Original Article

Transcription Factor FoxO1 Mediates Glucagon-Like Peptide-1 Effects on Pancreatic β-Cell Mass

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The glucoincretin hormone glucagon-like peptide-1 (GLP-1) increases pancreatic β-cell proliferation and survival through sequential activation of the epidermal growth factor receptor (EGFR), phosphatidylinositol-3 kinase (PI 3-kinase), and Akt. We investigated the role of transcription factor FoxO1 in the proliferative and antiapoptotic actions of GLP-1 in β-cells. GLP-1 inhibited FoxO1 through phosphorylation-dependent nuclear exclusion in pancreatic β (INS832/13) cells. The effect of GLP-1 was suppressed by inhibitors of EGFR (AG1478) and PI 3-kinase (LY294002). In contrast, LY294002 but not AG1478 suppressed insulin-induced FoxO1 phosphorylation. Expression of constitutively nuclear FoxO1 in β-cells prevented the proliferative and antiapoptotic actions of GLP-1 in cultured β-cells and the increase in pancreatic β-cell mass in response to Exendin4 in transgenic mice. Gene expression and chromatin immunoprecipitation assays demonstrated that GLP-1 increases pancreatic and duodenal homeobox gene-1 and *Foxa2* expression and inhibits FoxO1 binding to both promoters. We propose that FoxO1 mediates the pleiotropic effects of the glucoincretin hormone on cell proliferation and survival. *Diabetes* 55:1190–1196, 2006

The glucoincretin hormone glucagon-like peptide-1 (GLP-1) (1–3) improves insulin secretion in subjects with impaired glucose tolerance and type 2 diabetes (4–6). GLP-1 signals via cAMP/PKA and cAMP-regulated guanine nucleotide exchange factors of the Epac (7). The biological actions of GLP-1 include stimulation of *Insulin* expression and insulin biosynthesis (8), presumably via increased expression and activity of the β-cell–specific transcription factor pancreatic and duodenal homeobox gene-1 (PDX1) (9,10). In experimental animal models, GLP-1 increases islet mass (10,11). In cultured β (INS)-cells, GLP-1 acts as a growth factor (9,12,13) via proteolytic maturation of betacellulin (BTC), leading to transactivation of the epidermal growth factor receptor (EGFR), phosphatidylinositol-3 kinase (PI 3-kinase), protein kinase B/Akt, and protein kinase C ζ (9,12,13). This signaling cascade mediates the glucoincretin action of the hormone as well (14). Finally, GLP-1 has recently been shown to prevent β-cell apoptosis in animal models of diabetes (11,15) and glucolipotoxicity (16).

Forkhead transcription factors (Fox) of the O subclass are transcriptional effectors of insulin and IGF signaling. Their activity is inhibited by PI 3-kinase/Akt signaling via phosphorylation-dependent nuclear exclusion (17). We have shown that FoxO1 is a prominent mediator of growth factor signaling in β-cells and that FoxO1 controls β-cell mass through Pdx1 (18–21). Thus, the potential exists for a role of FoxO1 in GLP-1 action in the β-cell.

We report that GLP-1 inhibits the forkhead transcription factor FoxO1 through phosphorylation-dependent nuclear exclusion. Moreover, FoxO1 inhibition plays a role in the proliferative and antiapoptotic actions of GLP-1 in the β-cell. Finally, quantitative real-time PCR and chromatin immunoprecipitation assays reveal that GLP-1 alters the transcriptional profile of the β-cell through FoxO1 inhibition.

RESEARCH DESIGN AND METHODS

Reagents. LY294002 and AG1478 were purchased from Biomol (Plymouth Meeting, PA). Human GLP-1 fragment 7–36 amide and Exendin4 were obtained from Sigma (St. Louis, MO). RPMI 1640, FCS, and other culture media were purchased from Gibco BRL (Burlington, ON, Canada).

Cell culture and incubation. INS832/13 (22) cells (passage 36–70) were grown in RPMI 1640 medium supplemented with 10 mmol/l HEPES, 10% heat-inactivated FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Cells at 70% confluence were washed with PBS and preincubated in serum-free RPMI supplemented with 5 mmol/l glucose and 0.1% BSA (Fraction V; Sigma) for at least 4 h before treatment. LY294002 and AG1478 were applied 30 min before growth factor treatment.

Animal studies. Eight-week-old wild-type and FoxO1 transgenic mice were treated with daily intraperitoneal injections of Exendin4 (10 nmol/kg) or saline for 7 days ($n = 4$ each). Animals were killed and pancreas sections processed for insulin and Ki67 immunohistochemistry to assess β-cell proliferation. Cross-sectional islet area was measured using Image Pro Plus software (Media Cybernetics, Silver Spring, MD), and small β-cell clusters (<6 cells) were counted for each section. For each animal, at least three sections spaced 80 μm apart were studied (18).

Western blot. Proteins were extracted and quantified by BCA (bicinchoninic acid) assay (Roche, Rockford, IL) before fractionation on 8 or 10% polyacrylamide gels. Primary antibodies (anti–forkhead transcription factor Foxo1 [anti-FKHR], anti–phospho-FKHR, anti–extracellular signal–related kinase 1/2 [anti-ERK1/2], and anti–phospho-ERK1/2) were purchased from Cell Signaling (Beverly, MA). Western blotting was performed as described previously (19).

