

Regulation of MafA Expression in Pancreatic β -Cells in *db/db* Mice With Diabetes

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OBJECTIVE—Islet β -cells lose their ability to synthesize insulin under diabetic conditions, which is at least partially due to the decreased activity of insulin transcription factors such as MafA. Although an in vitro study showed that reactive oxygen species (ROS) decrease MafA expression, the underlying mechanism still remains unclear. In this study, we examined the effects of c-Jun, which is known to be upregulated by ROS, on the expression of MafA under diabetic conditions.

RESEARCH DESIGN AND METHODS—To examine the protein levels of MafA and c-Jun, we performed histological analysis and Western blotting using diabetic *db/db* mice. In addition, to evaluate the possible effects of c-Jun on MafA expression, we performed adenoviral overexpression of c-Jun in the MIN6 β -cell line and freshly isolated islets.

RESULTS—MafA expression was markedly decreased in the islets of *db/db* mice, while in contrast c-Jun expression was increased. Costaining of these factors in the islets of *db/db* mice clearly showed that MafA and insulin levels are decreased in c-Jun-positive cells. Consistent with these results, overexpression of c-Jun significantly decreased MafA expression, accompanied by suppression of insulin expression. Importantly, MafA overexpression restored the insulin promoter activity and protein levels that were suppressed by c-Jun. These results indicate that the decreased insulin biosynthesis induced by c-Jun is principally mediated by the suppression of MafA activity.

CONCLUSIONS—It is likely that the augmented expression of c-Jun in diabetic islets decreases MafA expression and thereby reduces insulin biosynthesis, which is often observed in type 2 diabetes. *Diabetes* 59:1709–1720, 2010

The development of type 2 diabetes is associated with islet β -cell dysfunction and insulin resistance. Normal β -cells can compensate for insulin resistance by increasing insulin secretion and/or β -cell mass (1), but insufficient compensation leads to the onset of glucose intolerance. Once hyperglycemia becomes apparent, β -cell function, including insulin biosynthesis, progressively deteriorates. This process is often observed in type 2 diabetic patients and is well known as β -cell glucose toxicity.

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Insulin biosynthesis is principally regulated by key insulin transcription factors including pancreatic and duodenal homeobox (PDX)-1 (formerly known as IPF-1, STF-1, and IDX-1) (2–4), BETA2 (5), and MafA (6–8). It was previously reported that chronic hyperglycemia suppresses insulin gene expression due to the decreased binding of PDX-1 and RIPE3b1 activator/MafA to its promoter region (9–12). It has been suggested that reactive oxygen species (ROS) are involved in the suppression of insulin biosynthesis, which is often observed in type 2 diabetes.

Islet β -cells are thought to be vulnerable to oxidative stress, since expression levels of antioxidant enzymes such as catalase and glutathione peroxidase are very low in β -cells compared with other tissues (13,14). In fact, it has been demonstrated that oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine, 4-hydroxy-2-nonenal (HNE)-modified proteins and heme oxygenase-1 are increased in islet β -cells of diabetic rats and mice (15,16). Some groups, including ourselves, previously showed that antioxidants restore the suppressed binding activity and expression of PDX-1 in the islets of different diabetic model animals (17,18). It was also reported that MafA protein levels are suppressed at the posttranslational level under chronic high-glucose conditions in cell line-based experiments and that antioxidant treatment restores the suppression of MafA by high glucose (18,19). However, it remains unclear as to whether MafA expression is decreased under diabetic conditions in vivo and, if so, what factor(s) mediate the suppression of MafA by oxidative stress.

It is well known that ROS activate several kinases, such as p38 mitogen-activated protein kinase (p38) and c-Jun NH₂-terminal kinase (JNK), in several tissues including pancreatic islets (20–22). The amount and activity of c-Jun, which is phosphorylated by JNK, are also increased by ROS (23,24). Importantly, expression and activity of c-Jun are upregulated by high glucose and glycation products in several tissues (25,26). However, it was still unclear as to whether c-Jun is actually upregulated in islets under diabetic conditions. So far, several reports have indicated the effects of these ROS-activated factors on insulin transcription factors; for example, p38 acts as a kinase activating MafA (27) and JNK activation translocates PDX-1 from the nucleus to the cytoplasm (28). In particular, two reports (29,30) clearly demonstrated that c-Jun dramatically suppressed the insulin promoter activity. These in vitro studies showed that c-Jun inhibits transcriptional activity of the human and mouse insulin gene through the cAMP response element (CRE) and RIPE3 element located in the insulin enhancer region. Interestingly, we previously reported that MafA binds not only RIPE3b1 but also to other Maf response elements (MAREs) including CRE (31). From these findings we speculated that c-Jun is a

negative regulator of MafA, although the expression pattern of c-Jun in islets under diabetic conditions has not been clearly reported.

In this study, we analyzed the c-Jun and MafA expression pattern and levels in *db/db* mice, a model of type 2 diabetes, and MafA protein expression was found to be low or almost undetectable in c-Jun-positive cells. Furthermore, we show that MafA expression is suppressed upon c-Jun overexpression in islet β -cells, coinciding with the reduction of insulin gene transcription. Significantly, MafA overexpression or c-Jun knockdown restored the insulin gene expression in diabetic islets.

RESEARCH DESIGN AND METHODS

Preparation of expression plasmids and reporter gene analysis. The -238 insulin firefly luciferase (-238 Insulin luc) expression plasmid contain rat insulin 2 gene sequences from -238 to +2 bp (32). The cytomegalovirus (CMV) enhancer-driven rat c-Jun, human JNK, p38 α (CSBP2), and mouse MafA expression vectors were constructed by subcloning coding sequences into the pAdTrack-CMV or pcDNA3.1 expression vector. Myc-tagged MafA was produced by subcloning mouse MafA coding sequences into pcDNA6/myc-His (Invitrogen). -238 insulin LUC (0.25 μ g) was transfected into cells with the control empty vector, pAdTrack, and/or the indicated expression vectors (1.0 μ g in total) using LIPOFECTamine (Invitrogen), together with the thymidine kinase promoter-driven renilla luciferase expression plasmid (20 ng pHRL-TK; Promega Madison, WI) serving as an internal control.

Preparation of adenoviruses. Recombinant adenoviruses expressing c-Jun, JNK, p38, and MafA were prepared using the AdEasy system as described previously (33). An adenovirus expressing a small-interfering RNA against mouse c-Jun (Ad-si-cJun) was constructed using the following oligonucleotides as described previously (34): 5'-CGCGTAAGCTGATTACTGTCAATAAATTCAGAGATTTATTGACAGTAATCAGCTTTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAAGCTGATTACTGTCAATAAATCTCTTGAATTTATTGACAGTAATCAGCTTA-3' (c-Jun sequences are underlined). These oligonucleotides were annealed and inserted into the *MluI/HindIII* sites of the pRNAT-H1.1/Adeno shuttle plasmid (GenScript, Piscataway, NJ). Adenovirus titer was roughly 10^{10} plaque-forming units (pfu)/ml after purification with the Adeno-X Virus Purification kit (Clontech), as estimated using the Adeno-X Titer kit (Clontech).

Isolation of mouse pancreatic islets. To isolate mouse islets, 1.5 mg/ml of collagenase plus protease inhibitor (Sigma-Aldrich) and hyaluronidase (Sigma-Aldrich) were injected into the pancreatic duct. The isolated pancreas was digested in a 37°C incubator for 20 min. After washing and precipitating with 0.25 mol/l sucrose, islets were hand picked.

Real-time PCR analysis. Real-time PCR analysis was performed as described previously (34). Primer sets for mouse insulin 1 (numbering relative to ATG, forward -47 GACCAGCTATAATCAGAGACC, reverse +331 AGTTG CAGTAGTCTCCAGCTG, 378 bp product), mouse insulin 2 (forward -57 AGCCCTAAGTGATCCGCTACAA, reverse +331 AGTTGCAGTAGTCTC CAGCTG, 388 bp), mouse MafA (forward +757 TTCAGCAAGGAGGAGGT CAT, reverse +973 CCGCCAACCTCTCGTATTTC, 217 bp), mouse PDX-1 (forward +192 CATCTCCCCATACGAAGTGC, reverse +526 GGGGCCGG GAGATGTATTG, 335 bp), mouse c-Jun (forward +736 TCCCTATCGACAT GGAGTC, reverse +881 TTTTGGCTTTCAAGGTTTT, 146 bp), and mouse β -actin (forward +778 GCTCTTTCCAGCCTTCCTT, reverse +945 CTTCTG CATCCTGTCAGCAA, 168 bp) were utilized to detect specific bands for each factor.

