FoxO Feedback Control of Basal IRS-2 Expression in Pancreatic β-Cells Is Distinct From That in Hepatocytes

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OBJECTIVE—Appropriate regulation of insulin receptor substrate 2 (IRS-2) expression in pancreatic β-cells is essential to adequately compensate for insulin resistance. In liver, basal IRS-2 expression is controlled via a temporal negative feedback of sterol regulatory element–binding protein 1 (SREBP-1) to antagonize transcription factors forkhead box class O (FoxO)1/FoxO3a at an insulin response element (IRE) on the IRS-2 promoter. The purpose of the study was to examine if a similar mechanism controlled IRS-2 expression in β-cells.

RESEARCH DESIGN AND METHODS—IRS-2 mRNA and protein expression, as well as IRS-2 gene promoter activity, were examined in isolated rat islets. Specific transcription factor association with the IRE on the IRS-2 promoter was examined by chromatin immunoprecipitation (ChIP) assay, and their nuclear translocation was examined by immunofluorescence. A direct in vivo effect of insulin on control of IRS-2 expression in liver and pancreatic islets was also investigated.

RESULTS—In IRS-2 promoter-reporter assays conducted in isolated islets, removal of the IRE decreased basal IRS-2 promoter activity in β-cells up to 80%. Activation of IRS signaling in isolated rat islets by insulin/IGF-I (used as an experimental in vitro tool) or downstream constitutive activation of protein kinase B (PKB) significantly decreased IRS-2 expression. In contrast, inhibition of phosphatidylinositol 3-kinase (PI3K) or PKB significantly increased IRS-2 levels in β-cells. ChIP assays indicated that transcription factors FoxO1 and FoxO3a associated with the IRE on the IRS-2 promoter in β-cells in a PI3K/PKB–dependent manner, whereas others, such as SREBP-1, the transcription factor binding to immunoglobulin heavy chain enhancer 3′, and the aryl hydrocarbon receptor nuclear translocator (ARNT), did not. However, only FoxO3a, not FoxO1, was capable of driving IRS-2 promoter activity via the IRE in β-cells. In vivo studies showed insulin was able to suppress IRS-2 expression via activation of SREBP-1 in the liver, but this mechanism was not apparent in pancreatic islets from the same animal.

CONCLUSIONS—The molecular mechanism for feedback control of IRS signaling to decrease IRS-2 expression in liver and β-cells is quite distinct, with a predominant role played by FoxO3a in β-cells. *Diabetes* **60:2883–2891, 2011**

See accompanying original article, p. 2892.

The onset of type 2 diabetes is marked by failure of the functional pancreatic β-cell mass to compensate for inherent insulin resistance (1). As such, type 2 diabetes is a disease of insulin insufficiency, and a means to preserve sufficient functional β-cell mass is a reasonable therapeutic approach to treat the condition. However, there is limited information on mechanisms that control β-cell survival, and few molecular targets have yet emerged. One exception is insulin receptor substrate 2 (IRS-2), which is essential for β-cell survival (2–4). When IRS-2 expression is specifically increased in β-cells, it is protective, maintains adequate functional β-cell mass, and avoids the onset of diabetes (5–7). However, these "proof of principal studies" using artificial transgenic means to raise IRS-2 expression in β-cells give little insight as to how IRS-2 expression is regulated endogenously, and such knowledge could reveal a more practical therapeutic means to specifically increase IRS-2 expression in β-cells.

IRS-1 and IRS-2 are adaptor molecules that interface between insulin and/or IGF-I receptors and two major downstream signaling pathways: *1*) the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) pathway and *2*) the growth factor receptor-bound protein 2/mammalian Son of Sevenless/Ras/Raf-1/extracellular signal–related kinase 1 and 2 mitogen-activated protein kinase pathway (8,9). In pancreatic β-cells, IRS-2 and downstream signaling pathways are key to controlling normal β-cell growth and survival (8). IRS-2 turnover is unusually rapid in β-cells, but this is balanced by its dynamically regulated expression (10). The major regulation of IRS-2 expression in β-cells is mediated at the transcriptional level (10), which can be specifically augmented in response to glucose (10,11) and elevation of [cAMP]$_i$ (12). However, there is little mechanistic information as to how basal expression of IRS-2 is controlled in β-cells at the molecular level.

In liver, transcription factors forkhead box class O (FoxO)1 and FoxO3a are major contributors to specific control of basal IRS-2 gene transcription (13,14), assisted by the transcription factor binding to immunoglobulin heavy chain enhancer 3′ (TFE3) (15). Insulin suppresses transcription of the IRS-2 gene in hepatocytes through an insulin response *cis*-element (IRE) on the IRS-2 gene promoter that is a FoxO binding site (13). Activation of insulin signal transduction via IRS-2/PI3K/PKB leads to PKB-mediated phosphorylation of FoxO1 that decreases DNA binding and excludes it from the nucleus (13). In parallel, insulin-mediated activation of sterol regulatory element–binding proteins (SREBPs), especially SREBP-1c, suppresses IRS-2 gene transcription by blocking the FoxO/TFE3 complex's access to the IRS-2 gene promoter's IRE (14). This represents a temporal feedback mechanism of IRS-2 signaling by

an insulin-induced delayed reduction of IRS-2 expression (13–16).

It is unclear whether the mechanism for control of basal IRS-2 gene transcription in liver is present in β-cells. Indeed, there is reason to believe this might not be so, since regulation and composition of certain transcriptional regulatory complexes varies among cell types. In this study, we found that IRS-2 expression in β-cells is not regulated by SREBP-1c/TFE3/FoxO as in liver but predominately by FoxO3a.

