

Repression of Glucagon Gene Transcription by Peroxisome Proliferator-activated Receptor γ through Inhibition of Pax6 Transcriptional Activity*

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Sven Schinner[‡], Claudia Dellas[‡], Margit Schröder[‡], Cynthia A. Heinlein[§], Chawnsang Chang[§], Janina Fischer[‡], and Willhart Knepel^{†¶}

From the [‡]Department of Molecular Pharmacology, University of Göttingen, D-37075 Göttingen, Germany and the [§]George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Radiation Oncology, University of Rochester Medical Center, Rochester, New York 14642

The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) is involved in glucose homeostasis and synthetic PPAR γ ligands, the thiazolidinediones, a new class of antidiabetic agents that reduce insulin resistance and, as a secondary effect, reduce hepatic glucose output. PPAR γ is highly expressed in normal human pancreatic islet α -cells that produce glucagon. This peptide hormone is a functional antagonist of insulin stimulating hepatic glucose output. Therefore, the effect of PPAR γ and thiazolidinediones on glucagon gene transcription was investigated. After transient transfection of a glucagon-reporter fusion gene into a glucagon-producing pancreatic islet cell line, thiazolidinediones inhibited glucagon gene transcription when PPAR γ was coexpressed. They also reduced glucagon secretion and glucagon tissue levels in primary pancreatic islets. A 5'/3'-deletion and internal mutation analysis indicated that a pancreatic islet cell-specific enhancer sequence (PISCES) motif within the proximal glucagon promoter element G1 was required for PPAR γ responsiveness. This sequence motif binds the paired domain transcription factor Pax6. When the PISCES motif within G1 was mutated into a GAL4 binding site, the expression of GAL4-Pax6 restored glucagon promoter activity and PPAR γ responsiveness. GAL4-Pax6 transcriptional activity was inhibited by PPAR γ in response to thiazolidinedione treatment also at a minimal viral promoter. These results suggest that PPAR γ in a ligand-dependent but DNA binding-independent manner inhibits Pax6 transcriptional activity, resulting in inhibition of glucagon gene transcription. These data thereby define Pax6 as a novel functional target of PPAR γ and suggest that inhibition of glucagon gene expression may be among the multiple mechanisms through which thiazolidinediones improve glycemic control in diabetic subjects.

Peroxisome proliferator-activated receptor γ (PPAR γ)¹ is a

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¶ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, University of Göttingen, Robert-Koch-Str. 40, Postfach 3742, D-37070 Göttingen, Germany. Tel.: 49-551-395787; Fax: 49-551-399652; E-mail: wknepel@med.uni-goettingen.de.

¹ The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor re-

sponse element; PISCES, pancreatic islet cell-specific enhancer sequence; RT, reverse transcriptase; CBP, cAMP-response element-binding protein-binding protein; GFP, green fluorescent protein; RXR, retinoid X receptor.

member of the ligand-regulated nuclear hormone receptor superfamily (1). Like other nuclear receptors, PPAR γ comprises an amino-terminal ligand-independent transactivation domain (AF-1), a central DNA-binding domain, and a carboxyl-terminal ligand-binding domain that contains a second, ligand-dependent transactivation surface (AF-2) (1). PPAR γ binds as a heterodimer with the 9-*cis*-retinoic acid receptor, RXR, to response elements in target genes to activate transcription. A typical PPRE consists of a direct repeat of hexamer half-sites, TGACCT, spaced by one nucleotide (DR-1) (1). PPAR and RXR occupy the 5' and 3' half-sites, respectively, and thus show a polarity in binding that is the opposite of that observed for other nuclear receptor-RXR heterodimers (1). Like other nuclear receptors, there is evidence that PPAR γ -RXR require the ligand-dependent recruitment of coactivator proteins like SRC-1, GRIP-1, pCIP, CBP, p300, DRIP205, and p120 (2–5) to effectively stimulate gene transcription. This recruitment is dependent on allosteric alterations in the AF-2 helical domain. A "mouse trap" model of receptor activation has been proposed, in which the AF-2 helix closes on the ligand-binding site in response to ligand and establishes a transcriptionally active form of the receptor (3). Cocystal studies (3, 4) indicated that two highly conserved amino acids, Glu-469 in the AF-2 helix and Lys-301 in helix 3 of the ligand-binding domain, form a charge clamp that places a helical LXXLL motif of SRC-1 class of coactivators into a hydrophobic pocket in the receptor. In addition to stimulation of transcription, PPAR γ has been shown to be capable of also negative regulation of gene transcription (6–14).

PPAR γ has been suggested to be involved in a broad range of cellular functions, including adipocyte differentiation, inflammatory responses, and apoptosis, as well as in chronic diseases such as obesity, atherosclerosis, and cancer (15, 16). Of particular importance is its role in glucose homeostasis and type 2 diabetes mellitus (15, 16). Human genetic studies support an important role of PPAR γ in mammalian metabolism (15, 17, 18). Thus, dominant negative mutations in human PPAR γ are associated with hypertension, severe insulin resistance, and diabetes mellitus (18). These physiologic and pathophysiologic actions suggest that synthetic PPAR γ ligands may be of use in the treatment of type 2 diabetes mellitus.

