The Repression of IRS2 Gene by ATF3, a Stress-Inducible Gene, Contributes to Pancreatic β-Cell Apoptosis

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OBJECTIVE—β-Cell failure is an essential component of all types of diabetes, and the insulin receptor substrate 2 (IRS2) branch of signaling plays a key role in β-cell survival and function. We tested the hypothesis that activating transcription factor 3 (ATF3), a stress-inducible proapoptotic gene, downregulates the expression of IRS2 in β-cells.

RESEARCH DESIGN AND METHODS—We used both the gain- and loss-of-function approaches to test the effects of ATF3 on IRS2 gene expression. We also analyzed the binding of ATF3 to the IRS2 promoter by chromatin immunoprecipitation assay and the transcription of the IRS2 gene by polymerase II occupancy assay. Furthermore, we tested the ability of IRS2 to alleviate the proapoptotic effects of ATF3 in cultured β-cells and in transgenic mice using the rat insulin promoter to drive the transgenes.

RESULTS—Expression of ATF3 is sufficient to reduce IRS2 gene expression; in contrast, knockdown or knockout of ATF3 reduces the ability of stress signals to downregulate IRS2 expression. ATF3 binds to the IRS2 promoter in vivo, and the binding of ATF3 correlates with decreased IRS2 gene transcription. Functionally, expression of IRS2 protects β-cells from ATF3-induced apoptosis.

CONCLUSIONS—IRS2 is a target gene of ATF3, and its repression by ATF3 contributes, at least partly, to the apoptosis induced by ATF3. Because ATF3 is a stress-inducible gene, our work provides a direct link to explain how environmental stress factors can modulate IRS2 gene transcription. ***Diabetes*** **57: 635–644, 2008**

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Received for publication 28 May 2007 and accepted in revised form 20 November 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 5 December 2007. DOI: 10.2337/db07-0717.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0717.

ATF3, activating transcription factor 3; ChIP, chromatin immunoprecipitation; CREB, cAMP response element-binding protein; FACS, fluorescence-activated cell sorting; GLP, glucagon-like peptide; IFN-γ, γ-interferon; IL-1β, interleukin-1β; IRS2, insulin receptor substrate 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; RIP, rat insulin promoter; TNF-α, tumor necrosis factor-α.

Insulin signaling plays an important role in the pathogenesis of diabetes. Upon activation, the insulin receptor recruits and phosphorylates its substrates, insulin receptor substrates (IRSs), to initiate signal transduction (rev. in 1 and 2). Although the IRS family of proteins includes IRS1–IRS6 and other proteins such as GAB1, GAB2, and CBL (2,3), the majority of the insulin action appears to be mediated by IRS1 and IRS2 (rev. in 3). Research using mice deficient in IRS1 or IRS2 has shed light on their respective roles in diabetes. Mice deficient in IRS1 develop peripheral insulin resistance and mild glucose intolerance but never develop diabetes, presumably due to the compensation by the increased pancreatic β-cell growth and insulin secretion (4,5). In contrast, mice deficient in IRS2 consistently develop diabetes (6,7). While insulin resistant at an early age, the IRS2$^{-/-}$ mice are initially able to compensate for this through increased insulin secretion. However, as they age, their β-cells fail to adequately compensate for insulin demand and begin to die, thus resulting in overt diabetes. This progression from insulin resistance to β-cell failure and finally diabetes mimics the natural development of type 2 diabetes. Thus, the IRS2 branch of signaling is a key pathway that regulates both the peripheral insulin signaling and β-cell mass/function (rev. in 8). Significantly, the diabetic phenotype in the IRS2 knockout mice can be prevented by crossing them with transgenic mice expressing IRS2 in their β-cells (9) or transgenic mice engineered to have increased β-cell mass and function by either expression of Pdx1 (10) or haploinsufficiency of FoxO1 (11).

Despite the above results from animal models indicating a potential role of IRS2 in type 2 diabetes, polymorphisms in IRS2 gene are rare and not associated with common type 2 diabetes in humans (12; rev. in 13), suggesting that genetic mutations of IRS2 are most likely not the causal factors for type 2 diabetes. One explanation is that the function of IRS2 can be compromised by various means without the mutations in its corresponding gene. These can include *1*) the inhibition of IRS2 expression and/or function by regulatory factors and *2*) the dysfunction of any steps downstream from IRS2 signaling. In this report, we demonstrate that activating transcription factor 3 (ATF3), a stress-inducible gene, downregulates the expression of IRS2, providing a molecular link from cellular stress response to IRS2 transcription.

ATF3 is a member of the ATF/cAMP response element-binding protein (CREB) family of transcription factors (rev. in 14–17). Overwhelming evidence indicates that ATF3 gene is induced in many tissues by a variety of stress signals, including in the pancreatic β-cells by proinflammatory cytokines, reactive oxygen species, and high concentrations of glucose or fatty acids (18–20). Recently, we

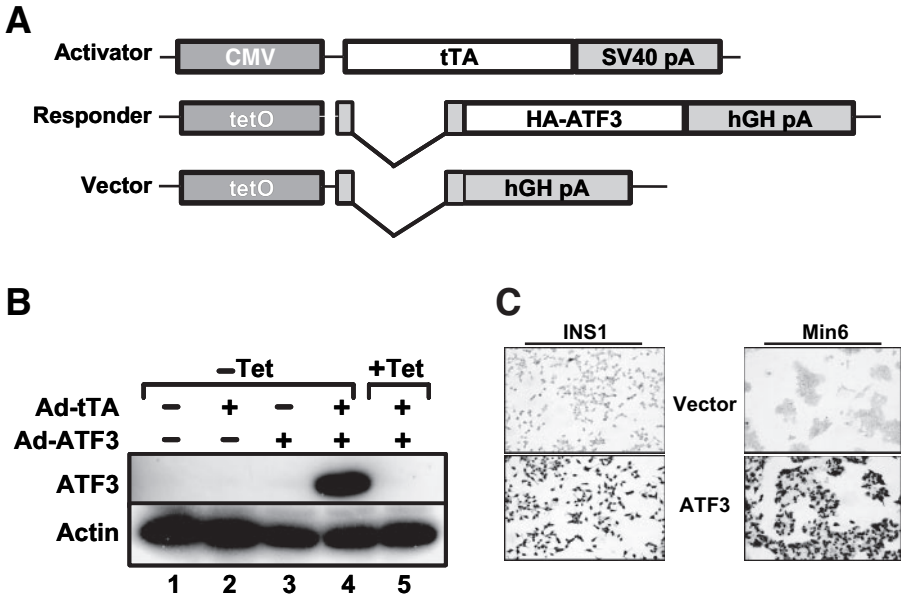

FIG. 1. A tet-off system to express ATF3. *A*: A schematic of the activator and responder. An exon-intron-exon junction from the β-globin gene is upstream from the ATF3 open reading frame, and the SV40 or human growth hormone (hGH) polyadenylation signals (pA) downstream. *B*: MIN6 β-cells were infected and treated with tetracycline (Tet) for 12 h followed by immunoblot. *C*: INS832/13 and MIN6 cells were infected with Ad-tTA plus Ad-Vector or Ad-ATF3 for 24 h, followed by immunocytochemistry for ATF3.