Northern blotting. Total RNA or Poly(A)⁺mRNA was separated under denaturing conditions on 1% agarose gels. The transferred RNA was hybridized with a ³²P-labeled mouse MafA (314–490 bp; numbering relative to ATG), mouse insulin (35), mouse PDX-1 (622–852 bp), or mouse- β actin (53–549 bp) cDNA probe.

Western blot analysis. MIN6 nuclear extracts were prepared as described previously (34) at the indicated times or 60 h after the adenovirus infection. Islet nuclear extracts were prepared with a nuclear extraction kit (Active Motif, Carlsbad, CA). Western blot analysis was performed as described previously (34), with the following antibodies and dilutions: rabbit anti-MafA antibody (8), 1:1,000; rabbit anti-c-Jun antibody (no. 9165; Cell Signaling Technology, Danvers, MA), 1:200; rabbit anti-PDX-1 antibody (17), 1:2,000; and goat anti-actin antibody (no. sc-1615; Santa Cruz Biotechnology), 1:2,000.

Enzyme-linked immunosorbent assay for insulin. Whole-cell extracts were obtained from >100 islets from each group of mice or adenovirus-infected MIN6 cells by treating for 24 h at 4°C in acid-ethanol. The insulin content of the extract was determined with the Mouse Insulin ELISA kit

(Morinaga Biochemicals, Yokohama, Japan). Insulin concentration was normalized with total cellular protein, as measured using the Bio-Rad protein assay kit (Bio Rad Laboratories, Richmond, CA).

Immunohistochemistry. Pancreata were dissected and fixed overnight in 4% paraformaldehyde. Fixed tissues were processed routinely for paraffin embedding and sectioned. After treatment with 1% blocking goat or donkey serum, sections were immunostained for MafA, c-Jun, and insulin with the following antibodies and dilutions: rabbit (8) and our newly produced goat antibodies against MafA, 1:1,000 and 1:200, respectively; rabbit anti-c-Jun (no. 9165; Cell Signaling Technology), 1:200; guinea pig anti-insulin (no. A0564; DAKO, Glostrup, Denmark), 1:2000; guinea pig anti-glucagon (Linco, St. Charles, MO), 1:500; sheep anti-somatostatin (American Research Products, Belmont, MA), 1:500; and guinea pig anti-pancreatic polypeptide antibodies (Linco), 1:500. For the single detection of MafA or c-Jun, a Vectastain Elite ABC kit (Vector Laboratories) was utilized. For the combined staining of c-Jun, MafA, and insulin, we used Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 555 donkey anti-goat IgG (Molecular Probes, Eugene, OR), and donkey Cy5-conjugated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively, at a dilution of 1:200. For the costaining of glucagon, somatostatin, and pancreatic polypeptide, we used donkey Cy5-conjugated anti-guinea pig IgG and donkey Cy5-conjugated anti-sheep IgG as secondary antibodies at a dilution of 1:200. Fluorescent images were captured using a BIO-RAD Radiance 2100 confocal microscope.

Immunocytochemistry. Sixty hours after the infection of adenoviruses or the transfection of expression plasmids, MIN6 cells were fixed on Lab-Tek chamber slides, and mouse islets were fixed on a poly-D-lysine cell culture plate with 4% paraformaldehyde. After the treatment with 0.1% Triton X-100 for the induction of cell permeability, the slides were incubated with 1% blocking goat serum. To detect MafA, MafA-myc fusion protein, c-Jun, and insulin, we performed immunocytochemistry with rabbit anti-MafA at 1:1,000 dilution (8), mouse anti-c-myc at 1:200 dilution (no. sc-40; Santa Cruz Biotechnology), rabbit anti-c-Jun at 1:200 dilution (no. 9165; Cell Signaling Technology), and guinea pig anti-insulin antibodies at 1:2,000 dilution (no. A0564; DAKO). Cells were then exposed to the secondary antibodies (Alexa Fluor 596 goat anti-rabbit IgG for MafA and Alexa Fluor 596 goat anti-guinea pig IgG for insulin at 1:200 dilution), and images were captured using a Nikon microscope digital camera (DXM1200F). Other secondary antibodies were used for detection with the Bio-Rad Radiance 2100 confocal microscope (Alexa Fluor 546 goat anti-mouse IgG for myc, Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes) for c-Jun, and donkey Cy5-conjugated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories) for insulin at 1:200 dilution.

Statistical analysis. Data are expressed as means \pm SD. Statistical analysis was performed using one-way ANOVA followed by the Scheffe test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Marked decrease of MafA expression under diabetic conditions. C57 BLKsJ *db/db* (*db/db*) mice have been established as a type 2 diabetic model with obesity. In *db/db* mice, hyperinsulinemia is observed to compensate for the increased insulin resistance. However, after ~12 weeks of age, plasma insulin levels begin to decrease, as shown in Fig. 1A. This alteration of insulin levels is similar to that observed in type 2 diabetic patients. To evaluate whether the key insulin transcription factor MafA is also affected under diabetic conditions in vivo, we performed immunohistochemistry with an anti-MafA antibody (Fig. 1A). In *db/db* mice, MafA expression in pancreatic islet nuclei was markedly decreased after 12 weeks of age and was not clearly detected at 17 and 22 weeks of age, whereas control *m/m* mice retained MafA expression at least up to 22 weeks old. These findings were consistent with the profile of plasma insulin and blood glucose levels. **Increased expression of c-Jun in pancreatic islets under diabetic conditions.** Since c-Jun activity and protein levels are increased in response to oxidative stress (23,24), which leads to suppression of insulin promoter activity in vitro, we evaluated in this study whether c-Jun expression is upregulated in the islets of diabetic mice that are exposed to oxidative stress. As shown in Fig. 1B, c-Jun expression was clearly detected in duct cells and a small

