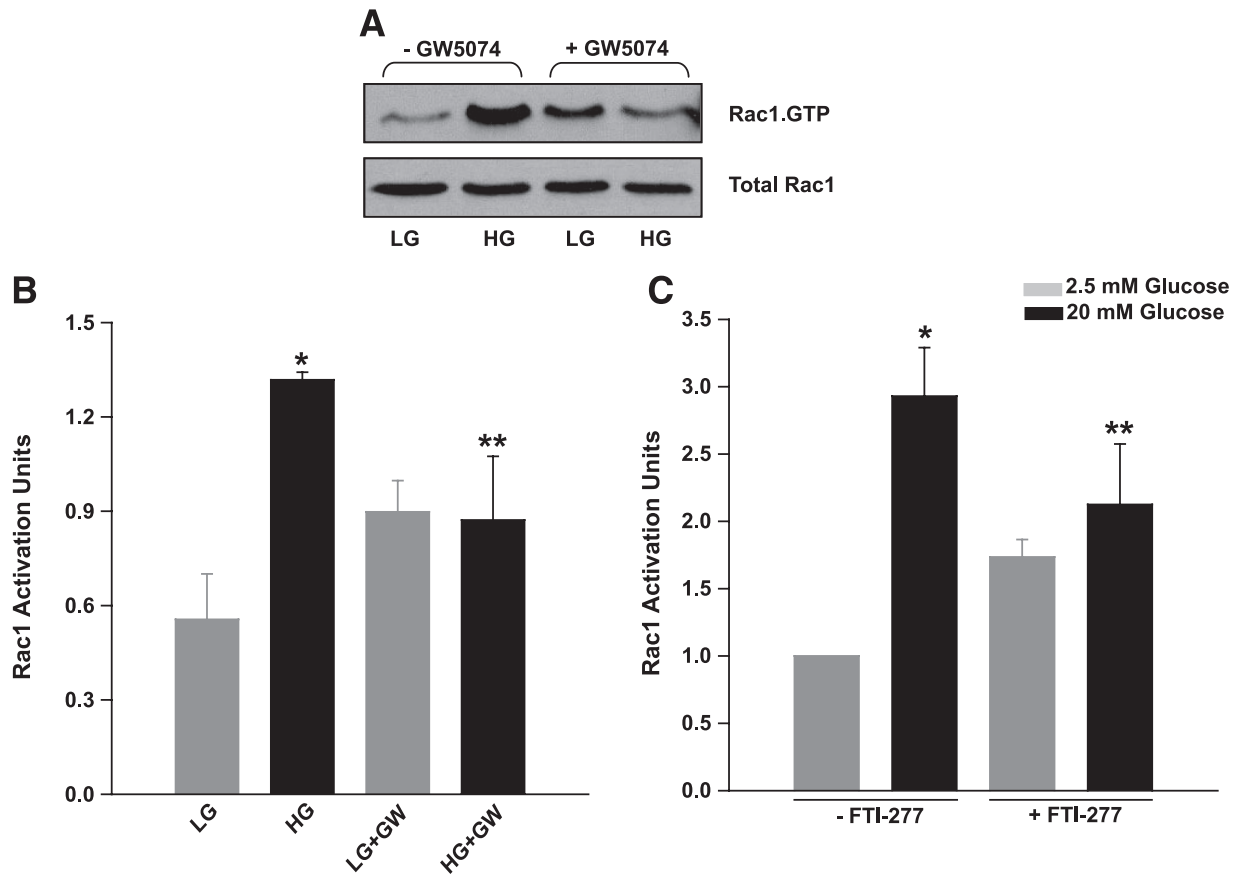Despite the uncertainty about the identity of the G-