RESEARCH DESIGN AND METHODS

Materials. Antibodies to the PI3K p83 and IRS-2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against FoxO1 and FoxO3a were acquired from Cell Signaling Technology, Inc. (Danvers, MA); those against SREBP-1 were from Thermo Scientific (Fremont, CA); and those against aryl hydrocarbon receptor nuclear translocator (ARNT) and TFE3, as well as control rabbit IgG, were from Santa Cruz Biotech Inc. (Santa Cruz, CA). IGF-I was obtained from GroPep Ltd. (Adelaide, Australia), and insulin came from Eli Lilly (Indianapolis, IN). LY294002 was acquired from Promega (Madison, WI), and adenoviruses expressing FoxO3a (AdV-FoxO3a-WT and AdV-FoxO3a-CA) were from Vector Biolabs (Philadelphia, PA,). Unless otherwise stated, all other chemicals were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).
Pancreatic islet isolation. Pancreatic islets were isolated from adult Wistar rats or C57Blk6/6 J mice from Charles River Laboratories (Wilmington, MA) as previously described (17). Isolated human islets from normal cadavers were provided by the Islet Cell Transplantation Unit at the University of Chicago. All animal care, use, and experiment protocols were approved by the University of Chicago Institutional Animal and Use Committee. The pancreatic β-cell line INS-1 was maintained as previously described (18).
Adenovirus vectors. Adenovirus vectors were generated, amplified, and purified as previously described (19). The human IRS-2 promoter constructs (⁻1051 to ⁻116 relative to the translation start site) were provided by Dr. Joseph Goldstein, University of Texas Southwestern Medical Center, Dallas, Texas, as described previously (13). These IRS-2 promoter regions were linked to a Firefly luciferase (FLuc) reporter and recombinant adenoviruses were generated (AdV-WT-IRS2-FLuc and AdV-Mut-IRS2-FLuc) (19). A control reporter adenovirus, where the thymidine kinase (TK) promoter was driving the *Renilla* luciferase (AdV-TK-RLuc), was also generated (20). The TK promoter–driven RLuc activity is readily detectable but not responsive to glucose, cAMP, Ca^{2+}, or IRS signaling in β-cells and, thus, serves as an excellent control reference standard.
Immunoblot and immunohistochemical analyses. Immunoblot and immunohistochemical analyses were conducted as previously outlined (5,6,19).
Luciferase assay. FLuc and RLuc assays were performed as previously described (20). IRS-2 promoter–driven FLuc activity was expressed as normalized to control TK promoter–driven RLuc activity in the same sample.
Real-time fluorescence-based RT-PCR. Real-time fluorescence-based quantitative RT-PCR (qRT-PCR) was conducted as previously described (10). Total RNA was extracted from rat islets or INS-1 cells using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and quantified by Spectrophotometer Nanodrop 2000 (Thermo Scientific). IRS-2 mRNA expression relative to PI3K p85 mRNA was quantified using a Power SYBR Green RNA-to-C_T 1-Step Kit in StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). To compare relative expression of several mRNAs, reverse transcription of RNA extracted from rat islets fasted for 16 h was performed using an iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA). Each cDNA was amplified using PCR SuperMIX from Invitrogen (Carlsbad, CA) and specific primer pairs for each gene. Amplified cDNA was isolated by 2% agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit from Qiagen. Standard curves for each cDNA were generated for a comparative expression among different mRNAs. Reverse transcript of extracted RNA was performed as for the standard sample preparation, the cDNA for the samples and standards of each mRNA were amplified using Fast SYBR Green Master Mix from Applied Biosystems, and the copy number of each product was calculated. Data are expressed as ratio to the copy number of FoxO1 mRNA expression. The sequences of the specific primer pairs are described in Supplementary Table 1.
Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit (Active Motif) from Millipore (Danvers, MA). INS-1 cells were cross-linked in 1% formaldehyde for 10 min prior to lysis. Nuclear pellets were isolated and then sheared by sonication (12 × 30-s pulses at 4°C) using a Bioruptor UCD-200 TM from Diagenode Inc. (Sparta, NJ). Next, 100 μg DNA was precleared with 25 μL of protein A agarose/salmon sperm DNA for 1 h at 4°C and incubated overnight

at 4°C with ~2 μg of control rabbit IgG or immunoprecipitated with antibodies against FoxO1, FoxO3a, SREBP-1, ARNT, or TFE3. Immune complexes were isolated at 4°C with protein A agarose/salmon sperm DNA. Bound DNA fragments were eluted and then purified by using QIAquick PCR Purification Kit from Qiagen. DNA isolated from the ChIP and "input" corresponding to DNA aliquot not submitted to immunoprecipitation were then analyzed by PCR for the region of the IRS-2 promoter containing the IRE, SREBP-1, TFE3, and ARNT binding sites with the following primers: 5′-GTAACGCAGAGTCAC-GTGTTGT-3′ and 5′-AACGACGACCTGGGTTTGT-3′. Amplified products were analyzed by agarose gel electrophoresis and qRT-PCR.
Statistical analysis. Data are expressed as the mean ± SE with number of experiments indicated. Statistical significance is indicated by an unpaired two-tailed Student t test. A P value ≤0.05 was considered statistically significant.

RESULTS

Feedback regulation of IRS signaling by inhibiting basal IRS-2 expression in β-cells. IRS-2 signaling is critical for β-cell survival and function (2,7), but the biologically relevant ligand/receptor interaction upstream of IRS-2 in β-cells in vivo remains unclear. It is contentious that this is an insulin/insulin receptor engagement on β-cells (21–25) and unlikely to be an autocrine feedback effect of secreted insulin (24,25). An IGF-I/IGF-I receptor interaction is also questionable (22,23,26). As such, the physiological means by which IRS-2 signal transduction is activated in primary β-cells in vivo remains an open question. Nonetheless, one can use the combination of a high concentration of insulin and IGF-I as an experimental tool to activate IRS-2 signaling in β-cells in vitro. Here, incubation of isolated rat islets and INS-1 cells with 100 nmol/L insulin plus 10 nmol/L IGF-I significantly decreased the expression of IRS-2 mRNA and protein, compared with that at basal 3 mmol/L glucose, by ~40% over a 6-h period ($P ≤ 0.05$) (Fig. 1A). In contrast, inhibition of the PI3K/PKB branch of IRS signaling using the PI3K inhibitor LY294002 (50 μmol/L) significantly increased IRS-2 mRNA and protein expression from three- to fivefold in rat islets and INS-1 cells ($P ≤ 0.01$) (Fig. 1A). IRS-2 expression in β-cells is specifically regulated by glucose (10). However, the effect of PI3K inhibition on increasing IRS-2 expression was independent of glucose, with LY294002 significantly increasing IRS-2 mRNA and protein expression in rat islets at both basal 3 mmol/L and stimulatory 15 mmol/L glucose concentrations (Fig. 1B). Inhibition of the extracellular signal–related kinase 1/2 branch of IRS-2 signaling by inhibition of MEK1 with either 50 μmol/L PD98059 or 10 μmol/L U0126 or by inhibition of mammalian target of rapamycin with 10 nmol/L rapamycin had no effect on IRS-2 mRNA (data not shown) or protein levels in isolated rat islets (Supplementary Fig. 1). Downstream of PI3K, the negative regulation of IRS-2 expression in rat islets appeared to be mediated by PKB (Fig. 1C). Adenoviral-mediated expression of wild type (WT) PKB-1 in isolated rat islets specifically decreased IRS-2 protein expression at both basal 3 mmol/L and stimulatory 15 mmol/L glucose levels relative to that in uninfected islets or the FLuc adenoviral-infected control islets (Fig. 1C). Adenoviral-mediated expression of constitutively activated (CA) PKB-1 in islets further depleted IRS-2 protein expression. Conversely, introduction of a kinase-dead PKB-1 variant specifically augmented IRS-2 protein expression independently of the glucose concentration (Fig. 1C).
PI3K/PKB negative regulation of IRS-2 expression in β-cells is via an IRE in the IRS-2 gene promoter. In hepatocytes, basal regulation of IRS-2 expression is mediated via an IRE in the IRS-2 gene promoter that is a conserved FoxO1 binding site at ⁻574 to ⁻568 in the human IRS-2 promoter where ⁺1 is the "A" of the ATG start codon (13).

