

MOL#35568

1

Title page

A Peroxisome Proliferator-activated Receptor γ – Retinoid X Receptor Heterodimer Physically
Interacts with the Transcriptional Activator PAX6 to Inhibit Glucagon Gene Transcription*

Ralph Krätzner, Florian Fröhlich, Katrin Lepler, Michaela Schröder, Katharina Röher, Corinna Dickel,
Mladen V. Tzvetkov, Thomas Quentin, Elke Oetjen and Willhart Knepel

Molecular Pharmacology, Clinical Pharmacology (MVT) and Pediatric Cardiology and Intensive Care
Medicine (TQ), University of Göttingen, D-37099 Göttingen, Germany.

Runnig title page

a) PPAR γ inhibits PAX6 by physical protein-protein interaction

b) Elke Oetjen, MD

Molecular Pharmacology

University of Göttingen

37099 Göttingen

Germany

Telephone: + 49 551-395771

Fax: + 49 551-395699

E-mail: eoetjen@med.uni-goettingen.de

c) number of text pages: 18

number of tables: 0

number of figures: 5

number of references: 40

number of words in *Abstract*: 238

number of words in *Introduction*: 729

number of words in *Discussion*: 1377

d) abbreviations: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; RXR, retinoid X receptor; PISCES, pancreatic islet cell-specific enhancer sequence; GFP, green fluorescent protein; GST, glutathione S-transferase; SV40, simian virus 40; PTEN, phosphatase and tensin homolog deleted from chromosome 10;

Abstract

The peptide hormone glucagon stimulates hepatic glucose output and its levels in the blood are elevated in type 2 diabetes mellitus. The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) has essential roles in glucose homeostasis and thiazolidinedione PPAR γ agonists are clinically important antidiabetic drugs. As part of their antidiabetic effect, thiazolidinediones like rosiglitazone have been shown to inhibit glucagon gene transcription through binding to PPAR γ and inhibition of the transcriptional activity of PAX6 that is required for cell-specific activation of the glucagon gene. However, how thiazolidinediones and PPAR γ inhibit PAX6 activity at the glucagon promoter remained unknown. After transient transfection of a glucagon promoter-reporter fusion gene into a glucagon-producing pancreatic islet α -cell line, ligand-bound PPAR γ was found in the present study to inhibit glucagon gene transcription also after deletion of its DNA-binding domain. Like PPAR γ ligands, also retinoid X receptor (RXR) agonists inhibited glucagon gene transcription in a PPAR γ -dependent manner. In glutathione *S*-transferase pull-down assays, the ligand-bound PPAR γ -RXR heterodimer bound to the transactivation domain of PAX6. This interaction depended on the presence of the ligand and RXR, but was independent of the PPAR γ DNA-binding domain. Chromatin immunoprecipitation experiments showed that PPAR γ is recruited to the PAX6-binding proximal glucagon promoter. When taken together, the results of the present study support a model in which a ligand-bound PPAR γ -RXR heterodimer physically interacts with promoter-bound PAX6 to inhibit glucagon gene transcription. These data define PAX6 as a novel physical target of PPAR γ -RXR.

Peroxisome proliferator-activated receptor γ (PPAR γ) belongs to the superfamily of ligand-regulated nuclear hormone receptors (Desvergne and Wahli, 1999). It can be structurally subdivided into an amino-terminal, ligand-independent transactivation domain (AF-1) followed by a DNA-binding domain and a carboxyl-terminal, ligand-binding domain that contains a second, ligand-dependent transactivation surface (AF-2) (Desvergne and Wahli, 1999). PPAR γ binds as a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) to response elements (PPREs) in target genes to activate transcription. Upon ligand binding and depending on the tissue specific cofactor environment the PPAR γ -RXR heterodimer recruits coactivators to stimulate promoter activity. This recruitment is dependent on ligand-induced allosteric alterations in the AF-2 helical domain (Nolte et al., 1998). In addition to transcriptional stimulation, PPAR γ has been shown to be also capable of repression of gene transcription (Ricote et al., 1998; Schinner et al., 2002)

PPAR γ is thought to be involved in a broad-range of cellular functions, including adipocyte differentiation, inflammation, blood pressure regulation, apoptosis, and in chronic diseases such as obesity, atherosclerosis, metabolic syndrome and cancer (Kersten et al., 2000). Of particular importance is its role in glucose homeostasis and type 2 diabetes mellitus (Kersten et al., 2000; Semple et al., 2006). Dominant negative mutations in PPAR γ are associated with insulin resistance and diabetes mellitus (Semple et al., 2006) suggesting that PPAR γ agonists may be useful in the treatment of type 2 diabetes mellitus.