Thiazolidinediones like rosiglitazone are PPAR γ ligands and a new class of orally active antidiabetic drugs (19–21). They

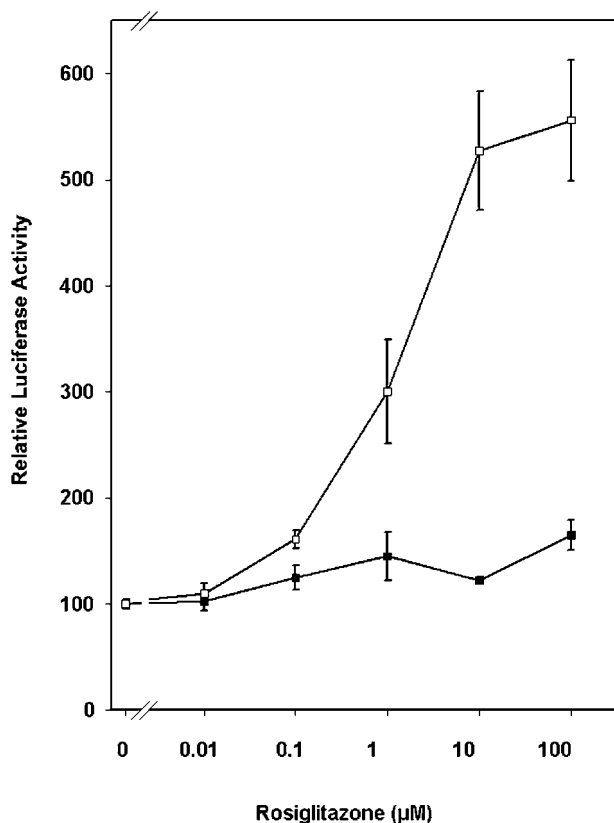


FIG. 1. Activation of a PPAR-dependent promoter by rosiglitazone and PPAR γ in InR1-G9 cells. A luciferase reporter gene under the control of three copies of a PPRE (plasmid PPRELuc) was transiently transfected into InR1-G9 cells together with an expression vector encoding PPAR γ (white squares) or without (black squares). Increasing concentrations of rosiglitazone were added 24 h before harvest. The expression of PPAR γ without rosiglitazone treatment had no effect on PPRELuc transcriptional activity (data not shown). Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means \pm S.E. of four independent experiments

decrease hepatic glucose output and reduce insulin resistance by increasing insulin-dependent peripheral glucose disposal (19). Thiazolidinediones thereby markedly decrease plasma glucose, insulin, and triglyceride levels in animal models of type II diabetes as well as in type II diabetic subjects (19). The antidiabetic effect of thiazolidinediones requires several days of treatment and does not produce overt hypoglycemia (19). Thiazolidinediones have been shown to decrease adipocyte tumor necrosis factor α /resistin secretion and circulating free fatty acid levels; to increase basal glucose uptake in 3T3-L1 adipocytes, L6 myocytes, and human muscle cultures derived from obese type II diabetic subjects; and to stimulate glucokinase gene transcription in HepG2 cells (19–28). The mechanism of action of these drugs has nevertheless remained unknown. The correlation between *in vivo* antihyperglycemic activity and *in vitro* PPAR γ activity (29) suggests that thiazolidinediones act as antidiabetic agents by regulating the transcription of a subset of genes through PPAR γ . However, the target genes involved are unclear.

It has been shown recently that high levels of PPAR γ are expressed in glucagon-producing α -cells of the endocrine pancreas (30–32). The pancreatic islet hormone glucagon is a biologic antagonist of insulin. The effects of glucagon on blood glucose levels balance those of insulin; glucagon increases hepatic glucose production and opposes hepatic glucose storage, whereas insulin increases peripheral glucose uptake and opposes glucagon-mediated hepatic glucose production. The met-

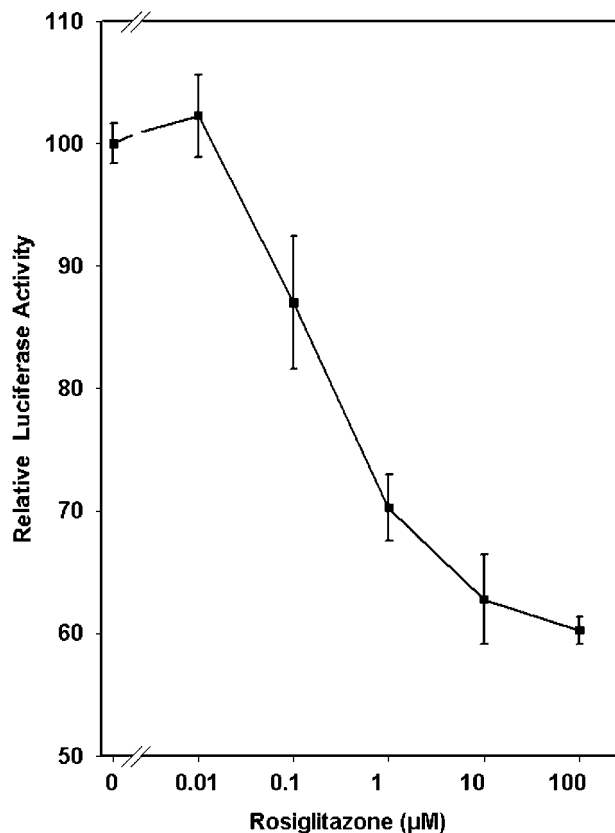


FIG. 2. Inhibition of glucagon gene transcription by rosiglitazone and PPAR γ . Plasmid -350GluLuc was transfected into InR1-G9 cells together with pPPAR γ . Rosiglitazone was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means \pm S.E. of three independent experiments, each done in duplicate.

abolic consequences of abnormal α -cell function in diabetes are well defined (33–35). In addition to hyperglycemia, insulin resistance, and impaired β -cell function, relative hyperglucagonemia is a common feature of patients with type II diabetes (33–35). The elevated glucagon levels in diabetes contribute to increased hepatic glucose output and hyperglycemia (33–35). Consequently, inhibition of glucagon secretion has been shown to reduce fasting hyperglycemia in diabetic animals (36) and patients (37, 38). Effects on the expression of glucagon in pancreatic islets are therefore important aspects in the treatment of diabetes mellitus.

