FoxO1 Is Required for the Regulation of Preproglucagon Gene Expression by Insulin in Pancreatic αTC1–9 Cells*

Caroline M. McKinnon, Magalie A. Ravier, and Guy A. Rutter

From the Henry Wellcome Laboratories for Integrated Cell Signalling and Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol, BS8 1TD, United Kingdom

Forkhead/winged helix box gene, group O-1 (FoxO1) is a member of a family of nuclear transcription factors regulated by insulin-dependent phosphorylation and implicated in the development of the endocrine pancreas. We show here firstly that FoxO1 protein is expressed in both primary mouse islet α and β cells. Examined in clonal αTC1–9 cells, insulin caused endogenous FoxO1 to translocate from the nucleus to the cytoplasm. Demonstrating the importance of nuclear exclusion of FoxO1 for the inhibition of preproglucagon gene expression, FoxO1 silencing by RNA interference reduced preproglucagon mRNA levels by >40% in the absence of insulin and abolished the decrease in mRNA levels elicited by the hormone. Electrophoretic mobility shift assay and chromatin immunoprecipitation revealed direct binding of FoxO1 to a forkhead consensus binding site, termed GL3, in the preproglucagon gene promoter region, localized −1798 bp upstream of the transcriptional start site. Deletion or mutation of this site diminished FoxO1 binding and eliminated transcriptional regulation by glucose or insulin. FoxO1 silencing also abolished the acute regulation by insulin, but not glucose, of glucagon secretion, demonstrating the importance of FoxO1 expression in maintaining the α-cell phenotype.

The forkhead family of transcription factors is implicated in the control of a wide range of cellular processes (1–5). Thus, members of this family have been shown to either repress or activate transcription after signaling by insulin (6). Insulin stimulates FoxO1 by engaging a phosphatidylinositol 3-kinase-protein kinase B (PKB/Akt) signaling cascade (7) that results in the phosphorylation by PKB of FoxO1 at least three sites, Thr-24, Ser-256, Ser-319 (8, 9). Protein kinase B-mediated phosphorylation at Ser-256 is crucial for the subsequent phosphorylation of Thr-24, which in turn allows nuclear export and sequestration of FoxO1 in the cytosol via binding to 14-3-3 proteins (8).

A role for several members of the forkhead family in the development of the endocrine pancreas has previously been described. For example, the forkhead protein Foxa2 (formerly called hepatocyte nuclear factor-3β) regulates transcription of the pdx-1 (10, 11) and slc2c2 genes (12) in the β-cell and is required for the development of a functional α-cell population (13). Moreover, mice in which the expression of forkhead protein Foxa1 is disrupted are hypoglycemic and display low levels of circulating glucagon despite lowered levels of insulin and an increase in the levels of cortisol and growth hormone, and proglucagon mRNA levels are depressed in these mice (14–16). However, the precise role and targets of forkhead proteins within the pancreatic α-cell are at present only partly defined.

There are at least four enhancer sites within the first 300 bp immediately upstream of the transcriptional start site of the proglucagon promoter, denoted G1–G4 (17–20). The G1 and G2 sites within the rat proglucagon gene bind Foxa proteins, predominantly Foxa2, which act as a repressor of proglucagon gene expression (18, 21). Foxa1 can also bind to G2 and, conversely, activate proglucagon gene transcription (16, 22). Although it is has been documented that FoxO1 acts in β cells as a repressor of Foxa2-mediated Pdx1 expression (23), the interplay, if any, between FoxO1 and Foxa2 within α cells has not been explored.

In the present study we used RNA silencing and overexpression approaches to determine the role of FoxO1 in the regulation of preproglucagon gene expression and glucagon secretion within clonal αTC1–9 cells. This line serves as a convenient glucose- and insulin-responsive model of the mature α-cell, although it also displays characteristics of less differentiated precursor cells, including a left-shifted dose response to glucose (24). Importantly, the use of these cells allows the effects of glucose and insulin on PPG gene expression to be examined independently, a goal that is difficult to achieve using intact isolated islets. We show that (a) FoxO1 is abundantly expressed in mature primary α cells as well as β cells, and its localization is regulated by insulin, but not glucose, in the former, (b) FoxO1 is required for the effects of insulin on PPG gene expression, (c) FoxO1 binds directly to an upstream region within the preproglucagon promoter, whose elimination or mutation abolishes transcriptional regulation by insulin or glucose. Together these data implicate FoxO1 as an important mediator of the effects of insulin on PPG gene expression and as a critical regulator of α-cell phenotype. Inappropriate nuclear inclusion of FoxO1 may, thus, serve to elevate glucagon gene expression in certain forms of diabetes.
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EXPERIMENTAL PROCEDURES

