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Glucose-regulated Glucagon Secretion Requires Insulin Receptor Expression in Pancreatic α-Cells*

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The insulin receptor (IR) and its signaling appear to be essential for insulin secretion from pancreatic β-cells. However, much less is known about the role of the IR in α-cells. To assess the role of the IR in glucagon and insulin secretion, we engineered adenoviruses for high efficiency small interference RNA (siRNA)-IR expression in isolated mouse pancreatic islets and lentiviruses for siRNA-IR expression in pancreatic α- and β-cell lines (α-TC6 and MIN6) with specific, long term stable IR knockdown. Western blot analysis showed that these strategies resulted in 60–80% reduction of IR protein in islets and α- and β-cell lines. Cell growth was reduced by 35–50% in α-TC and MIN6 cells stably expressing siRNA-IR, respectively. Importantly, glucagon secretion, in response to glucose (25 to 2.8 mM), was completely abolished in islets expressing siRNA-IR, whereas secretion increased 1.7-fold in islets expressing control siRNA. In contrast, there was no difference in glucose-stimulated insulin secretion when comparing siRNA-IR and siRNA control, with both groups showing a 1.7-fold increase. Islet glucagon and insulin content were also unaffected by IR knockdown. To further explore the role of the IR, siRNA-IR was stably expressed in pancreatic cell lines, which dramatically suppressed glucose-regulated glucagon secretion in α-TC6 cells (3.4-fold) but did not affect GSIS in MIN6 cells. Defects in siRNA-IR-expressing α-cells were associated with an alteration in the activity of Akt and $p70^{S6K}$ where insulin-induced phosphorylation of protein kinase B/AKt was greatly reduced while $p70^{S6K}$ activation was enhanced, suggesting that the related pathways play important roles in α cell function. This study provides direct evidence that appropriate expression of the IR in α-cells is required for glucose-dependent glucagon secretion.

The pathology of diabetes involves a combination of insulin resistance, defects in pancreatic β-cell function and mass, and serious abnormalities in glucagon secretion (1, 2). The β-cell is the predominant cell type in the pancreatic islet (~70%) and plays an essential role in glucose homeostasis through its secretion of insulin in response to nutrients (3). Insulin secretion is regulated by the products of glucose metabolism in the β-cell (1). More recent observations suggest that signaling through receptor tyrosine kinases in the β-cell also controls insulin synthesis and release (4). Both insulin receptor (IR)[3] and insulin-like growth factor-I receptor (IGF-IR) signaling pathways are known to play important roles in maintaining cell responsiveness to nutrients (5, 6). Ablation of IR, IGF-IR, and their main substrates (*i.e.* IRS1 and IRS2) impairs β-cell function, suggesting that insulin signaling is involved in insulin secretion and β-cell compensation in response to insulin resistance (4, 7, 8).

Pancreatic α-cells, which constitute ~20% of the islet, release glucagon to stimulate the production of glucose from the liver when blood glucose is low (3). In the liver, glucagon binds to its receptor and activates signal transduction networks and transcription factors, leading to fine regulation of glucose homeostasis through enhanced synthesis and mobilization of glucose (9). Poor regulation of glucagon secretion and biosynthesis is often found in patients with diabetes (type 1 and advanced type 2) and pancreatic tumors (glucagonoma) (10–13). Diabetic patients often display elevated levels of circulating glucagon despite hyperglycemia and become unresponsive to the stimulation of glucagon release by low glucose, suggesting that α-cell dysfunction occurs during the pathogenesis of diabetes (13–16). The mechanism of this functional defect is unknown but involves selective failure in cell signaling by glucose, because the glucagon secretory response to other stimuli, such as amino acid administration, remains intact (17).

Under normal physiological conditions, glucose stimulates insulin secretion from β-cells but suppresses the release of glucagon from α-cells in pancreatic islets. In addition, the islet microcirculation flows from β-cells to α-cells (18). Thus, substances released from the neighboring β-cells, such as insulin, islet amyloid polypeptide, Zn^{2+}, ATP, γ-aminobutyric acid, and possibly glutamate may suppress glucagon secretion (15, 16, 19). Among all these molecules, insulin is one of the most prominent, based on supporting reports from islet perfusion and *in vivo* studies (20–22). Recent evidence suggests that islets require insulin prior to responding to glucose deprivation by releasing glucagon (23–25). However, little is known about the mechanism of insulin action or its signaling effects on glucagon secretion from α-cells.

In the present study, to more clearly define the role of the insulin/IR-signaling pathway in the α-cell, we suppressed insulin signaling by IR silencing through a siRNA strategy. It was found that appropriate expression of the IR in isolated islets and clonal α-cells was required for glucose-regulated glucagon secretion. We also found that the IR may play an important role in α-cell growth. Interestingly, the biological changes in α-cells with suppressed insulin action were associated with an alteration in the activity of important multiple signaling kinases,

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[3] The abbreviations used are: IR, insulin receptor; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interference RNA; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced GFP; XTT, sodium 3′-1-(phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; KRB, Krebs-Ringer bicarbonate buffer; RIA, radioimmunoassay; FBS, fetal bovine serum; RSK, ribosomal S6 protein-serine kinase; GSK3α, glycogen synthase-serine kinase 3 α; PRK, PKC-related protein-serine kinase 1; PI3K, phosphatidylinositol 3-kinase; AMPK, AMP-activated protein kinase; JNK, c-Jun NH_2-terminal kinase.

including Akt and p70^{S6K}. This implies that not only the IR-PI3K-Akt pathway but also other pathways, especially the nutrient-related mTOR-signaling pathway, modulate α-cell function. This study reveals the importance of the IR in α-cell function and suggests that the insulin-related pathway in the α-cell could be a potential target for controlling glucagon secretion and glucose counter-regulation.

MATERIALS AND METHODS

Reagents—Antibodies against IRβ and p70^{S6K} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt, phospho-Akt (Thr-308), AMPK, phospho-AMPK-α (Thr-172), p70^{S6K}, and phospho-p70^{S6K} (Thr-389) antibodies and cell lysis buffer (10×) were from Cell Signaling Technology (Beverly, MA). The I^{125}-labeled insulin and glucagon RIA kits were from Linco Research (St. Charles, MO). Collagenase type V, bovine serum albumin, and all other reagents were purchased from Sigma.

Animals and Islet Cell Lines—Male CD1 and CD1-GFP-transgenic mice (kindly provided by Dr. M. Hara, University of Chicago, Chicago, IL) (26), 3–4 months of age, were used in the present study. Mice were fed with standard diet (Purina Chow 5001, Harlan Teklad). The Animal Care Committee at the University of Toronto approved all animal protocols. All animals were handled according to the guidelines of the Canadian Council on Animal Care. Pancreatic cell lines MIN6 (a gift from Dr. S. Seino, Chiba University, Japan) and α-TC6 (kindly provided by Dr. Y. Moriyama, Okayama University, Japan) were used in the present study (27). CRE8 (CRE293) and HEK293T cells were provided by Dr. David Johns (The Johns Hopkins University) and purchased from American Type Culture Collection (ATCC, Manassas, VA), respectively. All cells were cultured in standard medium as previously reported (28–31). To achieve consistent glucose-dependent glucagon secretion, the α-TC6 cells with compact oval morphology and not broad flattened morphology were assayed. In addition, for the secretion assay, a 70–80% confluence was maintained, and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, catalog no. 12430-054, Invitrogen, Burlington, Ontario, Canada) containing 25 mM glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere (5% CO_2, 95% air).

Pancreatic Islet Isolation and Islet Dispersion—Pancreatic islets were isolated as described previously (30). Briefly, the pancreatic duct was perfused with 3 ml of collagenase V (0.8 mg/ml). The pancreas was then cut into 2-mm pieces and digested for 15–20 min at 37 °C. Islets were partially isolated by sedimentation, hand-picked from the acinar tissue debris, and then transferred into RPMI islet culture medium containing 20 units/ml penicillin G sodium, 20 g/ml streptomycin sulfate, 5.6 mM glucose, 2% (w/v) bovine serum albumin, 10 mM HEPES, pH 7.4. Islet culture was carried out at 37 °C in 5% CO_2. To obtain isolated single cells, islets were dispersed with 0.25% trypsin for 30 min with gentle mixing every 5 min, as described previously (29). Cells were then washed with culture medium and plated onto glass coverslips for fluorescence microscopy.