Because PPAR γ is expressed in glucagon-producing α -cells but its function has been unknown, in the present study the effect of PPAR γ and thiazolidinediones on glucagon gene transcription was investigated. PPAR γ and thiazolidinediones were found to inhibit glucagon gene transcription in pancreatic islet cells. They also reduced glucagon secretion and tissue levels in pancreatic islets. Mapping studies and the use of GAL4 fusion proteins indicate that PPAR γ represses in a ligand-dependent but DNA binding-independent manner transactivation by the paired domain-containing transcription factor Pax6 leading to inhibition of glucagon gene transcription. This novel action of PPAR γ assigns a function to PPAR γ expressed in pancreatic islet α -cells and suggests that the mechanisms through which thiazolidinediones improve glycemic control in diabetic subjects may include the inhibition of glucagon gene expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmids pT81Luc (39), -350GluLuc (40), 5xGal4E1BLuc (41), -292GluLuc, -169GluLuc, -136GluLuc, -60GluLuc, -350/-48GluLuc, -350/-91GluLuc, -350/-150GluLuc, -350/

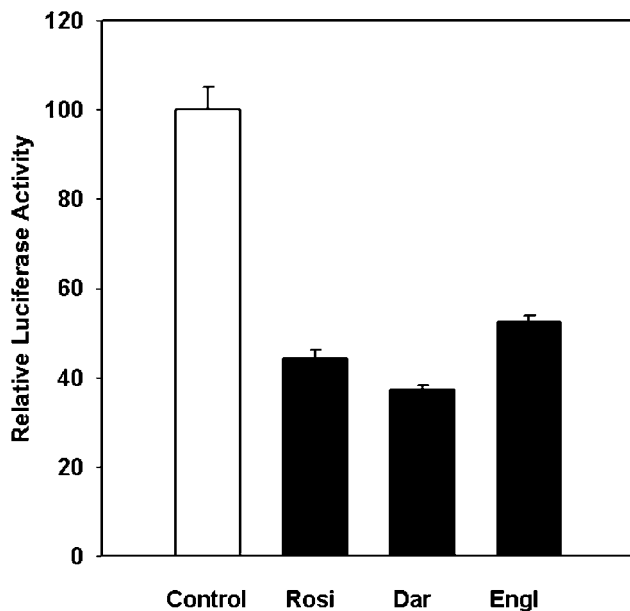


FIG. 3. Inhibition of glucagon gene transcription by the thiazolidinediones darglitazone and englitazone. InR1-G9 cells were transfected with -350GluLuc and pPPAR γ . They were treated with rosiglitazone (Rosi, 10 μ M), darglitazone (Dar, 30 μ M), or englitazone (Engl, 100 μ M) for 24 h before harvest. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the untreated controls. Values are means \pm S.E. of three experiments.

-210GluLuc, (42), -350(mutG1)GluLuc, pGAL4-Pax6 (43), PPRELuc, pPPAR γ , pRXR α (44), and pGAL4-PPAR γ (45) have been described previously. The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany).

RT-PCR—Total RNA was extracted from InR1-G9 cells using a commercial kit (RNeasy, Quiagen). For first strand cDNA synthesis, random hexamer primers (Amersham Biosciences, Inc.) were used. The RT enzyme was obtained from Invitrogen (Superscript II reverse transcriptase). For PCR amplification, the following primers were used: upstream primer, 5'-AGAGCTGACCCAATGGTTGC-3'; and downstream primer, 5'-ATCTCCGCCAACAGCTTCTC-3' (EMBL/GenBank[®]/DDBJ accession no. Z30972) (size of the expected product: 421 bp). PCR without RT step served as control for DNA contamination. After agarose gel electrophoresis, the RT-PCR product was identified by extraction, subcloning (TA-cloning kit, Promega), and cycle sequencing (Thermo Sequenase fluorescent labeled primer cycle sequencing kit, Amersham Biosciences, Inc.; IRD-800 labeled primers, MWG Biotech, Ebersberg, Germany).

Cell Culture and Transfection of DNA—The glucagon-producing pancreatic islet cell line InR1-G9 (46) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method (40) with 2 μ g of reporter gene plasmids and, when indicated, 1 μ g of expression vector per 6-cm dish. Cotransfections were carried out with a constant amount of DNA, which was maintained by adding Bluescript (Stratagene, La Jolla). In all experiments 0.5 μ g of cytomegalovirus-green fluorescent protein (GFP) (plasmid pCMV-GFPtpz) per 6-cm dish was cotransfected to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from luciferase/GFP ratios). Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. Cell extracts (40) were prepared 48 h after transfection. The luciferase assay was performed as described previously (40). Green fluorescent protein was measured in the cell extracts using the FluoroCount[™] microplate fluorometer (Packard).

Incubation of Isolated Pancreatic Islets—After the preparation of Langerhans pancreatic islets of NMRI mice (32), islets were cultured in RPMI medium supplemented with 5 mM glucose, 10% bovine serum albumin, 100 units/ml penicillin, and 100 μ g/ml streptomycin. After 48 h, glucagon levels were measured in the supernatants and the islet extracts (40) by radioimmunoassay using a commercial kit (IBL, Hamburg, Germany).

