Peroxisomal Proliferator–Activated Receptor-γ Upregulates Glucokinase Gene Expression in β-Cells

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Thiazolidinediones, synthetic ligands of peroxisomal proliferator–activated receptor-γ (PPAR-γ), improve peripheral insulin sensitivity and glucose-stimulated insulin secretion in pancreatic β-cells. To explore the role of PPAR-γ in glucose sensing of β-cells, we have dissected the β-cell–specific glucokinase (βGK) promoter, which constitutes glucose-sensing apparatus in pancreatic β-cells, and identified a peroxisomal proliferator response element (PPRE) in the promoter. The βGK-PPRE is located in the region between +47 and +68 bp. PPAR-γ/retinoid X receptor-α heterodimer binds to the element and activates the βGK promoter. The βGK promoter lacking or having mutations in PPRE cannot be activated by PPAR-γ. PPAR-γ activates the βGK promoter in β-cells as well as non–β-cells. Furthermore, troglitazone increases endogenous GK expression and its enzyme activity in β-cell lines. These results indicate that PPAR-γ can regulate GK expression in β-cells. Taking these results together with our previous work, we conclude that PPAR-γ regulates gene expression of glucose-sensing apparatus and thereby improves glucose-sensing ability of β-cells, contributing to the restoration of β-cell function in type 2 diabetic subjects by troglitazone. *Diabetes* 51:676–685, 2002

Insulin is the most important molecule among the regulators in glucose homeostasis. Glucose is the primary physiological stimulus for the regulation of insulin secretion in β-cells, the process that requires glucose sensing. The glucose-sensing apparatus of β-cells consists of glucose transporter isotype 2 (GLUT2) and glucokinase (GK), which play a critical role in glucose-stimulated insulin secretion (GSIS) (1). β-Cell–specific knockout of GLUT2 or GK results in infantile death because of severe hyperglycemia (2,3). Adenovirus-mediated expression of GLUT2 and GK in IL cells results in gaining of glucose sensitivity (4). Thus, GLUT2 and GK are important in glucose sensing of β-cells. However, GLUT2, being a low-affinity, high-capacity glucose transporter, is believed to play a more permissive role in glucose sensing, allowing rapid equilibration of glucose across the plasma membrane. GK traps glucose in β-cells by phosphorylation (5) and is the flux-controlling enzyme for glycolysis in β-cells (4). Thus, it serves as the gatekeeper for metabolic signaling, suggesting that GK rather than GLUT2 is directly responsible for the insulin secretion in response to increasing blood glucose levels (5).

Thiazolidinediones (TZDs) are a new class of antidiabetic agents that act by improving insulin sensitivity in various animal models of obesity and diabetes (6–9). The biological effects of TZDs are exerted by binding to and activating peroxisomal proliferator–activated receptor-γ (PPAR-γ). There is a strong correlation between TZD-PPAR-γ interaction and the antidiabetic action of TZDs; the relative potency of TZDs for binding to PPAR-γ and activation of PPAR-γ in vitro correlates perfectly with their antidiabetic potency in vivo (10). Patients with a dominant-negative mutation in the PPAR-γ gene show severe hyperglycemia, which provides a genetic link between PPAR-γ and type 2 diabetes (11). TZDs stimulate adipocyte differentiation, preferentially generating smaller adipocytes that are more sensitive to insulin and produce lower levels of free fatty acids, tumor necrosis factor-α, and leptin (10,12). TZDs are also known to restore the functions of β-cells, reduce intracellular fat deposition, and relieve β-cells from a lipotoxic environment (13,14). There are reports that TZDs improve glucose-sensing ability in isolated islets of diabetic ZDF rats and increase GLUT2 gene expression in β-cells (8,13,15). These data suggest that the expression of genes involved in glucose sensing of pancreatic β-cells may be modulated by PPAR-γ. However, the molecular targets of TZDs involved in their action on the physiological regulation of insulin secretion are yet to be identified (16).

We previously reported the presence of peroxisomal proliferator response element (PPRE) in the rat GLUT2 promoter and suggested its possible significance to explain the role of PPAR-γ in restoring GSIS (15). However, the general belief that GK may be more important than GLUT2 in the glucose sensing of β-cells led us to explore the presence of PPRE in the β-cell–specific GK (βGK) gene. In this report, we identify a PPRE in the βGK gene

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Received for publication 6 September 2001 and accepted in revised form 26 November 2001.

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βGK, β-cell–specific GK; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GK, glucokinase; GSIS, glucose-stimulated insulin secretion; LGK, rat liver–specific GK; MODY, maturity-onset diabetes of the young; PDX-1, pancreatic duodenal homeobox gene-1; PPAR, peroxisomal proliferator–activated receptor; PPRE, peroxisomal proliferator response element; RPA, RNase protection assay; RXR-α, retinoid X receptor-α; TZD, thiazolidinedione.

and show that this element is responsible for the upregulation of GK expression by PPAR-γ in β-cells.

RESEARCH DESIGN AND METHODS

Materials. Troglitazone was a gift from Sankyo (Tokyo, Japan). WY14643 was purchased from Cayman Chemical (Ann Arbor, MI), and *9-cis* retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO). Troglitazone concentration was adjusted to 10 mmol/l in 19% BSA (wt/vol), 5% DMSO (vol/vol). WY14643 (20 mmol/l) and *9-cis* retinoic acid (2 mmol/l) were prepared in 50% ethanol (vol/vol) and 50% DMSO (vol/vol), respectively. Expression plasmids pCMX-mPPAR-α, pCMX-mPPAR-γ, pCMX-mRXR-α, and pCMX-mRXR-γ were gifts from Drs. R.M. Evans and D.J. Mangelsdorf. Rat GK promoter region spanning –1,003/+196 bp of β-cell–specific gene (17) and –1,448/+127 of liver-specific gene (18) were cloned into pGL3 basic vector and named pRGP-1003 and pRGL-1448, respectively. 5′ serial deletion of βGK promoter reporter constructs pRGP-404, pRGP-128, pRGP+10, and pRGP+100 were constructed by amplifying rat GK promoter regions of −404/+196, −128/+196, +10/+196, and +100/+196 bp, respectively, and subcloning into pGL3 basic vector. For construction of PPRE-truncated promoter reporter constructs pRGPd+10/100, pRGPd+49/73, and pRGPd+73/100, *Kpn*I sites were introduced into the appropriate region by site-directed mutagenesis, and *Kpn*I-digested fragments were excised. Mutant constructs pRGP-1003m1, pRGP-1003m2, pRGP-1003m3, pRGP-1003m4, pRGP-1003m5, pRGP-1003m6, and pRGP-1003mT were produced by introducing substitution mutations into pRGP-1003 using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pRGP+10m5 and pRGP+10mT were also produced by introducing substitution mutations into pRGP+10. pGPRRE3-tk-LUC was produced by inserting three copies of the +44/+70-bp region of βGK gene into the *Sal*I site of ptkLUC reporter. pCRGKP3SP6 and pCRactin were cloned by inserting the PCR product amplifying the region +370/+696 bp of rat βGK gene and +224/+440 bp of rat β-actin gene, respectively. DNA sequences of all constructs were confirmed by T7 DNA sequencing. The primers used in site-directed mutagenesis, RGKP+43/75m1, RGKP+43/75m2, and RGKP+43/75m3→RGP+43/75m1, RGP+43/75m2, and RGP+43/75m3, were also used as probes in electrophoretic mobility shift assays (EMSAs).

Cell culture and transient transfection. CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Ins-1 cells (rat insulinoma cell line) were maintained in DMEM supplemented with 10% (vol/vol) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μmol/l 2-mercaptoethanol, and 1 mmol/l sodium pyruvate (19). HIT-T15 cells (hamster insulinoma cell line) were maintained in Ham's F12-K medium (Life Technologies) supplemented with 10% dialyzed horse serum, 2.5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Min6 cells (mouse insulinoma cell line) were maintained in DMEM supplemented with 15% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (20). Transient transfections were performed using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's protocol, and luciferase assay was performed as described previously (15).

In vitro transcription and translation. In vitro translated PPAR-γ and retinoid X receptor-α (RXR-α) were produced using TNT Quick Coupled Transcription/Translation systems (Promega, Madison, WI) following the manufacturer's protocol.