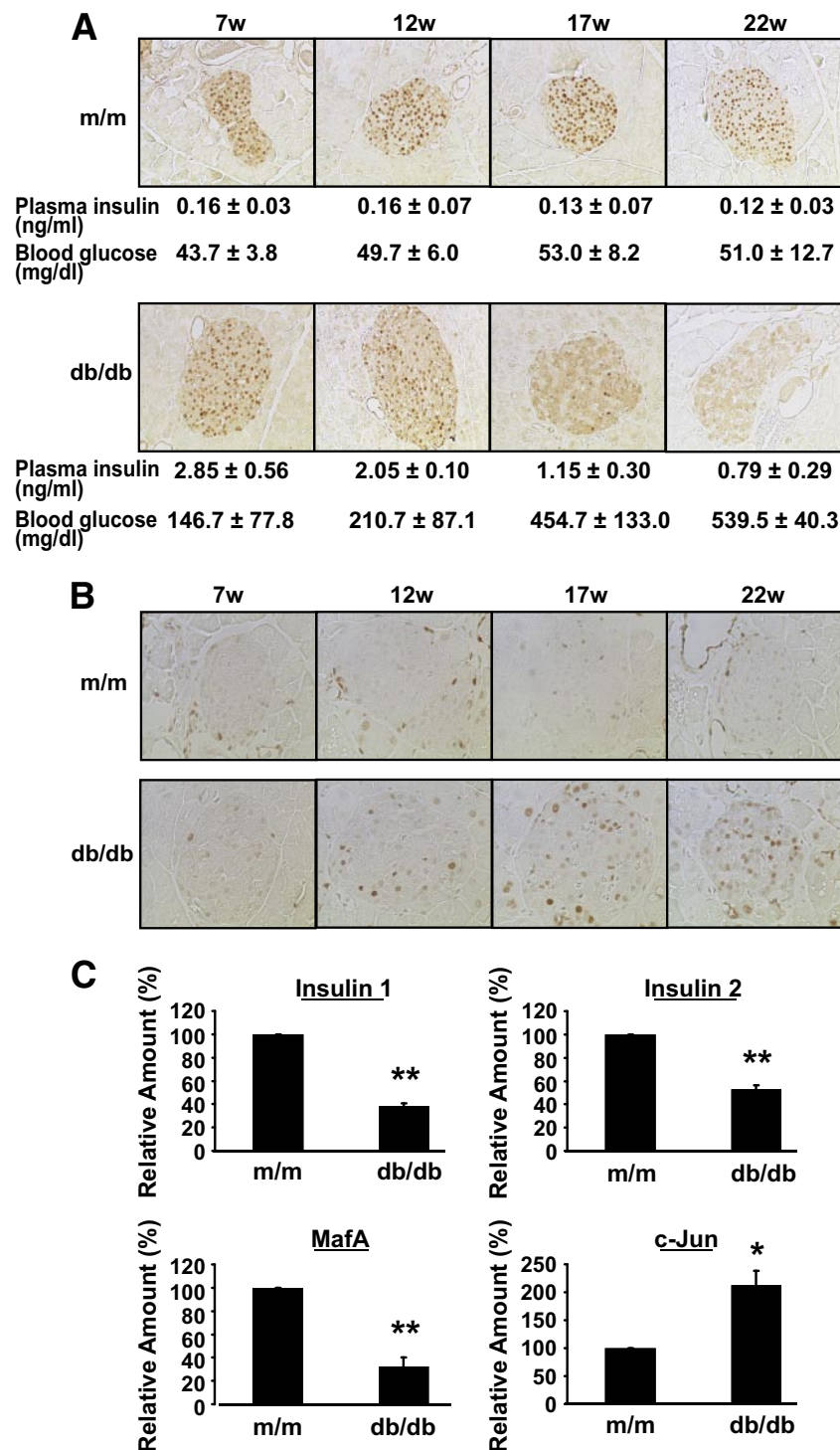


FIG. 1. Altered expression levels of MafA and c-Jun in diabetic islets. **A:** Pancreatic sections from 7-, 12-, 17-, and 22-week-old normal control *m/m* (upper panels) and diabetic *db/db* (lower panels) mice were immunostained with a MafA antibody. Fasting insulin and glucose levels in *m/m* and *db/db* mice at 7, 12, 17, and 22 weeks of age are indicated under each panel ($n = 3$). **B:** Pancreatic sections from 7-, 12-, 17-, and 22-week-old *m/m* (upper panels) and *db/db* (lower panels) mice were immunostained with a c-Jun antibody. **C:** Total RNA of freshly isolated islets from *m/m* and *db/db* mice at 17 weeks of age was utilized for real-time PCR analysis. mRNA levels of insulin 1, insulin 2, MafA, and c-Jun were quantified and normalized with that of β -actin. Each mRNA level of *db/db* mice was presented as the relative amount \pm SD with that of *m/m* being arbitrarily set at 100 ($n = 3$). * $P < 0.05$; ** $P < 0.001$ vs. each mRNA level of *m/m* mice.

number of islet cells in control *m/m* mice at 7–22 weeks of age and diabetic *db/db* mice at 7 weeks of age. However, the number of c-Jun–positive cells gradually increased with age in the islets of *db/db* mice. At 12 weeks of age, several c-Jun–positive islet cells were detected, and at 17 and 22 weeks of age a larger number of c-Jun–positive

cells were detected. Interestingly, this expression pattern of c-Jun paralleled the loss of MafA expression (Fig. 1A). Quantitative real-time PCR analysis, using freshly isolated islets from *m/m* and *db/db* mice at 17 weeks of age, clearly demonstrated that both MafA and insulin mRNA levels were markedly decreased (Fig. 1C). In contrast, c-Jun

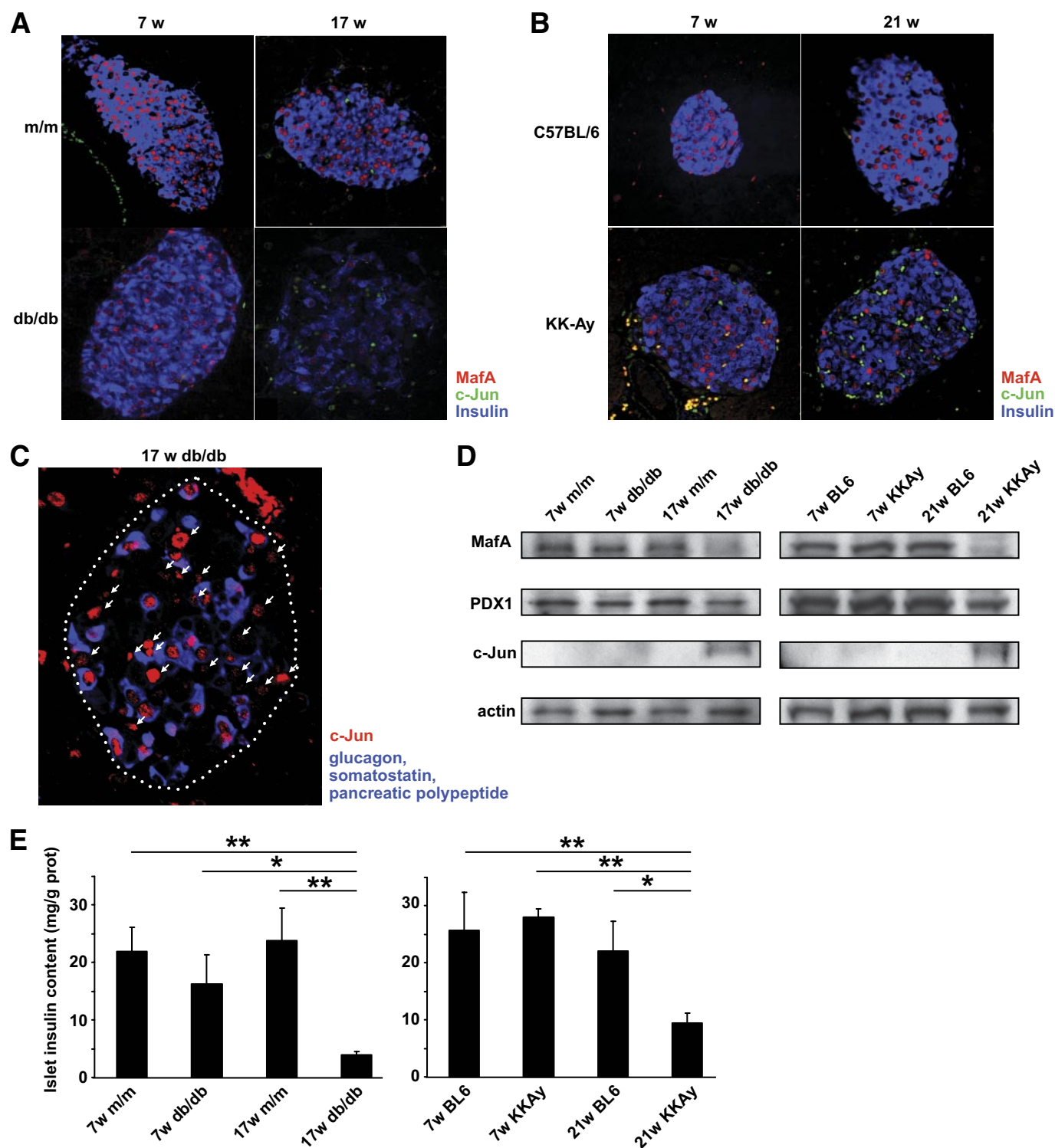


FIG. 2. Suppression of MafA and insulin expression in c-Jun-positive cells. **A:** Pancreatic sections from 7- and 17-week-old *m/m* (upper panel) and *db/db* (lower panel) mice were costained with anti-MafA (red), c-Jun (green), and insulin (blue) antibodies. **B:** Pancreatic islets of another diabetic model, *KK-Ay* mice, and C57BL/6 mice as normal control at 7 and 21 weeks of age were costained as described in **A**. **C:** A pancreatic islet (circled by the dotted line) of a 17-week-old *db/db* mouse was costained with antibodies against c-Jun (red) and non- β -cell hormones (blue) including glucagon, somatostatin, and PP. **D:** Five micrograms of islet nuclear protein from each group of *db/db* and *m/m* mice and 10 μ g of islet nuclear protein from each group of *KK-Ay* and C57BL/6 mice were applied for Western blotting analysis and probed with indicated antibodies. **E:** Insulin content in the islets from each group of mice was measured with the mouse insulin ELISA kit (Morinaga Biochemicals, Yokohama, Japan). Insulin concentration was normalized with total cellular protein. The normalized values \pm SD were calculated from four independent experiments. * $P < 0.05$; ** $P < 0.001$ vs. 17-week *db/db* or 21-week *KKAY*.

mRNA level in islets was significantly increased, which was consistent with the expression pattern of MafA and c-Jun observed by immunohistochemistry.