Materials

The Silencer<sup>®</sup> Small Interfering RNA (siRNA)<sup>2</sup> construction kit was from Ambion (Austin, TX). siRNA oligonucleotides and all primers were from Alta Bioscience (University of Birmingham, Birmingham, UK). TransIT-TKO transfection reagent was from Mirus (Madison, WI), and the rat glucagon radiolmmunoassay kit was from Linco Research Immunassay (St. Charles, MO). Tissue culture reagents were from Sigma or In vitro gen, and Lipofectamine 2000 was from Invitrogen. Polyclonal anti-FKHR antibody was from Cell Signaling Technology<sup>®</sup> (Beverly, MA). Rabbit polyclonal anti-carbohydrate response element-binding protein (ChREBP) antibody was kindly provided by Dr. I. Leclerc (University of Bristol). Anti-Foxa2 antibody was from Abcam. Horseradish peroxidase-conjugated anti-rabbit antibody was from Amersham Biosciences, and anti-insulin antibody was from Dako. Rabbit IgG polyclonal antibody was from Santa Cruz. SYBR<sup>®</sup> green was from Sigma.

<sup>2</sup>The abbreviations used are: siRNA, silenced RNA duplex; scRNA, scrambled RNA duplex; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; mRFP, monomeric red fluorescent protein; PPG, preproglucagon; FKHR, Forkhead-related transcription factors; ChREBP, carbohydrate response element-binding protein; fwd, forward; rev, reverse; DAPI, 4',6-diamidino-2-phenylindole; TRITC, tetramethylrhodamine isothiocyanate; HEK cells, human embryonic kidney cells; USF, upstream stimulatory factor.

Adenoviral and Plasmid Construction and Use

Plasmid pPPG(1.6)-LUC<sub>rf</sub> was generated as described previously (24). To prepare an adenovirus encoding monomeric red fluorescent protein under preproglucagon promoter control, luciferase cDNA was removed from pPPG(1.6)-LUC<sub>rf</sub> by digestion with Xbal and HindIII. cDNA encoding monomeric red fluorescent protein (mRFP; Clontech) was then ligated into the same Xbal-HindIII site of pPPG(1.6)-LUC<sub>rf</sub> to generate pPPG(1.6)-mRFP. Adenoviral generation was then as described (25). Plasmid pPPG(2.3)-LUC<sub>rf</sub> was constructed by subcloning a −2.3-kilobase fragment of the rat PPG promoter (kindly provided by B. Ritz-Laser and J. Philippe, Geneva) into the Smal-Xhol site of pGL3-basic (Promega). pCMV-LUC<sub>rf</sub> was as described (26). To generate a mutant PPG plasmid, pPPG(2.3mut)-LUC<sub>rf</sub>, site-directed mutagenesis was performed on pPPG(2.3)-LUC<sub>rf</sub> as described by the manufacturer’s instructions (Stratagene). Primer sequences designed to mutate the GL3 binding site from TGGTTTA to TTGAGG were as follows: mut1fwd, 5′-GCAAGACCTCTGTGCTATGGGACGCTTTGTATGC-3′; mut1rev, 5′-ACGGATGACTAAAAGCCGTCCCAT- AGCACACAGTCTTGC-3′. Plasmid pFoxO1.leGFP was a kind gift from Dr L. Fletcher and Professor J. Tavaré (ProXara, University of Bristol). Briefly, a cDNA encoding human FoxO1 was cloned in two overlapping fragments by PCR using a cDNA template prepared from HeLa cells. The N terminus was amplified using primer sequences 5′-TTTTCTCGAGATGGCCGAGGCTTGAGGCGCATCAGTGT-3′ and 5′-TTTTACCATCCACTCGTAGATCTGAGACG-3′, and the C terminus was amplified using primer sequences 5′-TTTTCTCAGATGCGGAGGTGGTAG-3′ and 5′-TTTTCTCGAGATGGCCGAGGCTTGAGGCGCATCAGTGT-3′. Both fragments were ligated into plasmid pGEM-T, sequenced in entirety, then ligated by virtue of a common BglII site.