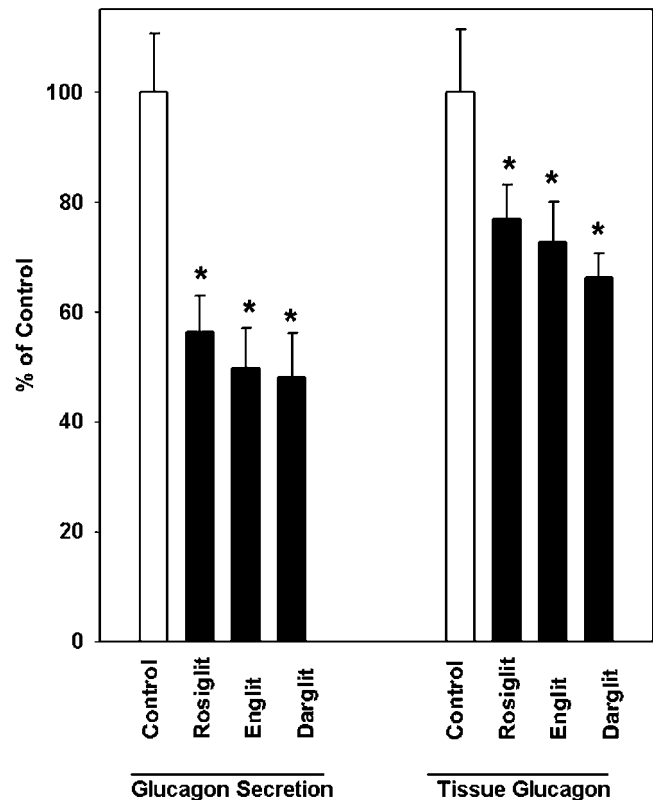


FIG. 4. Inhibition by thiazolidinediones of glucagon secretion and glucagon tissue levels in primary pancreatic islets. Isolated mouse pancreatic islets were treated with rosiglitazone (Rosiglit, 30 μ M), englitazone (Englit, 100 μ M) darglitazone (Darglit, 30 μ M), or the solvent (control) for 48 h. Glucagon secretion and tissue levels are expressed as percentage of the mean value, in each experiment, of the levels measured in the respective control. Values are means \pm S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test).

Materials—Rosiglitazone was kindly provided by SmithKline Beecham (Worthing, United Kingdom); darglitazone and englitazone (CP-72,467-02, sodium salt) was provided by Pfizer Inc. (Groton, CT). A stock solution (100 mM) was prepared in Me₂SO. Controls received the solvent only.

RESULTS

Inhibition of Glucagon Gene Transcription by PPAR γ in Response to Thiazolidinediones—PPAR γ was found by RT-PCR to be expressed in the glucagon-producing pancreatic islet cell line InR1-G9 (data not shown). In normal pancreatic islets, the expression of PPAR γ is very high, approximately two thirds of the expression level in white adipose tissue (30). In contrast, InR1-G9 cells express low levels of PPAR γ such that activation of a PPAR-dependent promoter (PPRELuc) required transfection of a PPAR γ expression plasmid (Fig. 1). This cell line therefore allowed a direct assessment of the role of PPAR γ in glucagon gene transcription. Similarly, low level expression of PPAR γ in cell lines derived from tissues with high level expression has been reported previously (6, 13). To study the effect of PPAR γ and thiazolidinediones on glucagon gene transcription, 350 base pairs of the 5'-flanking region of the rat glucagon gene were fused to the luciferase reporter gene (construct -350GluLuc) (40). This glucagon promoter fragment is sufficient to confer tissue-specific gene expression (47) and regulation of gene transcription by cAMP-, calcium-, protein kinase C-, and insulin-induced signaling pathways (40, 42, 43, 48-52). In the absence of a cotransfected PPAR γ expression plasmid, treatment of InR1-G9 cells with the thiazolidinedione rosiglitazone at concentrations up to 100 μ M had no effect on glucagon