MafA and insulin expression is reduced in islet c-Jun-positive cells. Our results implied that the increased level of c-Jun caused a decrease in MafA and insulin gene

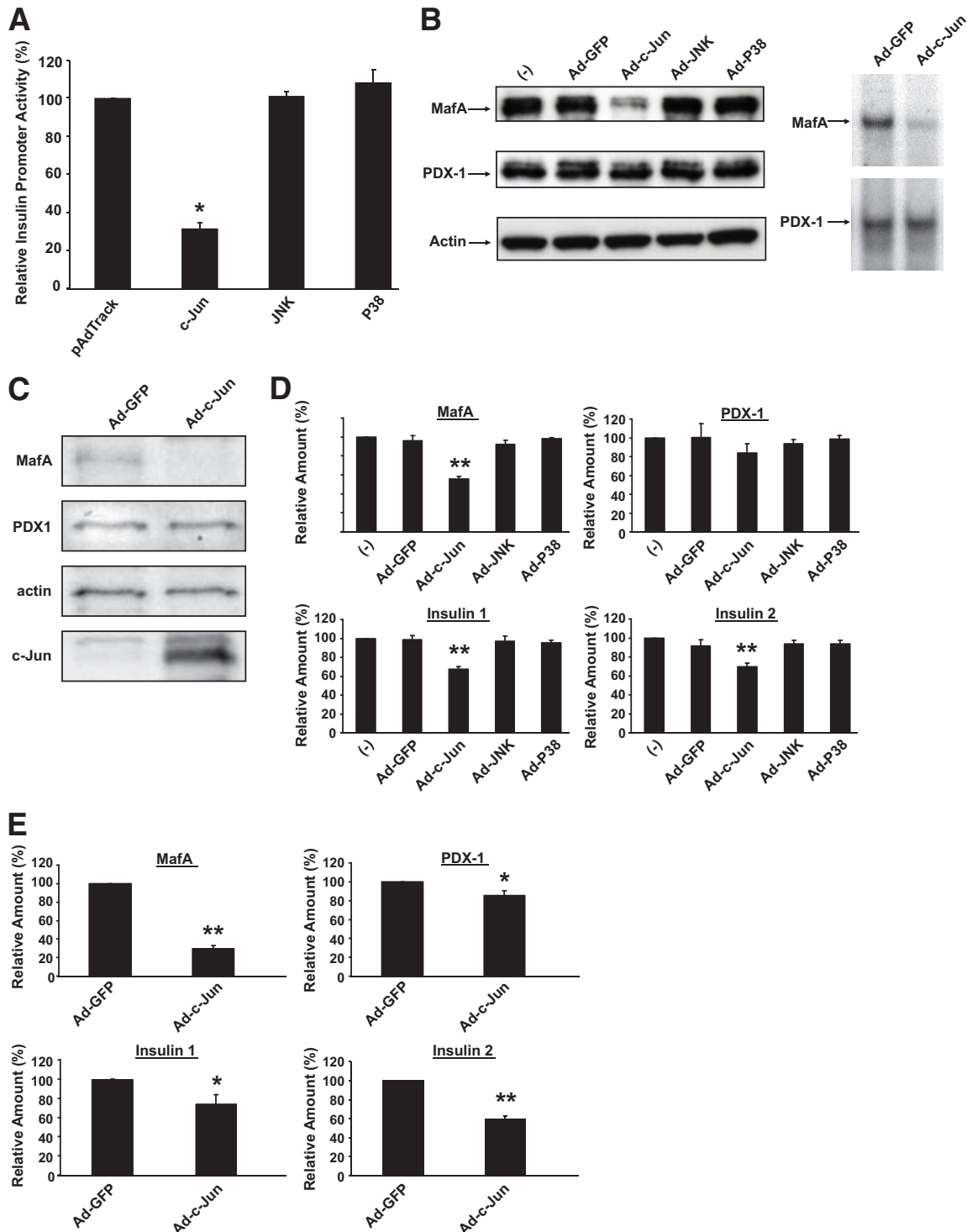


FIG. 3. Marked suppression of MafA by c-Jun overexpression. **A:** The rat insulin 2 (–238 to +2 bp)-driven firefly luciferase expression plasmid (–238 insulin luc) was cotransfected with c-Jun, JNK, p38/pAdTrack, or empty-vector pAdTrack into MIN6 cells. The firefly luciferase activity from –238 insulin luc was normalized with the cotransfected pRL-TK renilla luciferase signal. The relative –238 insulin luc activity was calculated as the ratio of luc activity after c-Jun, JNK, or p38/pAdTrack transfection to that after control pAdTrack transfection. Experiments were performed at least four times, and the relative values are expressed as means \pm SD. * $P < 0.001$ vs. control pAdTrack. **B:** MIN6 cells were infected with each indicated adenovirus. Sixty hours after the infection, nuclear protein was isolated and Western blotting (left panel) and gel-shift (right panel) analysis were performed using 10 μ g of nuclear protein to detect MafA and PDX-1. **C:** Isolated mouse islets were infected with Ad-c-Jun or control Ad-GFP. Sixty hours after the infection, nuclear protein was isolated and Western immunoblot analysis was performed using 2 μ g of nuclear protein. Each factor was probed with indicated antibodies on the same blotting. **D and E:** Sixty hours after infection of the indicated adenovirus to MIN6 (**D**) or cultured mouse islets (**E**), total RNA was isolated followed by real-time RT-PCR analysis to quantify the mRNA levels of MafA, PDX-1, insulin 1, insulin 2, and control β -actin. Each mRNA level was normalized with that of β -actin and is presented as relative amount \pm SD, with the ratio of each control mRNA level without adenovirus treatment (**C**) or Ad-GFP infection (**D**) being arbitrarily set at 100 ($n = 4$). * $P < 0.01$; ** $P < 0.001$ vs. each control mRNA.