Besides naturally existing ligands like 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, a number of synthetic ligands of PPAR γ as a new class of antidiabetic drugs have been synthesized including the thiazolidinediones rosiglitazone and pioglitazone (Lehmann et al., 1995; Ikeda et al., 1990) In type 2 diabetes mellitus, thiazolidinediones lower blood glucose levels by binding to PPAR γ leading to a reduction in hepatic glucose output and a decrease in insulin resistance. PPAR γ is expressed also in the α -cells of the pancreatic islets (Dubois et al., 2000) and we recently identified the glucagon gene as a novel thiazolidinedione target gene (Schinner et al., 2002).

The peptide hormone glucagon is synthesized in the α -cell of the endocrine pancreas (Unger and Orci, 1981). Glucagon stimulates glycogenolysis and gluconeogenesis, and thereby increases hepatic glucose output. These actions of glucagon are opposite to those of insulin, the peptide hormone from pancreatic islet β -cells. Important in the coordination of the secretion of these two hormones is the direct inhibition by insulin of glucagon synthesis and secretion (Unger and Orci, 1981; Grzeskowiak et al., 2000). In insulin resistant or deficient states, glucagon synthesis and secretion becomes disinhibited leading to hyperglucagonemia that contributes to hyperglycemia in type 2 diabetes mellitus (Unger and Orci, 1981). Thiazolidinediones/PPAR γ were shown to inhibit glucagon gene transcription and secretion (Schinner et al., 2002). This suggests that inhibition of glucagon gene expression may be among the multiple mechanisms through which thiazolidinediones improve glycemic control in diabetic subjects.

The PISCES sequence motif within the glucagon gene promoter element G1 is required for PPAR γ responsiveness (Schinner et al., 2002). This sequence motif does not bind PPAR γ but rather binds the transcription factor PAX6 (Grzeskowiak et al., 2000; St-Onge et al., 1997), which is required for α cell-specific activation of the glucagon gene (Grzeskowiak et al., 2000). PAX6 is composed of an amino terminal paired domain followed by a linker region, a homeodomain and a carboxyl-terminal transactivation domain. The PAX6 transactivation domain recruits cofactors like CBP/p300 and mediates the interaction with the general transcription machinery (Hussain and Habener, 1999). When the PISCES motif within G1 was mutated into a GAL4 binding site, the expression of GAL4-PAX6 restored glucagon promoter activity and thiazolidinedione/PPAR γ responsiveness (Schinner et al., 2002), suggesting that PPAR γ in a ligand-dependent but DNA binding-independent manner inhibits PAX6 transcriptional activity on glucagon gene transcription. However, how PPAR γ inhibits PAX6 transcriptional activity in molecular details remained unknown. The results of the present study suggest that thiazolidinediones and PPAR γ inhibit glucagon gene transcription through a mechanism that involves a direct interaction of PPAR γ with the PAX6 transactivation domain. In contrast to the glucocorticoid receptor, where transrepression is apparently mediated by glucocorticoid receptor

monomers (Reichardt et al., 1998), transrepression of PAX6 seems to be conferred by PPAR γ -RXR heterodimers. These data define PAX6 as a novel physical target of PPAR γ -RXR, which may have implications beyond the regulation of glucagon gene transcription and blood glucose control.

Materials and Methods

Plasmids. The plasmids -350GluLuc, pcDNA3-PPAR γ (Schinner et al., 2002), pGEX2T (GE Healthcare, Munich, Germany), pGST-PAX6(299-437) (Mikkola et al., 1999), and PPRE-Luc (Schinner et al., 2002) have been described previously. The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany). The expression vector pcDNA3-RXR α was generated by *EcoRI* digestion of pSG5-RXR α (Heinlein et al., 1999) and cloning of the insert into *EcoRI* digested pcDNA3. The plasmid pcDNA3-PPAR γ was used as PCR template for the generation of all described PPAR γ variants. PCR fragments coding for the described PPAR γ variants were amplified using primer pairs introducing a 5' *KpnI* and a 3' *XbaI* site for cloning into pcDNA3. All constructs were confirmed by sequencing.

In Vitro Transcription/Translation. The TNT T7-coupled reticulocyte lysate system (L4610) was purchased from Promega (Mannheim, Germany). The reactions were carried out as recommended by the manufacturer.

GST Pull-down Assay. The GST-PAX6(299-437) fusion protein and GST were expressed in *E. coli* BL 21 and extracted using glutathione-agarose beads (Sigma-Aldrich, Munich, Germany). The proteins were stored bound to agarose beads on ice in PBS buffer containing 1mM DTT and 1mM PMSF. To equalize amounts of GST-PAX6(299-437) and GST used in GST-pull down assays the amounts were estimated by Coomassie Blue staining after SDS-PAGE. For the GST-pull down assay the protein-covered beads were washed with NETN-buffer (20mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, Nonidet-P40 0,5 % (v/v)) containing 1 mM DTT and 1mM PMSF. For the binding step 30 μ l of GST-PAX6(299-437) or GST beads were added to 250 μ l NETN buffer and incubated with 7.5 μ l of radioactively labelled *in vitro* transcribed/translated protein at 4 °C overnight. The beads were washed three times with 500 μ l ice cold NETN buffer, after the last washing step the supernatant was carefully removed and the pellet was boiled in 30 μ l 2x Laemmli sample buffer for 10 min. After SDS-PAGE, the radioactively labelled proteins were visualized using a phosphorimager. Densitometry

of band intensities was done using the program TINA v 2.09g (Santa Cruz Biotechnology, Heidelberg, Germany).