A

B

C
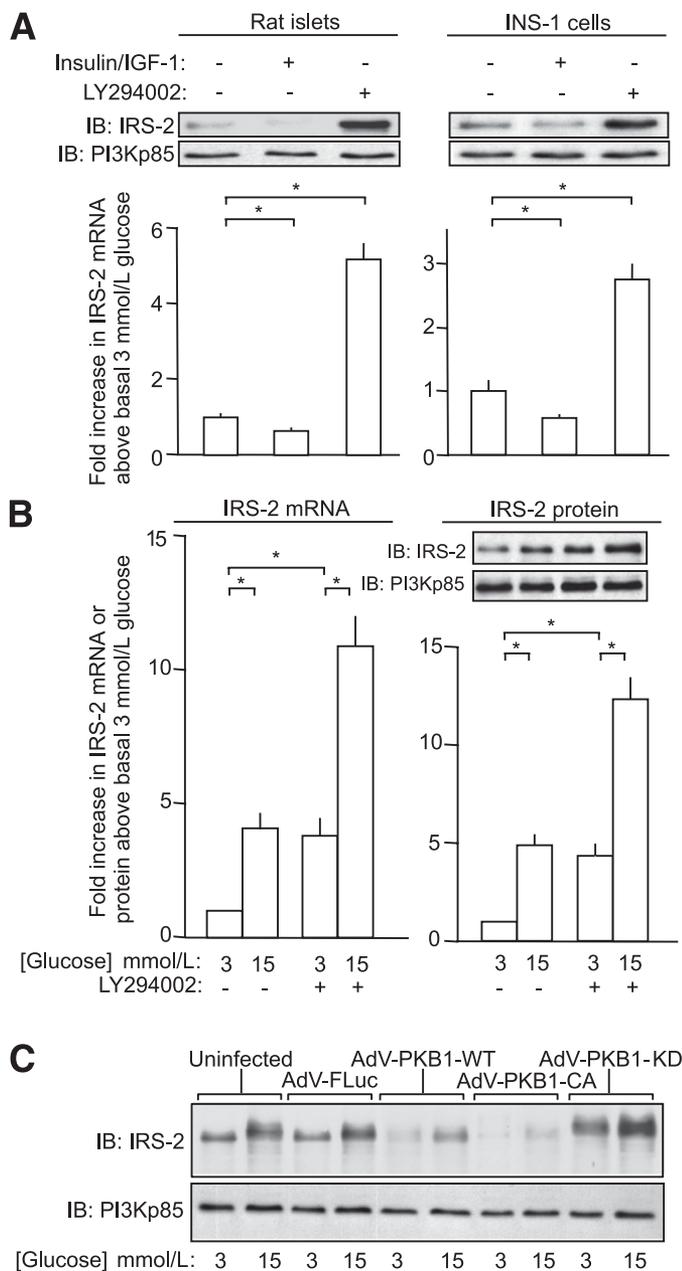
FIG. 1. Regulation of IRS-2 expression by IRS signaling in pancreatic islet β-cells. Isolated rat islets or INS-1 cells were incubated overnight at normal 5.6 mmol/L glucose and then incubated as indicated. Protein and mRNA expression of IRS-2 and PI3K p85 (control) were examined by immunoblot (IB) and qRT-PCR analyses, respectively, as outlined in RESEARCH DESIGN AND METHODS. ***A*: Isolated rat islets or INS-1 cells, as indicated, were incubated at basal 3 mmol/L glucose for 6 h in the presence or absence of insulin (100 nmol/L) plus IGF-I (10 nmol/L) or the PI3K inhibitor LY294002 (50 μmol/L). An example IB is shown for protein analysis and the qRT-PCR mRNA analysis results are mean ± SEM ($n \geq 4$). *B*: Isolated rat islets were incubated at basal 3 mmol/L or stimulatory 15 mmol/L glucose concentrations for 6 h in the presence or absence of LY294002 (50 μmol/L). Results of the qRT-PCR analysis are mean ± SEM ($n \geq 4$) with an example IB shown. *C*: Isolated rat islets either uninfected or infected with adenoviruses expressing FLuc, WT, CA, or kinase-dead (KD) PKB-1 as indicated, then incubated for 6 h at either basal 3 mmol/L or stimulatory 15 mmol/L glucose. An example IB of three independent experiments is shown. The * indicates statistically significant difference ($P \leq 0.05$).**

We generated recombinant adenoviruses where a FLuc reporter was driven by the WT human ($^-$1051 to $^-$116) IRS-2 promoter (AdV-WT-IRS2-FLuc) or where the IRE had been mutated (from 5′-TGTTTTG-3′ to 5′-<u>AG</u>AT<u>C</u>TG-3′;

AdV-Mut-IRS2-FLuc). Isolated human, mouse, or rat islets, and INS-1 cells, were infected with either AdV-WT-IRS2-FLuc or AdV-Mut-IRS2-FLuc, together with the control reporter adenovirus (AdV-TK-RLuc). Adenovirally infected islets/INS-1 cells were then incubated for 6 h at basal 3 mmol/L glucose, and the IRS-2 promoter–driven FLuc activity was measured relative to control RLuc reporter activity. Mutation of the IRE specifically decreased IRS-2 gene promoter activity by 60–80% in isolated human, rat, or mouse islets ($P \leq 0.01$) and by ~75% in INS-1 cells ($P < 0.01$) (Fig. 2*A*). These data indicate the importance of the IRE in the IRS-2 promoter for driving basal IRS-2 gene transcription.

In similar experiments, AdV-WT-IRS2-FLuc/AdV-TK-RLuc–infected rat islets showed a significant inhibition of IRS-2 promoter activity in the presence of insulin/IGF-I and a twofold increase in the presence of LY294002 ($P \leq 0.05$) (Fig. 2*B*). This complemented observations for IRS-2 mRNA and protein levels in isolated islets (Fig. 1). However, in AdV-Mut-IRS2-FLuc/AdV-TK-RLuc–infected rat islets, mutation of the IRE in the IRS-2 promoter resulted in loss of IRS-2 promoter activity regulated by insulin/IGF-I and LY294002 (Fig. 2*B*). This indicates control of basal IRS-2 gene expression by IRS signaling is mediated via the IRE on the IRS-2 gene promoter in pancreatic islet β-cells.