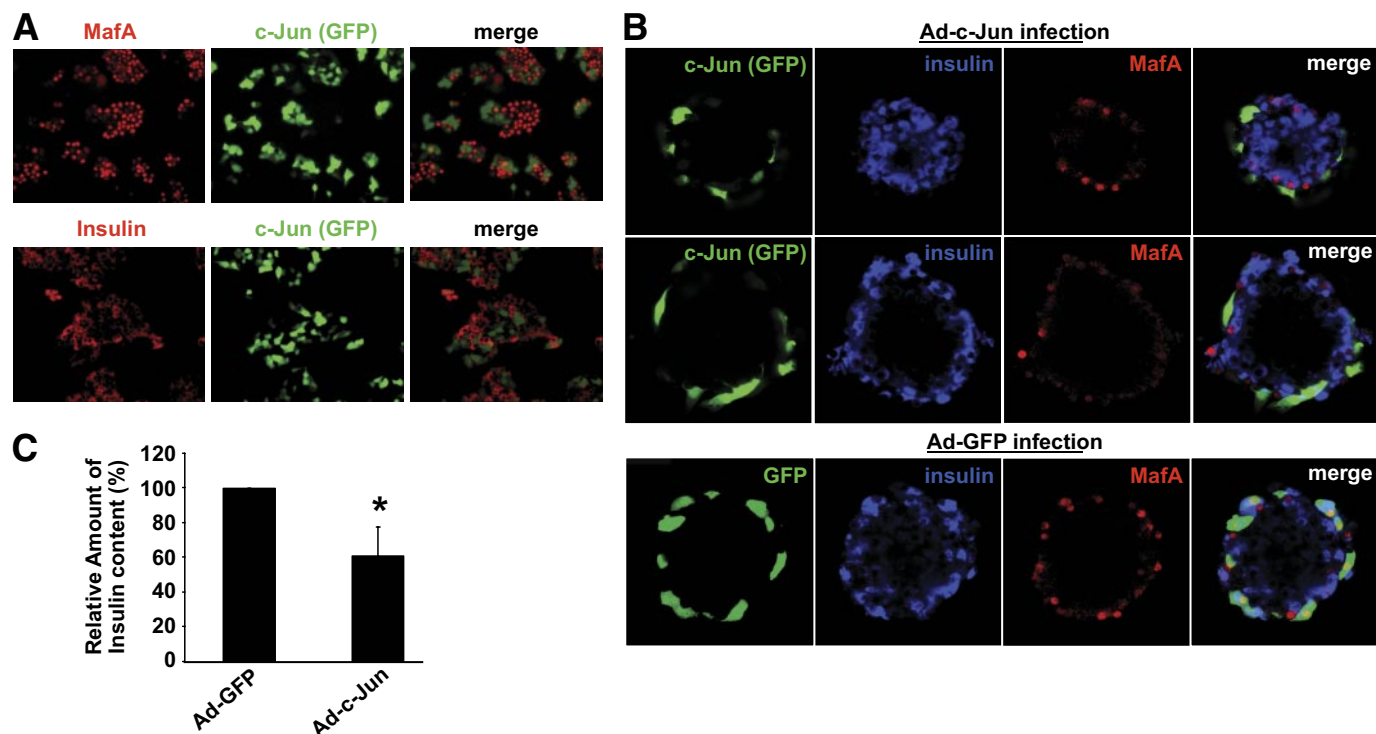


FIG. 4. Suppression of MafA and insulin protein expression by c-Jun overexpression. **A:** Immunostaining of MafA in red (upper panels) and insulin in red (lower panels) was performed with MIN6 cells, 60 h after infection of Ad-c-Jun, which expresses c-Jun and GFP simultaneously. **B:** Sixty hours after Ad-c-Jun (upper and middle panels) or Ad-GFP (lower panel) infection, normal mouse islets were immunostained with anti-MafA and insulin antibodies, and captured with confocal microscopy. MafA is shown in red, insulin is shown in blue and Ad-c-Jun-infected cells are shown in green by the GFP signal. Upper and middle panels showed the same islet but different sections. **C:** Insulin content of Ad-c-Jun-infected islets was measured with ELISA. After normalized with protein concentration, insulin protein level was presented as relative amount \pm SD, with the ratio of Ad-GFP treatment being arbitrarily set at 100 ($n = 4$). * $P < 0.05$ vs. Ad-GFP infection.

expression in older diabetic mice. To evaluate these association in further detail, we first performed immunohistochemistry with MafA, c-Jun, and insulin antibodies using 7- and 17-week-old diabetic mice (Fig. 2A), as MafA expression levels were markedly decreased between 7 and 17 weeks of age (Fig. 1A). Normal mice expressed a large amount of insulin and MafA without enhanced c-Jun expression as expected. In contrast, in *db/db* mice, insulin expression was decreased in some cells in which MafA was undetectable or weakly expressed compared with control *m/m* mice. Interestingly, MafA and insulin expression was suppressed in most c-Jun-positive cells, which became clearer in 17-week-old *db/db* mice. *KK-Ay* mice, in which the *Ay* mutation is introduced onto a *KK* strain, are also known as an obese diabetic model. Since *KK-Ay* mice get severe diabetes after \sim 20 weeks, we used older mice than *db/db* mice. In the *KK-Ay* islets, the number of c-Jun-positive cells was increased with marked hyperglycemia (\sim 200–300 mg/dl fasting glucose) at 21 weeks of age, and both MafA and insulin protein levels were decreased in those cells, as observed in *db/db* mice (Fig. 2B). To examine the distribution of c-Jun in islet cells, immunohistochemistry was performed with antibodies against c-Jun and non- β -cell islet hormones, including glucagon, somatostatin, and pancreatic polypeptide (PP). In the islets of 17-week-old *db/db* mice, many c-Jun-positive cells were observed without costaining of non- β -cell hormones (Fig. 2C), indicating that c-Jun was expressed in islet β -cells. To quantify the protein levels of MafA, c-Jun, and insulin in the islets of normal and diabetic mice, Western blotting using islet nuclear extracts and enzyme-linked immunosorbent assay (ELISA) for islet insulin were per-

formed (Fig. 2D and E). c-Jun protein level was clearly increased in the islets of 17-week-old *db/db* mice and of 21-week-old *KK-Ay* mice. In contrast, MafA protein level was decreased in those islets. Consistent with the suppressed expression of MafA, insulin content in the islets of 17-week-old *db/db* mice and 21-week-old *KK-Ay* mice were significantly decreased compared with control mice. These findings suggest that c-Jun is involved in the suppression of MafA and insulin expression under diabetic conditions.

c-Jun overexpression markedly suppresses both MafA and insulin expression. To evaluate the effects of c-Jun on insulin gene transcription, we examined the effects of c-Jun on insulin promoter activity in the MIN6 β -cell line by reporter gene analysis. As shown in Fig. 3A, c-Jun overexpression markedly decreased insulin promoter activity to 30% of that observed in control, which was consistent with previous reports (29,30). In addition, although it is known that several kinases such as JNK and p38 are activated by ROS in islet β -cells in vivo (15,16), their overexpression did not show significant effects.

To further examine the impact of c-Jun on MafA in islet β -cells, we generated an adenovirus expressing both c-Jun and green fluorescent protein (GFP) (Ad-c-Jun). We first performed Western blotting using nuclear extract of MIN6 and islet cells and found that c-Jun protein expression was almost undetectable in uninfected cells (data not shown). As shown in Fig. 3B, adenoviral c-Jun overexpression markedly suppressed MafA protein level and its DNA-binding activity 60 h after the infection in MIN6 cells. The protein level and DNA-binding activity of PDX-1 were not apparently altered under these conditions. Northern blot-