demonstrated that ATF3 is likely to play an important role in β-cell apoptosis and diabetes by both gain- and loss-of-function approaches. On the one hand, transgenic mice expressing ATF3 in the pancreatic β-cells have abnormal islet organization, reduced β-cell mass, and symptoms secondary to β-cell deficiency (18,19). On the other hand, knockout islets deficient in ATF3 are less vulnerable to stress-induced apoptosis than their wild-type counterparts (19). Significantly, ATF3 expression is elevated in the β-cells of diabetic patients (19), indicating that this pro-apoptotic gene is upregulated during the pathophysiological progression of diabetes. Despite the potential physiological relevance of ATF3 in β-cell apoptosis, the mechanisms by which ATF3 promotes β-apoptosis have not been elucidated. Because it is a transcription factor, ATF3 must exert its action, at least partly, by regulating downstream target genes. However, no target genes of ATF3 in β-cells have been identified. The following clues prompted us to hypothesize that IRS2 is a target of ATF3.

Jhala et al. (21) demonstrated that CREB, a prosurvival factor in many cell types (22–26), binds to the IRS2 promoter at TGACGCCCC and upregulates its expression. Because the binding site differs from the consensus CRE/ATF sequence TGACGTCAC by only two nucleotides (underlined), it could also be a binding site for ATF3. We hypothesize that ATF3, as a transcriptional repressor, may repress IRS2 expression, providing an explanation for its proapoptotic role in β-cells. Here, we present evidence supporting this hypothesis.

RESEARCH DESIGN AND METHODS

Cell culture and treatments. MIN6 and INS832/13 cells were grown as described previously (27,28). Both cells were used throughout the study, and the results are reproducible; only data from those with the most repeats are shown. Treatments, when indicated, are the following: 10 ng/ml interleukin-1β (IL-1β), 60 ng/ml tumor necrosis factor-α (TNF-α), 250 ng/ml γ-interferon (IFN-γ), 2.5 nmol/l exendin-4, 1 μmol/l thapsigargin, and 1 μg/ml tetracycline.
Animals and adenoviruses. The rat insulin promoter (RIP)-ATF3 transgenic mice were generated by pronuclear injection. The RIP-IRS2 transgenic mice and the ATF3 knockout mice were described previously (9,19). Double transgenic mice expressing ATF3 and IRS2 were obtained by crossing RIP-ATF3 with RIP-IRS2. Adenoviruses were generated using the Invitrogen Gateway technology and purified by cesium chloride ultracentrifugation (29).
Islets. Adult pancreata were removed after injecting Liberase (Roche) into the common bile duct; postnatal pancreata (<4 weeks) were removed and then digested with Liberase. After digestion, islets were isolated using gradient and filtration (30,31), followed by hand pick.
Cell viability and fluorescence-activated cell sorting analyses. Cell viability was measured by crystal violet or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) as described previously (32). For fluorescence-activated cell sorting (FACS) analyses, cells were stained with annexin V-fluorescein isothiocyanate (Pharmingen) and propidium iodide (Sigma), followed by flow cytometry (BD FACSCalibur).
RNA analyses and chromatin immunoprecipitation assay. Total RNA was isolated by Trizol (Invitrogen) and subjected to RT-PCR or quantitative RT-PCR using the QuantiTect SYBR Green PCR kit (Qiagen) in triplicate. Chromatin immunoprecipitation (ChIP) assay was carried out as described previously (32). All PCR primers are listed in supplemental Table 1 (available in an online appendix at http://dx.doi.org/10.2337/db07-0717).
Immunoblot, immunohistochemistry, and immunocytochemistry. Fifty to 100 μg protein was analyzed by immunoblot. The primary antibodies were the following: anti-ATF3 (Santa Cruz), anti-actin (Sigma), anti-IRS2 (Upstate), anti-activated caspase 3 (Cell Signaling), anti-insulin (Dako), and anti-Ki67 (Dako).
Area analysis and glucose tolerance test. Five pancreata from each group of mice were analyzed. Approximately 50 fields per genotype were digitally analyzed using the region measurement function of MetaVue. Mice were fasted for 15–18 h, injected intraperitoneally with glucose (1 g/kg body wt), and assayed for blood glucose (Bayer Glucometer).
Statistics. All quantitative data are expressed as means ± SD, and comparisons are made by the Student's *t* test.

RESULTS

Expression of ATF3 promotes apoptosis in pancreatic β-cells. To study the function of ATF3 in MIN6 and INS832/13 β-cells, we developed adenoviruses expressing hemagglutinin-tagged ATF3 using the tet-off system (Fig. 1*A*). As shown in Fig. 1*B*, infecting β-cells with both the activator and responder viruses resulted in ATF3 expression in the absence but not in the presence of tetracycline. Because adenoviral infection can be cytotoxic, we titrated the virus and found that multiplicity of infection of 100 gave rise to low cytotoxicity but high infection efficiency (>85%; Fig. 1*C*). Consistent with a proapoptotic role of ATF3, β-cells expressing ATF3 had reduced cell number (Fig. 2*B*) and enhanced caspase 3 activation (Fig. 2*C*). The reduced attachment to the plates (Fig. 2*A*) is consistent with the increased cell death and suggests that ATF3 may affect cell adhesion.