FIG. 7. Inhibition of Raf-1 kinase or FTase markedly attenuates glucose-induced activation of Rac1 in INS 832/13 cells. INS 832/13 cells were cultured overnight in low-serum, low-glucose media with either diluent alone or GW-5074 (10 μmol/l) as indicated in the figure. The cells were then incubated further (30 min) in the presence of either low (5 mmol/l) or high (20 mmol/l) glucose in the continuous presence of GW-5074 or diluent. The degree of Rac1 activation was determined by PAK/PBD pull-down assay. *A*: A representative blot from four independent experiments yielding similar results. *B*: Densitometric analysis of the ratio of total Rac1 and Rac1.GTP. Data are mean ± SEM from four determinations in each case. *$P < 0.05$ vs. diluent, **$P < 0.05$ vs. high glucose (20 mmol/l) alone and expressed as fold increase over basal. *C*: INS 832/13 cells were cultured overnight in low-serum, low-glucose media with either diluent alone or FTI-277 (10 μmol/l). The cells were then incubated further (30 min) in the presence of either low (2.5 mmol/l) or high (20 mmol/l) glucose in the continuous presence of FTI-277 or diluent. The degree of Rac1 activation was determined by PAK/PBD pull-down assay. Densitometric analysis of ratio of total Rac1 and Rac1.GTP. Data are mean ± SEM from two independent determinations in each case. *$P < 0.05$ vs. diluent, **$P < 0.05$ vs. high glucose (20 mmol/l) in the absence of FTI-277.

protein(s) mediating ERK1/2 activation by various insulin secretagogues, the majority of data appear to support involvement of Raf-1 kinase in ERK1/2 activation. Our current findings further confirm the above observations that Raf-1 kinase activation is necessary for glucose-mediated effects on ERK1/2 phosphorylation. In addition, they also implicate these signaling steps in glucose-induced Rac1 activation and insulin secretion. Based on our observations from H-Ras depletion experiments, it appears that H-Ras may not promote glucose-mediated effects on ERK1/2 activation and insulin secretion. These studies are in agreement with findings of Briaud et al. on lack of involvement of H-Ras on glucose-induced activation of ERK1/2 (25). The potential identity of the farnesylated protein involved in glucose-mediated regulation of ERK1/2, Rac1 activation, and insulin secretion remains to be identified. In this context, it should be noted that, in addition to Ras, specific forms of γ-subunits of trimeric G-proteins (e.g., Gγ$_1$) undergo farnesylation. We previously reported immunologic identification and carboxylmethylation of Gγ$_1$, Gγ$_2$, Gγ$_5$, and Gγ$_7$ in pancreatic β-cells, which was stimulated by GTPγS (in broken lysates) and glucose, KCl, or Mas (in intact cells [43]). Because the prenylation precedes the carboxylmethylation step, we speculate that glucose-induced carboxylmethylation of Gγ

subunit may, in part, be due to its ability to regulate protein prenylation. This remains to be verified further.