Transfection and confocal microscopy. Cells were transfected with FoxO1-GFP (23) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in LabTek chambered slides (Nalgene). In brief, 4×10^5 cells were transduced with 1 μg plasmid DNA and 2 μl Lipofectamine 2000 in 0.5 ml RPMI medium. The following day, cells were treated for the indicated time periods and fixed in 4% paraformaldehyde in PBS for 15 min. Image acquisition was performed using a Zeiss confocal microscope (23).

Recombinant adenovirus transduction. The constitutively nuclear mutant FoxO1 carries single amino acid substitutions replacing the three main

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β-Gal, β-galactosidase; BrdU, bromodeoxyuridine; BTC, betacellulin; EGFR, epidermal growth factor receptor; ERK, extracellular signal–related kinase; FKHR, forkhead transcription factor Foxo1; GLP-1, glucagon-like peptide-1; Pdx1, pancreatic and duodenal homeobox gene-1; PI, phosphatidylinositol.

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phosphorylation sites, Thr^{24}→Ala, Ser^{253}→Asp, and Ser^{316}→Ala^{24}. Constitutively nuclear FoxO1 transduction was carried out as previously described (24). INS832/13 cells were seeded 2 days before use in 100-mm Petri dishes and cultured in RPMI. Cells were then infected with constitutively nuclear FoxO1 or β-galactosidase (β-Gal) adenoviral constructs at a multiplicity of infection of 50 plaque-forming units/cell for 1 h in 1 ml complete medium. The viral solution was then replaced with complete medium, and cells were allowed to recover for 24 h before being used.

Cell proliferation. Proliferation of INS832/13 cells was evaluated using an enzyme-linked immunosorbent assay–based bromodeoxyuridine (BrdU) incorporation kit (Roche). In brief, INS832/13 cells were transduced for 1 h in 100-mm Petri dishes, trypsinized, seeded in 96-well plates at 70% confluence, and incubated overnight in minimal RPMI medium before the experiment. BrdU was added to the culture medium for 1 h before harvesting cells. Cells were then fixed and incubated with a peroxidase-conjugated anti-BrdU antibody, and the immune complexes were quantified using a spectrophotometer to measure absorbance (Bio-Rad, Hercules, CA).

Measurements of caspase activity and mitochondrial membrane potential. Cells were plated on chambers slide (Nalgene Nunc, Naperville, IL) and incubated for 24 h in complete RPMI. The following day, cells were washed and treated under different conditions in minimal RPMI medium for 4 h. Caspase activation was measured with the sulforhodamine multi-caspase activity kit and mitochondrial membrane potential with Mit-E-Ψ mitochondria permeability detection kit (Biomol). Briefly, SR-VAD-FMK, a sulforhodamine derivative of a potent caspase inhibitor, was added during the last 1 h of a 4-h incubation. SR-VAD-FMK is cell permeable and covalently binds to active caspases, allowing for detection by fluorescence microscopy. The lipophilic Mit-E-Ψ reagent was added for the last 15 min of a 90-min incubation. Mit-E-Ψ enters the mitochondria and aggregates to emit red fluorescence. In the presence of a loss of mitochondrial membrane potential (ΔΨ), red fluorescence is replaced by cytoplasmic green fluorescence.

Quantitative real-time PCR. RNA was isolated using Qiagen kits (Valencia, CA), and quantitative real-time PCR was performed as described previously (25).

Chromatin immunoprecipitation. Cells were fixed to isolate intact chromatin and treated as described previously. FoxO1 was immunoprecipitated with anti-FKHR (H-128) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and bound DNA was amplified by PCR. Primers sequences are available on request (18,19).

Calculations and statistics. Data are presented as means ± SE. Statistical analyses were performed with SPSS using ANOVA.

RESULTS

GLP-1 inhibits FoxO1 in β (INS832/13) cells. FoxO1 is a prominent effector of PI 3-kinase/Akt, a signaling cascade that is activated by GLP-1 in β-cells. We investigated whether GLP-1 affects FoxO1 phosphorylation and subcellular localization in pancreatic β (INS832/13) cells. In cultured β-cells, FoxO1 is constitutively phosphorylated and cytoplasmic, probably because of endogenously produced insulin (26). To measure the effects of GLP-1 on FoxO1 phosphorylation, we cultured INS832/13 cells in serum-free RPMI medium supplemented with 5 mmol/l glucose for 24 h. At the end of the incubation period, we added 10 nmol/l GLP-1 for various lengths of time (Fig. 1*A*). Under these conditions, GLP-1 induced FoxO1 phosphorylation in a time-dependent manner, with a maximal effect observed at 30 min. The action of GLP-1 was mimicked by the EGFR ligand BTC (not shown). The effect of GLP-1 was blocked by inhibitors of EGFR (AG1478, 250 nmol/l) and PI 3-kinase (LY294002, 50 μmol/l) signaling (Fig. 1*B*). Insulin also increased FoxO1 phosphorylation. Unlike the effect of GLP-1, that of insulin could be blocked by the PI 3-kinase inhibitor LY294002 but not by the EGFR inhibitor AG1478 (Fig. 1*C*).