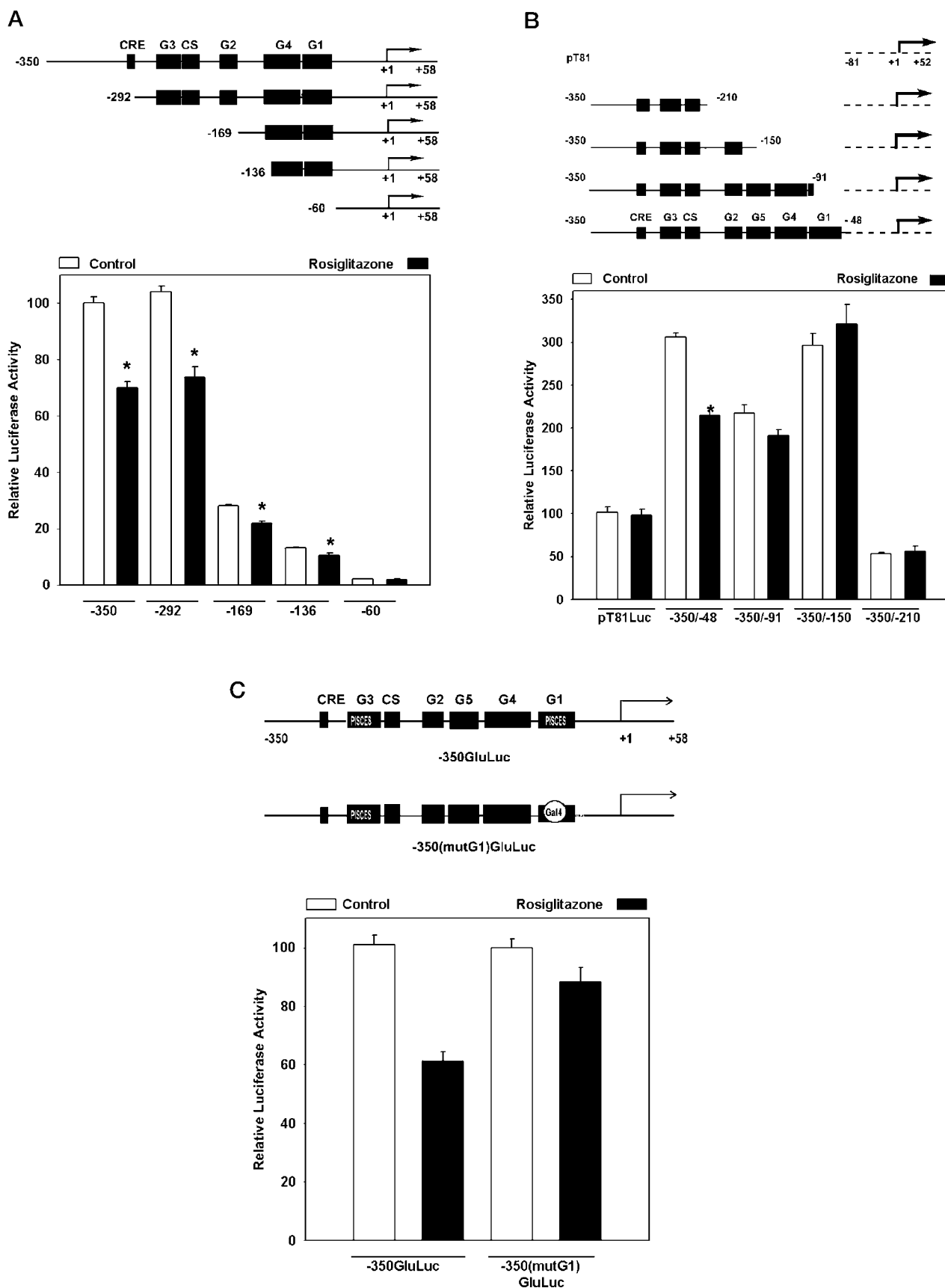


FIG. 5. Mapping of a negative PPAR γ response element in the glucagon gene promoter. *A*, 5'-deletion analysis. After cotransfection of the indicated constructs and a PPAR γ expression vector, InR1-G9 cells were treated with rosiglitazone (10 μ M) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the -350 control. Values are means \pm S.E. of three independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test). Control elements in the 5'-flanking region of the glucagon gene are indicated (see "Results" for explanation). *B*, 3'-deletion analysis. The indicated

promoter activity (data not shown). Additionally, the cotransfection of an expression plasmid encoding PPAR γ alone had no effect on -350GluLuc activity ($94 \pm 3\%$ of controls, $n = 6$). However, when a PPAR γ expression plasmid was transfected into these cells, rosiglitazone inhibited glucagon gene transcription (Fig. 2). Thus rosiglitazone inhibits glucagon gene transcription by a PPAR γ -dependent mechanism. Inhibition of glucagon gene transcription by rosiglitazone was concentration-dependent with an IC_{50} value of ~ 300 nM (Fig. 2). Cotransfection of an expression vector encoding RXR α together with PPAR γ did not alter the concentration-response curve for inhibition by rosiglitazone of -350GluLuc activity (data not shown). These concentrations of rosiglitazone are similar to those that activated a PPAR γ -dependent promoter (Fig. 1). The maximum inhibition of glucagon gene transcription by rosiglitazone was $\sim 40\%$ (Fig. 2). Like rosiglitazone, two other thiazolidinediones, darglitazone and englitazone, also inhibited glucagon gene transcription (Fig. 3). To assess the effect of thiazolidinediones in a natural context, the effect of thiazolidinediones on glucagon secretion and glucagon tissue levels was investigated in primary pancreatic islets. After 48 h of treatment with rosiglitazone, glucagon secretion from isolated pancreatic islets was inhibited by 44% (Fig. 4). Englitazone and darglitazone showed a similar inhibition (Fig. 4). Furthermore, glucagon tissue levels were significantly reduced by treatment with rosiglitazone, englitazone, or darglitazone (Fig. 4). These data indicate that PPAR γ inhibits glucagon gene transcription in response to binding of thiazolidinediones. Thiazolidinediones also reduce glucagon tissue levels and secretion in pancreatic islets.

Mapping of a Negative PPAR γ Response Element in the Glucagon Gene—PPAR γ is known to activate transcription through DR-1 motifs (1). The inhibition by PPAR γ of the glucagon reporter fusion gene -350GluLuc indicates that a negative PPAR γ response element resides within 350 base pairs of the 5'-flanking region of the glucagon gene (Figs. 2 and 3). This fragment of the glucagon gene contains the enhancer-like element G2 and G3 as well as a cAMP response element (53). The truncated glucagon gene promoter (136 base pairs) containing the proximal promoter elements G1 and G4 exhibits low transcriptional activity but is essential for proper enhancer function (53). To localize more precisely the *cis*-acting DNA sequences of the glucagon gene that mediate transcriptional repression by PPAR γ , a 5'/3'-deletion, and internal mutation analysis was performed.

Expression of 5'-deleted mutant plasmids in InR1-G9 cells revealed that the repression by PPAR γ in response to rosiglitazone was unimpaired when the 5' end was shortened from -350 to -169 (Fig. 5A). It was, if at all, only slightly diminished when the 5' end was shortened to -136 (Fig. 5A). However, truncation to -60 abolished the repression by PPAR γ (Fig. 5A). These results indicate that a DNA control element required for repression by PPAR γ may have its 5' boundary and reside between -136 and -60 .

The results of the 3'-deletion analysis are shown in Fig. 5B. Fragments of the glucagon promoter with deletions at their 3' ends were linked to the truncated thymidine kinase promoter (-81 to $+52$) of herpes simplex virus (pT81Luc). This promoter does not respond to PPAR γ and rosiglitazone (Fig. 5B). The

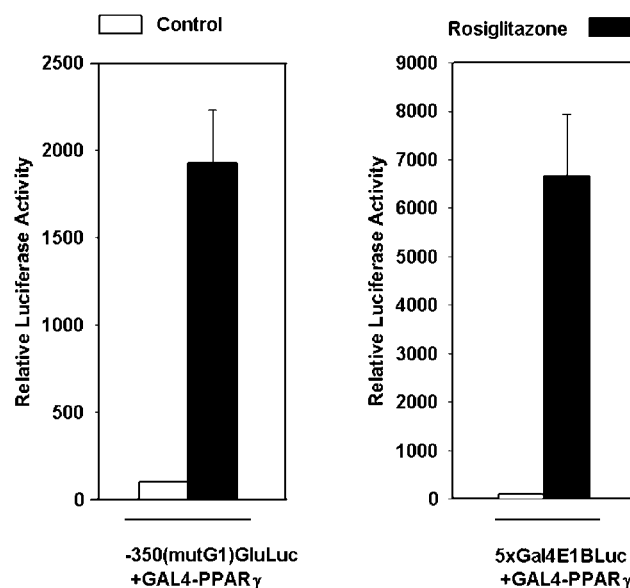


FIG. 6. GAL4-PPAR γ stimulates transcription, when anchored to the glucagon promoter. An expression vector encoding GAL4-PPAR γ was transfected into InR1-G9 cells together with $-350(\text{mutG1})\text{GluLuc}$ or $5x\text{Gal4E1BLuc}$ reporter gene. Cells were treated with rosiglitazone ($10 \mu\text{M}$) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means \pm S.E. of four experiments.

glucagon gene 5'-flanking region from -350 to -48 conferred repression by PPAR γ (Fig. 5B). When only sequences from -350 to -91 were fused to the viral promoter, PPAR γ in response to rosiglitazone no longer inhibited gene transcription (Fig. 5B). This deletion eliminates the G1 element (Fig. 5B). Further deletion to -150 or -210 had no effect (Fig. 5B). These data suggest that a DNA control element required for repression by PPAR γ may have its 3' boundary and reside between -48 and -91 .