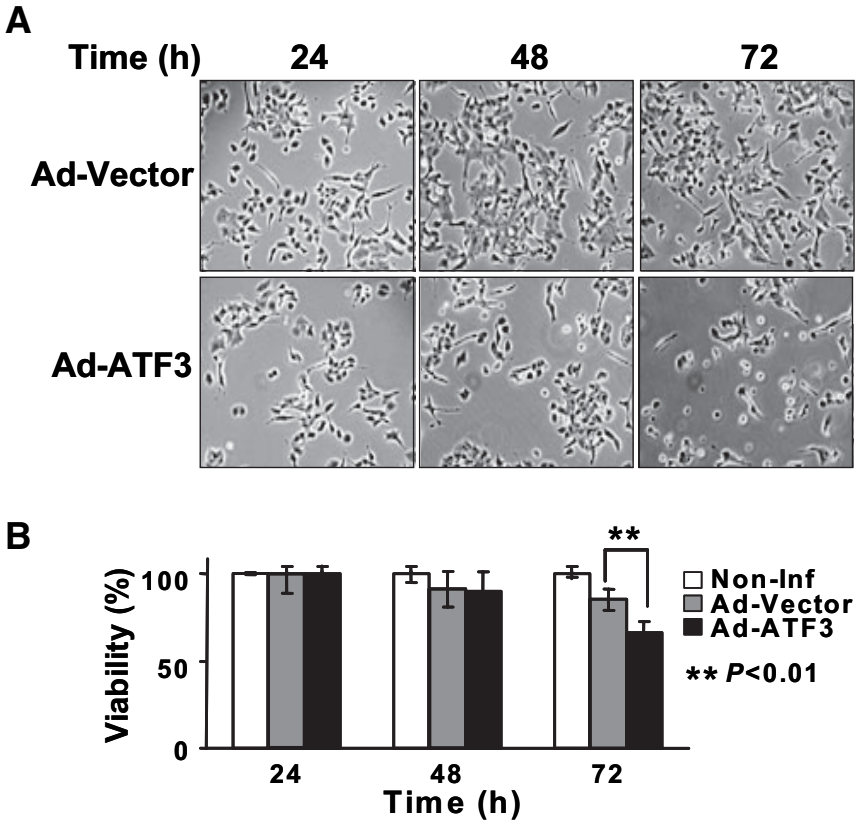

FIG. 2. Expression of ATF3 is proapoptotic in β-cells. *A*: MIN6 cells were infected with Ad-tTA plus the indicated viruses for the indicated times. Phase contrast images are shown. *B*: MIN6 cells were noninfected (Non-Inf) or infected as indicated and assayed by crystal violet. A_{595} from the noninfected cells at each time point was arbitrarily defined as 1. Triplicate samples were assayed for each experiment to obtain the means ± SD. Shown is a representative of four experiments. *C*: Same as *B* but immunoblot is shown.

ATF3 plays a necessary and sufficient role in repressing IRS2 expression. To determine whether ATF3 represses IRS2 expression, we expressed ATF3 and examined the IRS2 mRNA level by quantitative RT-PCR and protein level by immunoblot. As shown in Fig. 3*A* and *B*, MIN6 cells infected with Ad-tTA+Ad-ATF3 had reduced IRS2 mRNA (Fig. 3*A*) and protein (Fig. 3*B*) levels compared with cells infected with Ad-tTA+Ad-Vector virus. We note that ATF3 protein is detectable at 4 h after infection (supplemental Fig. 1); the lack of signal in Fig. 3*B*, *lane 4*, is due to the short exposure. Because IRS2 is upregulated by prosurvival signals such as glucagon-like peptide 1 (GLP-1; rev. in 33), we asked whether ATF3 can antagonize this induction. As shown in Fig. 3*C*, ATF3 decreased the expression of IRS2 under exendin-4 treatment. Thus, ATF3 is sufficient to repress both the basal (Fig. 3*A* and *B*) and induced (Fig. 3*C*) expression of IRS2.

Because ATF3 is induced by a variety of stress signals, our results suggest that the expression of IRS2 may be reduced under stress conditions. As shown in Fig. 3*D* and *E*, IL-1β reduced IRS2 mRNA levels (Fig. 3*D*) and protein levels (Fig. 3*E*). Control experiments confirmed that ATF3 was transiently induced by IL-1β as we reported previously (19). To test whether ATF3 is necessary for cytokines to reduce the expression of IRS2, we took a knockdown approach. Among three short hairpin RNAs designed to knockdown ATF3 (sh*ATF3*), one worked well (Fig. 3*G*; data not shown). Quantitative RT-PCR analysis showed that this sh*ATF3* reduced the ability of proinflammatory cytokines to inhibit the expression of IRS2 gene (Fig. 3*F*). Immunoblot confirmed the effect of sh*ATF3* on IRS2 protein level (Fig. 3*G*).

To determine whether the results from the β-cell lines are reproducible in primary islets, we examined islets isolated from transgenic mice expressing ATF3 in the pancreatic β-cells driven by the RIP. As shown in Fig. 4*A*, the RIP-ATF3 islets had lower IRS2 protein level than islets from the nontransgenic littermates. The RIP-ATF3 islets also had higher activated caspase 3 than the nontransgenic islets (Fig. 4*A*, ~2.5-fold increase based on three experiments), consistent with a proapoptotic activity of ATF3. The RIP-ATF3 islets were visibly distinguishable from the control islets, with a higher percentage displaying irregular borders and poor morphological integrity (Fig. 4*B*). Using a complementary approach, we examined the effects of ATF3 deficiency on IRS2 expression