We next studied the GLP-1 effect on the subcellular localization of FoxO1 by fluorescence microscopy. To this end, we transiently expressed a FoxO1-GFP fusion protein in INS832/13 cells (23). GLP-1 caused a time-dependent translocation of FoxO1 from the nucleus to the cytoplasm, consistent with FoxO1 phosphorylation (Fig. 2, *first row*).

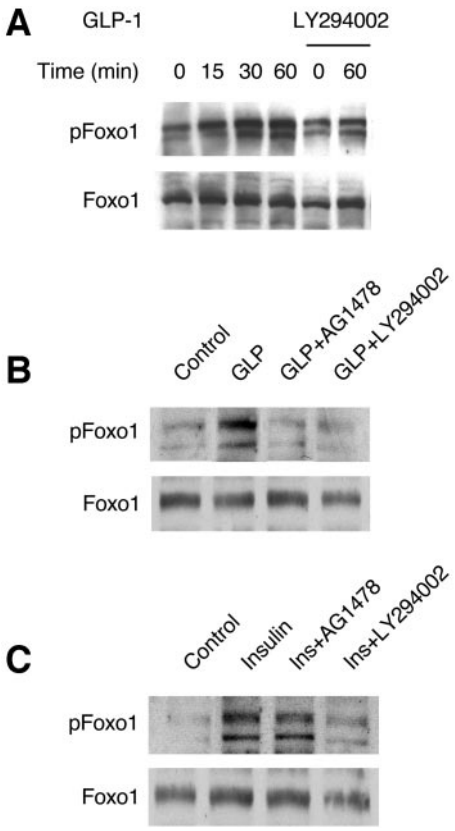

FIG. 1. GLP-1 increases FoxO1 phosphorylation via EGFR and PI 3-kinase signaling. *A*: INS832/13 cells were incubated overnight in serum-free medium and then treated with 10 nmol/l GLP-1 for the indicated time periods. *B*: Cells were treated with 10 nmol/l GLP-1 with or without 250 nmol/l AG1478 (AG) or 50 μmol/l LY294002 (LY) for 30 min. *C*: Cells were treated with 10 nmol/l insulin with or without 250 nmol/l AG1478 (AG) or 50 μmol/l LY294002 (LY) for 30 min. In both *B* and *C*, AG and LY were added 30 min before stimulation. FoxO1 phosphorylation was analyzed by immunoblotting using anti–phospho-FoxO1 and anti-FoxO1 antisera.

Insulin, BTC, and serum had comparable effects (Fig. 2, *second row*). Inhibition of PI 3-kinase by LY294002 preempted translocation induced by all ligands tested (Fig. 2, *third row*). In contrast, AG1478 was able to prevent translocation induced by GLP-1 and BTC but not that induced by insulin (Fig. 2, *fourth row*).

These data indicate that GLP-1 inhibits FoxO1 through phosphorylation-dependent nuclear exclusion. Given that GLP-1 has been shown to activate sequentially EGFR and PI 3-kinase (9,12,13,16), the observed changes in FoxO1 phosphorylation/subcellular localization suggest that FoxO1 mediates GLP-1 action on β-cells.

Proliferative and antiapoptotic actions of GLP-1 require FoxO1 inactivation. Because FoxO1 controls cell cycle progression (27), we examined whether its inhibition is required for GLP1-dependent β-cell proliferation. Incubation of serum-deprived cells with high glucose increased BrdU incorporation by ~50%, as did GLP-1 and BTC. Addition of 10% serum to the medium resulted in a 3.5-fold increase in BrdU incorporation (Fig. 3*A*). We next transduced cells with a constitutively nuclear FoxO1 mutant (20) or with control β-gal adenovirus. Overexpression of constitutively nuclear FoxO1 inhibited β-cell proliferation induced by glucose, GLP-1, BTC, and serum, without significantly affecting basal proliferation, whereas control virus encoding β-gal had no effect (Fig. 3*A*). This result