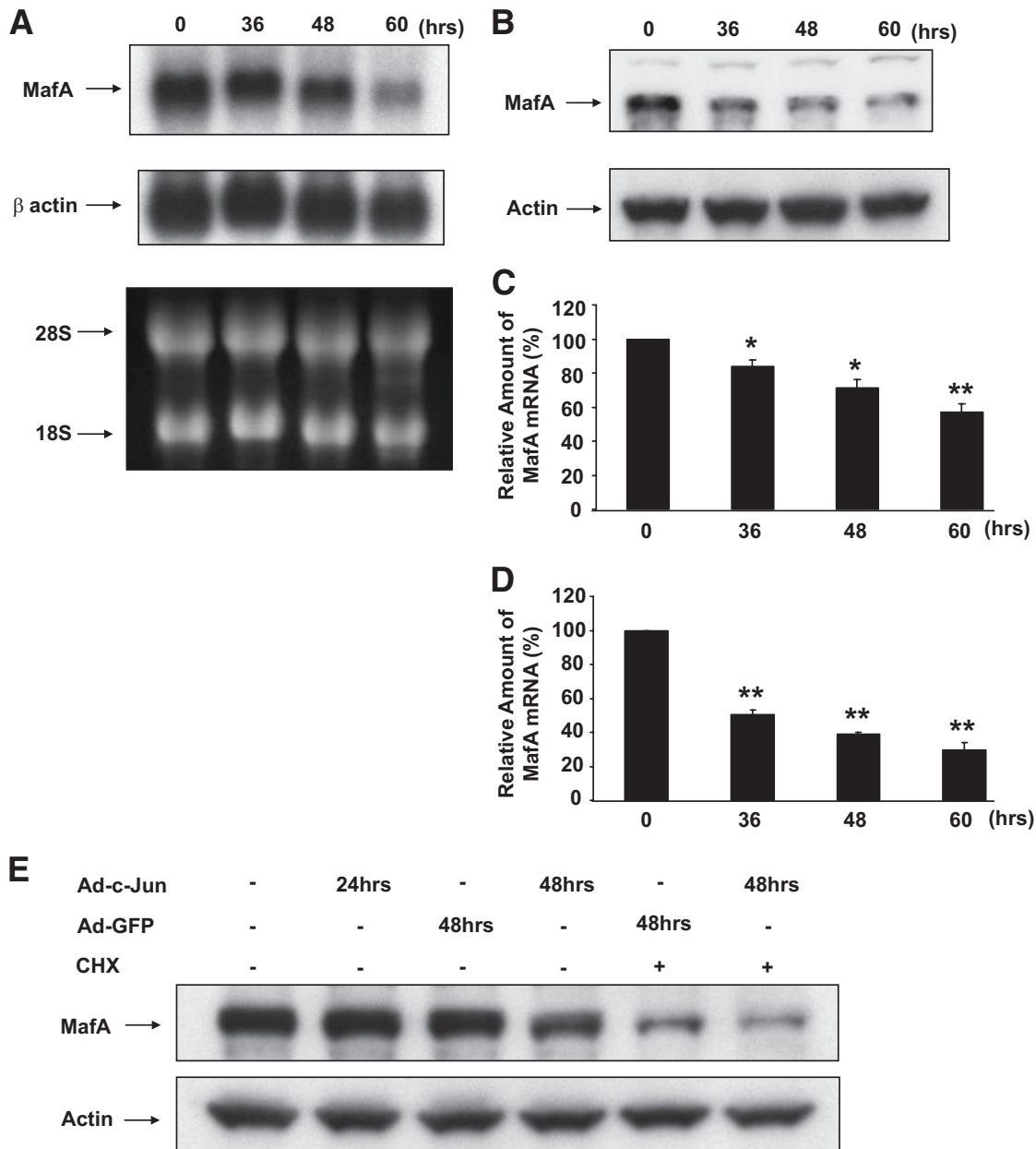


FIG. 5. c-Jun suppresses MafA partially at the posttranslational level. Total RNA and nuclear protein were isolated from MIN6 cells at 0, 36, 48, and 60 h after Ad-c-Jun infection. **A:** Northern blotting (**A**) and Western blotting (**B**) were performed using 10 μ g of total RNA and 10 μ g of nuclear protein to evaluate the amount of mRNA and protein levels of MafA and control β -actin. **C:** mRNA levels of MafA were quantified by real-time RT-PCR analysis. Each MafA mRNA level was normalized with that of β -actin. Data are presented as relative amounts \pm SD, with the ratio of MafA mRNA level without adenovirus treatment being arbitrarily set at 100 ($n = 4$). **D:** Protein levels of MafA were quantified by densitometry. Each MafA protein level was normalized with that of actin. Data are presented as relative amounts \pm SD, with the ratio of MafA protein level without adenovirus treatment being arbitrarily set at 100 ($n = 4$). * $P < 0.001$; ** $P < 0.001$ vs. without adenovirus infection. **E:** Western blotting was performed with 10 μ g of nuclear protein isolated from MIN6 cells incubated with Ad-c-Jun or control Ad-GFP for 0, 24, or 48 h with or without cycloheximide (CHX; 10 μ g/ml media). CHX was added to media 24 h after the adenovirus infection.

ting and real-time PCR analysis also showed that c-Jun overexpression significantly suppressed MafA mRNA level (supplemental Fig. 1, available in the online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db08-0693/DC1/>) (Fig. 3D). Adenoviral overexpression of c-Jun in isolated mouse islets also markedly suppressed MafA mRNA and protein levels (Fig. 3C and E). Consistent with these results, the mRNA levels of insulin 1 and 2 were suppressed by c-Jun overexpression in both MIN6 cells and islets. A much smaller defect on PDX-1 mRNA was also observed under these circumstances.

To directly observe and visualize the effect of c-Jun, we

performed immunocytochemistry using MIN6 and islet cells. Adding only a low level of Ad-c-Jun (1.0 μ l of 1×10^9 pfu/ml to 1 ml of culture medium) enabled us to compare MafA and insulin expression in c-Jun-expressing and nonexpressing cells. In c-Jun-positive MIN6 cells, both MafA and insulin expression were reduced (Fig. 4A), while the negative control Ad-GFP had no effect (supplemental Fig. 2). We also evaluated whether MafA and insulin were affected by c-Jun overexpression in islets isolated from the *m/m* mouse. As observed in MIN6 cells, protein levels of MafA were markedly reduced in most Ad-c-Jun-infected islet cells, while control Ad-GFP showed no effects on

MafA (Fig. 4B). To quantify the suppressive effects of c-Jun on insulin content in isolated islets, a large amount of Ad-c-Jun (1.0 μ l of 1×10^{10} pfu/ml to 1 ml of culture medium) was used to overexpress c-Jun in entire islet cells. Consistent with the results of immunocytochemistry, insulin content measured by ELISA was significantly decreased in c-Jun-overexpressed islets (Fig. 4C). These findings directly demonstrate that c-Jun suppresses the expression of both MafA and insulin.

c-Jun suppresses MafA expression at both transcriptional and posttranslational levels. It was previously reported that MafA protein level was suppressed at the posttranslational level under chronic high-glucose conditions in vitro (19). To examine how c-Jun suppresses expression of MafA, protein and mRNA levels of MafA were compared at different times after exposure to Ad-c-Jun in MIN6 cells (Fig. 5A and B). Quantification of mRNA and protein levels showed that MafA protein levels are markedly decreased with relatively small suppression of mRNA levels within the first 48 h after exposure (Fig. 5C and D). To further evaluate the possibility of posttranslational suppression of MafA, 24 h after Ad-c-Jun or Ad-GFP infection, MIN6 cells were incubated with 10 μ g/ml of cycloheximide (CHX) for another 24 h. As Fig. 5E shows, protein level of MafA was markedly decreased by the 24-h exposure to CHX with overexpression of control GFP (lane 5). In the same condition, the loss of MafA was more intensified with overexpression of c-Jun (lane 6), although c-Jun had no effect on protein level of MafA within first 24 h (lane 2). These results suggest that c-Jun possibly reduces MafA levels via a posttranslational mechanism, although it was also observed that c-Jun significantly suppressed MafA at the mRNA level.

MafA restores the c-Jun-induced suppression of insulin gene transcription and its protein level. Since c-Jun decreased the expression of both MafA and insulin, we speculated that c-Jun decreases insulin gene transcription at least partially by mediating the suppression of MafA expression in addition to E1 and CRE binding transcription factors (29,30). To investigate this speculation, reporter gene analysis was performed using MIN6 cells. Insulin promoter activity was reduced by c-Jun, which was significantly restored by MafA overexpression (Fig. 6). Importantly, MafA single overexpression had no additional effect on insulin promoter activity in MIN6 cells. These findings suggest that the downregulation of MafA is the main cause of the suppression of insulin gene transcription by c-Jun.

To evaluate whether MafA overexpression prevented insulin protein loss by c-Jun, we transfected MafA and/or c-Jun expression plasmids into MIN6 cells. Sixty hours after the transfection, immunocytochemistry was performed (Fig. 7A). As observed with the adenoviral c-Jun overexpression in Fig. 4A, c-Jun overexpression by a plasmid also reduced insulin protein levels (Fig. 7A, top panels) but was restored by MafA overexpression even in c-Jun-positive cells (Fig. 7A, bottom panels). To quantify the effects of MafA on restoration of insulin protein, c-Jun and MafA were simultaneously overexpressed with the adenoviral method. As indicated in Fig. 7B, c-Jun-induced suppression of insulin content was significantly prevented by MafA overexpression. Next, MafA was overexpressed with Ad-MafA in isolated islets from 17-week-old *db/db* mice, since both MafA and insulin expression levels were suppressed in these islets. Compared with control diabetic islets infected with Ad-GFP, both insulin 1 and 2 mRNA

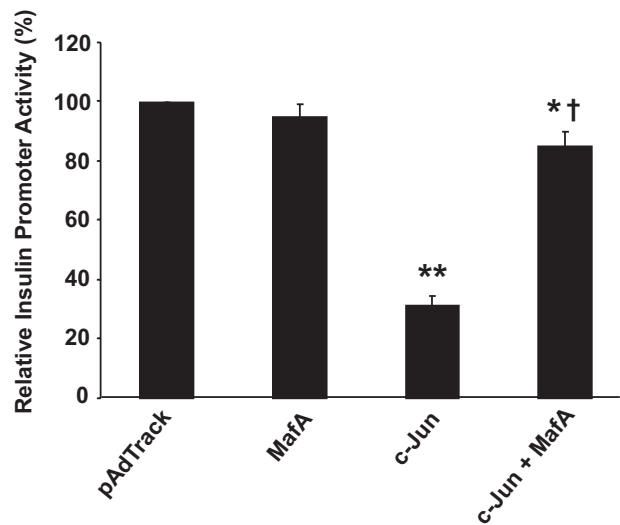


FIG. 6. MafA expression restores the c-Jun-induced suppression of insulin promoter activity. The -238 Insulin luc plasmid was cotransfected with c-Jun and/or MafA/pAdTrack into MIN6 cells. The firefly luciferase activity from -238 insulin luc was normalized with the cotransfected pRL-TK renilla luciferase signal. The relative -238 insulin luc activity was calculated as the ratio of luc activity after c-Jun and/or MafA/pAdTrack transfection to that after control pAdTrack transfection. Experiments were performed at least four times and the relative values are expressed as means \pm SD. * $P < 0.01$; ** $P < 0.001$ vs. control pAdTrack; † $P < 0.001$ vs. c-Jun.

levels were significantly increased by adenoviral MafA overexpression in isolated diabetic islets (Fig. 7C). To clarify that the increased expression of endogenous c-Jun in diabetic islets is the cause of suppression of insulin and MafA, c-Jun expression was suppressed by infection of adenovirus-expressing siRNA of c-Jun (Ad-si-c-Jun) using islets isolated from 14-week-old *db/db* mice in which c-Jun expression was upregulated. As shown in Fig. 7D, c-Jun mRNA level was effectively knocked down, and mRNA levels of insulin 1 and 2 and MafA were significantly increased in diabetic islets. These results clearly indicate that expression level of MafA and insulin are suppressed by physiologically increased c-Jun in the islets under diabetic conditions. Taken together, it is likely that overexpression of MafA or siRNA of c-Jun restores the c-Jun-induced suppression of insulin gene transcription and its protein level.