Construction of siRNA Expression Vectors—Expression of hairpin siRNA was based on pSUPER vector (OligoEngine, Seattle, WA) with H1 promoter (32). Briefly, 19-nucleotide sequences for targeting specific genes were designed based on the siRNA program provided by Integrated DNA Technologies (Coralville, IA). Then the 64-oligonucleotides for hairpin siRNA were designed containing sense and antisense targeting sequences with loop (TTCAAGAGA) in the middle and BglII/HindII overhangs (Fig. 1*A*). The oligonucleotides were synthesized by Integrated DNA Technologies, annealed, and ligated into BglII/HindIII-linearized pSUPER. The hairpin siRNA expression cassettes from pSUPER plasmids were cloned into adenoviral vector ADLOX.HTM and lentiviral vector pHR′-EF-GW-SIN through XbaI/KpnI and EcoRI/SalI sites, respectively (Fig. 1*A*). The target sense sequences were: ACACAACCTCACCATCACT (GI: 6754359) (siRNA-IR-1) and AGATGACAACGAGGAATGT (GI: 6754359) (siRNA-IR-2) for insulin receptor (IR). A control sequence (GAA-GAAGTCGTGCTGCTTCAT) was designed based on the sequence of an enhanced green fluorescence protein gene (pEGFP-N1, GI: 1377911) with two nucleotides missing (underlined letters). Another control vector containing a hairpin siRNA expression cassette without target sequence was also used.

Adenovirus Preparation—Adenoviruses (vector ADLOX.HTM) used in these studies are described elsewhere (33). Adenovirus expressing enhanced green fluorescent protein driven by a CMV promoter (AdEGFP) has been reported before (29). Generally, viral production was generated by transfection of CRE8 with the expressing constructs using Lipofectamine 2000 (Invitrogen) and the manufacturer's suggested method, followed by infection of the cells with helper virus (ψ5) (34). Then, the crude adenoviruses were propagated by re-infection of CRE8 cells. Preparation of purified adenovirus with high titer was performed by standard CsCl gradient centrifugations. Viral titers were determined by the end-point titration method (Clontech, Palo Alto, CA).

Lentivirus Preparation—The lentiviral transfer and packaging vectors were a generous gift from Dr. J. Medin (Ontario Research Institute, Toronto, Canada) (35). Lentivirus production was conducted by co-transfection of HEK293T cells with three plasmids, *i.e.* a packaging-defective helper construct (pCMV-ΔR8.91, 4 μg), a vesicular stomatitis virus glycoprotein envelop-coding construct (pMD.G, 0.4 μg), and a transfer vector (pHR′EF-GW-SIN, 3.2 μg) harboring a specific hairpin siRNA sequence under control of the H1 RNA polymerase III promoter. A control vector (pHR′EF-GW-SIN, without insertion of the hairpin siRNA expression cassettes) expressing EGFP was also used in this study. Transfection was conducted using Lipofectamine 2000 and the manufacturer's suggested method. At 48 h, supernatants containing the lentiviral recombinants were harvested and centrifuged twice or passed through 45-μm filters to eliminate transfer of cells. The viruses obtained were either used directly for cell infection or stored at −80 °C. Viral titers were assessed by transducing 293T cells with the control GFP-expressing virus and measuring the percentage of EGFP-positive cells.

Viral Infections—For islets, adenovirus infection was carried out on the same day as isolation. Briefly, freshly isolated islets were plated in 35-mm tissue culture plates, 50–100 islets/plate, with 2 ml of RPMI islet culture medium containing 5.6 mM glucose for 1–2 h at 37 °C. Purified adenovirus was then incubated with islets at 10^{10} virus particles/ml overnight at 37 °C. The next day, islets were transferred to new plates, washed twice, and further cultured at 37 °C with medium changed on a daily basis. At 72 h, islets were assayed as specified elsewhere. For generating stable cell lines expressing siRNA, MIN6 and α-TC6 cells were infected with lentiviruses. Cells were plated 16 h prior to viral infection, and then incubated with lentivirus in the present of protamine sulfate (4 μg/ml) for overnight. Cells were kept in culture for at least 1 week before performing any functional experiments. The amount of the siRNA lentivirus used was roughly calculated using a control lentivirus expressing GFP. Normally, the transduction of MIN6 and α-TC6 cells by control GFP lentivirus resulted in 50–60% of the cells expressing GFP initially (48–72 h post-infection). Approximately 7 days later, over 90% of cells were GFP-positive. In some cases, the cells were re-infected to maintain a high efficiency of transduction.

Confocal Fluorescence Microscopy—Islets and cells were mounted on slides in a fading retarder solution (0.1% *p*-phenylenediamine in glyc-

erol). Laser confocal fluorescence microscopy analysis was performed, and images were obtained using a Zeiss LSM-410 laser scanning confocal imaging system (Carl Zeiss, Oberkochen, Germany).

Cell Viability Assay—Cell proliferation and viability were measured by an XTT-based colorimetric assay (Cell Proliferation Kit II (XTT), Roche Applied Science). For cultured cell lines, cells were seeded into three 96-well plates starting with concentrations of 5×10^4 cells per well followed by serial dilutions on the plate to evaluate cell growth at different starting concentrations. One plate was subjected to an XTT assay at days 0, 2, or 4. For islets, cell viability was measured using 10–20 islets per sample 72 h post-infection. Generally, 150 or 200 μl of the XTT medium was applied to cell or islet samples for 4- or 6-h incubation at 37 °C, respectively. The XTT assay results were detected by spectrophotometric absorbance at 490 nm (A_{490}).

Islet Secretion Assay—Islet secretion experiments were designed to optimize glucagon secretion, based on the concept reported by Hope *et al.* (23). At 72-h post-islet isolation and viral infection, islets were used in secretion assays using Krebs-Ringer bicarbonate buffer (KRB) containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 2% bovine serum albumin. Islets were preincubated at 37 °C for 30 min in KRB supplemented with 5.6 mM glucose and then transferred to 1.5-ml tubes for assay (10–20 islets per sample). The islets were first incubated in 500 μl of KRB with 25 mM glucose for 1 h at 37 °C, and then in 500 μl of KRB with 2.7 mM glucose for an additional 1 h at 37 °C. Aliquots (450 μl) from each sample were taken at the end of the incubation period and stored at −20 °C until assayed for glucagon and insulin. After the secretion assay, islets were washed with RPMI medium, and cell viability was measured with the XTT assay. At the end of the XTT assay, islet protein was extracted in 75% ethanol containing 0.09 N HCl and then vacuum dried. Insulin and glucagon were then extracted from the protein pellet by adding H_2O and heating to 60 °C for 10 min. Samples were stored at −20 °C before being assayed for total DNA (micrograms as measured by A_{260}), insulin, and glucagon content. Insulin content was measured by a radioimmunoassay (RIA) described previously (36). Glucagon content was measured using an RIA kit (Linco Research, Inc.).

Cell Secretion Assays—To measure glucagon secretion, α-TC6 cells were plated in 24-well culture plates at 3×10^5 cells/well. The following day, cells were washed twice and preincubated in KRB with 25 mM glucose for 30 min at 37 °C. Subsequently, the cells were treated with 500 μl of KRB containing either 25 mM glucose or 2.8 mM glucose for 1 h at 37 °C. Aliquots (450 μl) from each treatment were taken at the end and stored at −20 °C until assayed for glucagon by RIA. Following the secretion assay, cells were collected for the measurement of total glucagon content by RIA and total DNA by A_{260}. Secretion data from the assays were normalized by the total glucagon content. For the insulin secretion measurements, MIN6 cells were plated in 24-well culture plates at $2–5 \times 10^5$ cells/well overnight. Cells were then washed twice and preincubated in KRB containing 2.8 mM glucose for 30 min at 37 °C. After the preincubation period, cells were washed thoroughly with KRB and incubated with 500 μl of KRB supplemented with glucose at 2.8 mM or 25 mM for 1 h at 37 °C. Aliquots (450 μl) were taken at the end of the incubation period and stored at −20 °C until assayed.