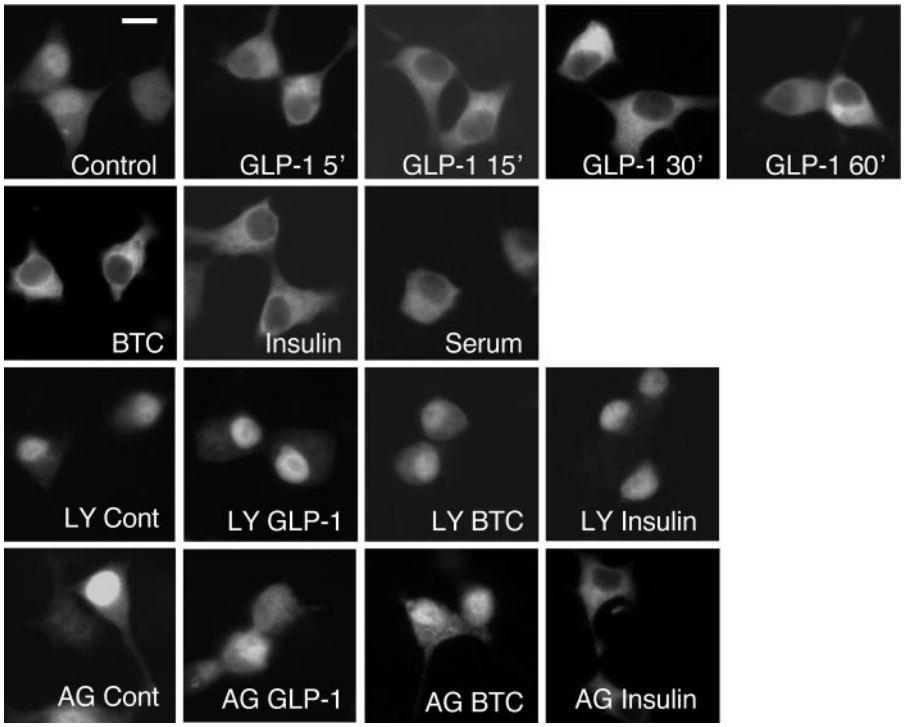

FIG. 2. GLP-1 induces FoxO1 nuclear exclusion. Immunocytochemical analysis of FoxO1 localization in INS832/13 cells after transient transfection with FoxO1-GFP. Representative images are shown. Bar = 10 μm.

indicates that FoxO1 nuclear exclusion is necessary for GLP-1– and BTC-induced proliferation.

To evaluate whether FoxO1 inactivation was required for the antiapoptotic effect of GLP-1, we examined caspase activation in response to H_2O_2, an inducer of oxidative stress (28), using a fluorescent probe in cells transduced with constitutively nuclear FoxO1 or β-Gal. Addition of H_2O_2 increased the number of cells expressing activated caspase by ~threefold (Fig. 3*B*). GLP-1 reversed the effects of H_2O_2. Transduction with constitutively nuclear FoxO1 blocked the ability of GLP-1 to decrease the number of caspase-positive cells (Fig. 3*B*). These data indicate that GLP-1 action on β-cell survival requires FoxO1 inhibition.

Our results indicate that constitutively nuclear FoxO1 inhibits GLP-1 action on proliferation and survival. To determine the mechanism by which FoxO mediates its effects in β-cells, we examined whether constitutively nuclear FoxO1 affects GLP-1–dependent increase in cAMP levels, preserves mitochondrial membrane potential, and prevents GLP-1–induced ERK1/2 activation, three metabolic actions of the hormone that do not require gene transcription. GLP-1 induced a rise in cAMP levels in β-Gal–transduced cells, as did treatment with 10 mmol/l nicotinamide (29). Basal cAMP levels were ~50% lower in cell transduced with constitutively nuclear FoxO1. This decrease is similar to that observed in growth-arrested cells and is likely to reflect the FoxO1-induced growth arrest. However, the magnitude of the GLP-1–induced rise was unaffected by constitutively nuclear FoxO1 (Fig. 4*A*). Next, we used a fluorescent probe (Mit-E-Ψ) to evaluate changes in mitochondrial membrane potential in cells transduced with constitutively nuclear FoxO1 after H_2O_2 treatment in the absence or presence of GLP-1. H_2O_2 caused a disruption of mitochondrial membrane potential, indicated by the reduction in red fluorescence and the appearance of green fluorescence. GLP-1 preserved the integrity of mitochondrial membrane potential in the presence of H_2O_2 and constitutively nuclear FoxO1 (Fig. 4*B*). Identical observations were made in untransduced cells (not shown). These data indicate that FoxO1 does not affect GLP-1 action on mitochondrial membrane potential. The mechanism by which GLP-1 acts on the mitochondria to preserve the membrane potential remains elusive but may be related to stimulation of mitochondrial ATP synthesis (30). Next, we investigated whether constitutively nuclear FoxO1 interferes with the ability of GLP-1 to activate ERK1/2. In untransduced cells, GLP-1 caused a moderate increase in ERK1/2 phosphorylation by 177 ± 34% ($P < 0.05$), as previously reported (12). Transduction of INS cells with constitutively nuclear FoxO1 did not prevent the GLP-1–induced rise in phospho-ERK (Fig. 4*C*). These results indicate that constitutively nuclear FoxO1 does not affect actions of GLP-1 that do not require gene transcription.