Isolation of Mouse Islets and Culture of Clonal α Cells

Islets were aseptically isolated from 3-month-old mice as previously described; αTC1–9 cells were also as previously described (24). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 25 mmol/liter glucose, 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Infection of primary cells with adenoviral pPPG(1.6)-mRFP was by an overnight infection of intact islets at 100 plaque-forming units/cell (within 2 h of islet isolation).
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Measurement of Glucagon Secretion

αTC1–9 cells were preincubated for 1 h at 37 °C in Krebs-Ringer bicarbonate medium containing 10 mmol/liter glucose (control medium). Cells were then incubated for 30 min in 700 μl of medium containing the test substances in a 12-well plate. At the end of the incubations, a 350-μl aliquot of medium was removed for measurement of secreted glucagon. To measure total cellular glucagon, cells were lysed in 500 μl of hydrochloric acid/ethanol (10 min room temperature) and passed through a 23-gauge needle three times. Measurements were performed by radioimmunoassay (RIA) using a glucagon RIA kit (Linco).

siRNA Construction and Introduction into Cells

siRNA was generated by using the Ambion Silencer™ siRNA construction kit as described elsewhere (27). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2). Target sequences were derived from the cDNA sequence for mouse FoxO1 (GenBank™ accession number NM_019739.2).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP analysis was performed as described elsewhere (28). PCR analysis was performed using the Expand High Fidelity PCR System™ (Roche Applied Science) using the following primer sequences: site 1 fwd, 5'-CTTTCCAAAAGCCTTTCCTC-3'; site 1 rev, 5'-GGGCTCCTCGGATCTTGGTT-3'; site 2 fwd, 5'-AGGCTCCCTGTAGCAGGT-3'; site 2 rev, 5'-CCAGCTAAATCTGGGTGTCC-3'; scrambled sequence, 5'-AAATTGCTCCCCTACGTGTCTGCTGCTC-3'. siRNA sequences were introduced into cells using TransIT-TKO transfection reagent according to the manufacturer's instructions (27).

Real-time PCR

RNA was isolated from adherent cells using the TRIzol® extraction method (Invitrogen) and quantified by spectrophotometry, and 50 μg was used for a reverse transcription step using Omniscript RTase (Qiagen) for 60 min at 37 °C. cDNAs were then subjected to real-time PCR using Platinum® SYBR® Green qPCR Supermix UDG (Invitrogen) using the following sequences for primers: cyclophilin A fwd, 5'-TATCTGCACT-GCCAAGACTG-3'; cyclophilin A rev, 5'-CCACAATGCTCATTGCCTTTTTCA-3'; glucagon fwd, 5'-GATCATTCAGTT-CCAGTCTCAG-3'; glucagon rev, 5'-CTGTGAAGAGGTCCTC-3' (29). Amplification was performed and detected using an Option2 (MJ Research) cycler, and data were analyzed using the comparative Ct method (ABI Biosystems) in Microsoft Excel™.

Preparation of Cell Extracts and Electrophoretic Mobility Shift Assays (EMSA)

Whole cell extracts were prepared using a modification of the method of Schreiber et al. (30) as described previously (31). EMSA was performed as described elsewhere (31, 32). Oligonucleotide sequences were complementary double-stranded 20-mers as follows: GL3, 5'-TGTGTGCTATGCCGCTT-3'; mutGL3, 5'-TGTGTGCTATGCCGCTT-3'; GL2, 5'-TCCTCCAGTTTTCATGG-3'; GL1, 5'-AAACCCCATATTATCACAGAT-3'. The sequence for USF was 5'-GGC-TTCCAGGTAAACACAGTG-3'. The specific activities of all labeled probes were quantified using an Isolabs Multifunction Scintillation Counter (Beckman Coulter). EMSAs were quantified using the Scion Image software package.

Immunocytochemistry

Clonal Cells—Cells were fixed with methanol at 4 °C for 10 min and incubated in 1 ml of “blocking buffer” (6.7% (v/v) glycerol, 0.2% (v/v) Tween 20, 2% (w/v) bovine serum albumin) at room temperature for 15 min. Cells were washed in phosphate-buffered saline 4 times, and endogenous FoxO1 was probed overnight using a polyclonal rabbit anti-FKHR antibody (Cell Signaling; 1:50 dilution in blocking buffer). Cells were then washed 4 times using immunocytochemistry wash buffer (6.7% (v/v) glycerol, 0.4% (v/v) Tween 20, 2% (w/v) bovine serum...
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Islets and Dissociated Islet Cells—Islet slices were obtained as described elsewhere (33). Cells were fixed 48 h after adenoviral infection and incubated with primary and secondary antibodies as previously described (34). Rabbit anti-FKHR, guinea pig anti-insulin, or rabbit IgG primary antibodies were used at a 1:50 dilution. Fluorescein isothiocyanate-labeled anti-rabbit IgG and TRITC-labeled anti-guinea pig IgG labeled secondary antibodies (Jackson) were used at a 1:200 dilution.