Production of recombinant PPAR-γ and anti–PPAR-γ antibody. Recombinant mouse PPAR-γ was expressed in *Escherichia coli* BL21(DE3)pLysS. PPAR-γ expression vector pETPγ was generated by inserting the cDNA fragment from pCMX-PPAR-γ into *Sac*I and *Xho*I sites of pET21a prokaryotic expression vector (Novagen, Madison, WI). The bacteria freshly transformed with expression vector were grown to mid-log phase, and recombinant protein was induced for 4 h with 1 mmol/l isopropyl-β-D-thiogalactopyranoside. The bacteria were harvested by centrifugation and disrupted by sonication. The recombinant protein containing NH_2-terminal T7 and COOH-terminal polyhistidine (His_6) tag were purified to homogeneity by Ni-NTA-agarose (Qiagen, Valencia, CA) chromatography. The purity and concentration of the recombinant protein were verified by SDS-PAGE followed by Coomassie brilliant blue staining. Anti–PPAR-γ antibody was produced using the recombinant PPAR-γ in rabbit. Quantity and quality of anti–PPAR-γ antibody was tested by enzyme-linked immunosorbent assay and Western blot analysis using recombinant PPAR-γ and in vitro translated PPAR-γ.

EMSA. Ten picomoles of single-stranded sense oligonucleotide were labeled with [^{32}P] using T4 polynucleotide kinase (TaKaRa, Shiga, Japan) and annealed with a 5-molar excess of antisense oligonucleotide. The resulting double-stranded oligonucleotides were purified by Sephadex G50 (Pharmacia, Piscataway, NJ) spin column. Probes (50,000 cpm; ~0.02 pmol) were incubated with 2 μl of in vitro translated proteins for 20 min on ice in a buffer containing 10 mmol/l HEPES (pH 7.9), 60 mmol/l KCl, 10% glycerol (vol/vol), and 1 mmol/l dithiothreitol. Poly(dI-dC) (1 μg) was added to each reaction to suppress nonspecific binding. Anti–PPAR-γ serum (2 μl) was added to the reaction for supershift assay. The protein-DNA complexes were resolved from the free probe by electrophoresis at 4°C on a 5% polyacrylamide gel in 0.5× TBE buffer (1× TBE contains 9 mmol/l Tris, 90 mmol/l boric acid, and 20 mmol/l EDTA, pH 8.0). The dried gels were exposed to X-ray film at −70°C with an intensifying screen.

The oligonucleotides used in EMSA were as follows: RGP+43/75, 5′-AGAGTTACCTGTTGCCTCATTACTCAAAAGCCA-3′; RGP+43/75m1, 5′-AGAGccgggGTTGCCTCATTACTCAAAAGCCA-3′; RGP+43/75m2, 5′-AGAGTTACCTGgatatcCATTACTCAAAAGCCA-3′; and RGP+43/75m3, 5′-AGAGTTACCTGTTGCCTCgaattcCAAAAGCCA-3′. The PPRE sequence is underlined, and mutated bases are shown in lowercase letters (21).

RNA preparation, RNase protection assay, and RT-PCR. Total RNA was isolated from β-cell lines treated with 20 μmol/l troglitazone and 1 μmol/l *9-cis* retinoic acid for 24 h using TRIzol reagent by the manufacturer's protocol (Life Technologies). Production of probes and RNase protection assay (RPA) were performed using Strip-EZ RNA probe synthesis kit and RPA III kit (Ambion, Austin, TX) according to the manufacturer's protocol. Briefly, antisense RNA probes, which covered the +370/+696-bp region of rat βGK and the +224/+440-bp region of rat β-actin, were produced from pCRGKP3SP6 and pCRactin using [^{32}P]UTP and T7 RNA polymerase, then template DNA and free nucleotides were removed by DNase I digestion and two successive ethanol precipitations. Total RNA (50 μg) and 300,000 cpm of probes were hybridized at 42°C for 24 h, then unhybridized RNA was digested by RNase A/RNase T1 mix. The remaining samples after RNase digestion were precipitated and resuspended in 10 μl gel loading buffer. Samples were incubated for 3 min at 94°C and subjected to electrophoresis on 5% denaturing polyacrylamide gel. The dried gels were exposed to X-ray film at –70°C with an intensifying screen.

For RT-PCR, first-strand cDNA was synthesized from 2 μg of total RNA in 20 μl volume using random hexamer and Superscript II reverse transcriptase (Life Technologies). Reverse transcription reaction mixture (1 μl) was amplified with primers specific for rat GK and β-actin in a total volume of 50 μl. Linearity of the PCR was tested by amplifying 100 ng of total RNA between amplification cycles 20 and 50. According to this amplification profile, samples were amplified for 30 cycles using the following parameters: 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s. β-Actin was used as an internal control for quality and quantity of RNA. The PCR products were subjected to electrophoresis on 1.4% agarose gel, and the quantities of PCR products were analyzed by Molecular Analyst II (Bio-Rad, Hercules, CA). The PCR product was confirmed by DNA sequencing. Primers used in PCR were as follows: GK sense, 5′-GTGGTGCTTTTGAGACCCGTT-3′; GK antisense, 5′-TTCGATGAAGGTGATTTCGCA-3′; β-actin sense, 5′-TTGTAACCAACTGGGACGATATGG-3′; and β-actin antisense, 5′-GACCAGAGGCATACAGGGACAAC-3′.

Measurement of GK activity. Cells were harvested and centrifuged at 1,200 rpm. Tissue pellets were lysed in 400 μl reporter lysis buffer (Promega) and vortexed, and cell membranes were disrupted by three freeze-thaw cycles. GK buffer (400 μl) consisting of 50 mmol/l Tris (pH 7.6), 4 mmol/l EDTA, 150 mmol/l KCl, 4 mmol/l Mg_2SO_4, and 2.5 mmol/l dithiothreitol, was added. The lysates were then centrifuged at 4°C for 1 h at 35,000*g* in a Beckman ultracentrifuge. Supernatants were used in GK enzyme assay, and GK activity was assayed as described by Walker and Parry (22), using NAD (Sigma) as coenzyme. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma) was used as coupling enzyme. Correction for low hexokinase activity was applied by subtracting the activity measured at 0.5 mmol/l glucose from the activity measured at 100 mmol/l glucose. Protein concentrations were determined by Bradford assay (23).

Statistical analysis. All transfection studies and GK enzyme assays were performed in triplicate and repeated more than three times. The data are presented as means ± SD except for GK enzyme assay. Statistical analysis was carried out using Excel software (Microsoft, Redmond, WA).

RESULTS

PPAR-γ activates the βGK promoter. GK is expressed in a cell type–specific manner by alternate promoter usage (24). To determine whether PPAR-γ can regulate GK gene expression, we cloned rat liver–specific GK (LGK) promoter and βGK promoter into luciferase reporter vector and tested the responsiveness to PPAR-α and PPAR-γ, respectively, in CV-1 cells. As shown in Fig. 1, PPAR-α did not activate either promoter, whereas PPAR-γ activated both. Moreover, the activation of the βGK promoter by