To investigate the role of FoxO1 in the proliferative actions of GLP-1 in vivo, we determined whether GLP1 affects β-cell mass in transgenic mice expressing constitutively nuclear FoxO1 in β-cells. The mutant transgene carries a single amino acid substitution replacing the main Akt phosphorylation site and is therefore constitutively nuclear (line 305) (19). We administered either saline or the long-acting GLP-1 analog Exendin4 (10 nmol/kg) daily for 7 days to either wild-type or 305 transgenic mice. We then measured β-cell proliferation by Ki67 immunostaining and islet mass by morphometry of pancreatic sections stained with anti-insulin antiserum (representative images are shown in Fig. 5*A*). Exendin4 administration resulted in a fourfold increase in Ki67-positive β-cells in control animals (Fig. 5*B*) and a twofold increase in cross-sectional islet area (Fig. 5*C*). We also observed a small increase in the number of small clusters of insulin-positive cells (<6 cells) in wild-type mice treated with Exendin4 compared with saline-treated littermates (Fig. 5*D*). In contrast, 305 transgenic mice failed to increase the number of Ki67-positive cells, islet size, and small β-cell clusters (Fig. 5*B–D*). These observations indicate that FoxO1 inactiva-

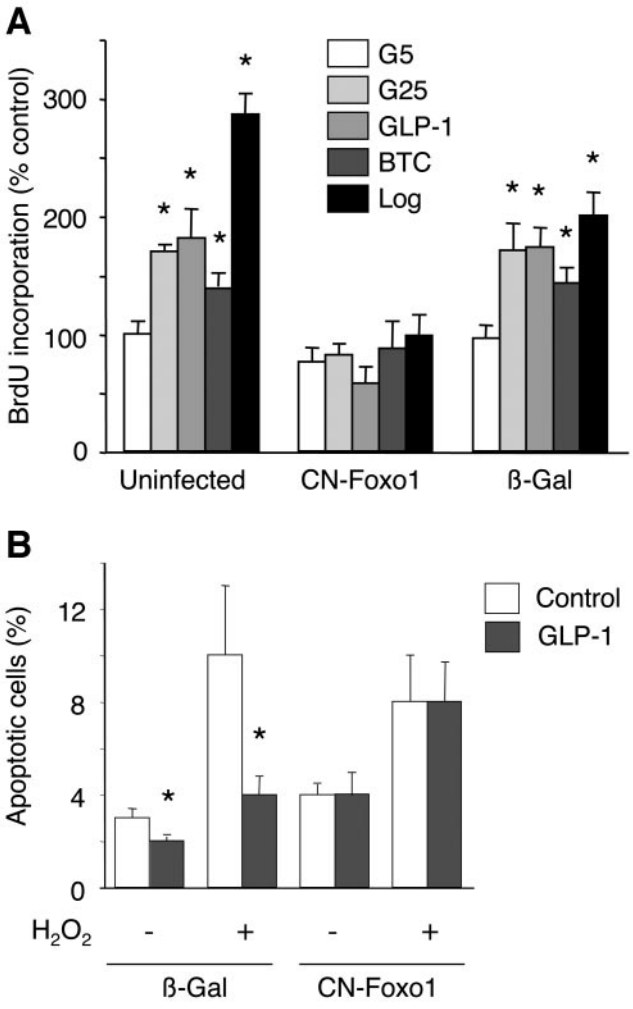

FIG. 3. GLP-1 effects on β-cell proliferation and survival require FoxO1 inactivation. *A*: Proliferation was evaluated by measuring BrdU incorporation in control cells or after transduction with constitutively nuclear FoxO1 or β-Gal. INS832/13 cells were incubated overnight in serum-free medium containing 5 mmol/l glucose (G5) and treated for 24 h with 25 mmol/l glucose (G25), GLP-1, BTC, or medium supplemented with 10% serum (log phase of growth [Log]). Results represent means ± SE of three separate experiments carried out at least in triplicate. *$P < 0.05$ by ANOVA. *B*: Apoptosis was evaluated in cells transduced with β-gal or constitutively nuclear FoxO1 after a 4-h incubation with or without 25 μmol/l H_2O_2 and in the absence or presence of 10 nmol/l GLP-1. The percentage of apoptotic nuclei detected in microscopic fields in different conditions is shown. *$P < 0.05$ by ANOVA.