Cell Culture and Transfection of DNA. The glucagon-producing pancreatic islet α -cell line InR1-G9 (Schinner et al., 2002) 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 (Schinner et al., 2002) 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. Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. Cell extracts (Schinner et al., 2002) were prepared 48 h after transfection. The luciferase assay was performed as described previously (Schinner et al., 2002). Green fluorescent protein was measured in the cell extracts using the FluoroCountTM microplate fluorometer (Packard).

Chromatin Immunoprecipitation (ChIP) and quantitative PCR. Two 16-cm dishes of confluent InR1-G9 cells transiently transfected with PPAR γ -HA were used for one ChIP experiment. For crosslinking, formaldehyde at a final concentration of 1 % was added; after 10 min at room temperature the reaction was stopped by adding glycine at a final concentration of 125 mM. After two washing steps in PBS buffer the cells were lysed on ice in 600 μ l lysis buffer (50 mM Tris/HCl pH 8.1, 10 mM EDTA, 1 % SDS (v/v), 1 mM of each inhibitor: PMSF, leupeptin, aprotinin, pepstatin) for 10 min. DNA was sonicated using 3 sets of 15 pulses at medium amplitude. After sonication lysates were precleared for 30 min at 4 °C with 50 μ l of equilibrated proteinA/G agarose (Santa Cruz, Heidelberg, Germany). 10 μ g of mouse anti-HA antibody (Sigma-Aldrich, Munich, Germany) or IgG were added to 250 μ l of the supernatant. The reaction was incubated overnight at 4 °C. 60 μ l of proteinA/G agarose slurry were added and samples were incubated on a rotor for 2 h at 4 °C. The precipitated complexes were washed twice in washing buffer 1 (20 mM Tris/HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1 % SDS (v/v), 1 % Triton-X (v/v)), once in washing buffer 2 (20 mM Tris/HCl

pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1 % SDS (v/v), 1 % Triton-X (v/v)), washing buffer 3 (20 mM Tris/HCl pH 8.1, 250 mM LiCl, 1 mM EDTA, 1 % NP40 (v/v), 1 % deoxycholate) and in TE buffer. The elution of DNA/protein complexes was performed in 10 mM Tris/HCl pH 8.1, 1mM EDTA, 1 % SDS. The crosslinks of DNA and protein were reversed by the addition of NaCl at a final concentration of 370 mM and overnight incubation at 65 °C. After proteinase K treatment DNA was extracted using a PCR purification kit (Qiagen, Hilden, Germany). The PCR was performed using a primer pair (hGlu-for 5'-CTGCCCTTT CCATTCCCAAAC-3', hGlu-rev 5'-TTCTGCACCAGGGTGCCGTGC-3') flanking the PAX6 binding site of the hamster glucagon gene promoter G1 element (annealing temperature 57°C, 25 cycles). Quantitative (real-time) PCR was performed using SYBRGreen qPCR Core Kit (Eurogentec, Seraing, Belgium) following the manufacturer's instructions and primers 5'-CTG CCC TTT CCA TTC CCA AAC-3' and 5'-TTC TGC ACC AGG GTG CCG TGC-3'. The reaction was carried out in a 7900HT (Applied Biosystems, Weiterstadt, Germany) real-time PCR machine under the following conditions: 95°C for 10 minutes, 40 cycles of 94 for 15 seconds, 62°C for 15 seconds, and 72°C for 1 minute. The samples were measured in triplicates and the average of the three threshold cycles (Ct) were used to calculate the relative amounts of template using the Δ Ct method.