FoxO1 and FoxO3a binding to the endogenous IRS-2 promoter IRE are regulated by IRS signaling in β-cells. Several transcription factors were predicted to bind in the region of the IRE on the IRS-2 gene promoter in addition to FoxO transcription factors (using rVista, available from http://rvista.dcode.org/), including SREBP-1c and TFE3, as well as the ARNT that has been linked to type 2 diabetes (27) (Fig. 3*A*). Relative mRNA expression of these transcription factors was examined in rat islets by qRT-PCR that were present at comparable levels, with the exception of FoxO4, which was negligibly expressed (Fig. 3*B*). ChIP assays were performed in INS-1 cells incubated for 6 h at basal 3 mmol/L glucose alone, with the addition of insulin/IGF-I or LY294002 to examine which of these factors were capable of associating to the IRE of the IRS-2 promoter. Only FoxO1 and FoxO3a were significantly bound to the IRE above that of the control (rabbit IgG) under basal conditions (Fig. 3*C*). In contrast, ARNT, SREBP-1, and TFE3 were not found to associate with this region of the IRS-2 promoter in β-cells (Fig. 3*C*), despite doing so in hepatocytes (13–15,27) (Supplementary Fig. 2). The association of FoxO1 and FoxO3a to the IRE of the IRS-2 promoter was decreased by insulin/IGF-I and increased by LY294002 at basal 3 mmol/L glucose (Fig. 3*C* and *D*), in correlation with the transcriptional control of basal IRS-2 expression previously observed (Figs. 1 and 2). Quantification of a series of ChIP analyses indicated that insulin/IGF-I decreased FoxO1 and FoxO3a association to the IRS-2 promoter's IRE by 75–80% ($P \leq 0.05$), and LY294002 increased this FoxO1 and FoxO3a association threefold ($P \leq 0.05$) (Fig. 3*D*). These data suggest that the IRS-2 transcriptional control in β-cells via an IRE in the IRS-2 promoter could be mediated by FoxO1 and/or FoxO3a, but unlike hepatocytes (14,15), SREBP-1 and TFE3 do not appear to be involved.

FoxO transcription factors are negatively regulated by PKB-mediated phosphorylation at three conserved Ser/Thr sites (16,28). This phosphorylation decreases FoxO DNA binding activity and promotes their export from the nucleus to the cytoplasm (29). FoxO1 and FoxO3a translocation was investigated in INS-1 cells by immunohistochemistry (Fig. 3*E*). It was found that FoxO3a was more prominently

A

B
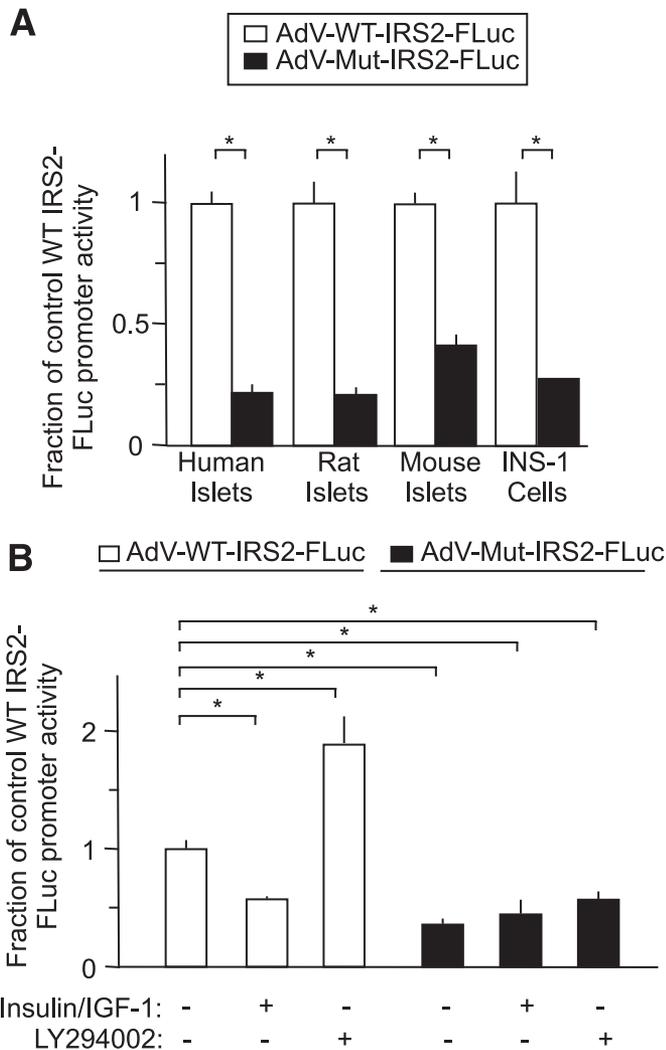
FIG. 2. The contribution of the IRS-2 promoter IRE to basal IRS-2 gene transcription. Isolated islets or INS-1 cells, as indicated, were infected with adenoviral vectors of either WT-IRS-2 promoter reporter (AdV-WT-IRS-2-FLuc) or IRE-mutated IRS-2 promoter reporter (AdV-Mut-IRS-2-FLuc) as indicated, together with the control TK promoter reporter (AdV-TK-RLuc). IRS-2 promoter activity was assessed as described in RESEARCH DESIGN AND METHODS. Results of relative luciferase activities are mean ± SEM ($n \geq 4$). A: IRS-2 promoter activity in isolated islet preparations from different species and INS-1 cells incubated for 6 h at basal 3 mmol/L glucose. B: IRS-2 promoter activity in isolated rat islets incubated at basal 3 mmol/L glucose plus or minus insulin (100 nmol/L)/IGF-I (10 nmol/L) or the PI3K inhibitor LY294002 (50 μmol/L). The * indicates statistically significant difference ($P \leq 0.05$).

located to the nucleus under basal 3 mmol/L glucose conditions compared with FoxO1, which was localized relatively more in the cytoplasm (Fig. 3E). Insulin/IGF-I further increased FoxO1 localization to the cytoplasm and promoted FoxO3a translocation out of the nucleus (Fig. 3E). Similar findings for insulin-mediated shuttling of FoxO1 recently have been shown in MIN6 cells (30). LY294002 enhanced a proportion of FoxO1 to the nucleus, whereas the majority of FoxO3a was located to the nucleus in the presence of LY249002 (Fig. 3E). These data suggest that in terms of nuclear translocation in β-cells, FoxO3a was more readily regulated by IRS signaling than FoxO1.