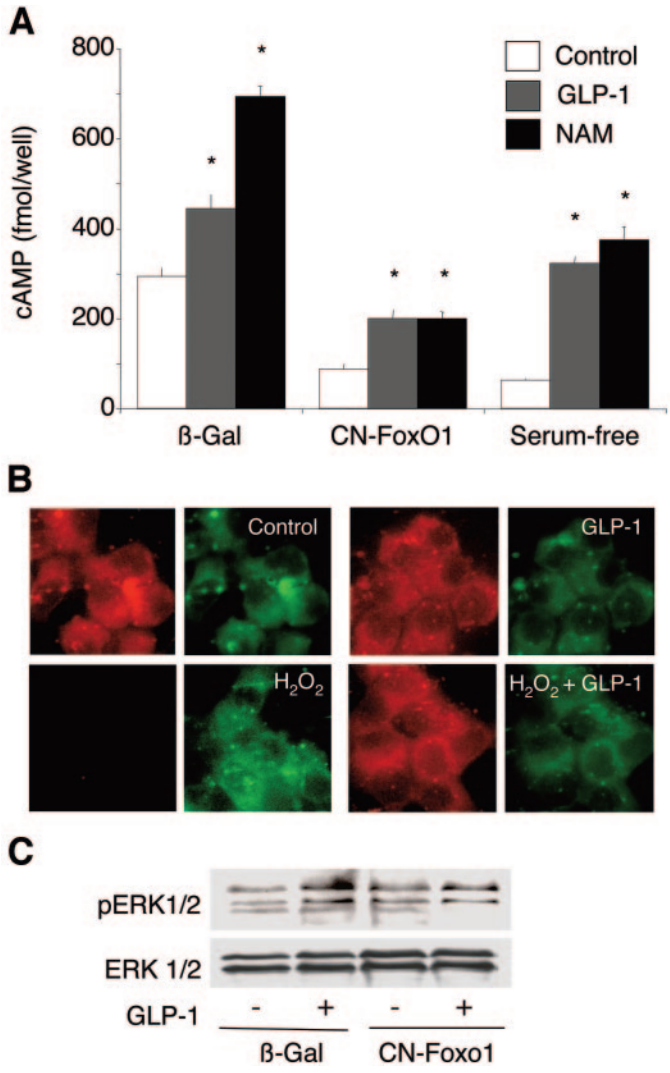

FIG. 4. Constitutively nuclear FoxO1 does not affect the ability of GLP-1 to increase cAMP and preserve mitochondrial membrane potential. *A*: INS832/13 cells were transduced with either β-Gal or constitutively nuclear FoxO1 in complete RPMI medium or incubated overnight in serum-free medium. The following day, cells were preincubated for 30 min in Krebs-Ringer bicarbonate HEPES buffer medium containing 5 mmol/l glucose and subsequently treated for 20 min with GLP-1 or 10 mmol/l nicotinamide. *$P < 0.05$ by ANOVA. *B*: Mitochondrial membrane potential was evaluated in INS832/13 cells transduced with constitutively nuclear FoxO1. The fluorescent compound Mit-E-Ψ enters the mitochondria and emits red fluorescence. A loss of mitochondrial membrane potential (ΔΨ) leads to Mit-E-Ψ leakage from the mitochondria and results in green fluorescence. *C*: INS cells, transduced with either β-Gal or constitutively nuclear FoxO1, were treated as described in Fig. 1*B* and *C*. FoxO1 phosphorylation was analyzed by immunoblotting using anti–phospho-ERK1/2 and anti-ERK1/2 antisera.

tion is required for the proliferative action of GLP-1 in vivo.

Constitutively nuclear FoxO1 prevents GLP-1 effect on gene expression. We next evaluated the contribution of FoxO1 nuclear exclusion to the GLP-1 transcriptional response in β-cells. GLP-1 increased the levels of mRNAs encoding *Pdx1*, *Foxa2*, and *Insulin2* and decreased the levels of *Igfbp1* (Fig. 6*A*). In all instances, constitutively nuclear FoxO1 prevented the effect of GLP-1. To determine whether this effect was due to a direct inhibition of FoxO1-dependent transcription, we measured occupancy of the forkhead binding site in the *Igfbp1* and *Foxa2* promoters using chromatin immunoprecipitation assays. Under basal conditions, we detected FoxO1 bound to both promoters. GLP-1 inhibited FoxO1 binding. GLP-1 was similarly able to decrease FoxO1 binding in H_2O_2-treated cells, consistent with its effect to inhibit H_2O_2-induced caspase activation (Fig. 3). In contrast, GLP-1 had no effect in cells expressing constitutively nuclear FoxO1 (Fig. 6*B*). It should be pointed out that GLP-1 inhibits *Igfbp1* and promotes *Foxa2* expression. Because FoxO1 binds to both promoters, the data are consistent with the notion that it acts as a transcriptional activator for some genes and as a repressor for others (18).

DISCUSSION

GLP-1 is a potent glucoincretin hormone with valuable properties for diabetes treatment. Although its proliferative and antiapoptotic properties have been demonstrated in the β-cell, the molecular mechanism by which GLP-1