Western Blot Analysis of Protein Expression—For extraction of total protein, cell or islet samples were homogenized in ice-cold lysis buffer (Cell Signaling Technology). Protein samples were mixed with electrophoresis sample buffer and resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF Membrane, Bio-Rad), incubated with 5% skim milk in Tris-buffered saline containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20, and then probed with specific primary antibodies. Bound antibodies were detected with goat anti-rabbit or anti-mouse antibodies that had been conjugated to horseradish peroxidase and ECL (Roche Applied Science). Band density was measured by densitometry, analyzed by using image analysis software (Scion Image version 4.02, Scion, Frederick, MD), and normalized to β-actin content in parallel samples.

Kinase Activation Assays—MIN6 and α-TC6 cells were serum-starved for 6 h or overnight in DMEM containing 0.05% FBS with 0 mM or 5.6 mM of glucose and subsequently stimulated in medium containing 25 mM of glucose with 10% FBS or 100 nM insulin for 15 min. Cell lysates were prepared as described above for Western blot analysis.

Protein Phosphorylation Profiling—α-TC6 cells stably expressing siRNA-IR and siRNA control were serum-starved for overnight in DMEM containing 0.05% FBS with 5.6 mM of glucose and subsequently stimulated in medium containing 25 mM of glucose with 100 nM insulin for 15 min. Whole cell proteins were extracted using lysis buffer provided by Kinexus Bioinformatics (Vancouver, Canada). Kinetworks KPSS-4.1 analysis was performed to track 41 phosphorylation sites in phosphoproteins with antibodies that recognize phosphorylated epitopes (Kinexus Bioinformatics).

Statistical Analysis—Data points represent means ± S.E. One-way or two-way analysis of variance tests were used to compare control values and treated groups. A p value of <0.05 was considered statistically significant.

RESULTS

Effectiveness of siRNA Transfer Using Adenoviral and Lentiviral Vectors—An adenovirus was used to achieve high efficiency siRNA expression in isolated islets. To verify the efficiency of viral transduction, a GFP-expressing adenovirus (ADLOX.HTM, the same vector for generating siRNA in this study) was used to infect islets isolated from CD-1 mice, employing the same protocol used for other islet experiments in this study. Intact islets were examined for GFP protein expression by confocal fluorescence microscopy. Sections 4.3–8 μm thick (whole islet thickness, 110–250 μm) revealed that most of the cells in the outer layer of the islet expressed GFP (at least 95%, Fig. 1*B*). This indicates that the outer layer of the islet, where most of the α-cells are located, can be efficiently infected by adenovirus. Greater than 50% of the cells from dispersed islet cells examined under fluorescence microscopy expressed GFP, amounting to an average adenoviral infection of 70–80% in isolated islets.

To achieve stable expression of siRNA in cultured cell lines, a lentivirus vector was used to transduce MIN6 and α-TC6 cells. The efficiency of viral transduction was evaluated by a control virus expressing GFP (*i.e.* the same amount of virus with the same viral vector was used to infect cells to generate stable cell lines and the same cell passage). Expression of GFP in the control cell lines was consistently monitored by imaging. The effectiveness of the knockdown in stable cell lines was also confirmed by Western blot analysis using cells 6–9 passages post-infection (2–3 weeks) (Fig. 2). In all functional experiments, control and siRNA-IR cell lines from the same passages were used.

Virus-mediated Specific RNA Interference Silencing in Isolated Mouse Islets and in Cultured Cells—Based on results from cultured cells, significant IR knockdown by siRNA-IR infection was seen as early as 48 h post-infection. However, optimal knockdown of the target was consistently observed 72 h post-infection (data not shown). To test the specificity of the knockdown, infected islets were analyzed by Western blotting. As shown in Fig. 2*A*, specific reduction of IR expression was observed in islets expressing either siRNA-IR-1 or siRNA-IR-2. The average IR-knockdown was ~70% when both viruses were co-infected, which was the condition used in experiments carried out in this study

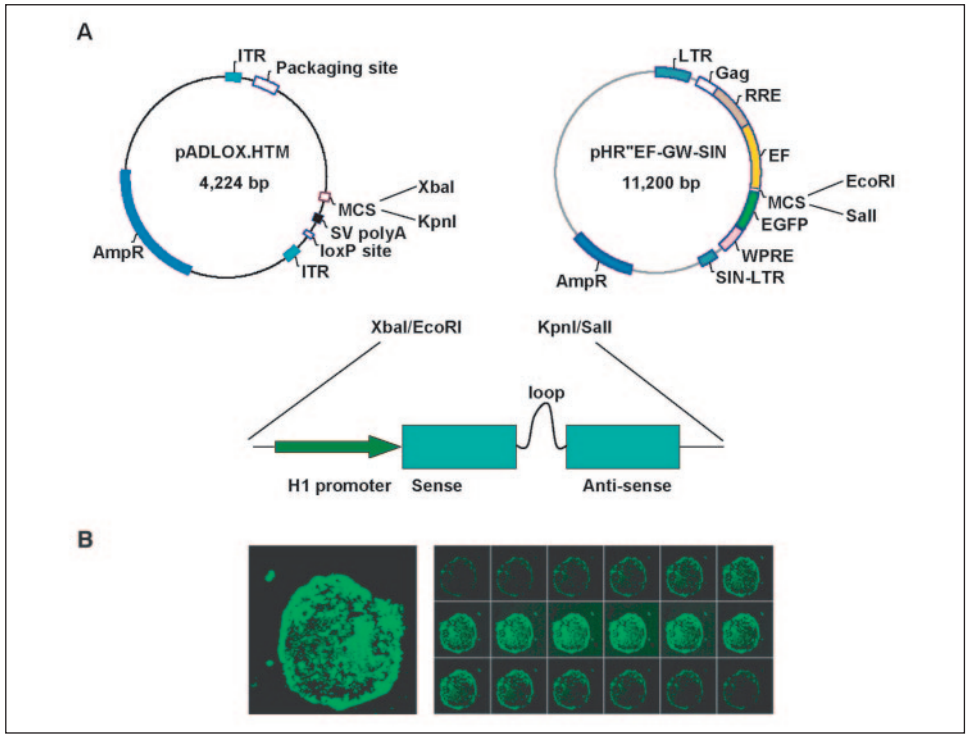

FIGURE 1. **siRNA expression cassettes and visualization by fluorescent confocal microscopy of an islet after transduction with adenovirus expressing EGFP.** *A*, expression of hairpin siRNA was based on the pSUPER vector (OligoEngine, Seattle, WA) with H1 promoter. 19-nucleotide sequences for targeting specific genes were designed based on the siRNA program provided by Integrated DNA Technologies. The 64 oligonucleotides for hairpin siRNA were designed containing sense and antisense targeting sequences with a loop (TTCAAGAGA) in the middle and BglII/HindII overhangs. The oligonucleotides were synthesized by Integrated DNA Technologies, annealed, and ligated into BglII/HindIII-linearized pSUPER vector. The hairpin siRNA expression cassettes from pSUPER vector were then cloned into the adenoviral vector ADLOX.HTM and the lentiviral vector pHR'EF-GW-SIN through XbaI/KpnI and EcoRI/SalI sites, respectively. *B*, islets were isolated from CD1 mice, infected with EGFP-expressing adenoviruses (10^{10} viral particles/ml), and cultured for 72 h. A transduced islet with EGFP expression was visualized by laser confocal fluorescence microscopy. Laser sections of the islet (8.0 μm per section) are shown in the *right panel*.