Measurement of Luciferase Activity

Cells seeded into 12-well plates were transfected with plasmids pFoxO-eGFP1, pPPG(2.3)-LUCFF, or pPPG(2.3mut)-LUCFF, encoding Photinus pyralis luciferase, and pCMV-LUCren, encoding Renilla reniformis luciferase activity using Lipofectamine2000 (Invitrogen). 4 μg of each plasmid was transfected into αTC1–9 cells as per the manufacturer's instructions. After stimulation with 0.5 mmol/liter glucose, cells were incubated in Dulbecco's modified Eagle's medium-based medium containing 10 or 0.5 mmol/liter glucose plus various concentrations of insulin as stated for 6 h. Cells were then scraped, and a 50-μl sample was removed for analysis. Luciferase activities were measured in a 96-well plate on a Packard Lumicount™ plate reader using the Dual-Glo™ luciferase assay kit (Promega) as per the manufacturer's instructions.

Statistical Analysis

Data are given as the means ± S.D. of three to four individual experiments. Comparisons between means were performed using Student's t test with Microsoft Excel™ or by analysis of variance using Graphpad Prism™ followed by a Newman-Keuls test when more than two groups were compared.

RESULTS

FoxO1 Immunoreactivity Is Present in Primary α and β Mouse Cells—The expression of FoxO1 protein was detected in both islet slices and dissociated mouse islet cells by immunostaining with an anti-FoxO1 antibody and confocal microscopy. First, islet slices were studied (Fig. 1). Nuclear regions were stained with DAPI, and endogenous FoxO1 was detected using an anti-FoxO1 antibody and confocal microscopy. B–D, αTC1–9 cells were preincubated in 0.5 mmol/liter glucose for 6 h before treatment with either 1, 5, or 17 nmol/liter insulin for 1 h as shown. Nuclear regions and endogenous FoxO1 was then detected as above. E, F, and G, αTC1–9 cells were preincubated in 0.5 mmol/liter glucose for 6 h and then incubated in either 0.5, 10, or 30 mmol/liter glucose, respectively, for 1 h before FoxO1 was detected as before. H, cells were fixed in the absence of an anti-FoxO1 antibody (Ab). I, subcellular translocation of endogenous FoxO1 in response to insulin was quantified by Velocity™ software, comparing the nuclear (nu, open bars) and cytoplasmic (cy, closed bars) regions. J, movement of endogenous FoxO1 in response to glucose was quantified as above. Values are the means ± S.D. for 71, 55, 60, 77, 65, and 77 cells respectively, taken from triplicate samples from at least three independent experiments.
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Insulin, but Not Glucose, Causes Endogenous FoxO1 to Translocate from the Nucleus to the Cytosol of αTC1–9 Cells—The subcellular localization of FoxO1 was determined in clonal αTC1–9 cells whose flattened morphology allowed the nuclear and cytoplasmic compartments to be readily distinguished. Cells were preincubated for 30 min in media containing 0.5 mmol/liter glucose before stimulation with either insulin or glucose as shown (Fig. 3). After treatment with either 1, 5, or 17 nmol/liter insulin for 1 h we observed a significant change in the ratio of immunoreactivity in the cytosolic and nuclear regions of the cell, as quantified by measuring the average intensities of individual pixels. For these measurements, both the total cellular area and nuclear areas were counted with the difference between these two values revealing the cytosolic component. Insulin increased the ratio of cytosolic:nuclear fluorescence from 15.79 ± 6.0 to 23.47 ± 10.45% and 75.59 ± 10.43% (Fig. 3,A, B, D, and I; p < 0.005 in each case; numbers of cells are given in the legend) at 1 and 17 nmol/liter, respectively. Incubation with 5 nmol/liter insulin led to an almost equal distribution in nuclear and cytosolic regions (Fig. 3C). Upon stimulation with either 10 or 30 mmol/liter glucose, no change in the ratio of the immunoreactivity in the cytosolic:nuclear compartments was apparent (Fig. 3, E, F, G, and J).