**FoxO3a is more effective than FoxO1 in driving IRS-2 gene expression in β-cells.** We examined if there was any difference between FoxO1 and FoxO3a in driving IRS-2 gene expression in β-cells. Recombinant adenoviruses were

generated to express WT FoxO1 (AdV-FoxO1-WT), WT FoxO3a (AdV-FoxO3a-WT), and green fluorescent protein (GFP) as a control (AdV-GFP). Equivalent levels of FoxO1 and FoxO3a were achieved using these vectors at a similar titer and dosage (Supplementary Fig. 3). In AdV-FoxO1-WT–infected rat islets incubated at basal 3 mmol/L glucose, IRS-2 mRNA levels were only modestly increased above AdV-GFP–infected control islets (Fig. 4A), and IRS-2 protein levels were not appreciably affected (Fig. 4B). In contrast, in AdV-FoxO3a-WT–infected rat islets, there was a threefold increase in basal IRS-2 mRNA levels ($P \leq 0.05$) (Fig. 4A) that reflected a similar increase in IRS-2 protein levels (Fig. 4B). In isolated islets infected with both AdV-FoxO1-WT and AdV-FoxO3a-WT, the basal IRS-2 mRNA (Fig. 4A) and protein (Fig. 4B) levels did not appreciably increase above that observed with increased FoxO3a expression alone, suggesting that this effect was mediated only by FoxO3a.

Recombinant adenoviruses were also generated to express constitutively active (CA) variants of FoxO1 (AdV-FoxO1-CA) and FoxO3 (AdV-FoxO3a-CA). These variants have Ser/Thr to Ala point mutations at the PKB phosphorylation sites and, thus, cannot be negatively regulated by PKB (16,28). In control AdV-GFP–infected rat islets, a significant reduction in basal IRS-2 mRNA levels was observed in the presence of insulin/IGF-I ($P \leq 0.05$) (Fig. 4C) that was reflected in an insulin/IGF-I–induced decrease in basal IRS-2 protein levels (Fig. 4D). In AdV-FoxO1-CA–infected rat islets, the insulin/IGF-I–induced decrease in basal IRS-2 mRNA (Fig. 4C) and protein (Fig. 4D) levels was partly overcome. In contrast, in AdV-FoxO3a-CA rat islets, the insulin/IGF-I–induced decrease in basal IRS-2 mRNA levels was bypassed to the extent that a threefold increase in basal IRS-2 mRNA levels was observed above that in control AdV-GFP rat islets in the presence of insulin/IGF-I ($P \leq 0.05$) (Fig. 4C). There was no additive effect of FoxO1-CA plus FoxO3a-CA to bypass insulin/IGF-I–induced inhibition of IRS-2 mRNA levels above that of FoxO3-CA alone (Fig. 4C). A similar effect of FoxO3a-CA to specifically overcome the insulin/IGF-I–induced decrease in basal IRS-2 protein levels was observed in isolated rat islets (Fig. 4D).

A direct effect of FoxO1 and FoxO3a to drive IRS-2 gene transcription via the IRS-2 gene promoter IRE was examined in isolated rat islets incubated at basal 3 mmol/L glucose using the IRS-2-FLuc/TK-RLuc promoter-reporter system. In AdV-WT-IRS2-FLuc/AdV-TK-RLuc–infected rat islets additionally infected with AdV-FoxO1-WT, a modest ~50% increase in specific WT IRS-2 promoter activity was observed above that of control islets infected with only AdV-GFP ($P \leq 0.05$) (Fig. 5). In contrast, in islets additionally infected with AdV-FoxO3-WT, a significant sixfold increase in specific WT IRS-2 promoter activity was observed above that of control islets ($P \leq 0.01$) (Fig. 5). In AdV-Mut-IRS2-FLuc/AdV-TK-RLuc–infected islets, where the IRE in the IRS-2 promoter-reporter vector has been mutated, the slight effect of FoxO1 to drive IRS-2 promoter activity was eliminated and the marked effect of FoxO3a inhibited ≥90% (Fig. 5). These data collectively indicate that FoxO3a drives basal IRS-2 gene transcription more effectively than FoxO1 in β-cells via the IRS-2 promoter's IRE.