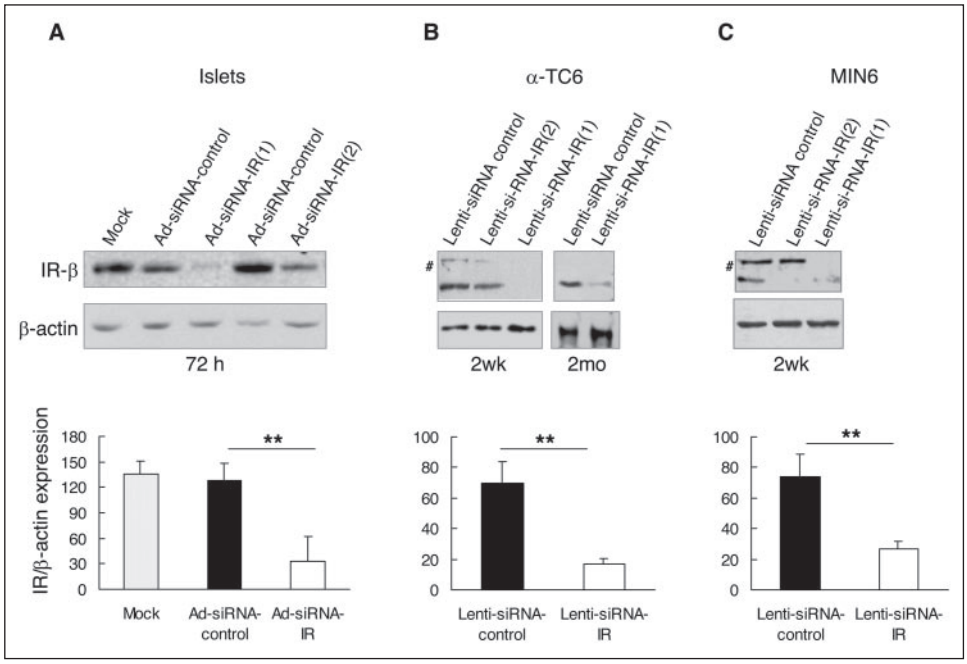

FIGURE 2. **Immunoblot analysis of IR expression in isolated islets, MIN6 cells, and α-TC6 cells after transduction with viruses expressing siRNA control and siRNA-IR.** *A*, islets isolated from CD1-GFP mice were infected with siRNA adenoviruses (10^{10} viral particles/ml). Islets were harvested 72 h post-infection/isolation, and analyzed by Western blotting using specific polyclonal antibodies against the β-subunit of the IR (IRβ). *B* and *C*, α-TC6 and MIN6 cells were infected with lentiviruses (see details under "Materials and Methods"), and cultured for 2 weeks (*2wk*)) to generate homogeneous stable cell lines. Cells were then analyzed by Western blotting using the same antibodies used in *A*. Stable α-TC6 cells maintained in culture for 2 months (*2mo*) were also examined. Blots are shown in the *top panels*, and the relative amount of IRβ protein normalized to cell β-actin is shown in the *bottom panels* ($n = 3$, **, $p < 0.01$). Please note the top band (#) in some samples corresponds to the IR precursor.

(Fig. 2*A*). In the MIN6 and α-TC6 cells stably transduced by lentiviruses, specific knockdown of IR protein expression was also observed (Fig. 2, *B* and *C*). In stable cell lines, a maximum of 80% IR-knockdown at the protein level was observed at any passage post-transduction. Interestingly, a higher percentage of knockdown dramatically inhibited cell growth and survival. These results indicated that our viral siRNA delivery systems were suitable for both isolated islets and pancreatic cell lines.

Effect of IR Knockdown on Cell Viability and Growth—One of the main biological functions of insulin is to promote cell survival and proliferation (37–39). To test the effect of adenovirus-delivered siRNA expression on the viability of islet cells, an XTT assay was performed. Islet DNA was then extracted and measured by A_{260}. Cell viability, detected by XTT readings (A_{490}), was normalized to DNA content. Results showed that there were no significant differences in cell viability among the islets expressing siRNA-IR and siRNA control ($p > 0.05$, $n = 4$) (Fig. 3*A*). In the cell lines, cell viability was also evaluated by an XTT assay by plating an equal number of cells in 96-well plates. Results showed that cell viability was not remarkably affected during the early stages of lentiviral transduction (*i.e.* 3–5 days after being incubated with viruses) in both MIN6 and α-TC cells (data not shown). The long term effects (cells stably transduced by lentiviruses) of expressing siRNA-IR on cell growth and cell proliferation were also evaluated at different time points (Fig. 3, *B* and *C*). Cells expressing siRNA-IR grew more slowly

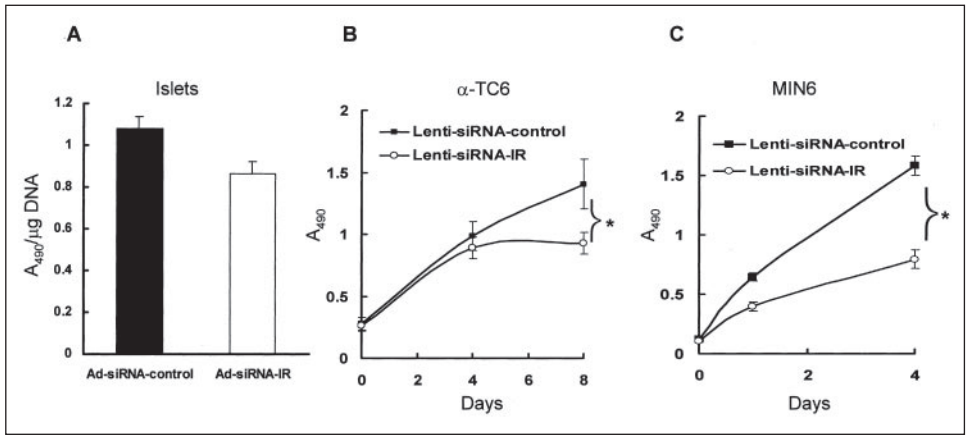

FIGURE 3. **Cell viability of isolated mouse islets and cell growth of stable cell lines after the expression of siRNA control and siRNA-IR.** *A*, isolated mouse islets were infected with adenoviruses expressing siRNA control or siRNA-IR (10^{10} viral particles/ml). Cell viability was measured by XTT assay 72 h post-islet isolation and virus infection. Results are presented as the XTT reading normalized to islet DNA (A_{490}/μg of DNA). *B* and *C*, α-TC6 (*B*) and MIN6 (*C*) stable cell lines expressing siRNA control and siRNA-IR were plated onto 96-well plates (10^4/well), and the growth rate was monitored by XTT assay for the time indicated. The results from XTT assays are presented as absorbance at 490 nm. Data from four experiments were averaged (*, $p < 0.05$).