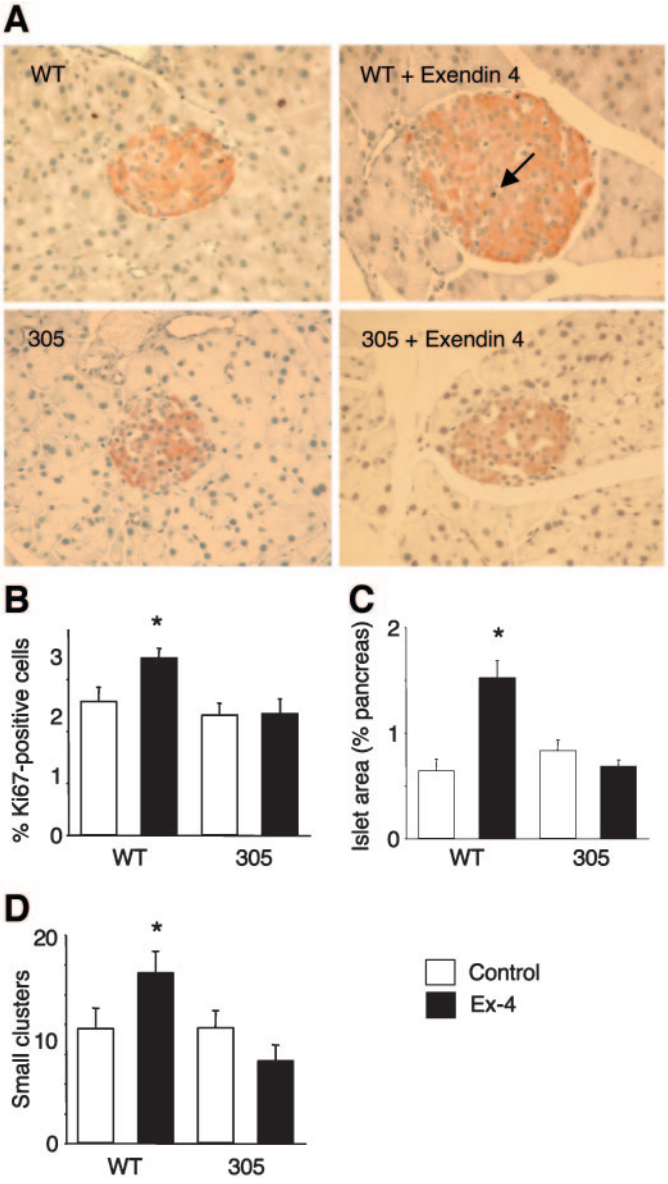

FIG. 5. Exendin4 action on pancreatic β-cell area is blunted in transgenic mice with β-cell–specific expression of constitutively nuclear FoxO1. Wild-type or 305 transgenic mice received daily injections of Exendin4 (10 nmol/kg body wt) or saline for 7 days. Pancreatic β-cell mass and β-cell replication were evaluated by morphometry and immunohistochemistry with anti-insulin (brown) and anti-Ki67 antisera (blue). Representative images of pancreas sections are shown in *A*. The arrow indicates a Ki67-positive nucleus. Bar graphs show the percentage of Ki67-positive β-cells (*B*), the relative cross-sectional islet area (*C*), and the number of small β-cell clusters, defined as groups consisting of less than six insulin-positive cells (*D*). **P* < 0.05 by ANOVA.

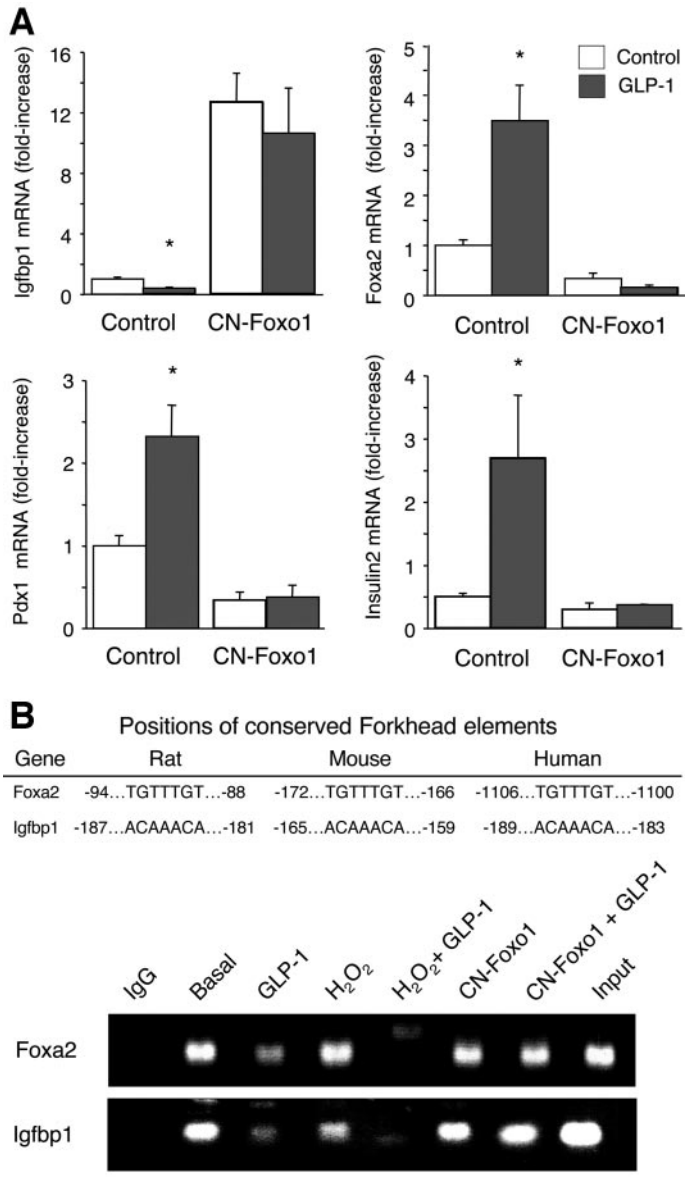

FIG. 6. FoxO1 plays a role in the transcriptional response of the β-cell to GLP-1. *A*: Total RNA was extracted from INS832/13 cells transduced with constitutively nuclear FoxO1 or β-Gal after a 3-h incubation period in the presence or absence of 10 nmol/l GLP-1. Gene expression was evaluated by quantitative real-time PCR and is plotted as fold change from basal levels. **P* < 0.05 by ANOVA. *B*: Chromatin immunoprecipitation assays. FoxO1 was immunoprecipitated from intact chromatin extracted from INS832/13 cells using anti-FoxO1 antiserum. An isotype-matched IgG was used as control. Eluted DNA was amplified using oligonucleotides flanking the forkhead binding site in the *Foxa2* and *Igfbp1* promoters. Cells were treated with GLP-1 or H_2O_2. Total input DNA is shown as control. A representative result from three separate experiments is shown except for the effect of GLP-1 in constitutively nuclear FoxO1–expressing cells, which was performed twice.