Anti-FoxO1 siRNAs Reduce FoxO1 Levels and Decrease Preproglucagon Gene Expression in αTC1–9 Cells—To study the potential significance of insulin-induced FoxO1 exclusion, we next examined the effects of down-regulating FoxO1 expression in αTC1–9 cells using anti-FoxO1 siRNAs. Immunocytochemistry was initially used to quantify FoxO1 levels in cells treated with either a scrambled (sc) or small interfering (si) RNA duplexes. To account for potential off-target effects, the impact of one scRNA and two siRNA duplexes, named si#1 and si#2, was examined (Fig. 4, A–C, respectively). For control purposes, incubations in the absence of primary antibody were also included (Fig. 4E, F, G, and J).
fected cells versus scRNA-transfected cells, whereas there was a 90 ± 0.45% decrease in PPG mRNA levels compared with untreated, scRNA-transfected cells (p < 0.05). Transfection with the anti-FoxO1 si#1 RNA duplex caused a 25 ± 0.1% decrease in PPG mRNA levels compared with untreated scRNA-transfected cells (p < 0.05; Fig. 5A), whereas transfection of the si#2 RNA duplex caused a 41 ± 0.31% decrease in PPG mRNA levels compared with untreated scRNA-transfected cells (p < 0.05; Fig. 5B). Incubation with insulin had no further effect on PPG mRNA levels in FoxO1-silenced cells. Because the anti-FoxO1 si#2 RNA duplex was the most efficient at silencing FoxO1 expression, this siRNA duplex was used in all subsequent experiments.

Binding of FoxO1 to the Preproglucagon Promoter—Given the apparent dependence of regulated PPG expression on FoxO1, we next sought to determine whether FoxO1 might bind directly to the PPG promoter. Examination of the rat promoter sequence revealed three putative FoxO1 binding sites that differed from the daf-16 consensus sequence (5'-TTGTTTAC) by only one nucleotide in each case (Fig. 6A and Table 1).

In vitro DNA binding studies were performed by EMSAs using double-stranded oligonucleotides corresponding to the three putative FoxO1 binding sites. EMSA analysis (Fig. 6B) revealed that a protein-DNA complex formed when aTC1–9 nuclear extracts were incubated with the site 3 (GL3) oligonucleotide, which could be competed out using unlabeled oligonucleotide and abolished by preincubation with anti-FoxO1 antibody. A further complex of lower molecular mass was observed upon incubation with the site 1 (GL1) oligonucleotide. Binding at this site was stronger than that observed at the other sites and could not be competed out using the FoxO1 antibody. At site GL2, two complexes were observed that appeared to be of similar molecular mass to the complexes observed at the other sites. The higher molecular mass complex could not be abolished with the FoxO1 antibody in this case (Fig. 6B). Furthermore, preincubation with an anti-Foxa2 antibody had no effect on the binding complexes formed at any of the sites studied (data not shown). To test the binding of FoxO1...
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to the GL3 site in a heterologous system, HEK293 cells were transfected with the plasmid pEGFP-FoxO1. As expected, the expression of FoxO1 protein was revealed by Western (immuno-) blotting only in the nuclear fractions of transfected HEK cells (Fig. 7A). In vitro binding to the GL3 site of FoxO1 from these nuclear extracts was measured by EMSA and compared with the binding in untransfected cells. In extracts from untransfected HEK cells, only the formation of an unspecific complex was observed, as shown by the inability of this complex to be competed out upon the addition of excess unlabeled GL3 oligonucleotide. In contrast, a complex was observed in FoxO1-transfected HEK cells that was competed out using unlabeled oligonucleotide and blocked by incubation with FoxO1 antibody (Fig. 7B). Correspondingly, αTC1–9 cells transfected with the anti-FoxO1 si#2 RNA for 48 h incubation showed a 71.4 ± 0.78% reduction in complex formation at site GL3 compared with either untransfected cells or cells transfected with a scRNA duplex (Fig. 7C). Apparent FoxO1 binding to the GL3 site was unaffected by the addition of an unrelated, unlabeled oligonucleotide (USF) or by preincubation with an irrelevant (anti-ChREBP) antibody (Fig. 7D). Finally, mutation of site GL3 abolished complex formation (Fig. 7D).