Taken together, the results of the 5'- and 3'-deletion analysis suggest that a DNA control element conferring PPAR γ repression to the glucagon gene may be located between -136 and -48 . This region contains the G1 element (Fig. 5, A and B). The G1 element contains a PISCES motif that is essential for promoter function (43, 47, 54, 55). To examine the role of the PISCES motif within G1 in the repression of glucagon gene transcription by PPAR γ , the PISCES motif in G1 was mutated (and thereby changed into a binding site of the yeast transcription factor GAL4) (Fig. 5C). The mutation of the PISCES motif within G1 decreased basal activity to low but detectable levels ($2.5 \pm 0.3\%$ of wild type, $n = 6$) and almost abolished the repression of transcription by PPAR γ in response to rosiglitazone (Fig. 5C). These results confirm that the PISCES motif within G1 is important for basal glucagon promoter activity. Although interpretation is difficult in view of the change in basal promoter activity, these results furthermore provide evidence that the PISCES motif within G1 is required for repression of glucagon gene transcription by PPAR γ .

constructs were transfected into InR1-G9 cells together with a PPAR γ expression vector. Cells were treated with rosiglitazone ($10 \mu\text{M}$) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the pT81 control. Values are means \pm S.E. of three independent experiments, each done in duplicate. *, $p < 0.005$ (Student's *t* test). C, internal mutation. Pax6 binds to PISCES motifs within G1 and G3. Bases including the PISCES motif within G1 were mutated into a GAL4 binding site. Plasmids -350GluLuc or $-350(\text{mutG1})\text{GluLuc}$ were transfected into InR1-G9 cells together with pPPAR γ . Cells were treated with rosiglitazone ($10 \mu\text{M}$) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means \pm S.E. of three independent experiments, each done in duplicate.

Inhibition of Pax6 Transcriptional Activity by PPAR γ in Response to Thiazolidinediones—The 5'/3'-deletion and internal mutation studies suggest that a proximal promoter fragment containing the G1 element and the PISCES motif is required for inhibition of glucagon gene transcription by PPAR γ in response to thiazolidinediones. To examine the function of PPAR γ when forced to bind to this promoter region, a GAL4-PPAR γ fusion protein was used (45). This GAL4-PPAR γ fusion protein was transfected into InR1-G9 cells together with -350(mutG1)GluLuc, in which the PISCES motif within G1 has been mutated into a binding site of GAL4. After cotransfection of GAL4-PPAR γ , rosiglitazone markedly stimulated -350(mutG1)GluLuc activity (Fig. 6, *left panel*), similar to the stimulation by rosiglitazone of a luciferase reporter gene placed under the control of multiple GAL4 DNA binding sites linked to a minimal viral E1B promoter (5xGal4E1BLuc) (Fig. 6, *right panel*). This enhancement of -350(mutG1)GluLuc transcriptional activity (Fig. 6, *left panel*) is in contrast to the inhibition of -350GluLuc transcriptional activity by PPAR γ in response to rosiglitazone (Figs. 2 and 3). These results therefore suggest that the ligand-dependent transcription control domain of PPAR γ stimulates transcription when anchored to the glucagon proximal promoter.

The PISCES motif has been shown to bind the paired-domain transcription factor Pax6 (55, 56). The observation that mutating the PISCES motif within G1 abolished the repression of glucagon gene transcription by ligand-activated PPAR γ (Fig. 5C) thus raises the possibility that PPAR γ may target Pax6 to inhibit glucagon gene transcription. However, because of potentially overlapping binding sites, the mutation of the PISCES motif within G1 may not only abolish Pax6 binding but also affect the binding of additional transcription factors like *cdx2/3* and *brain-4* (57–59). We therefore examined whether repression of the glucagon promoter by PPAR γ can be restored by Pax6 recruited to the mutant glucagon promoter through the GAL4 binding site. When 1 μ g of an expression vector encoding a GAL4-Pax6 fusion protein (43) per dish was transfected together with -350(mutG1)GluLuc, basal transcriptional activity of the mutant glucagon promoter was raised to a level similar to that of the wild-type promoter (Fig. 7A). The expression of GAL4-Pax6 also conferred repression by PPAR γ in response to rosiglitazone (Fig. 7A). After expression of GAL4-Pax6, PPAR γ inhibited the transcriptional activity of the mutated glucagon promoter in response to rosiglitazone by ~40% (Fig. 7A); this is similar to the inhibition by ligand-activated PPAR γ of wild-type glucagon promoter activity. This effect of GAL4-Pax6 seems to be specific and also not secondary to the restoration of basal activity, because the expression of GAL4-VP16 also elevated basal activity of the mutated glucagon promoter but did not confer PPAR γ responsiveness (7A). Likewise, when cotransfected with a reporter construct, in which multiple GAL4 binding sites had been placed in front of the truncated viral E1B promoter (5xGal4E1BLuc), GAL4-Pax6 transcriptional activity was inhibited by PPAR γ upon treatment of the cells with rosiglitazone (Fig. 7B, *left panel*). This effect was again specific because PPAR γ plus rosiglitazone had no effect on the transcriptional activity conferred by GAL4-VP16 to 5xGal4E1BLuc (Fig. 7B, *right panel*). This indicates that inhibition of Pax6 transcriptional activity by PPAR γ in response to thiazolidinediones does not depend on the glucagon promoter context. These results thus suggest that PPAR γ in response to thiazolidinediones inhibits Pax6 transcriptional activity and thereby reduces glucagon gene transcription.