exerts its effect on β-cell mass is not fully understood. Pharmacological and biochemical evidence indicates that PI 3-kinase/Akt signaling plays a central role in GLP-1 action on β-cell growth and survival. In the present study, we investigated the role of the transcription factor FoxO1 in the action of GLP-1 on β-cell proliferation and survival. As recently hypothesized (31), we show that GLP-1 inhibits FoxO1 transcriptional activity through phosphorylation-dependent nuclear exclusion in INS832/13 cells. Moreover, the ability of the long-acting GLP-1 derivative Exendin4 to increase β-cell mass was blunted in transgenic mice expressing constitutively nuclear FoxO1 in β-cells. Finally, we demonstrate that FoxO1 inactivation plays an important role in the effect of GLP-1 on the expression of important β-cell transcription factors, such as *Pdx1* and *Foxa2*. Our data indicate that FoxO1 inhibition is required for GLP1-dependent proliferation (Fig. 7).

Restoration of insulin secretion and β-cell mass is central to the treatment of diabetes (32–34). Through its pivotal role in PI 3-kinase/Akt signaling, FoxO1 has the potential to integrate the proliferative and antiapoptotic signals of β-cell growth factors. We have previously shown that FoxO1 plays an important role in metabolic control,

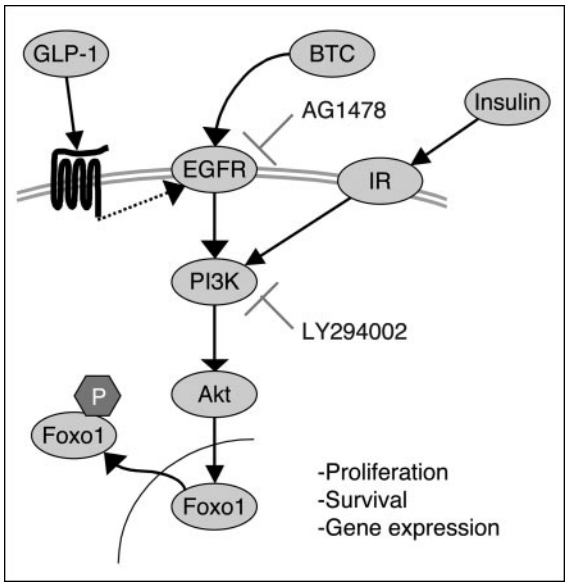

FIG. 7. Model of GLP-1 action on β-cell proliferation and survival. Glp-1 promotes EGFR trans-phosphorylation (dashed line), which in turn links GLP-1 signaling to activation of the PI 3-kinase→Akt pathway, leading to FoxoO1 phosphorylation and nuclear exclusion. The biochemical pathway linking GLP-1 receptors to EGFR remains to be elucidated.

but because of the complex interactions among different tissues, it remained unclear whether this effect is due to intrinsic actions of FoxO1 in the β-cell or to its regulation of peripheral insulin sensitivity (19). In this study, we show that expression of constitutively nuclear FoxO1 inhibits β-cell proliferation induced by glucose, GLP-1, BTC, and serum. Moreover, a recent publication demonstrates that FoxO1 mediates the action of glucose-dependent inhibitory peptide on β-cell survival by regulating *Bax* expression (35). Based on those results and on the present study, it appears that FoxO1 is a general regulator of β-cell mass in response to incretins.

The mechanism by which FoxO1 inhibition by GLP-1 increases β-cell mass is complex. We have previously shown that FoxO1 inhibits *Pdx1* expression, thereby providing a mechanism by which FoxO1 controls β-cell proliferation and insulin secretion (18). Several publications indicate that Pdx1 plays a role in β-cell differentiation, replication, and regeneration. Thus, *Pdx1* haploinsufficient mice fail to increase β-cell mass in response to insulin resistance (36), and increased *Pdx1* expression is associated with an early burst of β-cell proliferation after subtotal pancreatectomy (37). It has also been shown that GLP-1 increases Pdx1 expression, but the mechanism is unclear (9). The present findings indicate that GLP1 increases Pdx1 expression via inhibition of FoxO1. Moreover, we show that GLP-1 increases *Foxa2* expression, and this is associated with decreased FoxO1 binding to the *Foxa2* promoter. Thus, it appears that the mechanism by which FoxO1 controls Foxa2 expression is similar to the mechanism by which it controls Pdx1. Further work will be required to understand the molecular mechanism of transcriptional inhibition by FoxO1.

In conclusion, we show that FoxO1 is in the GLP-1 signal transduction pathway. As GLP receptor agonists enter the clinical arena, FoxO1 represents a potential target to modulate its beneficial effects in diabetes.

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