To determine whether FoxO1 was bound to any of the three putative forkhead binding sites in intact α cells, primers were next designed to allow amplification of the regions corresponding to sites GL1, GL2, and GL3 after ChIP assay (Fig. 8A). Whereas PCR analysis of input samples (IgG controls) revealed a product for all three sites, as expected, analysis of FoxO1 antibody-immunoprecipitated samples revealed a product with only one of the primer sets, corresponding to site GL3. Immunoprecipitation with an unrelated (anti-ChREBP) antibody revealed no PCR amplification products (Fig. 8A).

ChIP analysis was then used to determine the effect of insulin and glucose concentrations on the in vivo binding status of FoxO1 to the GL3 site (Fig. 8B). IgG input controls revealed PCR products regardless of the glucose or insulin concentration tested. However, for FoxO1 antibody-immunoprecipitated samples, whereas a product was detected for cells incubated at 0.5 mmol/liter glucose, none was detected in cells incubated at 0.5 mmol/liter glucose plus 17 nmol/liter insulin, 10 mmol/liter glucose, or 10 mmol/liter glucose plus 17 nmol/liter insulin. Quantification of the PCR products as performed by ImageQuant™ is shown in Fig. 8C.

siRNA against FoxO1 Abolishes Inhibition of the Preproglucagon Promoter by Insulin—We next used promoter/reporter assays to determine the importance for the regulation of PPG promoter activity of FoxO1 binding to the GL3 site. αTC1–9 cells were transfected simultaneously with either the scRNA duplex or anti-FoxO1 siRNA duplex for 48 h and either an empty plasmid (data not shown) or the pPPG(2.3)-LUCp plasmid, which included sites GL1–3 described above. A plasmid
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expressing *R. reniformis* luciferase under cytomegalovirus promoter control, pCMV-LLUC<sub>RR</sub> was used for normalization. Cells were either left untreated in 0.5 mmol/liter glucose or treated with 10 mmol/liter glucose or 17 mmol/liter insulin at 0.5 mmol/liter glucose for 6 h, as shown. Elevated insulin and glucose concentrations caused a significant decrease in the activity of pPPG(2.3)-LLUC<sub>FF</sub> when co-transfected with the scRNA duplex (p < 0.05 in both cases). However, after co-transfection with the anti-FoxO1 si#2 RNA duplex, the inhibitory effect of insulin was abolished, and a significant increase (72.9 ± 5.3%, p < 0.05) in apparent PPG promoter activity was now observed upon treatment with insulin (Fig. 9A). The effect of mutating site GL3 within the −2.3-kilobase PPG promoter was then studied. Introduction of reporter plasmid pPPG(2.3mut)-LUC<sub>FF</sub> with the scrambled RNA duplex caused an overall decrease in PPG promoter activity regardless of the conditions (p < 0.05 in all cases). When pPPG(2.3mut)-LUC<sub>FF</sub> was co-expressed with the anti-FoxO1 si#2 RNA, a further decrease in PPG activity was observed (p < 0.05 in all cases, Fig. 9A). Once again, a small increase due to insulin stimulation was observed when either pPPG(2.3mut)-LUC<sub>FF</sub> or anti-FoxO1 si#2 RNA was expressed (Fig. 9A). When a plasmid (pPPG(1.6)-LLUC<sub>FF</sub>) bearing a −1.6-kilobase PPG promoter fragment lacking the GL3 site was co-transfected with the scRNA duplex, insulin caused a 28.9 ± 2.6% (p < 0.05) decrease in promoter activity. By contrast, elevated glucose (10 versus 0.5 mmol/liter glucose) or 17 mmol/liter insulin (at 0.5 mmol/liter glucose) caused 30.2 ± 5.1 and 37.2 ± 2.3% decreases in promoter activity, respectively (p < 0.05 in both cases) after co-transfection with the anti-FoxO1 siRNA duplex (Fig. 9B). Finally, when a FoxO1-expressing plasmid, pEGFP-FoxO1, was co-transfected with the pPPG(2.3)-LLUC<sub>FF</sub> and pCMV-LLUC<sub>RR</sub> constructs, there was an increase in basal promoter activity observed in 0.5 mmol/liter glucose compared with control (empty vector-transfected) cells (15 ± 0.1%; Fig. 9C). Increased glucose concentrations or the addition of insulin caused a significant decrease in promoter activity with a more marked effect of insulin (47.56 ± 3.5, 68.29 ± 3.5, and 74.39 ± 3.3% for 1.0, 5.0, or 17 mmol/liter insulin, respectively) compared with that of elevated glucose concentrations (43.9 ± 3.77% inhibition at 10 mmol/liter glucose). After the overexpression of FoxO1, the most marked effect on the promoter was again found with elevated insulin concentrations (55.67 ± 3.6, 70.10 ± 3.6, and 77.32 ± 3.5% for 1.0, 5.0, and 17 mmol/liter respectively), with elevated glucose causing a 36.08 ± 4.0% decrease in promoter activity (Fig. 9C).