DISCUSSION

PPAR γ has been shown to be highly expressed in normal human glucagon-producing pancreatic islet α -cells (30), al-

though its function has been unknown. The present study now demonstrates that PPAR γ inhibits glucagon gene transcription in glucagon-producing pancreatic islet cells. This was followed by a decrease in glucagon tissue levels and secretion. This study thereby assigns a function to pancreatic PPAR γ receptors, further supporting a role of PPAR γ in glucose homeostasis. This action of PPAR γ is ligand-dependent, because it was observed only upon adding the thiazolidinedione PPAR γ ligands rosiglitazone, darglitazone, and englitazone. These synthetic compounds mimic the effect of endogenous PPAR γ ligands like fatty acids and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (16). The fact that rosiglitazone inhibited glucagon gene transcription over the same range of concentrations as it stimulated through PPAR γ the expression of a reporter gene directed by multiple PPAR γ DNA binding sites (IC₅₀ and EC₅₀ values of ~300 nM and 1 μ M, respectively) suggests that inhibition of glucagon gene transcription may accompany other PPAR γ -mediated effects. This effect may thus be therapeutically relevant for the action of thiazolidinediones. The IC₅₀ value of rosiglitazone for inhibition of glucagon gene transcription (~300 nM) is also similar to the reported affinity of rosiglitazone for PPAR γ binding (*K_i* 214 nM) (60).

It is now well established that, after several weeks of treatment of type II diabetic patients, thiazolidinediones diminish insulin resistance and reduce hepatic glucose production rates, resulting in lowering of both fasting and postprandial blood glucose as well as insulin levels (19, 61). The fasting plasma glucagon concentrations were not significantly changed by troglitazone treatment of type II diabetic patients (61). However, the fact that plasma glucagon concentrations were maintained despite decreased plasma glucose and insulin levels (61), which should disinhibit and, thus, enhance glucagon secretion (33–35), suggests that thiazolidinedione treatment imposed an inhibition on glucagon secretion. Indeed, troglitazone inhibited the glucagon response to a meal tolerance test in type II diabetic patients (61). By demonstrating an inhibition of glucagon gene transcription and expression by thiazolidinediones, the present study shows a novel action of PPAR γ and this class of antidiabetic agents. It offers a mechanism through which thiazolidinedione treatment of type II diabetic subjects could lead to decreased glucagon expression and secretion, thereby preventing a glucagon-induced increase in hepatic glucose output. The present study therefore suggests that inhibition of glucagon gene expression by thiazolidinediones could be part of the mechanisms through which these antidiabetic agents improve glycemic control in diabetic patients.

PPAR γ stimulates gene transcription by binding as an RXR heterodimer to DR-1-like DNA response elements, ligand binding, and coactivator recruitment (see Introduction). The glucagon gene provides an additional example that PPAR γ can also inhibit gene transcription (6–14). Several mechanisms have been described for negative regulation of gene transcription by nuclear receptors. The thyroid hormone receptor harbors ligand-independent repressor function and actively represses transcription upon binding to cognate sites within the promoter region of target genes. These active repressive function requires the recruitment of corepressor complexes that are dismissed upon ligand binding (62). PPAR γ inhibits the glucagon gene by a clearly distinct mechanism, because this inhibition is ligand-dependent and does not appear to involve direct binding to the glucagon promoter (see below). The mechanism may thus be more related to the ones best established for the glucocorticoid receptor, which in many cases mediates transrepression in a DNA binding-independent manner (63–67). Similarly, inhibition of inducible nitric-oxide synthase gene transcription by PPAR γ has been proposed to be achieved at least partially by