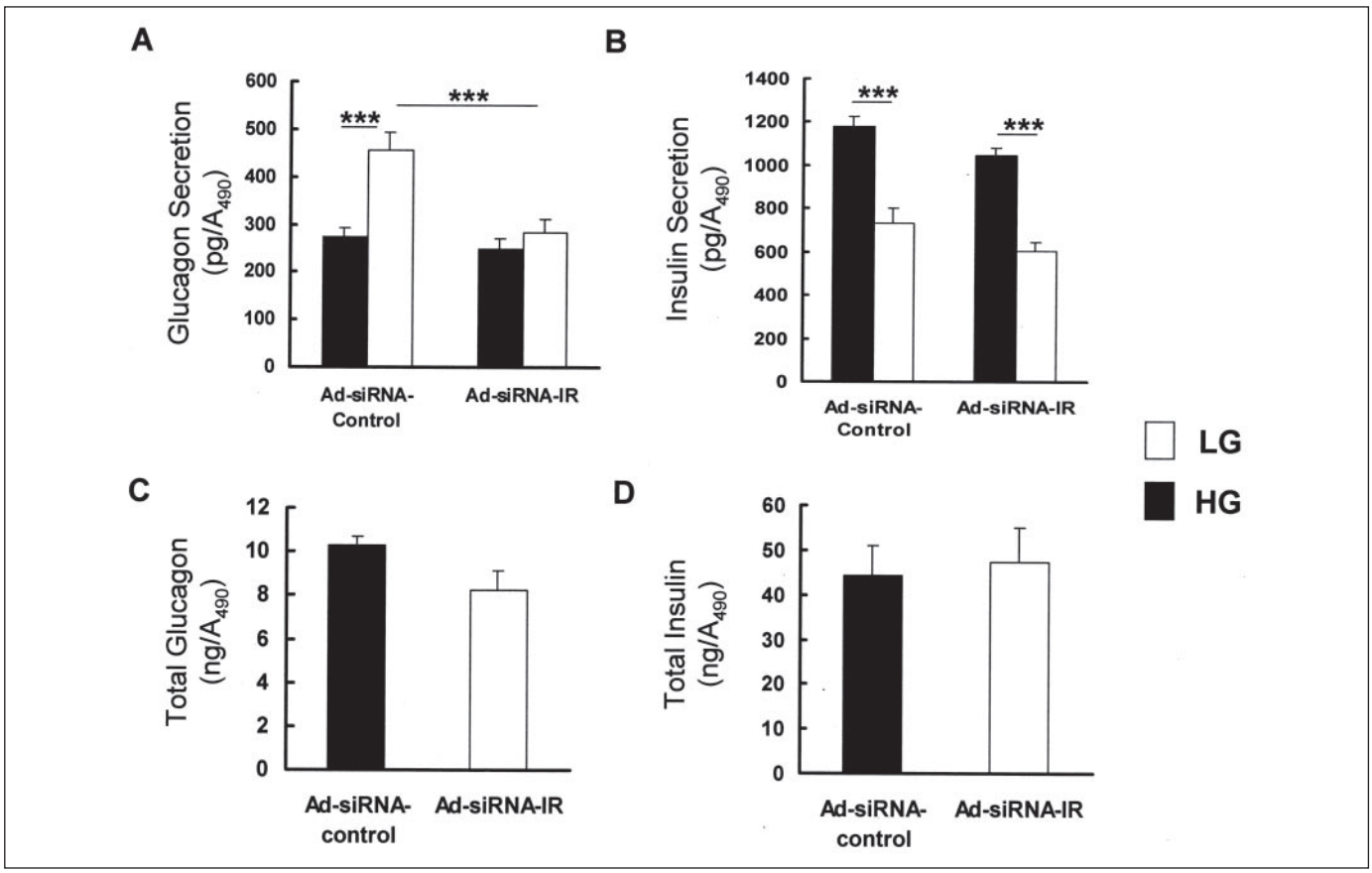

FIGURE 4. **Glucose-regulated glucagon and insulin secretion after the expression of siRNA control and siRNA-IR.** Islets were isolated from CD-1-GFP-transgenic mice ($n \geq 12$) and infected by adenoviruses expressing siRNA control or siRNA-IR (10^{10} viral particles/ml). After 72-h incubation, islets (10–20 islets/sample) were incubated in KRB with 25 mM glucose (*HG*) for 1 h, and then 2.7 mM (*LG*) for 1 h. Secreted and total glucagon (*A* and *C*) and insulin (*B* and *D*) were measured by RIA, and normalized to the islet cell viability as measured by XTT assay. Data from four experiments were averaged ($n \geq 12$, ***, $p < 0.001$).

than cells expressing siRNA control. Specifically, when expressing siRNA-IR, α-TC6 cell growth was reduced by ~35% with respect to cells expressing siRNA control (Fig. 3*B*), whereas MIN6 cell growth in cells expressing siRNA-IR was reduced by ~50% (Fig. 3*C*) ($p < 0.05$, $n = 4$).

Effect of IR Knockdown in Mouse Islets on Glucagon and Insulin Secretion—To test the functional effects of suppressing insulin receptor expression in isolated mouse islets, glucagon and insulin secretion were assessed. For all the secretion experiments, similar viability, as indicated by the A_{490}/DNA ratios, was maintained among all the samples expressing siRNA control or siRNA-IR. Thereby, in the present study normalizing the secretion data to either the DNA or XTT did not yield any difference. However, it may be more reliable to normalize the data based on cell metabolic activity in instances where treatment of cells, such as knockdown of growth factors, may affect cell viability or metabolism. Fig. 4 compares the effects of decreasing glucose concentrations from 25 to 2.8 mM on glucagon and insulin secretion from intact islets. Glucagon secretion in response to the change in glucose concentration from 25 to 2.8 mM was completely abolished in islets expressing siRNA-IR (1.13 ± 0.16-fold), whereas glucagon secretion remained in the islets expressing siRNA control (1.71 ± 0.19-fold) ($p < 0.001$, $n = 12$, Fig. 4*A*). Knockdown of IR had no effect on basal glucagon secretion at the higher glucose concentration (25 mM) ($p > 0.05$, $n = 12$) (Fig. 4*A*). In contrast, in the same islets, there was no significant effect on glucose-induced insulin secretion (1.75 ± 0.10- and 1.63 ± 0.11-fold for siRNA-IR and

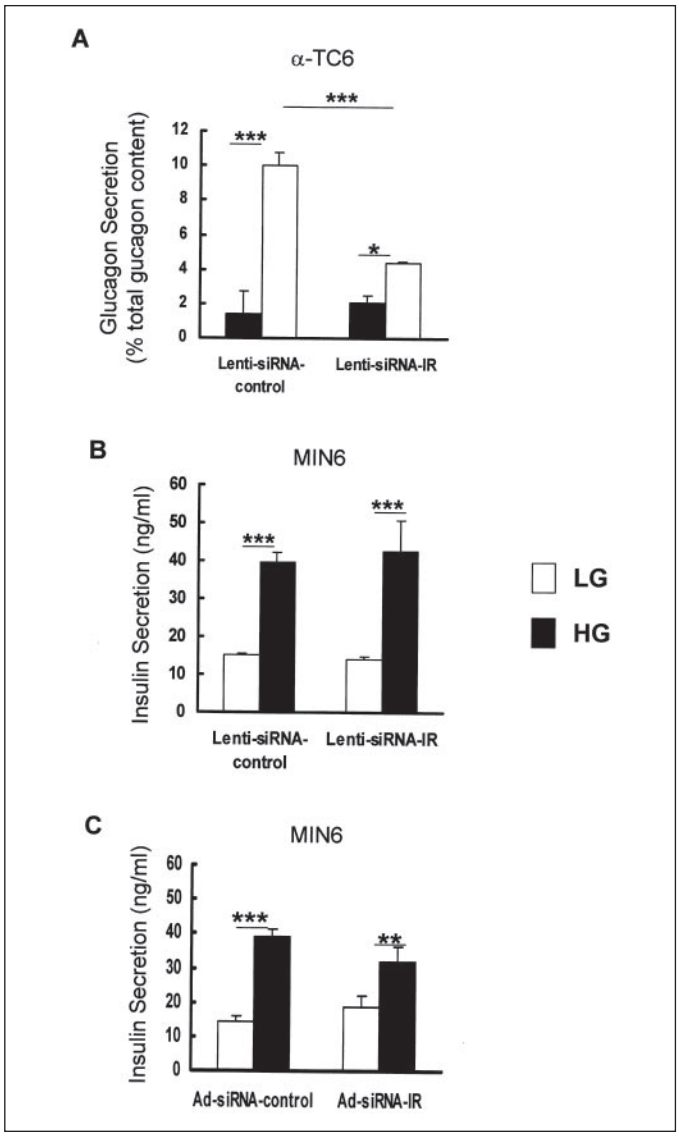

FIGURE 5. **Glucose-dependent glucagon secretion from stable α-TC6 cell lines and insulin secretion from stable MIN6 cell lines after transduction with viruses expressing siRNA control and siRNA-IR.** *A*, equal numbers of stable α-TC6 cells expressing siRNA control and siRNA-IR were plated onto 24-well plates (~5×10^4/well), and glucagon secretion was measured at high glucose (*HG*, 25 mM) or low glucose (*LG*, 2.7 mM). *B* and *C*, MIN6 cell lines stably transduced by lentiviruses (*B*) and MIN6 acutely infected with adenoviruses (10^{10} viral particles/ml, 72 h post-viral infection) (*C*) were studied for insulin secretion in response to HG (25 mM) or LG (2.7 mM). Equal numbers of cells were plated onto 24-well plates for overnight culture before the secretion assay. Data are presented as the insulin secreted per ml of KRB. See details under "Materials and Methods." Data from three experiments were averaged (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

siRNA control, respectively) ($p > 0.05$, $n \geq 16$, Fig. 4*B*), suggesting that insulin secretion in islet β-cells remained unaltered when IR expression was attenuated. Total islet glucagon and insulin content were also not affected by the knockdown of IR expression (siRNA-IR *versus* siRNA control glucagon: 4.7 ± 0.9 *versus* 5.8 ± 0.4 ng/μg DNA; insulin: 29.4 ± 7.9 *versus* 25.0 ± 6.7 ng/μg DNA) ($p > 0.05$, $n \geq 5$, Fig. 4, *C* and *D*).