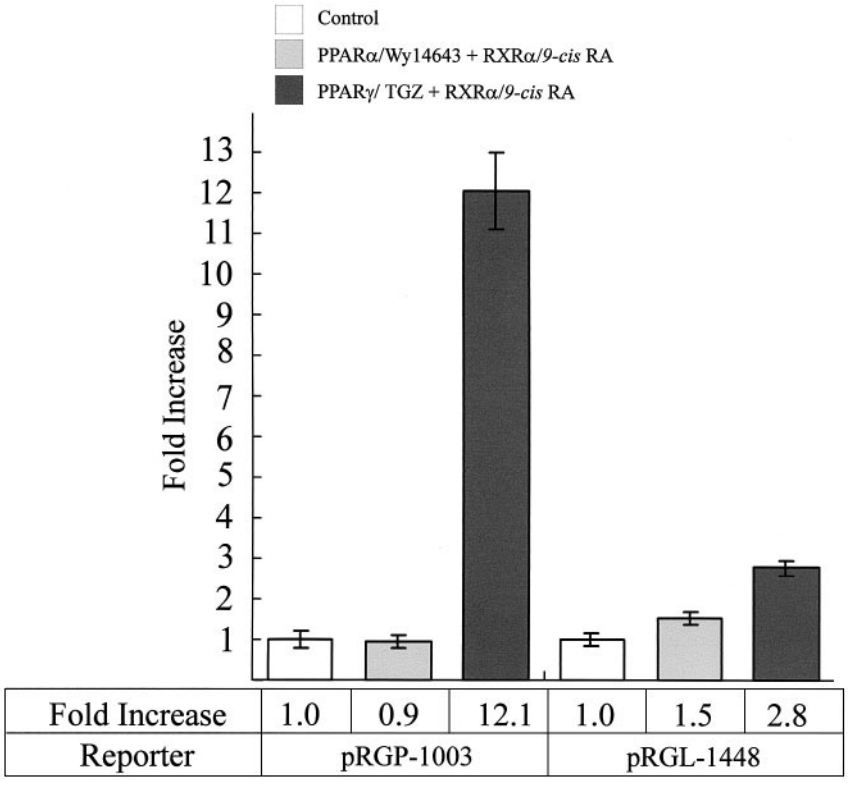


FIG. 1. Comparison of PPAR responsiveness between LGK and BGK promoters. Luciferase reporter under the control of rat βGK (pRGP-1003) or LGK (pRGL-1448) promoter was cotransfected into CV-1 cells with expression vectors of PPARs and RXR-α. Appropriate ligands for receptors were treated after transfection: 10 μmol/l WY-14643 for PPAR-α, 20 μmol/l troglitazone (TGZ) for PPAR-γ, and 1 μmol/l *9-cis* retinoic acid (*9-cis* RA) for RXR-α. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity of pRGP-1003 and pRGL-1448 in the absence of expression vectors and ligands.

PPAR-γ was >12-fold and that of the LGK promoter 2.8-fold, suggesting the importance of PPAR-γ in the regulation of the βGK promoter. These results led us to search for the presence of PPRE in the promoter region of the βGK gene.

To identify a functional PPRE in the βGK promoter, we prepared 5′ serial deletion constructs and tested their responsiveness to PPAR-γ in CV-1 cells. As shown in Fig. 2, pRGP-1003, pRGP-404, and pRGP+10 were activated by coexpression of PPAR-γ and RXR-α in the presence of troglitazone and *9-cis* retinoic acid. However, deletion down to +100 bp (pRGP+100) resulted in loss of ligand-dependent activation. Thus, the 5′ serial deletion study suggested that PPAR-γ, heterodimerized with RXR-α, activated the βGK promoter in a ligand-dependent manner, and the activation required the sequences between +10 and +100 bp of the βGK promoter. To localize precisely the region responsible for the transactivation by PPAR-γ, we prepared several truncated promoter-luciferase constructs lacking +10/+100 (pRGPd+10/100), +49/+73 (pRGPd+49/73), and +73/+100 (pRGPd+73/100) (Fig. 3*A*). The constructs pRGPd+10/100 and pRGPd+49/73 lost their responsiveness to PPAR-γ, whereas pRGPd+73/100 retained PPAR-γ responsiveness. Thus, we could narrow down the location of PPRE to the region including +49/+73 bp. In addition, the region between +34 and +80 bp was highly conserved between species, matching with the –383/–337-bp region of mice (25) and the +67/+113-bp region of humans (26) (Fig. 3*B*). This evolutionary conservation suggests the importance of the region in regulation of βGK gene expression.

PPAR-γ/RXR-α heterodimer binds to and activates the βGK-PPRE. Although the region containing +49/+73 bp was responsible for the transactivation of the βGK promoter by PPAR-γ, there was no conventional PPRE known as DR+1, a hexameric consensus sequence (AGGTCA) in a direct repeat spaced by one nucleotide (21). Thus, to characterize the composition of the element recognized by PPAR-γ, we constructed several mutant forms of the promoter and examined PPAR-γ responsiveness in CV-1 cells (Fig. 4*A*). Initially, we tested three scanning mutants of the promoter having six basepair substitutions in the region between +47 and +66 bp, named pRGP-1003m1, pRGP-1003m2, and pRGP-1003m3. These mutants were not activated by PPAR-γ/RXR-α heterodimer, indicating that the +47/+66-bp region constitutes at least part of the PPRE. Then we constructed four more mutants to map the PPRE and named them pRGP-1003m4, pRGP-1003m5, pRGP-1003m6, and pRGP-1003mT. The pRGP-1003m5 and pRGP-1003m6 constructs lost their responsiveness to PPAR-γ/RXR-α heterodimer, but pRGP-1003m4 and pRGP-1003mT retained PPAR-γ responsiveness. From these results, the βGK-PPRE could be localized at the region between +47 and +68 bp, even though there was little sequence similarity with conventional DR+1.

To confirm that the βGK-PPRE mediates PPAR-γ–dependent transactivation of the βGK promoter through DNA binding of PPAR-γ, we performed EMSA with in vitro translated PPAR-γ and RXR-α using the oligonucleotide covering +43/+75 bp of the βGK gene. As shown in Fig. 4*B*, PPAR-γ or RXR-α alone did not bind to the probe (*lanes 1* and *2*). However, incubation of the probe with