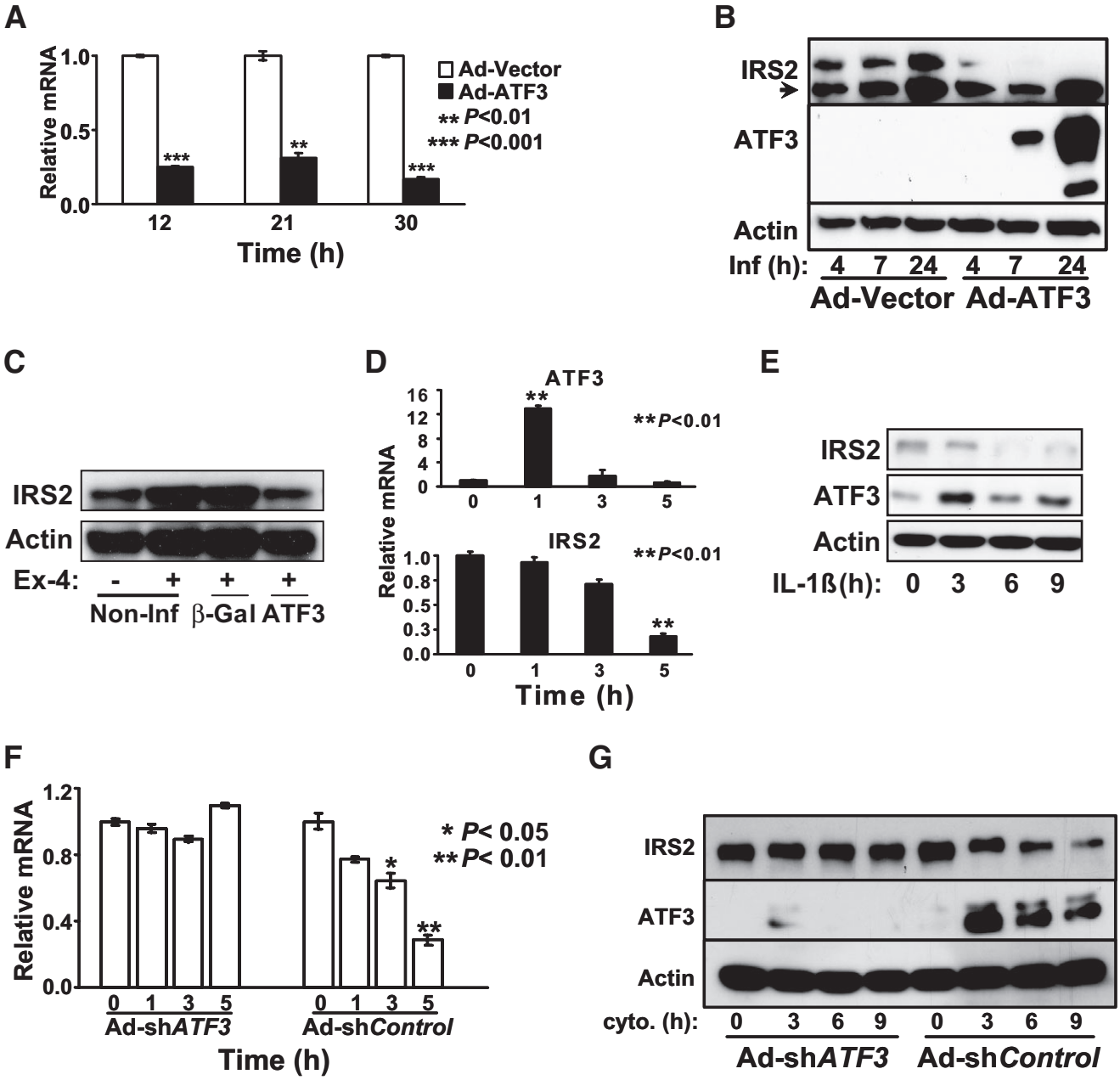

FIG. 3. ATF3 plays a sufficient and necessary role in repressing IRS2 in β-cell lines. *A*: MIN6 cells were infected with the indicated viruses and assayed by quantitative RT-PCR for IRS2. The signals were standardized against actin and the standardized signals from the vector control for each time point were arbitrarily defined as 1. **, ***Versus vector. *B*: Same as *A*, except immunoblot is shown. Arrow, nonspecific. *C*: MIN6 cells were noninfected or infected with the indicated virus for 12 h and treated with exendin-4 (Ex-4) for 6 h before immunoblot. *D*: MIN6 cells were treated with IL-1β for the indicated times and assayed for ATF3 and IRS2 by quantitative RT-PCR. The standardized signals at time point 0 were arbitrarily defined as 1. **Versus time 0. *E*: Same as *D*, except immunoblot is shown. *F*: INS832/13 cells were infected with control virus (sh*Control*) or sh*ATF3* virus for 24 h to knockdown ATF3, treated with IL-1β plus TNF-α for the indicated times, and assayed by quantitative RT-PCR for IRS2. The signals (standardized against actin) at time point 0 were arbitrarily defined as 1. *, **Versus time 0. *G*: Same as *F*, except immunoblot is shown. For all quantitative RT-PCR results, the mean ± SD from triplicate samples is shown. Shown are representatives of at least three independent experiments.

by comparing the wild-type ($ATF3^{+/+}$) and knockout ($ATF3^{-/-}$) islets. Although pro-inflammatory cytokines reduced IRS2 expression in the β-cell lines (see above), they did not repress IRS2 expression in the primary islets in a consistent manner in our hand. Thus, we used the endoplasmic reticulum stress paradigm, which was shown to downregulate IRS2 gene expression (34). We confirmed that thapsigargin, an endoplasmic reticulum stress inducer, induced ATF3 expression in the islets, consistent with its ability to induce ATF3 in other cell types (35,36). Importantly, the reduction of IRS2 expression by thapsigargin was absent in the $ATF3^{-/-}$ islets (Fig. 4*C*). Thus, ATF3 plays a necessary role for endoplasmic reticulum stress to downregulate IRS2 in the primary islets.

ATF3 binds to the IRS2 promoter and inhibits its transcription. Although the above results showed a necessary and sufficient role of ATF3 in downregulating IRS2 in both β-cell lines and primary islets, they do not indicate whether IRS2 is a direct target gene of ATF3. To address this issue, we examined the binding of ATF3 to the IRS2 promoter using ChIP assay. As shown in Fig. 5*A*, expres-

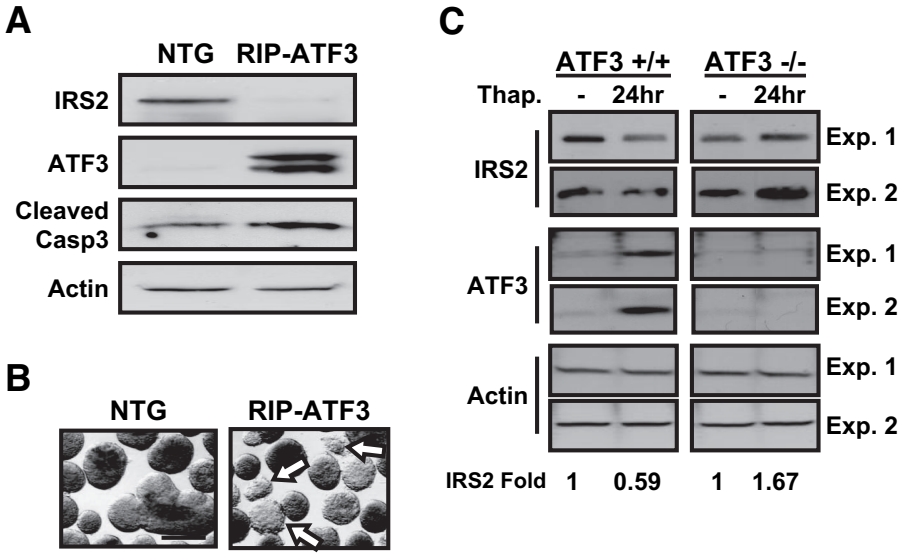

FIG. 4. ATF3 plays a sufficient and necessary role in repressing IRS2 in primary islets. *A*: Primary islets from postnatal nontransgenic (NTG) or RIP-ATF3 mice were analyzed by immunoblot (100 μg/lane from seven mice). *B*: A representative phase contrast image of islets is shown. Islet size and yield from the RIP-ATF3 mice were much lower than that from the nontransgenic mice. The image was taken after hand pick of islets with comparable sizes. Three RIP-ATF3 islets showing irregular boarders are indicated by arrows. Bar = 200 μm. *C*: Primary islets from ATF3$^{+/+}$ or ATF3$^{-/-}$ mice were treated with thapsigargin (Thap.) or vehicle (−), followed by immunoblot. Shown are data from two experiments (Exp. 1 and Exp. 2). The IRS2 signals were standardized against the actin signals, and the fold change was calculated by arbitrarily defining the normalized signals from vehicle control as 1.

sion of ATF3 by adenovirus resulted in a ChIP signal derived from primers flanking the CRE/ATF-like site on the IRS2 promoter (primers 2 in Fig. 5*C* schematic). The specificity of the band was demonstrated by the lack of signals when control IgG or Ad-Vector was used. We also examined the binding of endogenous ATF3 to the IRS2 promoter. We treated the cells with proinflammatory cytokines to induce ATF3 expression (as reported previously; data not shown) and examined the binding of ATF3 on the IRS2 promoter by ChIP. As shown in Fig. 5*B*, at 4 h after cytokine treatment, ATF3 bound to the IRS2 promoter (Fig. 5*B*, *top*, *lane 1*). Control primers for a region ~5 kb downstream from the binding site (primers 1) did not show any specific signal (Fig. 5*B*, *bottom*). A faint band was present in all samples and was not specific to ATF3 expression or ATF3 antibody. Taken together, our data indicate that both exogenously and endogenously expressed ATF3 binds to the IRS2 promoter in vivo.