Different molecular mechanisms control basal IRS-2 gene expression in liver and pancreatic islets. In liver, SREBP-1 suppresses IRS-2 gene transcription by preventing FoxO1 from binding to the IRE and its association with TFE3 under fed conditions (15,16). However, in this study, unlike hepatocytes, SREBP-1, TFE3, or ARNT did not associate with the IRE region of the IRS-2 promoter in islet
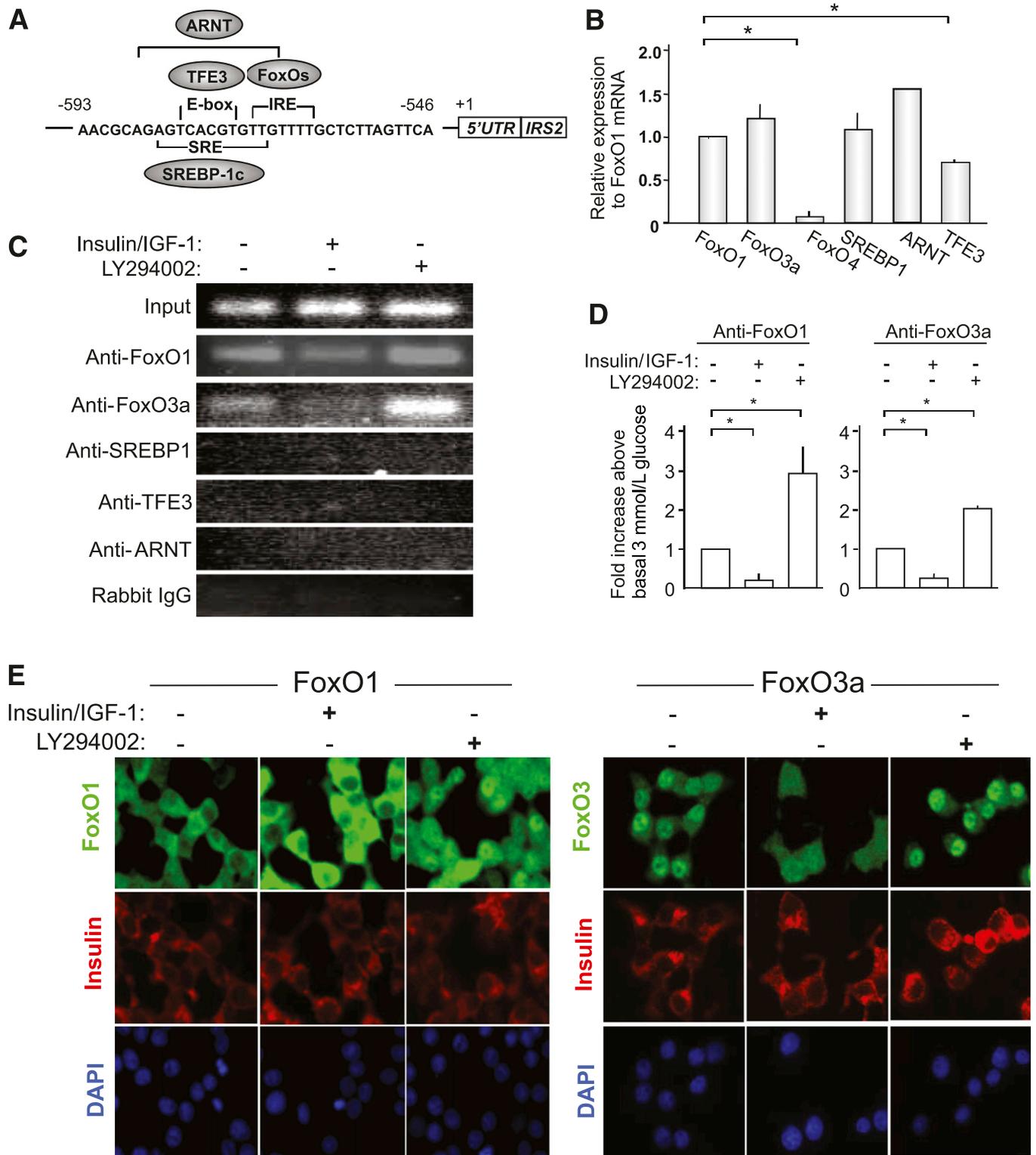
FIG. 3. IRS-2 signaling regulates FoxO1 and FoxO3a binding to the IRS-2 promoter IRE in β-cells. Isolated rat islets and INS-1 cells were examined for FoxO transcription factor expression association to the IRS-2 promoter's IRE and nuclear translocation. A: Schematic representation of candidate transcription factors predicted to associate to the $^-546$ to $^-593$ region of the IRS-2 promoter around the IRE, E-box, and SREBP response element (SRE). B: Relative mRNA expression of the candidate transcription factors in isolated rat islets was assessed by qRT-PCR. A mean ± SEM ($n \geq 4$) is shown. C and D: INS-1 cells were incubated at basal 3 mmol/L glucose for 6 h plus or minus insulin (100 nmol/L)/IGF-I (10 nmol/L) or PI3K inhibitor LY294002 (50 μmol/L) as indicated. ChIP assays for binding FoxO1, FoxO3a, SREBP-1, TFE3, or ARNT to the IRS-2 promoter IRE region were conducted (using rabbit IgG as a negative control) as described in RESEARCH DESIGN AND METHODS and analyzed by electrophoresis (C) or qRT-PCR (D). A mean ± SEM ($n \geq 3$) is shown (D). The * indicates statistically significant difference ($P \leq 0.05$). E: INS-1 cells were incubated at 3 mmol/L glucose for 6 h plus or minus insulin/IGF-I or LY294002 (50 μmol/L) and analyzed by immunofluorescence for FoxO1, FoxO3a, insulin, and DAPI to determine nuclear localization. Example images from three independent experiments are shown. (A high-quality digital representation of this figure is available in the online issue.)
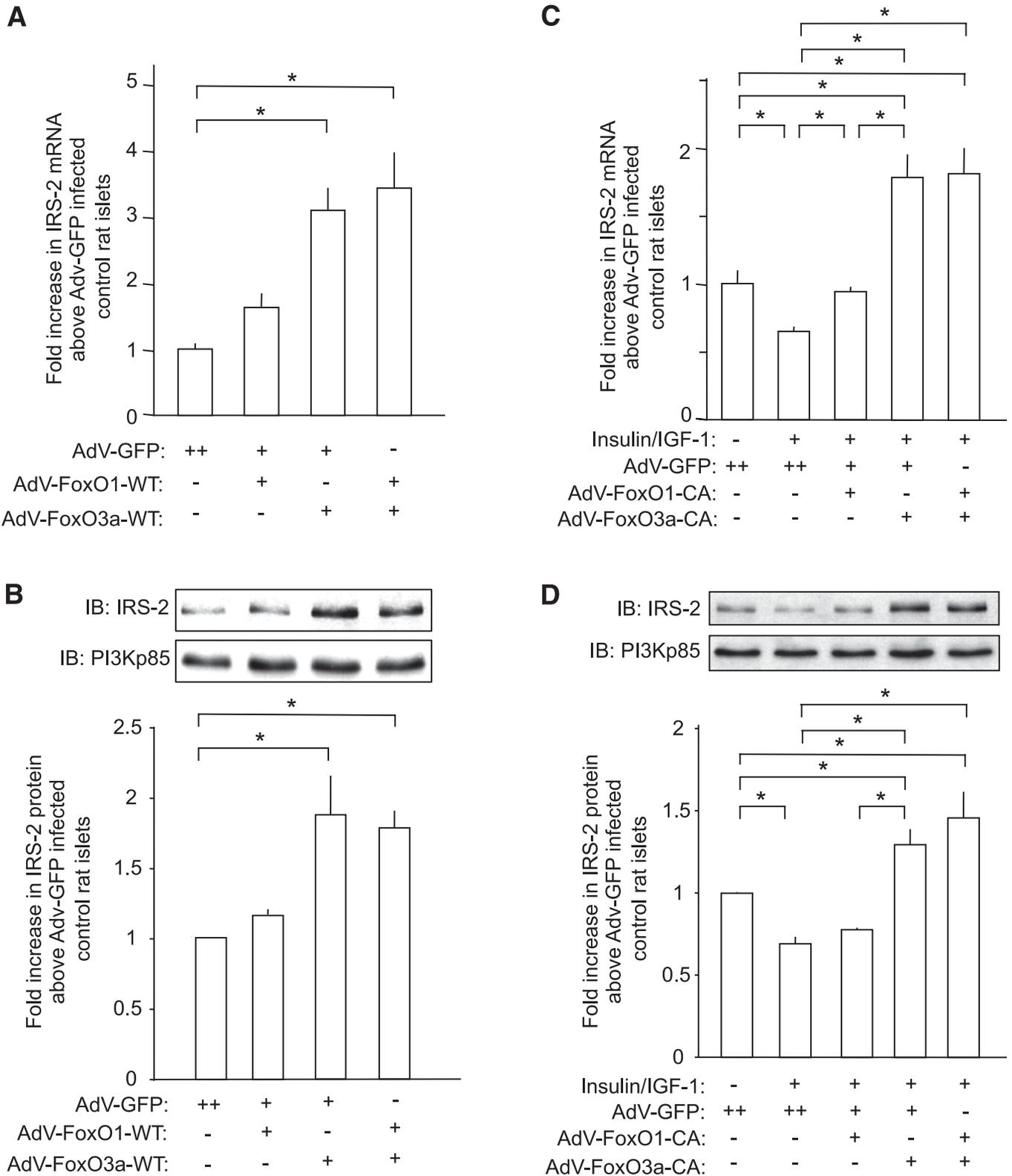
FIG. 4. FoxO3a predominately drives basal IRS-2 expression in β-cells. Adenovirally infected isolated rat islets were incubated as indicated, where "+" represents an equivalent dose of each adenoviral vector. The rat islet mRNA (A and C) and protein (B and D) levels of IRS-2 and PI3K p85 (control) were examined by qRT-PCR and immunoblot (IB) analyses, respectively, as described in RESEARCH DESIGN AND METHODS. The qRT-PCR data are shown as mean ± SEM ($n \geq 4$), and an example immunoblot for protein analysis is shown. A and B: Isolated rat islets were infected with adenoviruses expressing GFP (control), FoxO1-WT, FoxO3a-WT, or in combination as indicated at basal 3 mmol/L glucose for 6 h. C and D: Isolated rat islets were infected with adenoviruses expressing GFP, FoxO1-CA, FoxO3a-CA, or in combination as indicated at basal 3 mmol/L glucose for 6 h plus or minus insulin (100 nmol/L)/IGF-I (10 nmol/L). The * indicates statistically significant difference ($P \leq 0.05$).
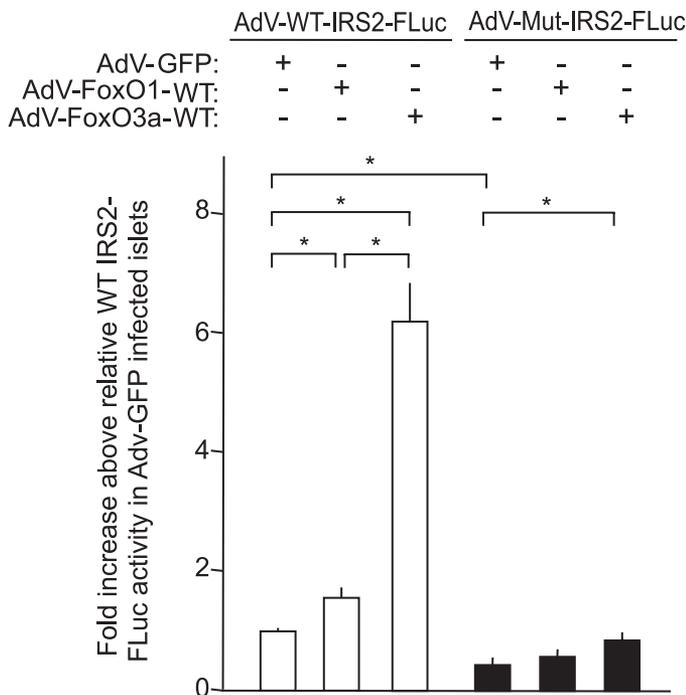
FIG. 5. FoxO3a predominately drives basal IRS-2 promoter activity β-cells. Relative FLuc activities mediated by either WT (AdV-WT-IRS-2-Luc) or IRE mutated (AdV-Mut-IRS-2-Luc) were measured as outlined in RESEARCH DESIGN AND METHODS in adenovirally infected isolated rat islets expressing GFP (AdV-GFP; control), WT FoxO1 (AdV-FoxO1-WT), or WT FoxO3a (AdV-FoxO3a-WT). Results are mean ± SEM ($n \geq 3$). The * indicates statistically significant difference ($P \leq 0.05$).