**Anti-FoxO1 siRNA Abolishes Insulin-regulated Glucagon Secretion**—The importance of FoxO1 expression for glucose- or insulin-regulated glucagon secretion was measured using αTC1–9 cells transfected with either the scRNA duplex or anti-FoxO1 si#2 RNA duplex. Cells were either left untreated, incubated for 30 min in 0.5 mmol/liter glucose, or treated with 10 mmol/liter glucose or 1.0, 5.0, or 17 mmol/liter insulin in 0.5...
mmol/liter glucose as shown. Release of glucagon from scRNA-transfected cells was inhibited by elevated glucose or insulin concentrations ($p < 0.05$ in each case; Fig. 10). By contrast, in cells treated with anti-FoxO1 si#2 RNA, the inhibitory effect of insulin was abolished (Fig. 10).

**DISCUSSION**

Expression of FoxO1 in Isolated Mouse Islet Cells—The principal aim of the present study was to determine the role of FoxO1 in the regulation by insulin and glucose of PPG gene expression. Whereas the role of FoxO1 in the pancreatic $\beta$-cell has been the subject of several previous investigations (15, 23), its presence and importance in $\alpha$ cells has remained unclear. Indeed, previous reports have suggested that FoxO1 expression may be restricted to $\alpha$ cells in the adult islet (15). In contrast, we show here using immunocytochemistry that FoxO1 protein is present in both $\beta$ and $\alpha$ cells from adult mouse islets and dissociated islet cells. As in a previous study, FoxO1 immunoreactivity was found to be nuclear in some cells while showing a more diffuse pattern in other cells (23). We go on to show that,
within αTC1–9 cells, FoxO1 undergoes subcellular translocation after stimulation with insulin but not elevated glucose concentrations, whereas either insulin or high glucose suppressed binding of FoxO1 to the PPG promoter (see below).

To determine the significance of these changes for the regulation of the PPG gene, we next deployed an RNA interference strategy. The level of PPG mRNA was found to decrease after FoxO1 silencing, suggesting that FoxO1 acts in a positive fashion on the expression of the PPG promoter. Indirect evidence for a role or FoxO1 in the α cells comes from studies of pre-morbid diabetic Irs2−/− mice. These animals display a disruption in islet cell morphology, with a severe decrease in the number of β cells and an increase in α cells. Haplo-insufficiency of the 3′ lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) in Irs2−/− mice increases the phosphorylation of Akt and, in turn, the phosphorylation and nuclear exclusion of FoxO1, leading to a restoration of β-cell mass and a reduction in α-cell mass (35). These data, therefore, suggest that FoxO1 acts negatively on the development of pancreatic β cells and, conversely, acts positively in the development of α cells, observations supported by the present results in clonal α cells.

Physical Interaction of FoxO1 with the Preproglucagon Promoter—

We used here both EMSA and chromatin immunoprecipitation to show that FoxO1 binds to a putative daf-16 consensus sequence within the PPG promoter in vitro and in living cells. There have been several previous studies that have examined the role of forkhead proteins on the PPG gene. Although no binding of Foxa3 (hepatocyte nuclear factor-3β) to the PPG gene G2 element using islet InR1-G9 extracts was detected, transfection of adenoviral Foxa3 into heterologous BHK cells caused enhanced G2 binding activity and increased PPG promoter activity in a G2-dependent manner. However, no increase in activity with adenoviral transduced GLUTag cells was observed. It, therefore, appears that although Foxa3 has the ability to bind to and activate G2-dependent transcription within fibroblasts, it does not regulate PPG gene expression in cell lines that express the endogenous PPG gene in vitro (36).