The domains of c-Jun critical for its suppressive effects on MafA. The functional domains of c-Jun are well characterized, and previous reports (30,36) have demonstrated effective mutants that eliminate the activity of each domain. We generated expression plasmids and adenoviruses of various c-Jun mutants, namely c-Jun transactivation domain (TAD), c-Jun basic region (BS), and c-Jun leucine zipper domain (LZ), which contain a deletion or mutation of in the transactivation, basic DNA binding, and leucine zipper dimerization domain, respectively (Fig. 8A). We first confirmed that each plasmid and adenovirus expressed proteins of the mutant c-Jun expected size and at adequate amounts by Western blot analysis (data not shown). As shown by Western blotting in Fig. 8B, adenoviral expression of c-Jun TAD and c-Jun BS demonstrated that these mutants did not possess the activity of c-Jun against the MafA protein, while c-Jun LZ retained this activity. Furthermore, as shown in Fig. 8C, insulin transcription activity was also affected by these mutants in the same way as their alteration of MafA

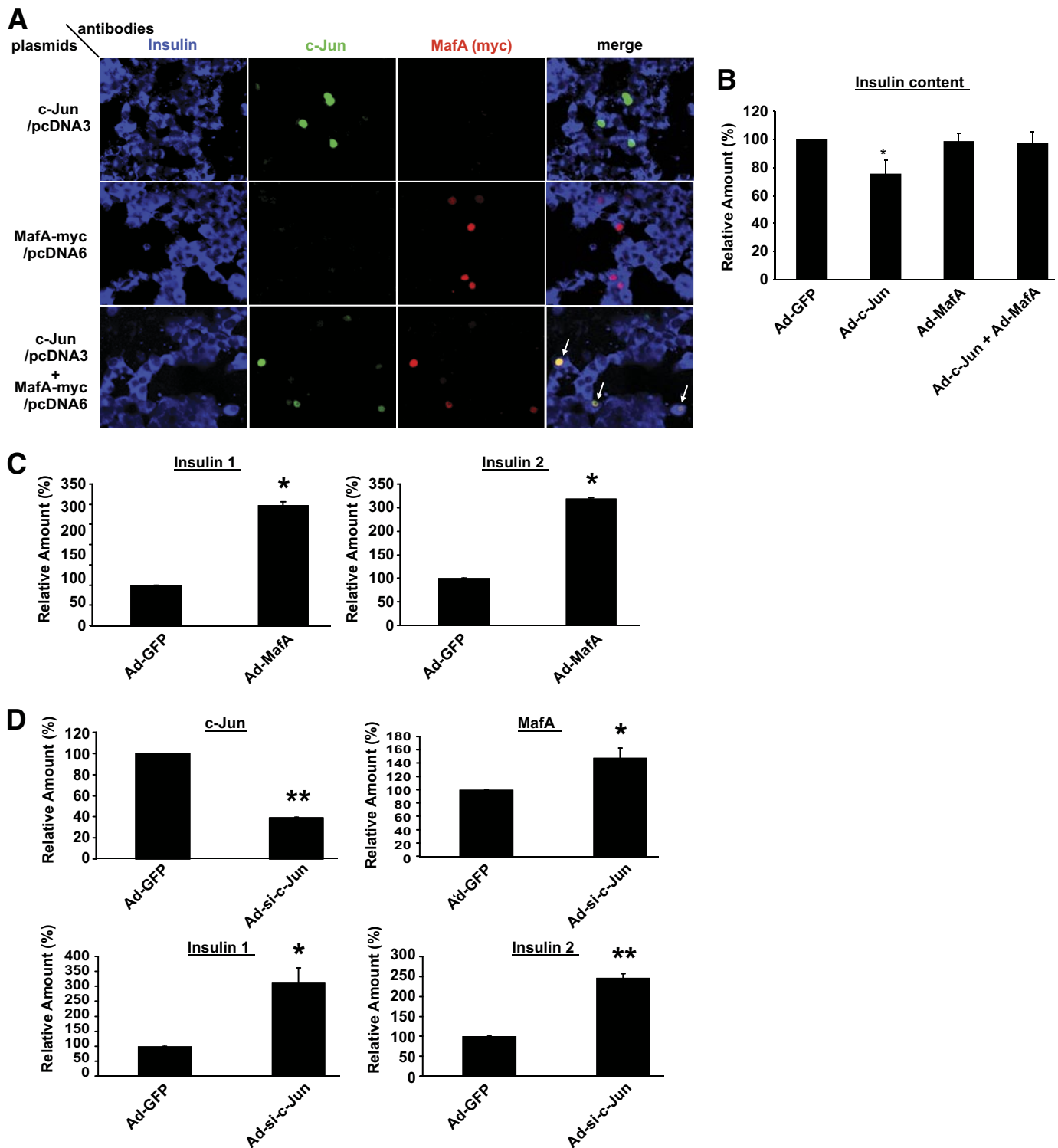


FIG. 7. MafA restores the c-Jun-induced suppression of insulin expression. **A:** Sixty hours after the transfection of c-Jun (top and bottom panels) and/or MafA-myc (middle and bottom panels) expression plasmids into MIN6 cells, immunocytochemistry was performed with anti-insulin (left), c-Jun (second from left), c-myc (second from right), or all three antibodies (right panels) as described in RESEARCH DESIGN AND METHODS. Insulin is shown in blue, c-Jun in green, and exogenously expressed MafA in red. Colocalization of c-Jun and MafA-myc is shown in yellow (indicated by arrows, right panels). **B:** Sixty hours after infection of MIN6 cells with Ad-c-Jun and/or Ad-MafA ($0.5 \mu\text{l}$ of 1×10^{10} pfu/ml each adenovirus) was added in 1 ml of culture medium and total amount of adenovirus was adjusted with Ad-GFP control adenovirus, amounts of insulin eluted by acid-ethanol method were measured with ELISA. Results are presented as relative amounts \pm SD, with the ratio of Ad-GFP treatment being arbitrarily set at 100 ($n = 4$). * $P < 0.001$ vs. Ad-GFP, Ad-MafA, or Ad-c-Jun + Ad-MafA infection. **C:** Sixty hours after infection of 17-week-old *db/db* islets with Ad-GFP or Ad-MafA ($1.0 \mu\text{l}$ of 1×10^{10} pfu/ml adenovirus) was added in 1 ml of culture medium, total RNA was isolated followed by real-time RT-PCR analysis with insulin 1 and 2 primers. Results are presented as relative amounts \pm SD, with the ratio of Ad-GFP treatment being arbitrarily set at 100 ($n = 3$). * $P < 0.001$ vs. Ad-GFP infection. **D:** Seventy-two hours after infection of 17-week-old *db/db* islets with Ad-GFP or Ad-si-c-Jun ($1.0 \mu\text{l}$ of 1×10^{10} pfu/ml adenovirus) was added in 1 ml of culture medium, total RNA was isolated followed by real-time RT-PCR analysis with c-Jun, MafA, and insulin 1 and 2 primers. Results are presented as relative amounts \pm SD, with the ratio of Ad-GFP treatment being arbitrarily set at 100 ($n = 3$). * $P < 0.01$; ** $P < 0.001$ vs. Ad-GFP infection.