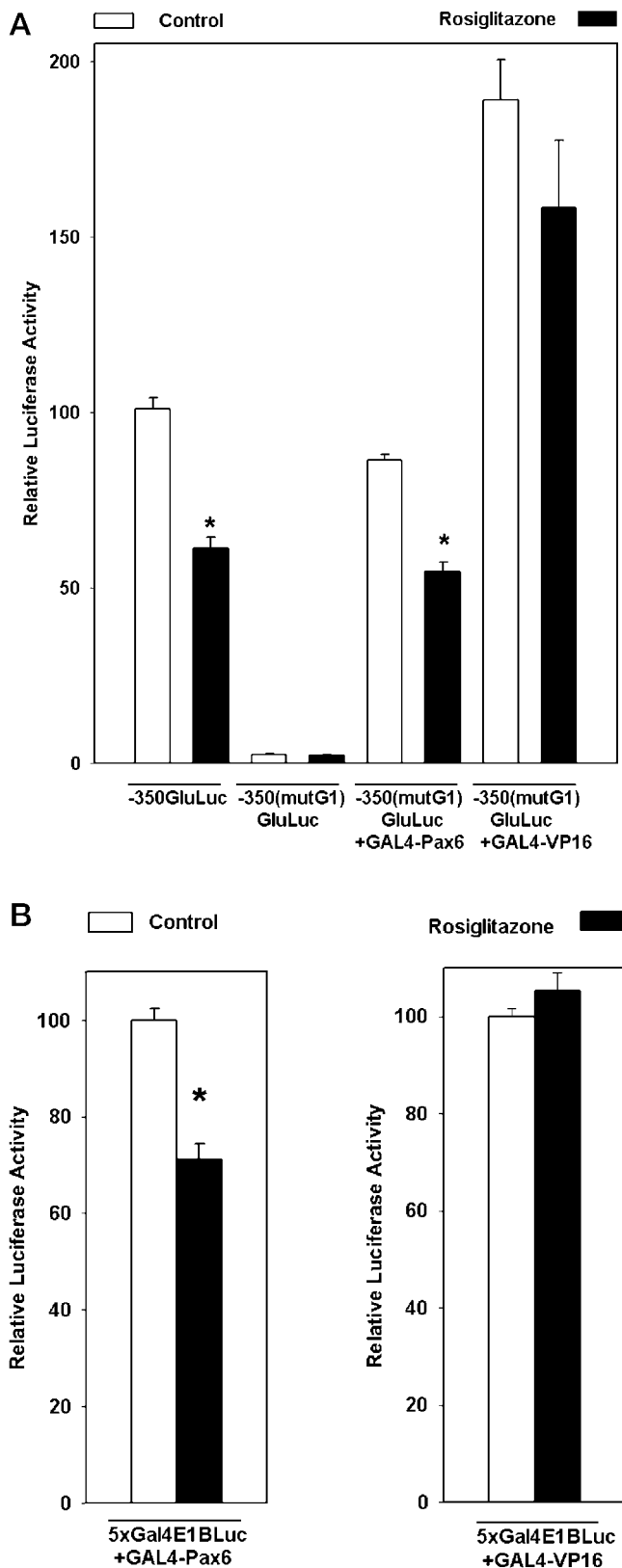


Fig. 7. Inhibition of Pax6 transcriptional activity by PPAR γ in response to rosiglitazone. A, Pax6 restores basal activity and confers PPAR γ responsiveness to the mutant glucagon promoter. Expression vectors encoding PPAR γ and GAL4-Pax6 or GAL4-VP16 were transfected into InR1-G9 cells together with -350(mutG1)GluLuc. Cells were treated with rosiglitazone (10 μ M) or solvent (control) for 24 h before harvest. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the -350 control. Values are means \pm S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test). B, Pax6 confers

antagonizing the activities of STAT1, NF- κ B, and AP-1 without binding of PPAR γ to the promoter (6, 7). By 5'/3'-deletion and internal mutation analysis as well as by using GAL4 fusion proteins, the present study provides evidence that PPAR γ inhibits glucagon gene transcription by inhibition of Pax6 transcriptional activity. These results thus define Pax6 as a novel functional target of PPAR γ .

Pax6 is a member of the paired box-containing genes that play important roles during development (68). Pax6 is expressed early in pancreas development defining endocrine cell lineages. Inactivation of Pax6 results in the absence of glucagon-producing α cells (69). Pax6 is expressed also in adult islets (55, 56, 70). It binds and strongly activates the glucagon promoter (43, 54–56, 71, 72). Mutational analyses further support the view that Pax6 is essential for activation of the glucagon gene in pancreatic islet cells (53). The inhibition by PPAR γ of Pax6 transcriptional activity thus appears to be sufficient to explain the inhibition of glucagon gene transcription by PPAR γ in response to thiazolidinediones. The glucagon promoter contains two Pax6 binding sites, a PISCES motif within the enhancer-like element G3 and another PISCES motif in the proximal promoter element G1 (53, 54, 71). The mapping experiments of the present study suggest that PPAR γ responsiveness is conferred by Pax6 at the proximal promoter site, indicating that Pax6 may function differently at the two binding sites.

The present study suggests that PPAR γ inhibits glucagon gene transcription through inhibition of transactivation by Pax6. Previous reports have shown that repression of gene transcription by PPAR γ can involve a change in transcription factor binding. Thus, PPAR γ activators were found to induce ATF3 in human vascular endothelial cells, which bound to and repressed E-selectin gene expression (9). Furthermore, inhibition of NFAT and NRF2 DNA binding was found when PPAR γ agonists inhibited interleukin-2 gene transcription in T-cells and thromboxane synthase gene transcription in macrophages, respectively (13, 14). In contrast, the results of the present study indicate that inhibition of glucagon gene transcription by PPAR γ does not depend on inhibition of Pax6 DNA binding but instead involves the inhibition of transactivation by Pax6, produced by PPAR γ in a ligand-dependent manner without binding of PPAR γ to the glucagon promoter. First, PPAR γ stimulated glucagon promoter activity in response to thiazolidinediones when fused to the GAL4 DNA binding domain and anchored to the proximal promoter element G1. Second, PPAR γ inhibited Pax6 transcriptional activity when Pax6 bound through a heterologous DNA-binding domain (GAL4-Pax6) to the G1 element of the glucagon gene (-350(mutG1)GluLuc) or to multiple binding sites in front of a minimal viral promoter (5xGal4E1BLuc). The mechanism of transcriptional repression of Pax6 by PPAR γ remains to be defined but could include direct binding of PPAR γ to Pax6 or protein-protein interactions of PPAR γ with coactivators or signaling molecules. The glucocorticoid receptor was found to bind directly to transcription factors like AP-1 and NF- κ B (63–67). On the other hand, single amino acid mutations in PPAR γ that abolished ligand-dependent interactions with SRC-1 and CBP also abolished transre-

transcriptional activity and PPAR γ responsiveness to a minimal viral promoter. Expression vectors encoding PPAR γ and GAL4-Pax6 or GAL4-VP16 were transfected into InR1-G9 cells together with 5xGal4E1BLuc reporter gene. Cells were treated with rosiglitazone (10 μ M) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means \pm S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test).

pression by PPAR γ of the inducible nitric-oxide synthase gene (7). This suggests that, in this case transrepression by PPAR γ may involve competition for limiting amounts of the general coactivators CBP and p300, which is achieved by targeting CBP through direct interaction with its NH₂-terminal domain and via SRC-1-like bridge factors (7).

When taken together, the results of the present study define Pax6 as a novel functional target of PPAR γ . Our results are consistent with a model in which PPAR γ in a ligand-dependent but DNA binding-independent manner inhibits transactivation by Pax6 leading to inhibition of glucagon gene transcription in pancreatic islet cells. This function of PPAR γ further supports the role of PPAR γ in glucose homeostasis and suggests that inhibition of glucagon gene expression and secretion could be part of the mechanisms through which thiazolidinediones like rosiglitazone improve glycemic control in diabetic subjects.

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