Effect of IR Knockdown in α-TC6 and MIN6 on Glucagon and Insulin Secretion, Respectively—To confirm the effect of IR on islet glucagon and insulin secretion, we extended our studies to homogeneous stable pancreatic cell lines expressing siRNAs. Equal numbers of cells were plated in 24-well plates for secretion assays. The glucagon secretion data were normalized by total cellular glucagon content, because α-TC6 cells are easily detached from the culture plates during the assay. Results demonstrated that stable siRNA-IR expression suppressed glucagon release from α-TC6 at 2.8 mM of glucose by 71.0 ± 7% ($p < 0.01$, $n = 4$, Fig. 5*A*). To confirm the absence of insulin secretion from α-TC6 cells, insulin was measured from cell extracts and the assay buffer using samples from glucagon secretion experiments. As expected, insulin was neither expressed nor secreted from α-TC6 cells (data not shown). To determine if insulin modulated glucagon secretion from α-TC6 cells, glucagon secretion assays were performed using KRB containing insulin from 10–100 ng/ml. We could not detect any effect of insulin on glucose-regulated glucagon release from α-TC6 cells (data not shown).

The expression of siRNA against IR did not affect insulin secretion from MIN6 cells ($p > 0.05$, $n \geq 4$, Fig. 5*B*). Interestingly, unlike in stable cell lines expressing siRNAs, an acute transient knockdown of the IR via siRNA-IR adenoviral transduction in MIN6 cells for 72 h did significantly reduce GSIS (37.2% ± 6.3%, $p < 0.05$, $n = 3$, Fig. 5*C*).

The Effect of IR Knockdown on Insulin Signaling—Our results (Figs. 4*A* and 5*A*) indicate that appropriate expression of IR in α-cells is essential for glucose-regulated glucagon secretion, suggesting that insulin signaling plays a role in regulating glucagon release. To identify the biological effects of IR knockdown at the molecular level, cell signaling pathways were examined using mouse islets and stable cell lines expressing siRNA. Two kinases, Akt and $p70^{S6K}$, and related pathways have been strongly implicated as important players in the pathogenesis of type 2 diabetes (40, 41). Because insulin activates both Akt and $p70^{S6K}$ through different but linked pathways (37–39), we measured the activation of both kinases. Results from the kinase assays, using serum as a stimulus, showed that Akt could be activated in islets, MIN6 and α-TC6 cells expressing either siRNA control or siRNA-IR (Figs. 6*A*, 6*B*, and 7*A*, respectively). A ~2-fold increased $p70^{S6K}$ activation by serum was detected in all the stable cells expressing siRNA-IR (Figs. 6*B* and 7*B*). These results suggested that the related pathways could still be activated by serum in cells expressing siRNA-IR. To test the specific effect of IR knockdown in α-TC6 cells, we measured Akt activation using 200 nM of insulin as stimuli. Results showed that expression of siRNA-IR abolished downstream Akt activation in an insulin-specific manner compared with siRNA control cells (Fig. 7*A*). However, insulin-induced $p70^{S6K}$ activation in the cells expressing siRNA-IR was the same as in the control cells (Fig. 7*B*). This suggested that those cells expressing siRNA-IR could produce higher $p70^{S6K}$ activities, even though insulin signaling was attenuated by a reduction in IR. As expected, knockdown of IR did not affect the insulin-independent AMPK pathway. In both islets and cells, AMPK was activated in response to stress caused by serum withdrawal and low glucose concentrations (Figs. 6 and 7*A*), indicating that IR silencing in the α-TC cells affects insulin-specific signaling pathways.

To further examine the insulin-related pathways in the α-TC6 cells stably expressing siRNA-IR and siRNA control, a Kinetwork™ Phospho-site Screen 4.1 (Kinexus Bioinformatics, Vancouver, Canada) was carried out to track 41 phosphorylation sites in 35 phosphoproteins. TABLE ONE shows the proteins with 1- to 2-fold (>100%) changes in insulin-induced phosphorylation after expressing siRNA-IR. This result confirmed that our stable siRNA-IR expression system worked in the α-TC6 cells, because other insulin-related pathways (*i.e.* protein kinase C-related and retinoblastoma protein (Rb)-related) were reduced after IR silencing. Data revealed more kinases could be the downstream targets of IR, including protein kinase C-related protein-serine kinase 1, Rb, ribosomal S6 protein-serine kinase 1/2/3 (RSK), and glycogen synthase-serine kinase 3 α (GSK3α). The result of phospho-site screen also showed that the phosphorylations of some proteins in the α-TC6 cells were very low (*i.e.* MAPK protein-serine kinase 1/2, bone marrow X protein-tyrosine kinase, Bruton's tyrosine kinase, calcium/calmodulin-