It may be germane to point out that although there is a general agreement that stimulatory concentrations of glucose promote activation of ERK1/2 in pancreatic β-cells, potential roles of this signaling event(s) in GSIS remain unclear. For example, studies by Khoo and Cobb demonstrated that pharmacologic inhibition of ERK1/2 activation had minimal effects on GSIS in INS-1 cells, suggesting that ERK activation is not necessary for GSIS to occur (44). Similar conclusions were drawn by Burns et al. in their studies involving adult rodent islets (45). More recent studies by Gray et al. (46) have suggested involvement of ERK1/2 in signaling cascade leading to insulin secretion after activation of the extracellular calcium-sensing receptor. Using pharmacologic and molecular biological approaches, Longuet et al. (47) have demonstrated a pivotal role of ERK1/2 in GSIS in MIN6 β-cells. The underlying reasons for such differential effects or roles for ERK1/2 in GSIS remain unknown, but could be explained on the basis of yet to be identified differences between β-cell preparations used in these studies. Our current findings in INS 832/13 cells and normal rat islets provide evidence for a direct regulatory role for ERK1/2 in GSIS, specifically at the level of activation of Rac1, which has been implicated
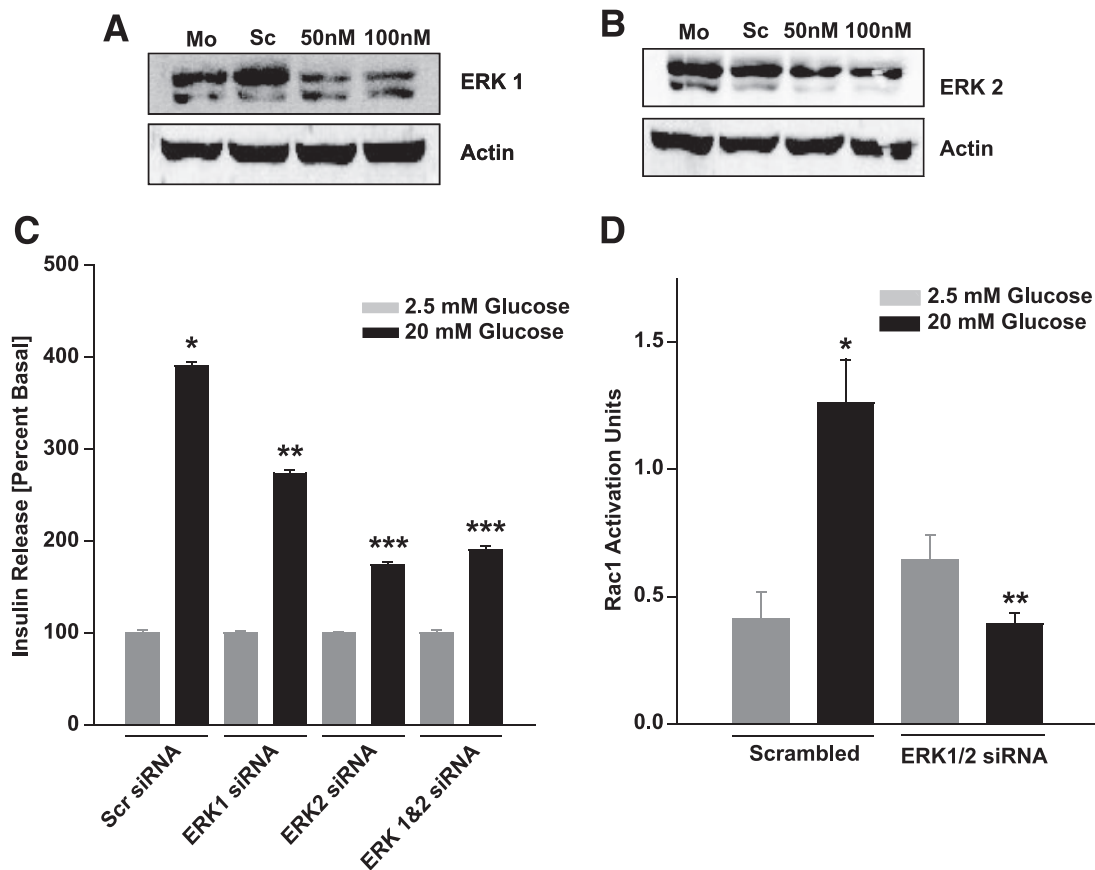
FIG. 8. Glucose-induced insulin secretion and Rac1 activation are markedly reduced in INS 832/13 cells after siRNA-mediated depletion of ERK1 and ERK2 expression. INS 832/13 cells were cultured in a 24-well plate overnight and transfected with ERK1 or ERK2 siRNA using HiPerfect transfection reagent (see the RESEARCH DESIGN AND METHODS section for additional details). After 48 h, the cells were harvested and homogenized, and 10 μg protein was separated in 12% SDS-PAGE, transferred to membrane, and then probed with ERK1 (*A*) or ERK2 (*B*) antibody to determine the efficiency of knockdown. Actin was used as reference protein. Mo, mock transfected; Sc, scrambled siRNA transfected. ERK1 or ERK2 siRNA at 50 or 100 nmol/l. *C*: INS 832/13 cells were cultured in a 24-well plate and transfected with either ERK1 (100 nmol/l) or ERK2 (100 nmol/l) or 50 nmol/l each of ERK1 and ERK2 siRNA. After 48 h, the cells were cultured overnight in low-glucose media followed by further incubation with either low (2.5 mmol/l) or high (20 mmol/l) glucose for 45 min at 37°C. Insulin released into the medium was quantified using ELISA. Data are expressed as nanograms per milliliter of insulin released and are mean ± SEM from eight independent determinations. *$P <$ 0.001 vs. low glucose, ** and ***$P <$ 0.001 vs. high glucose-induced insulin release in scrambled siRNA-transfected cells. *D*: INS 832/13 cells were cultured in a 24-well plate overnight and transfected with 50 nmol/l each of ERK1 and ERK2 siRNAs (as above). Glucose-induced activation of Rac1 was determined by PAK/PBD pull-down assay (see the RESEARCH DESIGN AND METHODS section for additional details). Data are mean ± SEM from two pull-down assays. *$P <$ 0.05 vs. low glucose, **$P <$ 0.05 vs. Rac1 activation seen in the presence of high glucose (20 mmol/l) in scrambled siRNA–transfected cells.

in cytoskeletal reorganization, vesicular transport, and exocytosis of insulin (8,14,23,31,42).