Because ATF3 is a transcriptional factor, the binding of ATF3 to the IRS2 promoter suggests (but does not prove) that ATF3 modulates its transcription. To address this issue, we examined RNA polymerase II (Pol II) occupancy on the IRS2 gene by ChIP using three sets of primers (Fig. 5*C* schematic). *1*) Primer set 1 targets to a region ~4 kb downstream from the transcriptional start site (+1). Pol II occupancy in this region would indicate active transcription of the IRS2 gene. *2*) Primer set 4 is similar to primer set 2 but produces a smaller amplicon than primer set 2 and is suitable for quantitative PCR. ChIP signals from this primer set indicate the Pol II loading on the promoter but not transcription. This primer set also can detect ATF3 binding, thus allowing a side-by-side comparison of the binding of ATF3 to the promoter with the transcription of the IRS2 gene (Pol II occupancy using primer set 1). *3*) Primer set 3 targets to a region ~6 kb upstream from the +1 site and is used as a control to rule out false-positive signals due to insufficient shearing of the DNAs.

As demonstrated in Fig. 5*C* (*left panel*), the ATF3 antibody only precipitated DNA fragment around the CRE/ATF-like site but not at the upstream (−6 kb) or downstream (+4 kb) region. The Pol II antibody precipitated DNA fragment containing the proximal promoter region (indicating Pol II loading) and DNA in the +4 kb region (indicating transcription). We also carried out controls to demonstrate target specificity using primers for the promoter regions of β- and α-actin. As indicated by the ChIP data, Pol II bound to the β-actin but not α-actin promoter (Fig. 5*C*, *right panel*), consistent with the fact that β-actin is expressed in β-cells but α-actin is not. We also carried out ChIP using ATF3 antibodies and demonstrated that ATF3 did not bind to the actin promoters. Binding of Pol II and ATF3 to the IRS2 promoter using primer set 4 was included as control. After validating the assays, we examined IRS2 gene transcription (using Pol II antibodies and primer set 1) at various times after cytokine treatment and quantified the data by quantitative RT-PCR. In parallel, we examined ATF3 binding to the IRS2 promoter using primer set 4. As shown in Fig. 5*D*, IRS2 gene transcription decreased on cytokine treatment, and the decline correlated with ATF3 binding. Taken together, our results from in vivo DNA binding and transcription assays (Fig. 5), combined with those from expression assays (Figs. 3 and 4), strongly support the notion that IRS2 is a direct target gene of ATF3 and that ATF3 represses its transcription.

Expression of IRS2 ameliorates the proapoptotic effects of ATF3. Because IRS2 is a prosurvival gene, the repression of IRS2 by ATF3 is likely to be functionally important. To test this idea, we carried out an add-back experiment. We infected INS832/13 β-cells with adenovirus-expressing βGal (control), ATF3 alone, IRS2 alone, or both proteins. As shown in Fig. 6*A*, expression of ATF3 resulted in reduced cell viability, consistent with the results from MIN6 β-cells (Fig. 2). Expression of IRS2 alone did not reduce viability; however, when coexpressed with ATF3, IRS2 inhibited the ability of ATF3 to reduce viability. We also added cytokine treatment to viral transduction to increase the stress intensity. Consistently, we observed enhanced reduction in viability. However, the trend remained the same: ATF3 had cytotoxic effect, IRS2 did not, and coexpression inhibited the cytotoxicity (Fig. 6*B*). Examination of caspase 3 activation confirmed that coexpression of IRS2 reduced caspase 3 activation induced by ATF3 (Fig. 6*C*). The βGal control adenovirus also induced caspase 3 activation to some extent, presumably due to the cytotoxic effect of viral infection. We also examined the cells by annexin V combined with propidium iodide stain followed by FACS. Consistent with the above results, IRS2 expression reduced the cytotoxic effect of ATF3. Figure 6*D* shows representative flow cytometry