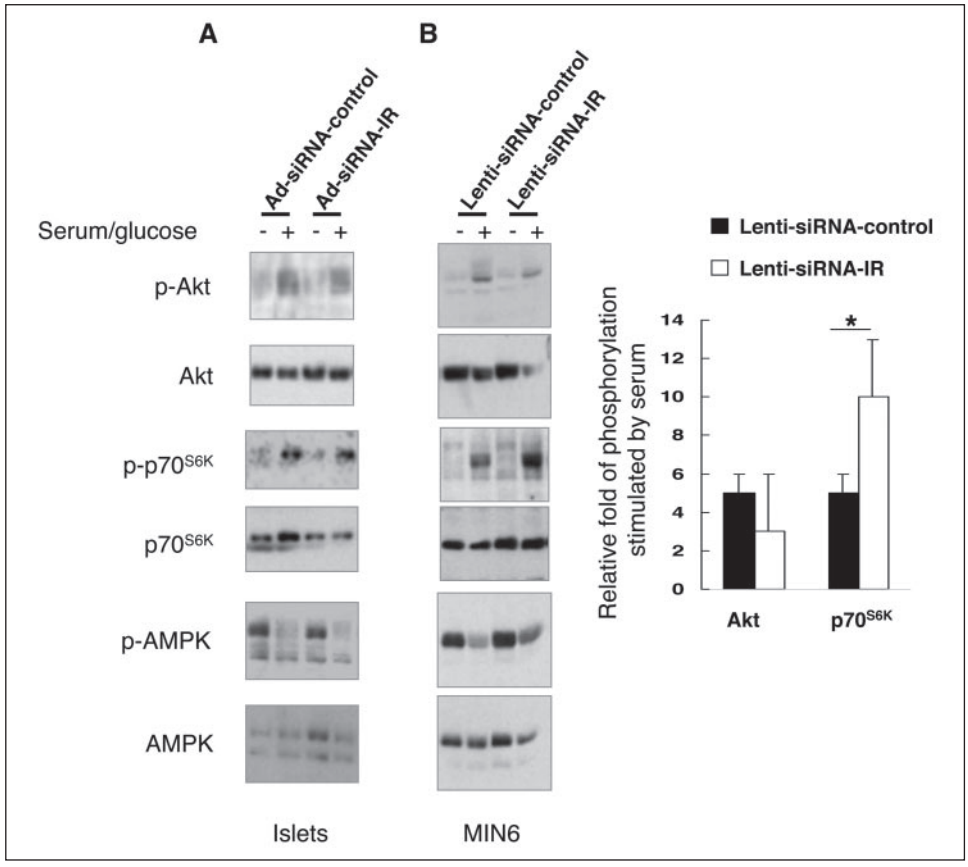

FIGURE 6. **Immunoblot analysis of the activation of signal transduction pathways in isolated mouse islets and MIN6 cells stably expressing siRNA control and siRNA-IR.** Isolated islets, 72 h post-infected by adenoviruses (*A*) and stable MIN6 cells (*B*) were cultured in DMEM containing 0.05% FBS and 5.6 mM glucose overnight. The islets or cells were treated with or without (+ or −) DMEM containing 10% FBS and 25 mM glucose, for 15 min before harvest. Samples were analyzed by Western blotting using specific antibodies against the phospho-sites of the target kinases as indicated. The blots were then re-probed using specific antibodies against the non-phosphorylated form. The -fold increase in phospho-Akt and phospho-p70^{S6K}, normalized to total Akt and p70^{S6K}, respectively, and compared with non-stimulated, based on the results from three experiments (*, $p < 0.05$).

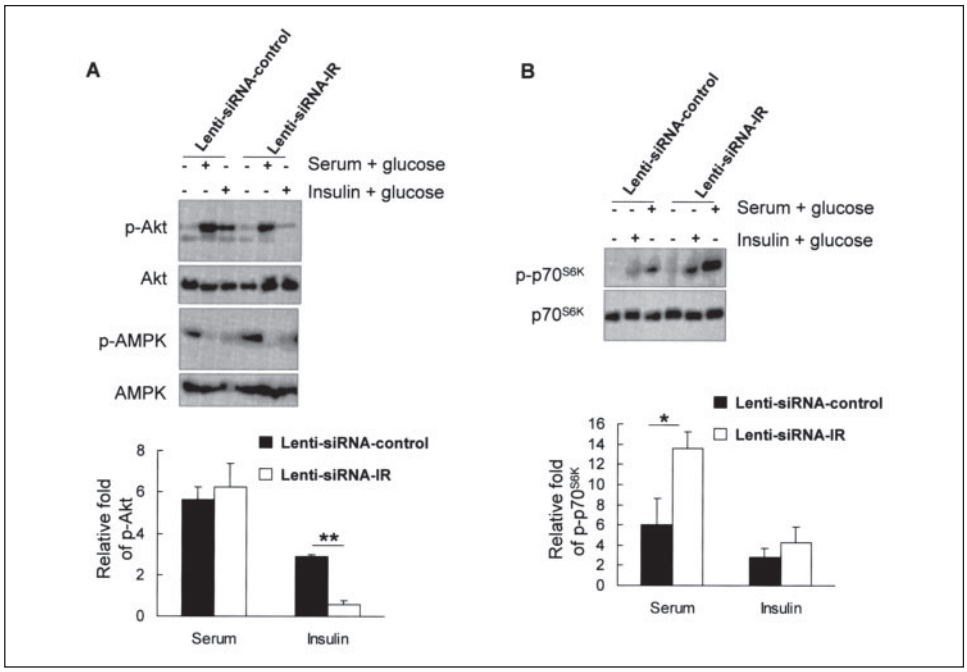

FIGURE 7. **Immunoblot analysis of the activation of signal transduction pathways in isolated mouse α-TC6 stably expressing siRNA control and siRNA-IR.** Stable α-TC6 cells were cultured with DMEM containing 0.05% FBS and 5.6 mM glucose overnight. The cells were treated with or without (+ or −) DMEM containing 10% FBS and 25 mM glucose, for 15 min (*A*). Cells were also stimulated with DMEM containing 200 nM insulin and 25 mM glucose for 15 min (*B*). Samples were analyzed by Western blotting using specific antibodies against the phospho-sites of the target kinases as indicated. The blots were then re-probed using specific antibodies against the non-phosphorylated form. The -fold increase in phospho-Akt and phospho-p70^{S6K}, normalized to total Akt and p70^{S6K}, respectively, and compared with non-stimulated, based on results from three to four experiments (*, $p < 0.05$; **, $p < 0.01$).

dependent protein-serine kinase 2α, eukaryotic translation initiation factor 4E binding protein1, inhibitor of NF-κB kinase α/β, Lyn protein-tyrosine kinase, protein kinase C θ, and ζ chain-associated protein-tyrosine kinase), suggesting that these proteins either were expressed at low levels or do not respond to insulin stimulation.

DISCUSSION

To more clearly define the role of insulin receptors (IRs) in the α-cell and their role in regulating glucagon secretion, we used adenoviral vectors to achieve acute high efficiency delivery of siRNA-expression cassettes in isolated mouse islets. We also used lentiviral vectors to efficiently generate stable islet α- and β-cell lines expressing siRNAs for persistent gene knockdown studies. Viral expression of small hairpin RNAs mediated by the H1 promoter resulted in specific reduction of IR protein levels in isolated islets and cell lines. Our results indicated that IR expression is critical for controlling glucagon secretion at low glucose concentrations. However, the IR is not required for glucose-mediated suppression of glucagon release at high glucose concentrations.

TABLE ONE

Protein phosphorylation induced by insulin stimulation in α-TC6 (Kinetworks™ phospho-site screen 4.1)

Protein (phospho-site)	Percent changes in phosphorylation (insulin-stimulated *versus* unstimulated)	
	Lenti-siRNA control	Lenti-siRNA-IR
PRK1 (Thr-774)	91.5 ± 24.5	NC[a]
Rb (Ser-780)	111.25 ± 30	NC[a]
RSK1/2/3 (Thr-573/Thr-577/Thr-570)	288.5 ± 17.5	50 ± 74
GSK3α (Ser-21)	38.3 ± 35.2	133.3 ± 70.8
GSK3β (Ser-9)	NC[a]	57.4 ± 33.5

[a] No change.

RNA interference has proven to be of great value as a genetic tool for studying gene function. However, the transfection approach for introducing RNA interference into islets is limited by low efficiency and poor cellular viability. The short term effect of transfection also limits its use for RNA interference in cultured pancreatic cells. Although drug-resistance selection can be utilized, this is time-consuming and can confound functional analysis. To bypass these difficulties, adenoviral and lentiviral vectors have been used to deliver expression cassettes designed for RNA interference-mediated gene knockdown. The adenoviral delivery approach has been well established for gene expression in isolated islets (42–44). Bain *et al.* (45) have demonstrated RNA silencing in isolated islets transduced by an adenovirus encoding a similar siRNA-expression vector as used in the present studies. Here, we successfully targeted the mRNA of IR in both islets and cell lines, with the aim of studying the effects of reducing these molecules on insulin/glucagon secretion and cell viability/proliferation.

Insulin has been considered as a negative regulator of glucagon release. Initial observations in human subjects show that insulin infusion can suppress plasma glucagon (20, 21). Experiments *in vivo* elaborated on these findings, demonstrating that insulin infusion alone did not suppress glucagon release but rather that insulin in combination with hyperglycemia was required for suppression of glucagon secretion (22, 46). In addition, a clamp study in healthy humans showed that a decrease in intra-islet insulin is a signal for the glucagon response to hypoglycemia (25). Other evidence also exists to support insulin as a critical component of glucose-dependent glucagon release. Specifically, streptozotocin mediated destruction of β-cells in Wistar rats resulted in a failure of hypoglycemia-stimulated glucagon release, although α-cells remained responsive to arginine (24). *In vitro* perfusion experiments showed that both low glucose concentrations and switching off α-cell exposure to insulin are required for the glucagon response in either human or rat islets (23). Therefore, the evidence suggests that, if there is no insulin in the microenvironment of α-cells or if there is no appropriate IR expression in α-cells, glucagon secretion defects may occur. However, the studies carried out to date implicating the role of insulin in glucose-dependent glucagon release have been performed in the whole animal or isolated islets. There has been little molecular biological research to address the mechanism of insulin action in glucose-dependent glucagon release. Our findings not only provide evidence for a positive role for insulin in glucagon release at low glucose but also demonstrate, through knockdown of its receptor, the direct effect of insulin on long term α-cell function. Our data cannot totally exclude the inhibitory role of insulin-related downstream signaling in the regulation of basal glucagon release at high glucose since Akt signaling could still be activated after IR expression silencing (Fig. 6), and IR knockdown had no effect on basal glucagon secretion (Figs. 4 and 5).