Recently, a novel Foxa site (Foxa site-A) within the PPG gene has been described (37). The previously mentioned rat G2 enhancer element, which binds Foxa proteins, is not conserved in humans, with the human G2 site lacking basal activity (37). Sharma et al. (37) also describe a novel Foxa binding site within the PPG promoter that is conserved between rat, mouse, and humans and mediates the activation of the glucagon gene by Foxa proteins and confers cell-specific promoter activity in glucagon-producing cells. This site preferentially binds Foxa1 (hepatocyte nuclear factor-3β) in nuclear extracts (37), offering an explanation as to why there is a decrease in glucagon gene expression in Foxa1-deficient mice (14, 15, 23).

We first analyzed the binding of FoxO1 to three putative daf-16 sites, termed GL1, GL2, and GL3, which are conserved in the rat, mouse, and human PPG promoters (Table 1) by EMSA. We provide direct evidence that the GL3 site is able to bind FoxO1 both in vitro and in living cells and that mutation of this site leads to dysregulation of the PPG promoter by insulin but not glucose. Binding at this site was also observed for FoxO1

![FIGURE 10. Effects of FoxO1 silencing on regulated glucagon secretion.](image-url)
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overexpressed in a non-α-cell line, HEK293. It is likely that this site does not bind Foxa2 because EMSA analysis in αTC1–9 only showed one protein-DNA complex being formed, which could be abolished using an anti-FoxO1 antibody. Furthermore, there was no effect of the addition of a Foxa2 antibody on complex formation (not shown). Furthermore, the complex formed at the GL1 was unaffected by incubation with anti-FoxO1 antibody. However, whether this site is also able to bind other forkhead family members is presently unclear. At site GL2, two protein complexes were observed. Each appeared to be of similar molecular mass to those found within the other sites. Again, these complexes were unaffected by anti-FoxO1 antibody and, thus, are unlikely to involve FoxO1.

Direct evidence for an interaction of FoxO1 at the GL3 site in situ was provided by ChIP assay. PCR analysis showed that only the GL3 site gave a PCR product after FoxO1 immunoprecipitation. Moreover, it was found that either insulin or elevated glucose concentrations blocked this binding. Interestingly, although insulin, but not glucose, caused a cellular translocation of FoxO1 from the nuclear to the cytosol, either agent abolished FoxO1 binding at the GL3 site. Thus, distinct mechanisms appear to be responsible for the effects of each stimulus on the interaction of FoxO1 with the PPG promoter.

Consistent with the above binding data, we also show here that an anti-FoxO1 siRNA abolishes the ability of insulin to decrease PPG promoter activity. Moreover, the results obtained with a PPG-expressing plasmid containing a mutation at the GL3 binding site (pPPG(2.3mut)-LUCFF) further supported the finding that FoxO1 acts positively on the PPG promoter by causing a substantial decrease in PPG activity while abolishing the negative effect of insulin. Nevertheless, we note that regulation by insulin, although not glucose, of PPG promoter activity was still observed in a construct containing only a 1.6-kilobase promoter fragment (pPPG(1.6)-LUCFF) and, therefore, lacking the distal FoxO1 binding site GL3. It is, therefore, evident that other elements are capable of conferring transcriptional responsiveness to this truncated promoter. Because the response of this shortened promoter to insulin was unaffected by FoxO1 silencing (Fig. 9B), it is also evident that other transcription factors, perhaps including Foxa1–3, must convey the responses to insulin in this setting. However, the abolition of changes in the PPG mRNA level in response to insulin when FoxO1 was silent (Fig. 5) points to the distal GL3 site and FoxO1 as a principal mediator of the insulin effect of the endogenous gene. It is, thus, conceivable that the proximal (GL1/GL2 containing) and distal (GL3 containing) regions of the promoter may physically interact to provide cooperative regulation of transcription. This is in agreement with previous studies that have suggested that insulin responsiveness of glucagon gene expression is dependent on interactions between proximal and distal elements (38), possibly through the formation of a DNA looping structure as found in previous studies (39, 40). Finally, expression of a FoxO1.eGFP chimera increased the basal activity of the PPG promoter. As expected, the effects of insulin on PPG promoter activity were still observed in cells overexpressing wild-type FoxO1, presumably reflecting preserved insulin-independent phosphorylation of FoxO1 and shuttling to the cytosol.

Might FoxO1 silencing also affect the expression of other α-cell genes and, thus, the overall responsiveness to insulin and/or glucose? Consistent with this view, silencing of FoxO1 blocked insulin inhibition of glucagon release. The identity of the gene products whose altered expression contributes to this phenotypic change will require further investigation in future.

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