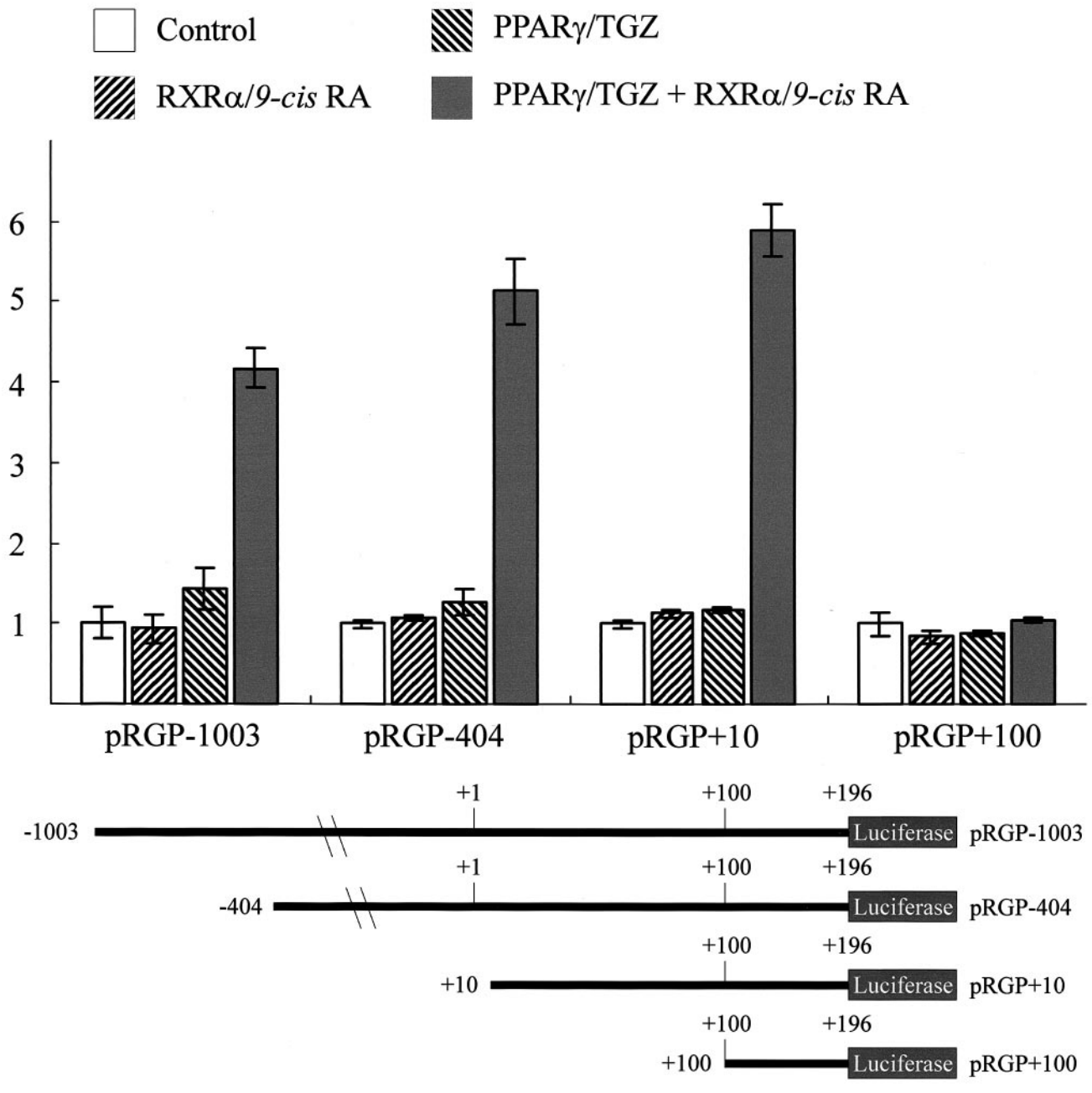


FIG. 2. 5′deletion study of βGK promoter. Luciferase reporter constructs under the control of rat βGK promoter spanning from −1,003, −404, +10, or +100 bp to +196 bp were cotransfected into CV-1 cells with or without PPAR-γ and/or RXR-α expression vectors as indicated. The cells were incubated in the presence of appropriate ligands as indicated: 20 μmol/l troglitazone (TGZ) for PPAR-γ and 1 μmol/l *9-cis* retinoic acid (*9-cis* RA) for RXR-α. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity in the absence of expression vectors and ligands.

PPAR-γ and RXR-α formed DNA-protein complexes (*lanes 3* and *5*), suggesting that PPAR-γ heterodimerized with RXR-α binds to the +43/+75-bp region of βGK gene, and the binding of PPAR-γ/RXR-α heterodimer was further confirmed by anti–PPAR-γ antibody (*lane 4*). In contrast, heterodimer of PPAR-γ and RXR-α did not bind to mutant probes m1, m2, or m3, which contained the same mutations as pRGP-1003m1, -m2, and -m3, respectively (*lanes 6*, *7*, and *8*). From these results, it can be concluded that the region between +47 and +68 bp is a variant form of PPRE, and transactivation of βGK promoter by PPAR-γ depends on the binding of PPAR-γ/RXR-α heterodimer.

Although CV-1 cells were suitable for testing PPAR-γ responsiveness, it would be more reasonable to examine the PPAR-γ responsiveness of the βGK promoter in β-cell lines that are known to express both GK and insulin. Therefore, we transfected the promoter reporter constructs into HIT-T15 cells. The pRGP-1003 construct resulted in only a 1.5-fold increase in coexpressions of PPAR-γ and RXR-α (Fig. 5). This marginal activation might be due to higher basal activity of the βGK promoter, which depends on β-cell–specific transcription factors in HIT-T15 cells. Thus, we transfected 5′ deletion constructs of the promoter into HIT-T15 cells to minimize the effects of β-cell–specific transcription factors. The pRGP-128 construct was transactivated by PPAR-γ more highly than

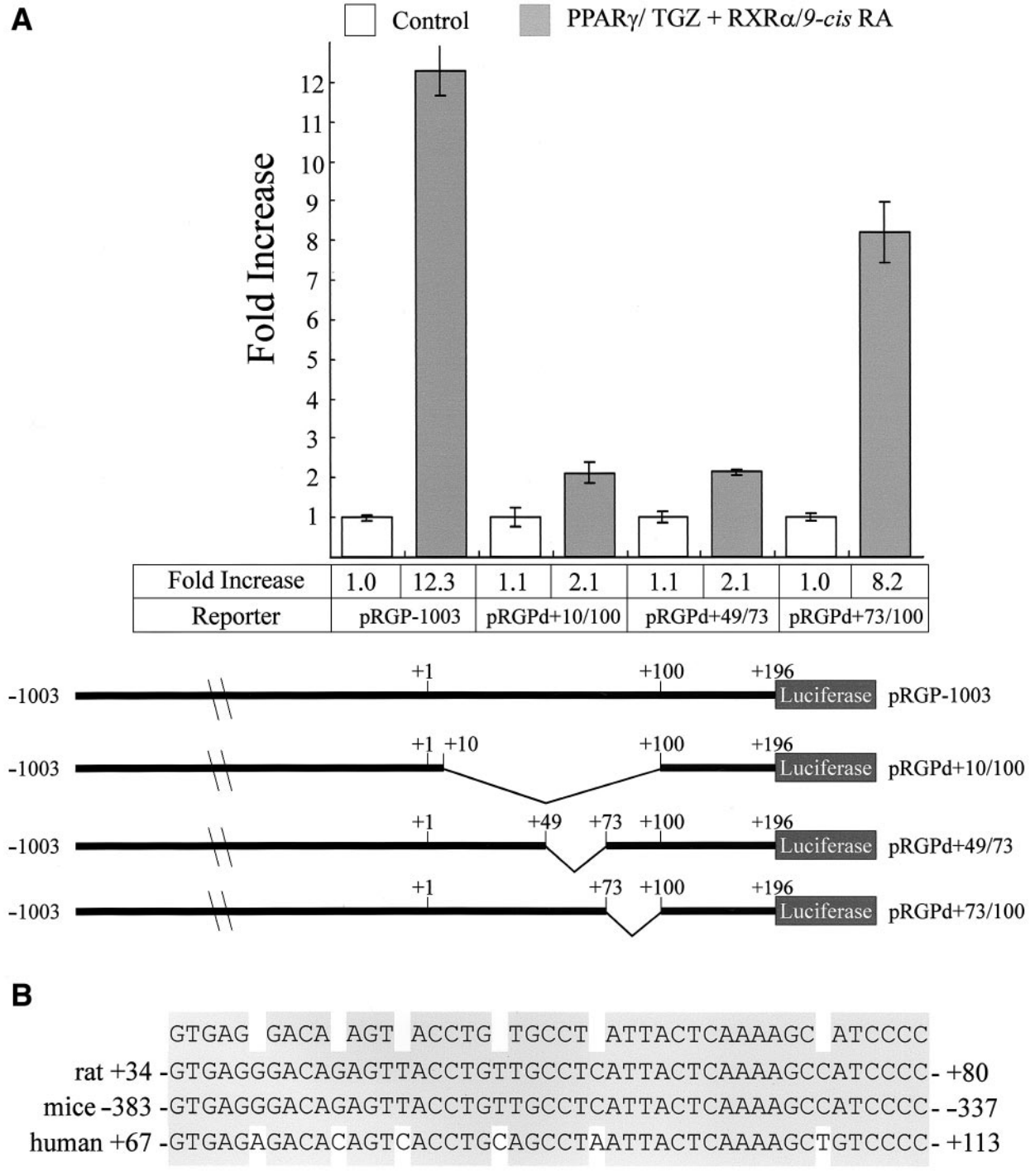
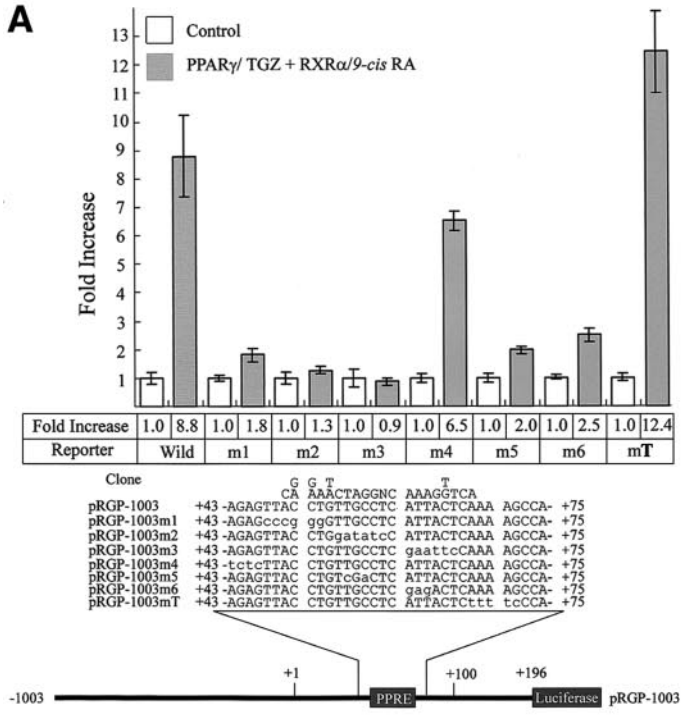
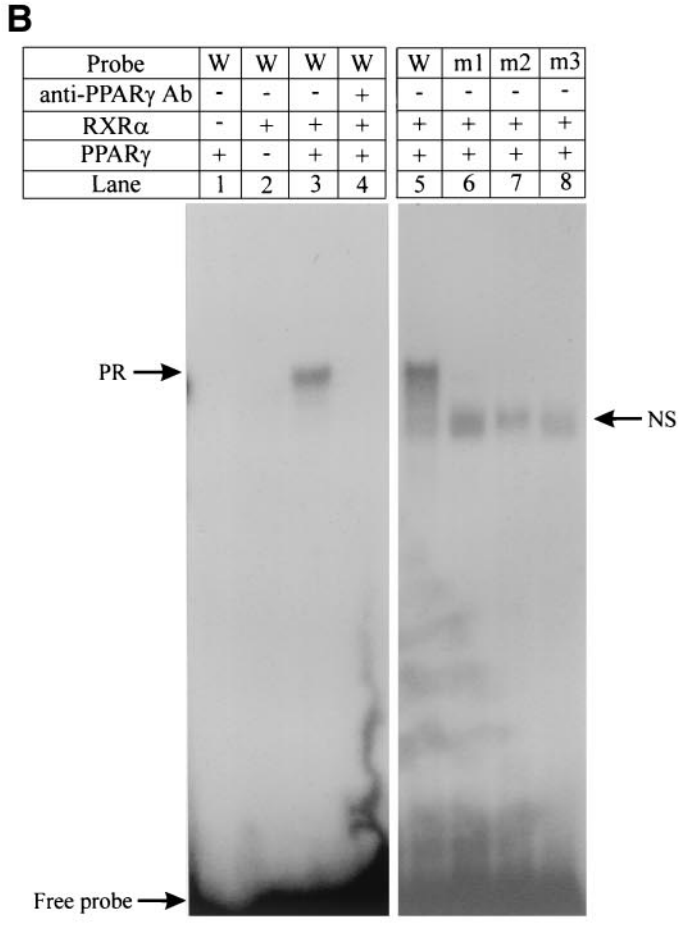