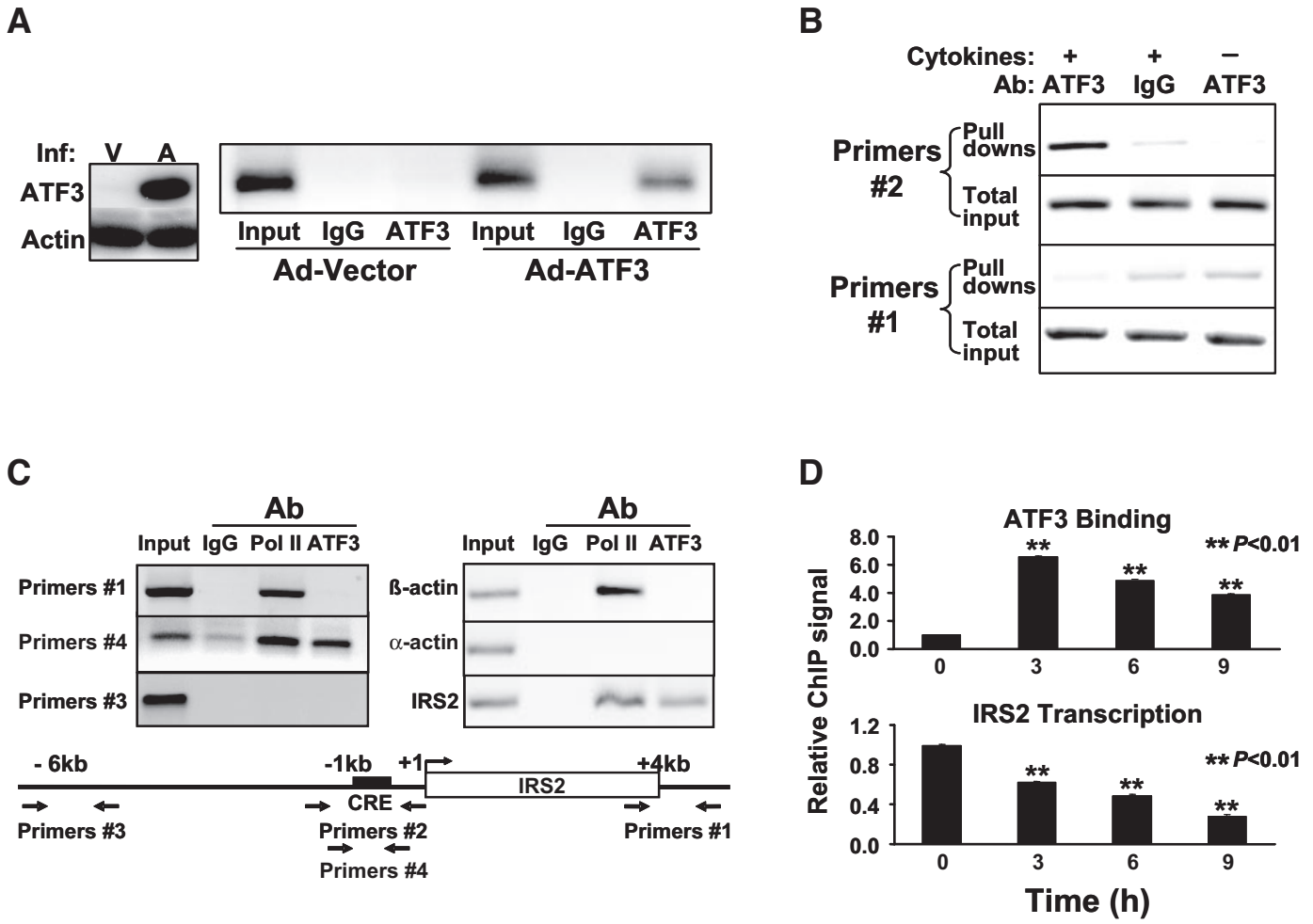

FIG. 5. ATF3 binds to the IRS2 promoter and downregulates the transcription of IRS2 gene. *A*: MIN6 cells were infected with Ad-tTA+Ad-Vector (V) or Ad-tTA+Ad-ATF3 (A) for 8 h and analyzed by immunoblot (*left*) or ChIP using primers 2 (*right*). Input: 5% of genomic DNAs. *B*: MIN6 cells were treated with IL-1β plus TNF-α plus IFN-γ for 4 h and analyzed by ChIP. *C*: INS832/13 cells were analyzed by ChIP. *Left*: IRS2 primers; *right*: IRS2 primers 4 and actin primers. *D*: INS832/13 cells were treated with IL-1β plus TNF-α for the indicated times and ChIP analyses using quantitative PCR. Signal for each assay at time point 0 was arbitrarily defined as 1. Shown is a representative of three independent experiments, with mean ± SD calculated from triplicate samples. **Versus time 0.

data, and Fig. 6*E* shows the quantitation of three independent experiments.

Previously, we generated transgenic mice expressing IRS2 in the pancreatic β-cells driven by RIP: the RIP-IRS2 mice (9). We showed that the mice had increased pancreatic insulin content and were resistant to the development of diabetes in several models: age-induced, high-fat diet–induced, and streptozotocin-induced diabetes (9). In contrast, transgenic mice expressing ATF3 in the β-cells (driven by the Pdx-1 or the transthyretin promoter) had abnormal islet organization, reduced β-cell mass, and symptoms secondary to β-cell deficiency (18,19). The add-back experiments using cultured β-cells (above) predicted that expression of IRS2 in the β-cells would also ameliorate the defects elicited by ATF3 in the transgenic mice. To test this prediction, we generated transgenic mice expressing ATF3 under the control of RIP: the RIP-ATF3 mice. These mice would express ATF3 under the control of the same promoter that drives the expression of IRS2 in RIP-IRS2. The RIP-ATF3 mice survived to adulthood and were fertile. This feature is different from the Pdx-ATF3 transgenic mice, which died before mating (19). Presumably, this is because RIP has a later and more restricted expression pattern than the Pdx-1 promoter. Immunohistochemistry analyses demonstrated the expression of ATF3 and the reduced IRS2 level in the RIP-ATF3 islets (Fig. 7*A*), consistent with the data from the isolated islets (Fig. 4*A*). Furthermore, the RIP-ATF3 mice had a significant reduction in their β-cell surface areas (Fig. 7*A* and *B*). Consistent with these findings, the RIP-ATF3 mice developed glucose intolerance, as shown by the glucose tolerance test (Fig. 7*C*).

To address the potential mechanisms for the reduced islet size in the RIP-ATF3 mice, we examined apoptosis by immunohistochemistry using antibodies against activated caspase 3. As shown in Fig. 7*A*, the RIP-ATF3 islets had higher activated caspase 3 than the nontransgenic islets, consistent with the data from the isolated islets (Fig. 4*A*) and further supporting a proapoptotic role of ATF3. We also examined β-cell replication by assaying Ki67, a proliferation marker. Analyses of multiple sections from at least three mice per group indicated that the RIP-ATF3 mice had reduced proliferation: Ki67-positive cells per 10,000 μm^2 islet area was 2.1 ± 0.6, compared with 4.6 ± 1.5 in the nontransgenic mice ($P < 0.05$). To test whether IRS2 can rescue the mice, we crossed RIP-ATF3 with RIP-IRS2 and analyzed the double transgenic mice in parallel with other mice. As shown in Fig. 7*A–C*, the