Our findings also implicate Raf-1 kinase activation as an obligatory step for glucose-induced activation of Rac1 in the pancreatic β-cell. Potential connection between these two signaling events remains unknown at this time. Along these lines, Goalstone et al. (19) reported a significant stimulation of the phosphorylation of the α-subunit of FTase/GGTase-I leading to its functional activation and farnesylation of Ras protein by insulin in 3T3-L1 fibroblasts and adipocytes. Interestingly, insulin-stimulated phosphorylation of the FTase/GGTase-I α-subunit was inhibited by PD98059, suggesting that FTase/GGTase-I α-subunit phosphorylation may be mediated by ERK kinase. Further, increased phosphorylation of FTase/GGTase-I α-subunit in cells overexpressing the constitutively active Raf-1 kinase mutant was also observed. Based on these findings, these investigators concluded that insulin-mediated activation involves a Ras/Raf-1/ERK signaling pathway. Similar FTase activation mechanisms involving phosphorylation of FTase α-subunits have been proposed during the transforming growth factor β receptor-1 activa-

tion (48). Our current findings are suggestive of such a regulatory mechanism(s) for glucose-stimulated activation of Rac1 as a consequence of Raf-1/ERK–mediated activation of the phosphorylation of the α-subunit of FTase/GGTase. This postulation is based on our recent findings demonstrating a significant inhibition in glucose-induced activation and membrane association of Rac1 and insulin secretion in INS 832/13 cells overexpressing a phosphorylation-deficient dominant-negative mutant of FTase/GGTase-I α-subunit (23). Based on the data accrued in the current investigations and evidence in the literature (49), we propose that a farnesylation-sensitive Raf-1/ERK1/2 signaling pathway is required for Rac1 activation leading to GSIS. Along these lines, using recombinant substrates, we recently reported a significant stimulation (2.5- to 4.0-fold over basal) of FTase and GGTase-I activities in INS 823/13 cells and rodent islets (50). Potential involvement of ERK1/2 in the phosphorylation and functional activation (19) of FTase/GGTase-I by glucose remains to be determined.

It is important to note that our data accrued from the overexpression of a constitutively active Raf-1 kinase

mutant (BXB; Fig. 6) indicated modest effects on ERK1/2 activation while exerting a marked potentiation of GSIS in INS 832/13 cells. Under these conditions, we failed to detect any potentiating effects of this mutant on glucose-induced Rac1 activation (i.e., 1.55 ± 0.02-fold stimulation by glucose in mock-transfected cells vs. 1.455 ± 0.1-fold stimulation by glucose in BXB-overexpressing cells; $n = 2$ experiments). These data raise an interesting possibility that additional (i.e., Raf-1/ERK1/2/Rac1 independent) mechanisms might underlie the potentiating effects of BXB on GSIS. These are being investigated in our laboratory currently.

In conclusion, our current findings involving site-specific inhibitors of FTase (FTI-277 and FTI-2628) and FTase siRNA provide direct evidence for the requirement for a farnesylation step in glucose-induced ERK1/2 activation and insulin secretion in pancreatic β-cells. Using the above approaches, we have also identified Rac1 as one of the target G-proteins for Raf-1/ERK1/2, the activation of which has been implicated in GSIS. The identity of the farnesylated protein remains unknown, but appears to be different from H-Ras because knockdown of H-Ras exerted minimal effects on glucose-induced ERK1/2 activation and insulin secretion. Studies are in progress to determine the identity of the putative farnesylated protein, which is involved in this regulatory pathway. Together, these findings support our model that protein farnesylation–dependent Raf/ERK signaling links to cytoskeletal remodeling and organization to promote insulin secretion in β-cells.

ACKNOWLEDGMENTS

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