FIG. 3. Localization of PPRE in β-cell–specific GK promoter. *A*: Truncation of the region from +49 to +73 bp resulted in the loss of responsiveness to PPAR-γ. The structure of truncated mutants of βGK promoter luciferase reporter constructs is shown. Luciferase reporter constructs under control of the βGK promoter spanning from −1,003 to +196 bp were cotransfected into CV-1 cells with or without PPAR-γ and RXR-α expression vectors. Truncated mutant constructs (pRGPd+10/73, pRGPd+10/135, and pRGPd+49/73) were also transfected into CV-1 cells and incubated in the presence of respective ligands as indicated. Troglitazone (TGZ) (20 μmol/l) for PPAR-γ and *9-cis* retinoic acid (*9-cis* RA) (1 μmol/l) for RXR-α were used as ligands. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity in the absence of expression vectors and ligands. *B*: Comparison of DNA sequences between species. DNA sequences of rat βGK +34/+80-bp region were compared with the mouse βGK –383/–337-bp and human +67/+113-bp regions. The shading indicates homologous sequences.

pRGP-1003, and the pRGP+10 construct, containing minimal region responsible for PPAR-γ response, showed the highest transactivation. To further confirm the functionality of the βGK-PPRE, we introduced the same mutations into pRGP+10 as in pRGP-1003m5 and pRGP-1003mT and named them pRGP+10m5 and pRGP+10mT, respectively. When transfected into HIT-T15 cells, pRGP+10mT was activated by PPAR-γ, whereas pRGP+10m5 was not. These results are consistent with the results from CV-1 cells and suggest that the βGK-PPRE is functioning in both β-cell lines and non–β-cell lines.

Although we showed the functionality of the βGK-PPRE





in the promoter context, it was necessary to confirm whether the βGK-PPRE itself could respond to PPAR-γ, because its sequence was very different from the consensus sequence. To this end, we constructed pGPRE3-tk-LUC, which had three copies of the βGK-PPRE in front of the thymidine kinase minimal promoter, and tested PPAR-γ responsiveness in CV-1 and HIT-T15 cells (Fig. 6). As expected, pGPRE3-tk-LUC reporter was well activated by PPAR-γ/RXR-α heterodimer in CV-1 and HIT-T15 cells. These results indicated that the βGK-PPRE is fully functional in both the βGK promoter context and the artificial promoter context.

Troglitazone increases GK expression and activity in β-cell lines. Troglitazone is known to have multiple effects on many genes involved in glucose and lipid metabolism, either directly or indirectly. Thus, it was necessary to confirm the effect of troglitazone on endogenous βGK expression in vitro. Total RNA was isolated from Min6 and Ins-1 cells after 24-h incubation in the presence or absence of troglitazone and *9-cis* retinoic acid, and RPA and semiquantitative RT-PCR were performed. In RPA, we used the region between +370 and +696 bp, which had a β-cell–specific exon, as a probe, and we amplified the COOH-terminal region by RT-PCR, which is conserved between LGK and βGK. As shown in Fig. 7, combined treatment of troglitazone and *9-cis* retinoic acid increased endogenous βGK expression in both Min6 and Ins-1 cells. Thus, activation of the βGK promoter induced endogenous βGK transcription. However, glucose is an important regulator of GK activity, and the regulation of GK activity by glucose predominantly depends on post-transcriptional mechanisms (27). Thus, we measured GK activity to confirm that activation of βGK transcription can increase GK activity in this system. Troglitazone increased GK activity by 40% in Ins-1 cells and 20% in Min6 cells (Fig. 8). This result clearly shows that PPAR-γ directly increases GK expression in β-cells through the βGK-PPRE, resulting in the increase of GK activity.

DISCUSSION

In the present study, we demonstrate that PPAR-γ stimulates βGK gene expression and enzyme activity in β-cells. The βGK-PPRE was localized within +47/+68 bp, containing the sequence TTACCTGTTGCCTCATTACTCA by promoter analysis performed in CV-1 cells. The pRGP-1003

FIG. 4. Localization of the PPRE and the binding of PPAR-γ. *A*: DNA sequences of the wild-type and mutant versions of the promoter element were shown to define the PPRE. The consensus sequence of PPRE known as DR+1 is also shown, and mutated bases are shown in lowercase. The oligonucleotide covering the indicated region (+43/+75 bp) was used as a probe (*B*), and mutant oligonucleotides (RGP+43/75m1, RGP+43/75m2, and RGP+43/75m3) were also used as probes for EMSA. Luciferase reporter constructs under control of the βGK promoter or mutated promoters were transfected into CV-1 cells with or without coexpression of PPAR-γ/RXR-α. The cells were incubated for 12 h after transfection in the presence of appropriate ligands: 20 μmol/l troglitazone (TGZ) for PPAR-γ and 1 μmol/l *9-cis* retinoic acid (*9-cis* RA) for RXR-α. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity in the absence of the expression vectors and ligands. *B*: EMSA of RGP+43/75 using in vitro translated PPAR-γ and RXR-α. [^{32}P]-labeled double-strand oligonucleotides (w, m1, m2, and m3) were incubated with in vitro translated PPAR-γ (3 μl) and/or RXR-α (3 μl) as indicated. Anti–PPAR-γ serum (2 μl) was added into the reaction mixture (*lane 4*). Ab, antibody; NS, nonspecific band; PR, shifted band by PPAR-γ/RXR-α heterodimer.