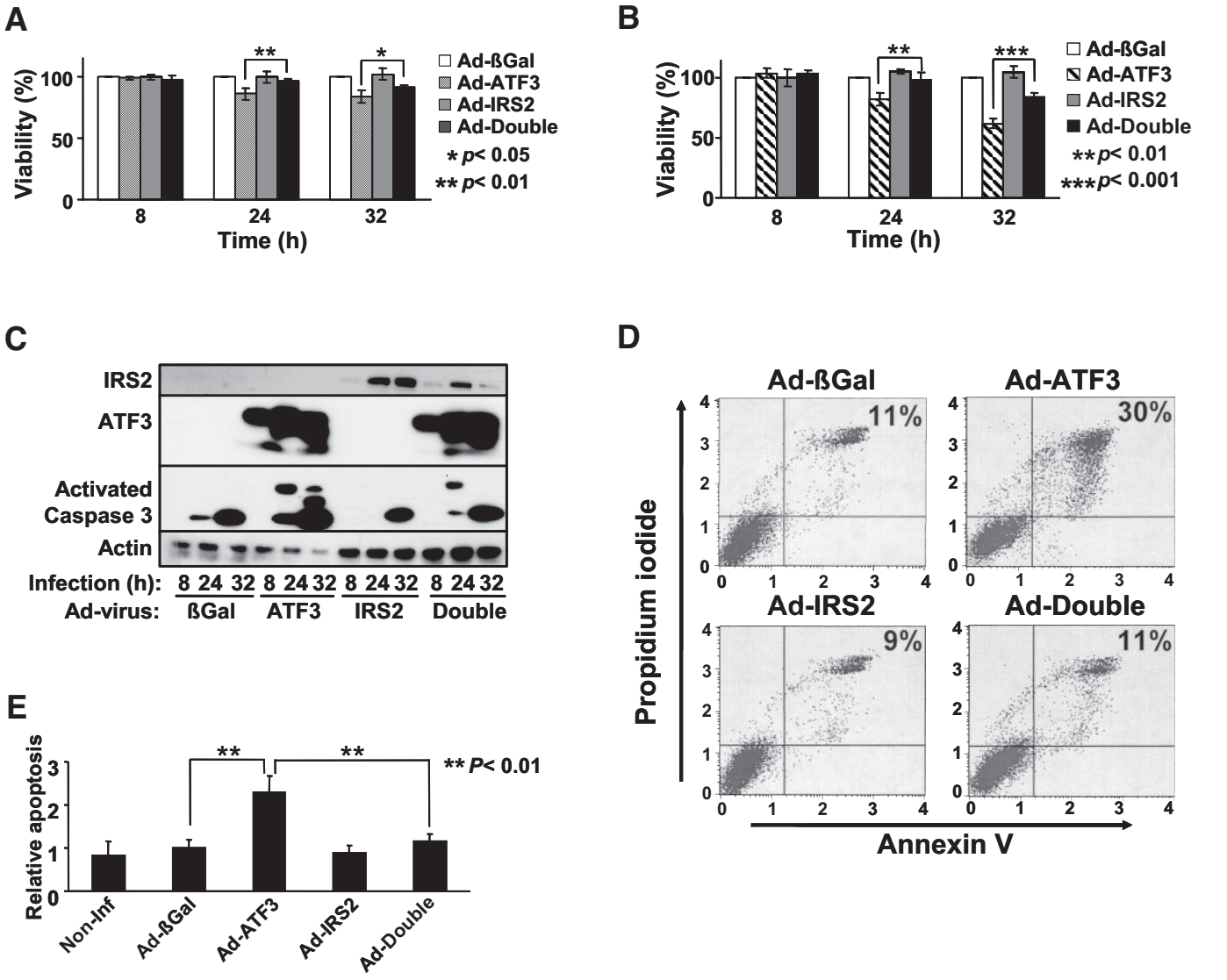

FIG. 6. Expression of IRS2 ameliorates the effects of ATF3 in cultured β-cells. *A*: INS832/13 cells were infected with the indicated adenoviruses for the indicated times and analyzed by MTT. A_{570} reading of βGal-expressing cells at each time point was arbitrarily defined as 1. Triplicate samples were assayed for each experiment to obtain the mean ± SD. Shown is a representative of four experiments. *B*: Same as *A*, except IL-1β plus TNF-α were added at 1 h after infection. *C*: Same as *A*, except immunoblot is shown. *D*: Same as *A*, except harvested at 24 h, stained, and analyzed by FACS. Shown is a representative of three experiments. Numbers indicate the percentages of cells in the *top right quadrant*. *E*: Derived from *top right quadrant* of *D*. The number from βGal-expressing cells was arbitrarily defined as 1.

double transgenic mice behaved almost identically to the nontransgenic or the IRS2 single transgenic littermates.

DISCUSSION

Accumulating evidence indicates that β-cell destruction in various pathophysiological conditions can be viewed as a consequence of stress response. First, all signals that are known to be relevant to type 1 diabetes, type 2 diabetes, and graft rejection in islet transplantation can also be considered as stress signals. These include *1*) proinflammatory cytokines, *2*) elevated glucose and elevated free fatty acids, and *3*) ischemia, hypoxia, lack of nutrients, and immune rejection. Second, many of the signaling pathways activated by these signals are similar, including Jun NH_2-terminal kinase–, nuclear factor-κB–, p38-, Janus tyrosine kinase/Stat–, and Fas-associated death domain/caspase 8–mediated pathways. Third, many of these signals have been demonstrated to affect the same regulators of cell death machinery, such as cytochrome c, Bcl family of proteins, and caspases. Thus, an emerging picture from a vast amount of literature is that the molecules, signaling pathways, and cellular machineries involved in the demise of β-cells under seemingly divergent pathophysiological conditions—type 1 diabetes, type 2 diabetes, and islet transplantation—are, to a large extent, the same. The current view is that the balance between the proapoptotic and anti-apoptotic (protective) processes determines the fate of β-cells.

In this report, we present evidence that ATF3, a stress-inducible proapoptotic gene, represses the expression of IRS2, a prosurvival gene, thus providing a direct link between stress response and a potent prosurvival pathway. Although the best-known mechanism to regulate IRS2 is its activation by the insulin receptor upon insulin/IGF treatment (rev. in 1 and 2), recent studies indicate that regulation of IRS2 expression also constitutes an important mechanism (21,34). Previously, IRS2 was demonstrated to be upregulated by GLP-1 via CREB (21). Here,