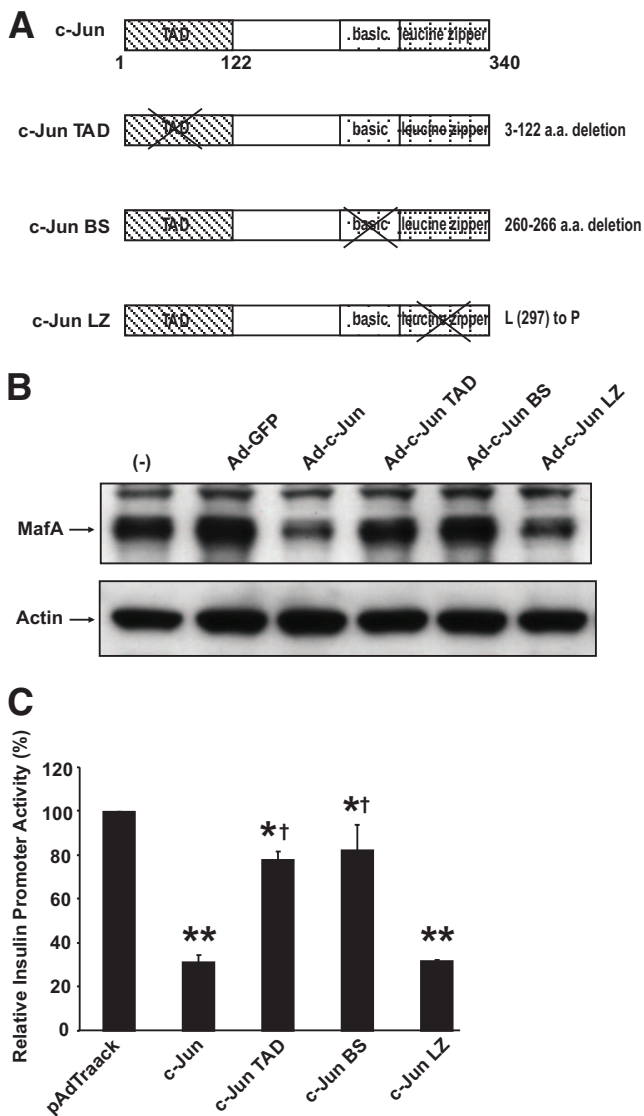


FIG. 8. Critical domain in c-Jun protein affecting MafA and insulin gene expression. **A:** Diagrammatic representation of the c-Jun protein showing the positions of the transactivation (TAD) and basic leucine zipper domains. Locations of the mutations within the c-Jun transactivation domain (c-Jun TAD, 3–122 a.a. deletion), the basic region (c-Jun BS, 260–266 a.a. deletion), and leucine zipper domain (c-Jun LZ, L(297) to P), are shown. **B:** MIN6 cells were exposed to an adenovirus expressing either c-Jun, c-Jun TAD (Ad-c-Jun TAD), c-Jun BS (Ad-c-Jun BS), c-Jun LZ (Ad-c-Jun LZ), or control Ad-GFP for 60 h. Ten micrograms of nuclear protein isolated from the cells were used for Western blotting with an anti-MafA (upper panel) or control anti-actin (lower panel) antibody. **C:** The -238 insulin luc plasmid was cotransfected into MIN6 cells with wild type c-Jun, c-Jun TAD, c-Jun BS, or c-Jun LZ expression plasmid. The firefly luciferase activity from -238 insulin luc was normalized with the cotransfected pRL-TK renilla luciferase signal. Forty-eight hours after the transfection, the relative -238 insulin luc activity was calculated as the ratio of luc activity after c-Jun or c-Jun mutants/pAdTrack transfection to that after control pAdTrack transfection. Experiments were performed at least four times and the relative values are expressed as means \pm SD. * $P < 0.01$; ** $P < 0.001$ vs. control pAd-Track; † $P < 0.001$ vs. wild-type c-Jun.

protein levels. In addition, to confirm that the leucine zipper domain is not required for this activity, we generated another mutant c-Jun that has two mutations in amino acids 290 and 297 of the leucine zipper domain. However, this mutant c-Jun also decreased insulin promoter activity to the same degree as wild-type c-Jun (data not shown). These findings indicate that the transactiva-

tion and basic region domains are critical for c-Jun to suppress MafA and insulin expression.

DISCUSSION

It is well known that insulin gene transcription is down-regulated under diabetic conditions, although its precise mechanism is still unclear. One possibility is that ROS induced by hyperglycemia and/or hyperlipidemia suppress insulin gene transcription and biosynthesis, as we and other groups previously suggested. In this study, we showed that protein levels of c-Jun, whose amount and activity are upregulated by ROS, are increased in diabetic islets in vivo, and that, in contrast, MafA protein levels are markedly decreased (Fig. 1). In addition, immunostaining of MafA and c-Jun in diabetic islets showed that both MafA and insulin expression are markedly suppressed in most c-Jun-positive cells (Fig. 2). These findings imply that c-Jun is involved in the suppression of MafA expression. We further directly demonstrated that c-Jun overexpression with an expression plasmid or adenovirus markedly decreases MafA and insulin expression in isolated islets as well as MIN6 cells (Figs. 3 and 4) and that overexpression of MafA markedly, but not completely, recovered insulin promoter activity suppressed by c-Jun (Fig. 6). These results indicate that increased expression of c-Jun suppresses insulin gene expression via the decrease of MafA expression, besides its direct suppressive effect on insulin gene promoter activity through the cyclic AMP response element as reported previously. Since MafA appears to not only regulate insulin expression but also to be involved in insulin secretion (37,38), it is likely that the suppression of MafA protein levels by c-Jun leads to insulin secretory defects that are often observed under diabetic conditions.

c-Jun is well known to induce apoptosis in a variety of cells. On the other hand, disruption of the *c-jun* gene in mice showed that c-Jun is required for cell proliferation and differentiation (39,40). These opposite effects of c-Jun on both the promotion of and protection from apoptosis might depend on the type, severity, and length of stimuli, as well as the effects of additional factors and/or cell types. First, to examine whether the suppressive effect of c-Jun on MafA and insulin expression is simply due to the induction of apoptosis, we isolated DNA from MIN6 cells infected with Ad-c-Jun for 1, 2, or 3 days and analyzed DNA fragmentation by electrophoresis. However, DNA ladders were not detected in these experiments (supplemental Fig. 3). Furthermore, since overexpression of MafA prevented the suppressive effects of c-Jun on insulin promoter activity (Fig. 6) and protein levels (Fig. 7), it is unlikely that c-Jun induced apoptosis, at least in our model. Interestingly, some of the increased Ki67-positive cells also expressed c-Jun in the islets of *db/db* mice (supplemental Fig. 4), which indicates that c-Jun-positive cells are able to proliferate. These results suggest that c-Jun-mediated β -cell dysfunction is not simply due to the induction of apoptosis, although the physiological reason as to why c-Jun is induced in pancreatic islets under diabetic conditions remains unknown.

As reported previously, MafA is a phosphorylated protein, and phosphorylation is critical for its activity (41–43). In this study, p38 and JNK, both of which are activated by ROS, were evaluated by these overexpression experiments; however, we observed no effects on insulin and MafA expression. This is consistent with the previous findings that MafA is not phosphorylated by p38 in vitro

(43). Concerning JNK, which phosphorylates and activates c-Jun, we predicted that the JNK pathway might also be involved in the alteration of MafA activity. However, the effects of c-Jun on insulin promoter activity and MafA protein levels were not inhibited by a dominant negative form of JNK (data not shown). Therefore, we assume that the induction of c-Jun expression, per se, is more important for the suppression of MafA expression. However, as shown in Fig. 8, the TAD of c-Jun, which includes its phosphorylation site by JNK, is apparently important for its suppression of MafA. These findings suggest that a relatively small amount of JNK is enough to activate c-Jun and suppress MafA.

In this study, we showed that the protein levels of MafA and insulin are simultaneously reduced in the islets of diabetic mice. These results are consistent with the previous findings that RIPE3b1-binding activity is reduced in diabetic islets (18) and that chronic high glucose decreases insulin promoter activity through the reduction of RIPE3b1 activity in HIT-T15 cells (10,19). However, this is the first report to show the increase of c-Jun expression and the decrease of both MafA and insulin expression under diabetic conditions in vivo and to directly demonstrate that the increased c-Jun expression suppresses both MafA and insulin expression.

In conclusion, the findings reported in this study suggest that the augmented expression of c-Jun in diabetic islets decreases MafA activity followed by reduced insulin biosynthesis and thereby explains, at least in part, the molecular mechanism for β -cell glucose toxicity that is often observed in type 2 diabetic patients.

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