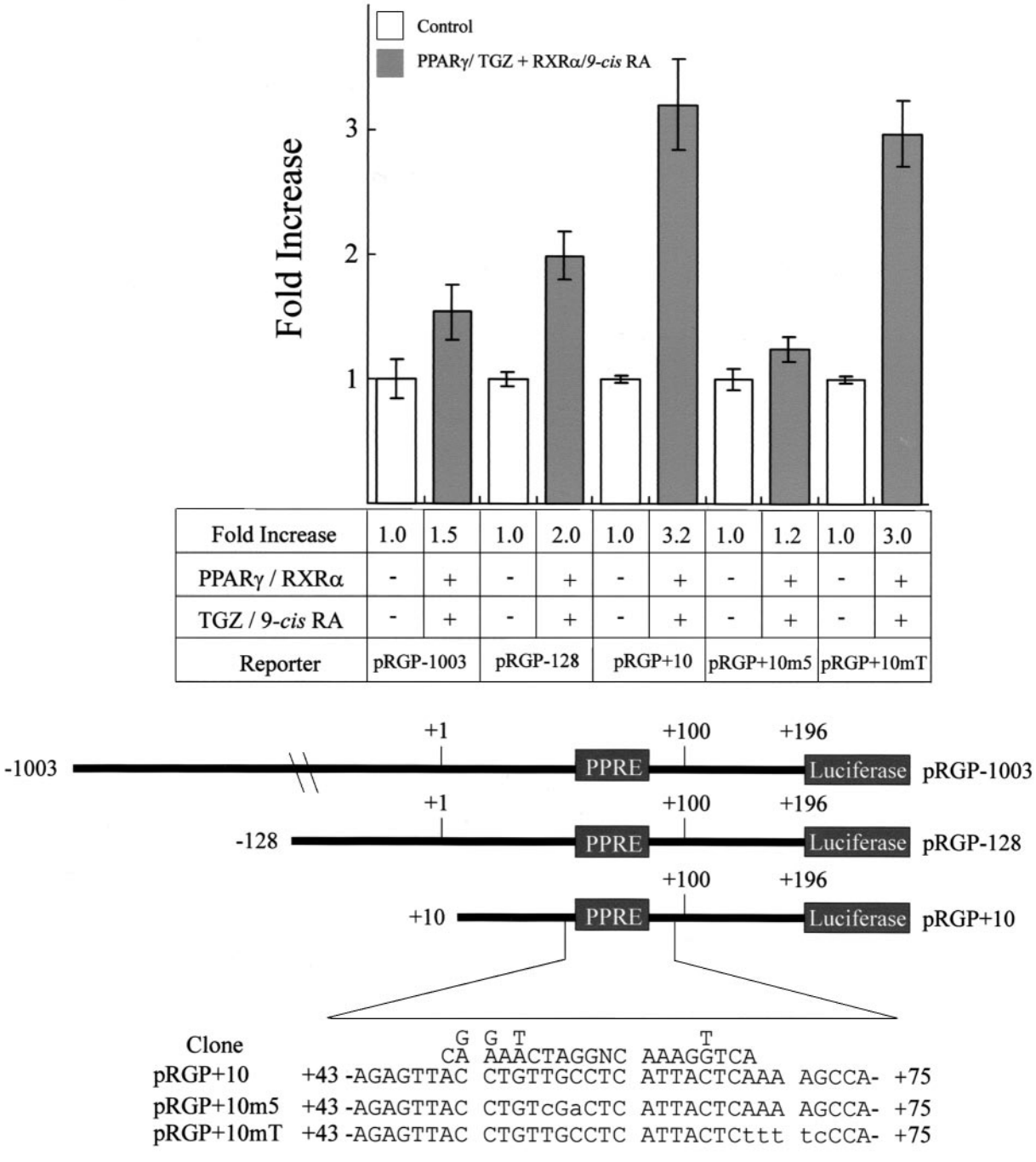


FIG. 5. PPAR-γ responsiveness of βGK promoter in HIT-T15 cells. βGK promoter–luciferase reporter constructs were transfected into HIT-T15 cells to test their responsiveness to PPAR-γ in β-cells. The structures are given. Expression vectors of PPAR-γ and RXR-α were cotransfected, and 20 μmol/l troglitazone (TGZ) and 1 μmol/l *9-cis* retinoic acid (*9-cis* RA) were added as indicated. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity in the absence of expression vectors and ligands.

construct was activated by PPAR-γ and RXR-α in the presence of their ligands. This transactivation disappeared upon truncation of the βGK-PPRE. The mutations introduced into the βGK-PPRE abolished the transactivation of the βGK promoter by PPAR-γ and the binding of PPAR-γ/RXR-α heterodimer to the βGK-PPRE. We also showed functionality of the βGK-PPRE in insulin-secreting HIT-T15 cells. However, the fold activation in HIT-T15 cells was much smaller than in CV-1 cells. This difference in fold activation could be explained by the tissue specificity of the βGK promoter. The basal activity of the βGK promoter in HIT-T15 cells was more than 5 times higher than that in CV-1 cells. Thus, the absolute amount of the activation in HIT-T15 cells is as much as that in CV-1 cells, in spite of the small fold activation. This higher basal activity of the βGK promoter predominantly depends on the UPE3 site, which is located in the region between −104/–95 bp and the binding site for pancreatic duodenal homeobox gene-1 (PDX-1), the most well-known β-cell–specific transcription factor (28,29). Thus, deletion constructs were better

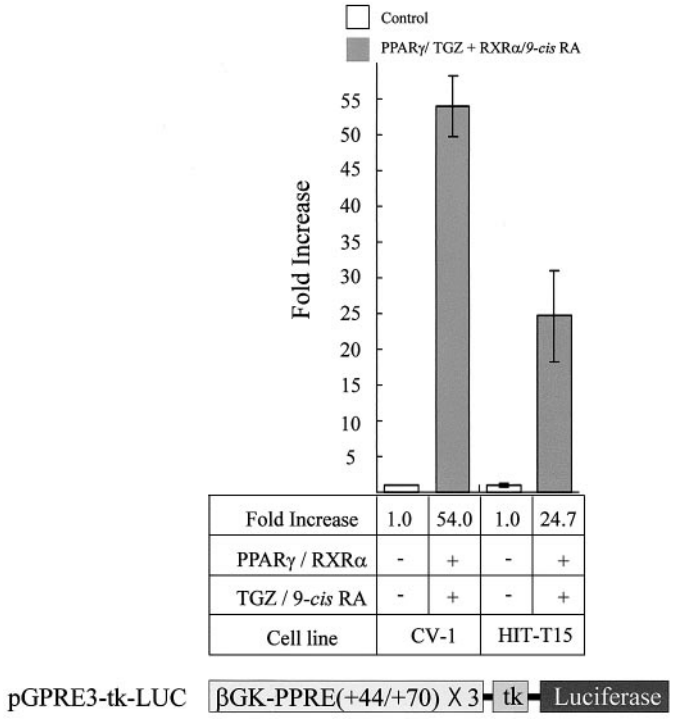


FIG. 6. PPAR-γ responsiveness of βGK-PPRE in heterologous promoter context. The luciferase reporter construct, containing three copies of the +44/+70-bp region of βGK gene (βGK-PPRE) in front of thymidine kinase (tk) minimal promoter, was transfected in CV-1 and HIT-T15 cells. Expression vectors of PPAR-γ and RXR-α were cotransfected, and 20 μmol/l troglitazone (TGZ) and 1 μmol/l *9-cis* retinoic acid (*9-cis* RA) were treated as indicated. Normalized luciferase activities are shown as means ± SD of three independent experiments in triplicate and are expressed as the fold increase relative to basal activity in the absence of expression vectors and ligands.