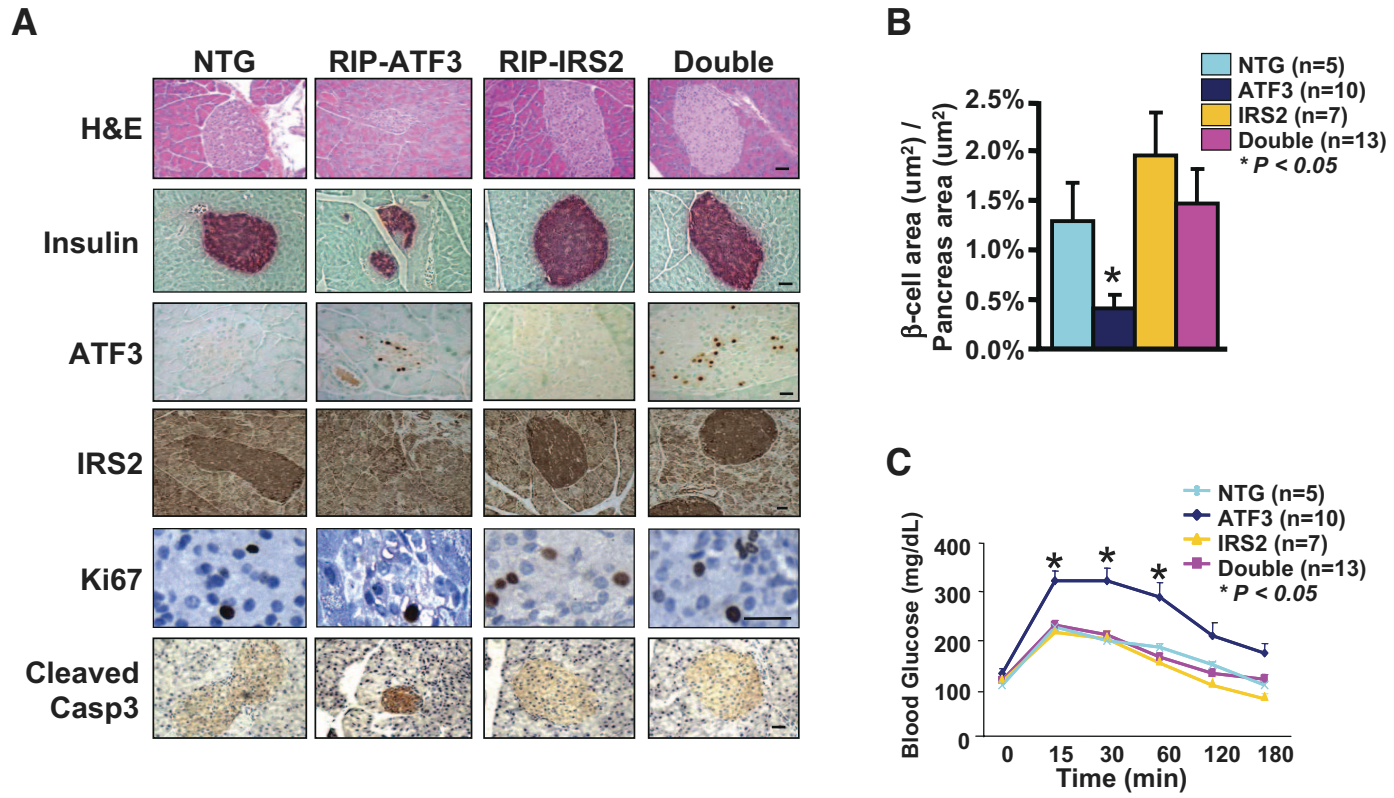

FIG. 7. IRS2 ameliorates the phenotype of the RIP-ATF3 mice. *A*: Pancreata from NTG, RIP-ATF3, RIP-IRS2, and the double transgenic mice at around 4 weeks of age were analyzed by hematoxylin-eosin staining or immunohistochemistry. Bar = 100 μm. *B*: Average percent β-cell surface area (β-cell area/pancreatic area). *C*: Glucose tolerance test. *ATF3 versus all others.

we demonstrate that ATF3, another member of the ATF/CREB family of transcription factors, has an opposite effect from CREB and represses the transcription of IRS2 gene. Thus, the CRE/ATF-like site on the IRS2 promoter acts as an integration point for survival signals (such as GLP-1) and stress signals (such as proinflammatory cytokines) to exert their opposite effects on IRS2 transcription (Fig. 8). Because ATF3 is induced by a variety of stress signals, it can function as a conduit for stress signals to dampen a potent prosurvival pathway in β-cells. In addition to transcriptional regulation, IRS2 expression can be modulated by its protein stability (37,38). Thus, it is possible to dysregulate IRS2 by multiple mechanisms, including its transcription, protein stability, and activity. These observations provide an explanation for the lack of IRS2 gene polymorphisms associated with common type 2 diabetes in humans (12), despite the potential importance of IRS2 (see the introduction).

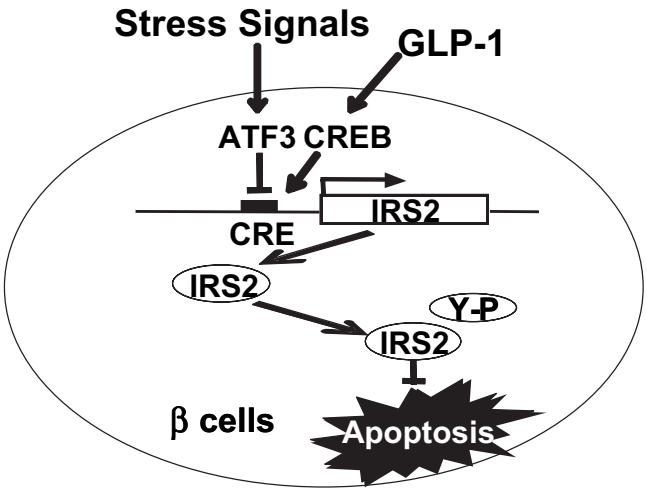

FIG. 8. A model for the opposite regulation of IRS2 transcription via the CRE/ATF site (see DISCUSSION).

Thus far, a handful of target genes of ATF3 have been reported, including Id-1 in keratinocytes (39), PEPCK and asparagine synthetase in hepatocytes (40,41), phospholamban in cardiomyocytes (42), adiponectin in adipocytes (43), and inflammatory genes in macrophages (44,45). However, target genes for ATF3 in β-cells have not been identified. Our report is the first to demonstrate a target gene of ATF3 in β-cells. Previously, the IRS2$^{-/-}$ β-cells were demonstrated to have reduced Pdx1 expression (10,11), an important gene for β-cell development and function. We thus examined the potential impact of ATF3 on Pdx1 expression. Our preliminary results suggest that expression of ATF3 leads to a reduced Pdx1 mRNA level; however, this effect was not observed in cells treated with exendin-4 (supplemental Fig. 2). Thus, a potential interplay between ATF3 and Pdx1 exists; however, it is not clear whether ATF3 reduces Pdx1 gene expression directly or indirectly through IRS2. Importantly, the add-back experiments indicated that the downregulation of IRS2 contributes to the proapoptotic effect of ATF3. The mechanisms by which IRS2 contributes to β-cell growth and survival can be explained, at least partly, through its activation of the phosphatidylinositol 3-kinase–Akt signaling pathways (rev. in 2). Akt has been demonstrated to phosphorylate and inactivate several proapoptotic molecules, such as BAD, Foxo1, and GSK-3β (rev. in 2 and 3). Thus, downregulation of IRS2 by ATF3 provides a mech-

anistic understanding for the proapoptotic role of ATF3 in β-cells and supports our previous conclusion that ATF3 may play an important role in diabetes (19). Clearly, ATF3 is most likely to affect β-cells via multiple mechanisms. Further investigation is required to understand its potential biological significance in β-cell biology.

ACKNOWLEDGMENTS

T.H. has received grants from the American Diabetes Association (7-05-RA-52) and the National Institutes of Health (RO1-DK-064938 and RO1-DK-59605). This work has received additional support from National Institutes of Health Grant P30-NS-045758.

We thank Dr. P. Quinn for insightful suggestions, Dr. M. Magnuson for the RIP enhancer construct, Dr. H. Bujard for the tTA construct, Dr. N. Hernandez for the U6 promoter construct, Dr. S.O. Yoon for Ad-βGal, The W.M. Keck Genetic Research Facility at the Ohio State University for generating the RIP-ATF3 mice, and a previous member, Dr. M. Hartman, for the initial analyses of the RIP-ATF3 mice.

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