β-cells under conditions where regulated FoxO1 and FoxO3a association were observed (Fig. 3). This discrepancy was further investigated in vivo, in liver and islets isolated from the very same rats that were either starved overnight or allowed to feed ad libitum and subjected to an intraperitoneal insulin injection (0.75 mU/g body wt) 2 h prior. In liver, there was an ~80% decrease in IRS-2 protein expression in the plus insulin/fed condition, compared with that in the fasted condition (Fig. 6). This was correlated by a massive increase in expression and proteolytic activation of SREBP-1 in liver, a modest increase in TFE3 protein expression, and a reduction in FoxO1 protein levels, but no change in ARNT or FoxO3a protein expression levels relative to the PI3K p85 loading control in the liver plus insulin/fed condition compared with the fasted condition (Fig. 6). In contrast, in isolated islets from the same animal, IRS-2, SREBP-1, FoxO1, FoxO3a, TFE3, and ARNT protein levels did not change relative to the PI3K p85 under the plus insulin/fed condition compared with the fasted condition (Fig. 6). It should also be noted that relative protein expression levels of IRS-2, FoxO1, FoxO3a, and ARNT were higher in islets compared with liver, and SREBP-1, TFE3, and PI3K p85 were lower (Fig. 6). Indeed, to make sure that SREBP-1 was detectably present in islets, the liver X receptor agonist TO-901317 was used as a positive control to increase SREBP-1 expression (31). Although TO-901317 instigated a specific increase in the precursor form of SREBP-1 protein in rat islets, there was no change in proteolytic cleavage activation of SREBP-1 or basal IRS-2 protein expression (Supplementary Fig. 4). These data collectively indicate that there is different regulation of SREBP-1 expression/activation between islets and liver, and SREBP-1 does not participate in the regulation of IRS-2

expression in islet β-cells. As such, the molecular mechanism behind regulation of IRS-2 gene transcription is distinct in these different cell types in vivo.

DISCUSSION

Control of basal IRS-2 gene transcription is critical to normal β-cell function (2,8). However, there has been little mechanistic insight into the regulation of IRS-2 expression in β-cells other than assumed analogies to that in other cell types. In liver, basal IRS-2 gene transcription is mostly driven by FoxO1 and FoxO3a via the IRE in the IRS-2 promoter, assisted by TFE3 (13–16). When IRS signal transduction is activated in hepatocytes by insulin, downstream activation of PKB leads to phosphorylation inactivation of FoxO1 and FoxO3a and induction of SREBP-1 that blocks further transcriptional activity of FoxO1 and FoxO3a at the IRE (13–15). This, in turn, lowers IRS-2 gene expression and acts as a delayed negative feedback loop to dampen IRS-2 signaling (16).