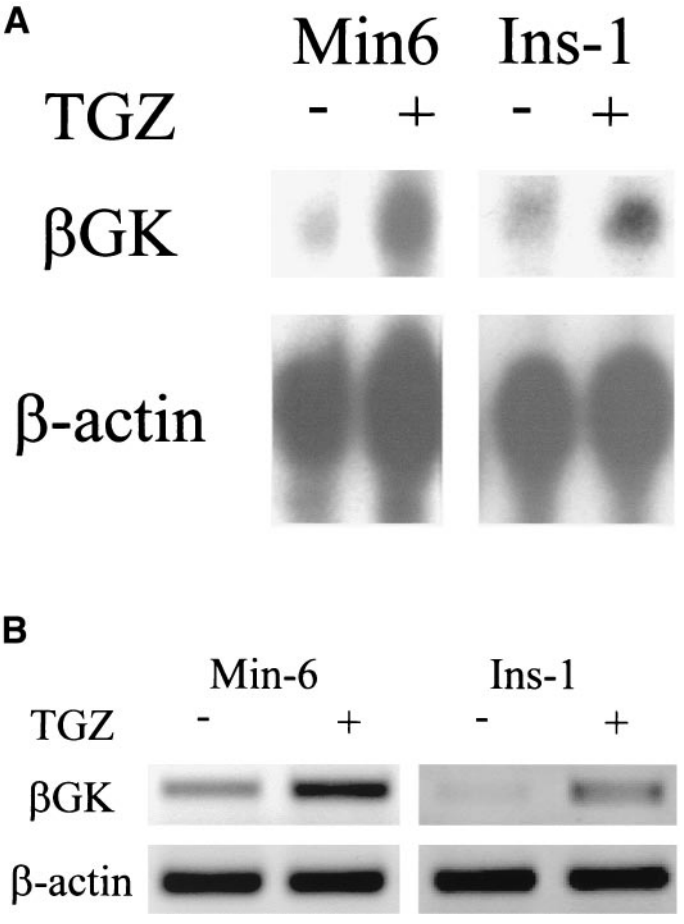


FIG. 7. PPAR-γ increased endogenous βGK expression in β-cell lines. *A*: RPA of βGK. Total RNA (50 μg) was isolated from Min6 and Ins-1 cells cultured in the presence or absence of troglitazone (20 μmol/l) and *9-cis* retinoic acid (1 μmol/l) and subjected to RPA. The protected fragment contained the β-cell–specific exon of GK. *B*: RT-PCR of GK. Total RNA was isolated from Min6 and Ins-1 cells. The amplified region of GK was the COOH terminus of the genes. In *A* and *B*, β-actin was used as an internal control, and detailed methods are described in RESEARCH DESIGN AND METHODS.

activated by PPAR-γ than the pRGP-1003 construct. In addition, PPAR-γ activated the UPE3-mutated βGK promoter by >5 times (data not shown). These results suggested that the transactivation of the βGK promoter by PPAR-γ in HIT-T15 cells is significant, in spite of its small fold activation, and that βGK-PPRE may have an important function in diabetic β-cells, where PDX-1 expression is reduced (30). We also showed that troglitazone increased endogenous βGK expression in β-cell lines. However, posttranscriptional regulation is known to be important in the regulation of βGK activity (27). Despite the report that increased GK transcription could increase GK activity in β-cells (31), we assumed that it was necessary to measure the GK activity level to consolidate the effect of PPAR-γ and confirmed the increase of GK activity by troglitazone in β-cell lines. Thus, it is concluded that the activation of the βGK-PPRE by PPAR-γ stimulates GK enzyme activity as well as GK expression.

Troglitazone can restore βGK expression and the integrity of islets in diabetic ZDF rats (data not shown). Direct regulation of βGK promoter by PPAR-γ can contribute to restoration of GK expression in diabetic ZDF rats. But the direct activation of the βGK promoter by PPAR-γ may not be the sole factor to restore βGK expression in the β-cells of troglitazone-treated type 2 diabetic animals, because troglitazone has a large number of possible effects on βGK expression (10). Troglitazone improves insulin sensitivity and decreases plasma glucose levels in type 2 diabetic subjects (7,8). It can also prevent glucotoxic effects, resulting in increased PDX-1 expression (32). Insulin can also regulate βGK expression (33). Taking these data together, the role of PPAR-γ on βGK expression in vivo can be summarized as follows: *1*) increased insulin sensitivity can stimulate βGK expression; *2*) reduced plasma glucose concentration can restore PDX-1 expression in β-cells and thus increase βGK expression; and *3*) PPAR-γ can directly activate βGK expression.

Insulin resistance is the common feature of type 2 diabetes and appears years before the onset of diabetes (34). In the prediabetic stage, β-cells secrete enough insulin to overcome the insulin resistance and maintain euglycemia. Once the balance of insulin secretion and action is disrupted, the clinical phenotype of type 2 diabetes develops, with defects of β-cell function (35). In the type 2 diabetic subject, insulin secretion is not adequately stimulated by glucose, and glucose-sensing apparatus is downregulated in β-cells. In maturity-onset diabetes of the young (MODY), loss of glucose sensing is the primary event in developing diabetes (5). Thus, failure of the β-cells to respond appropriately to glucose may be the central event in the development of β-cell defects, although the precise mechanism is still obscure.

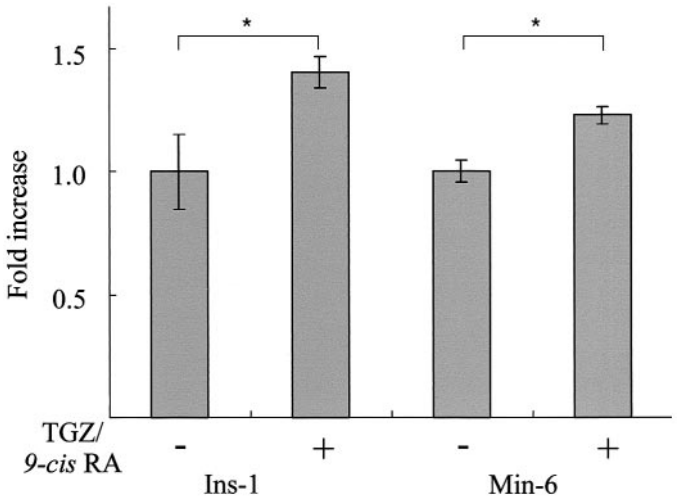


FIG. 8. Troglitazone increases GK activity in β-cell lines. Min6 and Ins-1 cells were incubated in the presence or absence of troglitazone (TGZ) (20 μmol/l) and *9-cis* retinoic acid (*9-cis* RA) (1 μmol/l). GK activity was normalized by protein concentration. Normalized GK activities are shown as the means ± SE of three independent experiments in triplicate and are expressed as the fold increase relative to GK activity in the absence of troglitazone and *9-cis* retinoic acid. Significance was assessed by Student's unpaired *t* test. *$P \leq 0.05$.

βGK has an important role in glucose homeostasis because it works as a glucose sensor for GSIS under physiological conditions. Any change of βGK activity causes alteration of the glucose threshold for insulin secretion (5). β-Cells may gradually lose their predominant position in blood glucose regulation in insulin-resistant states, for example, in obesity and late pregnancy. The glucose threshold of β-cells is usually lower, and thereby basal insulin secretion is increased, in primary islets isolated from animals with insulin resistance. Troglitazone decreases basal insulin secretion but increases GSIS in the primary islets isolated from diabetic ZDF rats (14). These troglitazone-induced changes of GSIS in isolated diabetic β-cells resemble the hyperinsulinemic pattern of compensated β-cells. The way in which troglitazone decreases basal insulin secretion levels in isolated islets is still unclear. However, considering that the first step of GSIS is glucose sensing, increased GK expression may contribute to restoration of glucose threshold and GSIS. Thus, the antidiabetic action of troglitazone might be the result of the combinatorial effects on several target tissues. Enhanced insulin sensitivity improves peripheral glucose disposal, reduces insulin secretory demand, and decreases hepatic glucose output. Enhanced glucose-sensing ability of β-cells improves the function and compensatory capacity of β-cells. Thus, troglitazone decreases blood glucose and insulin levels efficiently. Furthermore, considering that glucose is known to play important roles in the maturation of β-cells (36), the restoration of glucose-sensing ability might be involved in the functional and morphological restoration of β-cells by troglitazone.

In this study, we have characterized atypical PPRE in the βGK gene. The βGK-PPRE is uncommon in that it is located downstream of the transcription initiation site, and the sequence composition is much different from conventional PPRE. There are many reports of *cis*-elements located in exons and introns, but no PPRE is known to be located in exons except for the GLUT2-PPRE. The PPREs in GLUT2 and βGK are located in the first exon, and these two genes have relatively long 5′ untranslated regions. At this point, we don't know whether the location of PPREs of the glucose-sensing apparatus has special implication.