It is worth emphasizing that IR knockdown in stable cell lines mimics the effect of a chronic lack of insulin and related signals, which may well reflect the pathogenesis of insulin resistance *in vivo*. As discussed in the following paragraphs, many changes in signal pathways and transcriptional regulation occur in cells with IR knockdown. We did not observe any inhibitory effect of insulin on glucagon secretion from α-TC6 cells at low concentrations of glucose when insulin was added acutely to the secretion buffer (data not shown). However, a recent study reported that insulin could directly inhibit glucagon secretion from αTC1–9 cells in a PI3K-dependent manner (47).

There have been two reports examining the effects of IR/IGF-IR silencing on the pancreatic β-cell line MIN6 (48, 49). Consistent with our findings, results from these studies indicated that acute silencing of the IR in MIN6 cells led to a reduction in glucose-stimulated insulin secretion, whereas chronic silencing of the IR had no effect, suggesting the ability of MIN6 cells to compensate for long term loss of the IR. Moreover, the IR appears to be crucial to MIN6 cell growth due to its effect on the expression of cell cycle regulatory genes (49). However, only IGF-IR silencing, but not IR silencing, markedly inhibited glucose-stimulated increases in cytosolic and mitochondrial ATP, thus greatly decreasing the energy source driving cell growth (48). Our current studies show that persistent reduction in IR expression slows cell growth not only in MIN6 but also α-TC cells, suggesting that these cells, which are derived from a common precursor (50–52), share signaling networks that control cell proliferation as expected. In addition, IR knockdown had little influence on the survival of either islets or cell lines in our studies, suggesting that other survival pathways may compensate for the loss of IR or longer periods of IR deficiency are required. Interestingly, increasing the IR knockdown to >90% (by increasing the infection to $>10^{12}$ virus particles/ml) led to significant apoptosis not seen with control virus at the same titer (data not shown). Our phospho-site screen data also showed that insulin-induced Rb phosphorylation was decreased in α-TC6 cells expressing siRNA-IR, suggesting that Rb inactivation and subsequent cell cycle progression were blocked in these cells. Additionally, reduced phosphorylation of ribosomal S6 kinases (RSK1/2/3) and protein kinase C-related kinases (protein-serine kinase 1) was also found through phospho-site screen. RSK, which is also known as p90rsk or MAPK-activated protein kinase-1, is the substrate and mediator of ERK signal transduction (53). PRKs are a subfamily of Ser/Thr-specific kinases with a catalytic domain highly homologous to the protein kinase C family (54). They are effectors of Rho family GTPases and are activated by fatty acids and phospholipids *in vitro* (55). Therefore, the reduction of RSK and PRK phosphorylation in cells with IR silencing indicates that both ERK and protein kinase C pathways are involved in α-cell functions, probably cell proliferation.

Results from kinase activation experiments showed that the Akt signaling pathway downstream of the IR could still be activated by many factors present in serum (FBS) in cells with IR knockdown (Figs. 6*A*, 6*B*, and 7*A*), although IR knockdown reduced Akt activation by insulin (Fig. 7*A*). It could therefore be argued that the effects of IR knockdown on cell growth and glucagon secretion at low glucose are mainly through insulin-specific pathways. However, the $p70^{S6K}$ activity in MIN6 and α-TC6 was enhanced after the removal of IR (Figs. 6*B* and 7*B*). The signaling pathways downstream of the IR responsible for regulating α-cell function are unclear, whereas in many cells the primary pathway for growth factors and cytokine activation of $p70^{S6K}$ appears to be the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway (56). mTOR controls various cellular responses to mitogens; thus other growth factors may be compensating for IR knockdown, leading to the increase in $p70^{S6K}$ activation. In addition, because mTOR functions as an "ATP sensor," increased glucose and branched-chain amino acid metabolism in cells

can lead to a direct activation of mTOR (57, 58). Thus, accumulation of nutrients, including amino acids, fatty acids, and their related metabolic substances may occur in the α-cells with IR knockdown, which could contribute to an increase of $p70^{S6K}$ phosphorylation independent of IRS signaling (59).

mTOR also provides negative feedback signals through downstream $p70^{S6K}$ to down-regulate the IRS-1/2-PI3K-Akt pathway (60–62). $p70^{S6K}$ cooperates with multiple pathways (such as JNK and NF-κB) that make the cells unresponsive to insulin (63–66). Chronic activation of $p70^{S6K}$ is related to insulin resistance and could result from genetic loss of certain tumor suppressor genes (40, 59, 67, 68). When the IR-IRS-PI3K/Akt pathway is down-regulated in the muscle and liver of rats (*i.e.* insulin-resistant, hyperinsulinemic fructose-hypertensive rats) and mice (*i.e.* wild-type mice on a high fat diet and genetically obesity mice (K/K A^y and ob/ob), $p70^{S6K}$ shows increased activity (59, 69, 70). Therefore, in the context of the current study, $p70^{S6K}$ may play a critical role in reducing insulin sensitivity in α-cells. Further investigation is required to examine the possible link between $p70^{S6K}$ activation and glucagon regulation.

Like $p70^{S6K}$, GSK3 phosphorylation was unexpectedly increased in cells expressing siRNA-IR as indicated by the phospho-site screen study. Insulin activates glycogen synthase by inducing its dephosphorylation at a cluster of C-terminal residues, which are phosphorylated by glycogen synthase kinase-3α (GSK3α) and GSK3β (71). In muscle, insulin stimulates the dephosphorylation of glycogen synthase at these residues by inducing the inactivation of GSK3α and GSK3β via phosphorylation of an N-terminal Ser residue (Ser-21 in GSK3α and Ser-9 in GSK3β), which is catalyzed by Akt and reversed by the muscle glycogen-associated protein phosphatase-1 (72). In the liver, phosphorylation of the sites targeted by GSK3 also modifies hepatic glycogen synthesis and GSK3 inhibitors stimulate hepatic glycogen synthase (73, 74). However, the possibility that insulin may regulate glycogen synthase by a mechanism that is independent of GSK3 has not been excluded. The relative contributions made by the inactivation of GSK3 *versus* potential activation of protein phosphatase or regulation via other pathways to the activation of glycogen synthase is unknown. And here, the role of GSK3 and the regulation of GSK3 activation in α-cells remain to be examined.

Activation of another energy sensor, AMPK (AMP-activated protein kinase), was unaltered in both MIN6 and α-TC cells with IR knockdown. AMPK responds to stress caused by hypoxia, heat shock, and low glucose and becomes activated by phosphorylation (75, 76). Our findings suggest that the AMPK glucose-sensing pathway, and therefore cellular glucose metabolism/ATP production, remains intact in α- and β-cells despite IR knockdown, consistent with the finding that IR silencing has no effect on glucose-stimulated increases in ATP production (48).

The dysfunction of islet cells is central to the pathogenesis of type 2 diabetes (14). IR signaling is critical for maintaining β-cell function (7), and we have extended these findings to the α-cell. Our studies suggest that both α- and β-cell proliferation is controlled by a common insulin signaling pathway and that secretion from these cell types may also be regulated by insulin pathways that overlap at certain levels. Thus, the evidence is further strengthened that defects leading to reduced insulin secretion from the β-cells might be associated with the failure of glucose-regulated glucagon secretion from the α-cells, as suggested by others (77, 78). Consequently, insulin-related pathways in the α-cell could be potential targets for controlling glucagon secretion and glucose counter-regulation.

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