In β-cells, the IRE on the IRS-2 gene promoter is also critical, accounting for as much as 80% of basal IRS-2 expression control. But unlike hepatocytes, SREBP-1 is not involved in negative regulation of basal IRS-2 expression in β-cells. This is reflected by a marked in vivo insulin-induced upregulation of SREBP-1 expression and activation in liver but not in pancreatic islets of the same animal. This further indicates the lack of a direct effect of insulin on β-cells in vivo (25) and that although certain members of the FoxO transcription factor family are involved in control of basal IRS-2 expression in β-cells, the mechanism is quite different from that in hepatocytes. In β-cells, FoxO1 and FoxO3a are expressed but not FoxO4 (32). Both FoxO1 and FoxO3a are capable of binding to the IRE on the IRS-2 gene promoter, but only FoxO3a was able to significantly drive basal IRS-2 gene promoter activity and maintain IRS-2 expression in primary β-cells. There did not appear to be competition between FoxO1 and FoxO3a for transcriptional activity. The inhibitory effect of insulin/IGF-I on β-cell IRS-2 expression was overcome by introduction of constitutively activated FoxO3a, but not FoxO1, emphasizing that FoxO3a is the major driver of basal IRS-2 expression in β-cells. Since FoxO3a is also negatively regulated downstream in IRS-signaling pathways (28,32), a delayed feedback control loop to dampen IRS-2 signaling via FoxO3a-mediated regulation of basal IRS-2 expression is maintained in β-cells. However, this regulation is likely to be more complex in detail, since FoxO1 and FoxO3a will likely partner with distinct coactivators and/or corepressors to be able to drive basal IRS-2 expression in β-cells. These transcriptional partners have yet to be identified, but they are not SREBP-1 and are unlikely to be TFE3, which is not very highly expressed in islets compared with liver. Further experiments will elucidate which particular factors differentially partner with FoxO1 and FoxO3a in islet β-cells to gain the ability (or not) to drive IRS-2 gene transcription. Notwithstanding, this study highlights that the molecular mechanism that controls basal IRS-2 expression in β-cells is quite distinct from that in liver.

In general, FoxO proteins can play important roles influencing cellular metabolism, growth, and/or differentiation, but their action is specific to a given cell type (28). In β-cells, the effect of FoxO1 tends to be circumstantial (28,32). FoxO1 is thought to be important developmentally by influencing expression of a key factor required for pancreatic formation and β-cell neogenesis, Pdx-1 (28,32). In adult β-cells, FoxO1
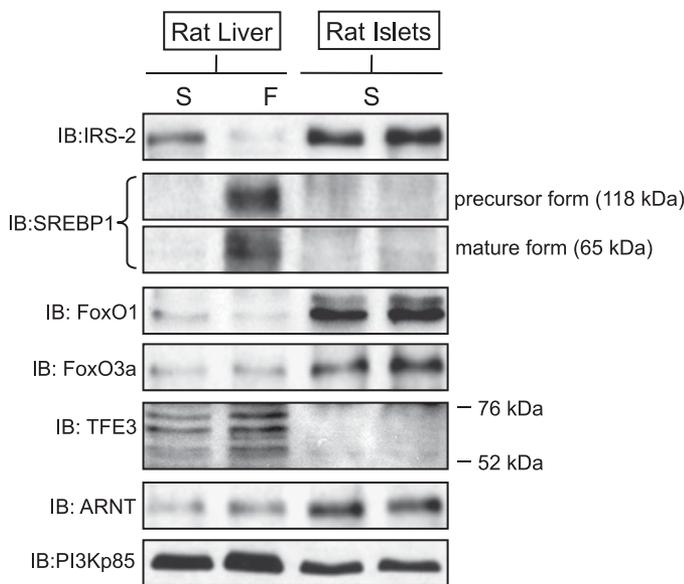
FIG. 6. Distinct mechanisms regulate IRS-2 expression in vivo in liver and in islets. Normal rats were either fasted for 12 h (S) or allowed ad libitum feeding and subjected to an intraperitoneal injection of insulin (0.75 mU/g body wt) 2 h prior to tissue harvesting (F). The liver and pancreatic islets from the same rats were then harvested and analyzed in parallel. The protein expression levels of IRS-2, SREBP-1 (precursor and proteolyzed activated forms), FoxO1, FoxO3a, TFE3, ARNT, and PI3K p85 (control) were measured in parallel by immunoblotting. An example immunoblot (IB) analysis of a single animal is shown from three independent experiments.

has been shown to inhibit β-cell proliferation via induction of cell cycle inhibitors like p27^{KIP1} (32). Indeed, haploinsufficiency of FoxO1 can recover a degree of β-cell mass and proliferation to alleviate the diabetes phenotype of IRS-2$^{-/-}$ mice (32,33). Under some circumstances, however, FoxO1 can have a beneficial impact on β-cell function. In pathophysiological oxidative stress conditions, FoxO1 can maintain insulin gene expression as well as key transcription factors MafA and NeuroD/Beta2 that are necessary for normal β-cell function (28,32). Thus, FoxO1 can play key roles in the β-cell's adaptive responses to differing in vivo metabolic conditions. A role of FoxO3a in the β-cell has been essentially unknown until now, whereas in this study, a critical role for FoxO3a driving basal IRS-2 expression has been revealed. As FoxO3a predominately drives basal IRS-2 expression in β-cells over FoxO1, it would be predicted that without appropriate FoxO3a regulation, IRS-2 levels would likely decrease and thereby compromise β-cell survival (2,4). In this regard, it is intriguing to note that selective deletion of the FoxO1 gene in β-cells does not affect glucose homeostasis (32), yet FoxO3a *null* mice are reported to be glucose intolerant (34). How FoxO1 and FoxO3a differ in their regulation and function in β-cells will also require further experimentation, but indications to date suggest that interplay between these FoxO family members could have contrasting outcomes for β-cell function (28).

Finally, it has been noted that manipulation of IRS-2 expression in β-cells could be a means to promote β-cell survival and as such, a potential therapeutic target to treat type 2 diabetes (1,8). With a mechanistic difference for control of IRS-2 expression in β-cells versus hepatocytes unveiled here, it furthers the idea that therapeutic control of IRS-2 expression could be tailored to be β-cell specific. However, one would probably want to keep this delayed

FoxO3a-mediated feedback regulation of IRS-2 signaling intact so as to avoid sustained IRS-2/PI3K/PKB signaling in β-cells that could lead to oncogenesis (35,36). Thus, alternative mechanisms for regulating IRS-2 expression in β-cells, such as by acute physiological fluctuations in glucose or glucagon-like peptide 1 analogs (10,12), would be better pursued for therapeutic potential. However, while limited evidence for incretin-based therapies to promote β-cell survival in rodent models is encouraging, this needs to be much better substantiated in humans.

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