In conclusion, our results demonstrate that troglitazone directly activates βGK expression, as it does GLUT2 expression, and PPAR-γ is the direct regulator of the glucose-sensing apparatus of β-cells, suggesting that βGK gene may be one of the sought-for molecular targets of PPAR-γ on the restoration of β-cell function in type 2 diabetic subjects. In addition, considering the contribution of PPAR-γ to the restoration of glucose-sensing ability of β-cells, the ligands of PPAR-γ may be suitable for treating MODY or early-stage type 2 diabetes.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Next Generation New Technology Development Program funded by the Ministry of Commerce, Industry, and Energy of Korea (2001). H.-I.K., J.-Y.C., and S.-Y.K. are graduate students supported by Brain Korea 21 Project for Medical Science, Yonsei University.

We thank Drs. R.M. Evans and D.J. Mangelsdorf for providing expression plasmids and reporter plasmids; Drs. Jun-ichi Miyazaki and Myung-Shik Lee for providing Min6 cells and Dr. Claes B. Wollheim for Ins-1 cells; and Dr. Chan Y. Jung and Dr. Mohsen Lachaal, SUNY at Buffalo, NY, for critical reading of the manuscript and valuable suggestions.

REFERENCES

1. Schuit FC, Huypen P, Heimberg H, Pipeleers DG: Glucose sensing in pancreatic β-cells. *Diabetes* 50:1–11, 2001
2. Thorens B, Guillam MT, Beermann F, Burcelin R, Jaquet M: Transgenic reexpression of GLUT1 or GLUT2 in pancreatic beta cells rescues GLUT2-null mice from early death and restores normal glucose-stimulated insulin secretion. *J Biol Chem* 275:23751–23758, 2000
3. Faradji RN, Havari E, Chen Q, Gray JT, Tornheim K, Corkey BE, Mulligan RC, Lipes MA: Glucose-induced toxicity in insulin-producing pituitary cells that coexpress GLUT2 and glucokinase: implication for metabolic engineering. *J Biol Chem* 276:36695–36702, 2001
4. Meglasson MD, Matschinsky FM: Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab Rev* 2:163–214, 1986
5. Matschinsky FM, Magnuson MA: Molecular pathogenesis of MODYs. In *Frontiers in Diabetes*, vol. 15. Belfiore F., Ed., Karger, Basel, Switzerland, 2000
6. Fujiwara T, Yoshioka S, Yoshioka T, Ushiyama I, Horikoshi H: Characterization of new oral antidiabetic agent CS-045: studies in KK and *ob/ob* mice and Zucker fatty rats. *Diabetes* 37:1549–1558, 1988
7. Nolan JJ, Ludvik B, Beerdsen P, Loyce M, Olefsky J: Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 33:1188–1193, 1994
8. Cavaghan MK, Ehrmann DA, Byrne MM, Polonsky KS: Treatment with oral antidiabetic agent troglitazone improves β-cell responses to glucose in subjects with impaired glucose tolerance. *J Clin Invest* 100:530–537, 1997
9. Sreenan S, Sturis J, Pugh W, Burant CF, Polonsky KS: Prevention of hyperglycemia in the Zucker diabetic fatty rat by treatment with metformin or troglitazone. *Am J Physiol* 271:E742–E747, 1999
10. Willson TM, Lambert MH, Kliewer SA: Peroxisome proliferator–activated receptor γ and metabolic disease. *Annu Rev Biochem* 70:341–367, 2001
11. Barroso I, Gurnell M, Crowley VE, Agostini M, Schwabe JW, Soos MA, Maslen GL, Williams TD, Lewis H, Schafer AJ, Chatterjee VK, O'Rahilly S: Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 402:880–883, 1999
12. Spiegelman BM: PPARγ: adipogenic regulator and thiazolidinedione receptor. *Diabetes* 47:507–514, 1998
13. Higa M, Zhou YT, Ravazzola M, Baetens D, Orci L, Unger RH: Troglitazone

prevents mitochondrial alterations, beta cell destruction, and diabetes in obese prediabetic rats. *Proc Natl Acad Sci U S A* 96:11513–11518, 1999
14. Shimabukuro M, Zhou YT, Lee Y, Unger RH: Troglitazone lowers islet fat and restores beta cell function of Zucker diabetic fatty rats. *J Biol Chem* 273:3547–3550, 1998
15. Kim H, Kim J, Kim SH, Cha JY, Kim KS, Ahn Y: Identification and functional characterization of the peroxisomal proliferator reponse element in rat GLUT2 promoter. *Diabetes* 49:1517–1524, 2000
16. Kahn CR, Chen C, Cohen SE: Unraveling the mechanism of action of thiazolidinediones. *J Clin Invest* 106:1305–1307, 2000
17. Magnuson MA, Shelton KD: An alternate promoter in the glucokinase gene is active in the pancreatic cell. *J Biol Chem* 264:15936–15942, 1989
18. Magnuson MA, Andreone TL, Printz RL, Koch S, Granner DK: Rat glucokinase gene: structure and regulation by insulin. *Proc Natl Acad Sci U S A* 86:4838–4842, 1989
19. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
20. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
21. Palmer CN, Hsu MH, Griffin HJ, Johnson EF: Novel sequence determinants in peroxisomal proliferator signaling. *J Biol Chem* 270:16114–16121, 1995
22. Walker DG, Parry MJ: Glucokinase. *Methods Enzymol* 9:381–383, 1966
23. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–264, 1976
24. Magnuson MA: Glucokinase gene structure: functional implications of molecular genetic studies. *Diabetes* 39:523–527, 1990
25. Hughes SD, Quaade C, Milburn JL, Cassidy L, Newgard CB: Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells. *J Biol Chem* 266:4521–4530, 1991
26. Tanizawa Y, Matsutani A, Chiu KC, Permutt MA: Human glucokinase gene: isolation, structural characterization, and identification of a microsatellite repeat polymorphism. *Mol Endocrinol* 6:1070–1081, 1992
27. Liang Y, Najafi H, Smith RM, Zimmerman EC, Magnuson MA, Tal M, Matschinsky FM: Concordant glucose induction of glucokinase, glucose usage, and glucose-stimulated insulin release in pancreatic islets maintained in organ culture. *Diabetes* 41:792–806, 1992
28. Shelton KD, Franklin AJ, Khoor A, Beechem J, Magnuson MA: Multiple elements in the upstream glucokinase promoter contribute to transcription in insulinoma cells. *Mol Cell Biol* 12:4578–4589, 1992
29. Watada H, Kajimoto Y, Umayahara Y, Matsuoka T, Kaneto H, Fujitani Y, Kamada T, Kawamori R, Yamasaki Y: The human glucokinase gene beta-cell-type promoter: an essential role of insulin promoter factor 1/PDX-1 in its activation in HIT-T15 cells. *Diabetes* 45:1478–1488, 1996
30. Seufert J, Weir GC, Habener JF: Differential expression of the insulin gene transcriptional repressor CCAAT/enhancer-binding protein beta and transactivator islet duodenum homeobox-1 in rat pancreatic beta cells during the development of diabetes mellitus. *J Clin Invest* 101:2528–2539, 1998
31. Fernandez-Mejia C, Vega-Allende J, Rojas-Ochoa A, Rodriguez-Dorantes M, Romero-Navarro G, Matschinsky FM, Wang J, German MS: Cyclic adenosine 3′,5′-monophosphate increases pancreatic glucokinase activity and gene expression. *Endocrinology* 142:1448–1452, 2001
32. Harmon JS, Gleason CE, Tanaka Y, Oseid EA, Hunter-Berger KK, Robertson RP: In vivo prevention of hyperglycemia also prevents glucotoxic effects on PDX-1 and insulin gene expression. *Diabetes* 48:1995–2000, 1999
33. Leibiger B, Leibiger IB, Moede T, Kemper S, Kulkarni RN, Kahn CR, de Vargas LM, Berggren PO: Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic beta cells. *Mol Cell* 7:559–570, 2001
34. Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. *N Engl J Med* 329:1988–1992, 1993
35. DeFronzo RA: The triumvirate B-cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes* 37:667–687, 1988
36. Dudek RW, Kawabe T, Brinn JE, O'Brien K, Poole MC, Morgan CR: Glucose affects in vitro maturation of fetal rat islets. *Endocrinology* 114:582–587, 1984