RNA isolation, RT-PCR and mRNA quantification. RNA was extracted from InR1-G9 cells using Trizol reagent (SIGMA, Taufkirchen, Germany) according to standard procedures. RNA-phase was purified using RNeasy kit columns (Quiagen, Hilden, Germany), and 1 μ g of total RNA was transcribed into cDNA using random hexamer primers and Superscript II reverse transcriptase according to the manufacturer's protocol (Invitrogen). The mRNA expression was quantified by iQ Sybr-Green Supermix in an iQ-thermocycler (Biorad Laboratories, Munich, Germany). For each reaction 3 ng of cDNA and the primers 5'-GCCCAAGATTTTGTGCAGTG-3' as sense and 5'-CAATGAATTCCTTTGCTGCC-3' as anti-sense for glucagon gene amplification and 5'-CTCATGACCACAGTCCATGC-3' as sense and 5'-CGACATGTGAGATCCACGAC-3' as anti-sense primer in a final concentration of 0.2pM, each, for the amplification of the GAPDH gene were used. An initial Taq-polymerase activating step at 95°C for 15 minutes was followed by 30 seconds at

95°C, 30 seconds at 58°C, and 30 seconds at 72°C. After each run a melting curve analysis was performed to ensure amplification of correct products. The mRNA expression of glucagon was normalized to the mRNA expression of GAPDH.

Materials. BMS 649 was kindly provided by Hinrich Gronemeyer, IGBMC, Strasbourg, France. Rosiglitazone was kindly provided by SmithKline Beecham (Worthing, United Kingdom). Luciferin was purchased from Promega (Mannheim, Germany) and 9-*cis*-retinoic acid and GW9662 were from Sigma (Taufkirchen, Germany), respectively.

Results

PPAR γ Inhibits Glucagon Gene Transcription Independent of Its DNA-binding Domain.

Glucagon gene transcription was studied in the pancreatic islet α -cell line InR1-G9. Primary pancreatic islet α -cells express high levels of PPAR γ (Dubois et al., 2000) and PPAR γ agonists have been shown to inhibit glucagon synthesis and secretion in primary islets (Schinner et al., 2002). In contrast, InR1-G9 cells express low levels of PPAR γ such that activation of a PPRE-linked reporter gene as well as inhibition of glucagon gene transcription by PPAR γ agonists requires transfection of a PPAR γ expression plasmid (Schinner et al., 2002). Furthermore, in this cell line in the presence of overexpressed PPAR γ treatment with 50 μ M rosiglitazone decreased the mRNA level of endogenous glucagon by 37 % \pm 11.4 ($P < 0.05$; n=4) (data not shown). This cell line therefore allows a direct assessment of the role of PPAR γ and PPAR γ variants in glucagon gene transcription. Glucagon gene transcription was studied in InR1-G9 cells using the reporter gene construct -350GluLuc (Schinner et al., 2002), which contains the rat glucagon gene promoter from -350 to +58 fused to the luciferase reporter gene. This glucagon promoter fragment is sufficient to confer tissue-specific gene expression and regulation of gene transcription by cAMP-, calcium-, protein kinase C-, and insulin-induced signaling pathways (Unger and Orci, 1981; Schwaninger et al. 1993a,b; Fürstenau et al., 1997; Grzeskowiak et al., 2000; Oetjen et al., 1994; Knepel et al., 1990). It also confers responsiveness to thiazolidinediones and PPAR γ (Schinner et al., 2002). To examine which domain of PPAR γ is involved in the repression by PPAR γ of glucagon gene transcription, we constructed the PPAR γ variant PPAR γ -(175-475)N. This variant lacks the DNA-binding domain and carries a carboxyl-terminal extension of eight amino acids representing the nuclear localization sequence of the SV40 large T antigen (PKKKRKVE) (Kalderon et al., 1984). To evaluate the effect of this carboxyl-terminal nuclear localization signal on the inhibition of glucagon gene transcription by thiazolidinediones and PPAR γ , we fused that sequence also to PPAR γ full-length (construct PPAR γ -(1-475)N). All PPAR γ variants were cotransfected with -350GluLuc into InR1-G9 cells. After treatment with rosiglitazone, all PPAR γ variants inhibited glucagon gene transcription by 50-70 % (Fig. 1). These data show that the ligand binding domain of PPAR γ is sufficient to inhibit glucagon gene transcription. These results

thus support the previous conclusion that PPAR γ does not bind to the glucagon promoter to inhibit glucagon gene transcription (Schinner et al., 2002), and show furthermore that transrepression by PPAR γ is also independent of its DNA-binding domain.

RXR Agonists Inhibit Glucagon Gene Transcription in a PPAR γ -dependent Manner. PPAR γ activates gene transcription by binding as a heterodimer with RXR to PPREs in the promoter region of target genes (Desvergne and Wahli, 1999). PPAR γ -RXR is a permissive heterodimer, the transcriptional activity of which can be activated by both, PPAR γ agonists and RXR agonists (Kliwer et al., 1992). To examine whether RXR is involved in the repression of glucagon gene transcription by thiazolidinediones and PPAR γ , the effect of RXR agonists on glucagon gene transcription was studied. In the absence of PPAR γ , the RXR agonist 9-*cis*-retinoic acid at concentrations up to 1 μ M had no effect on glucagon gene transcription (Fig. 2B, left panel). However, in the presence of PPAR γ , 9-*cis*-retinoic acid inhibited glucagon gene transcription (Fig. 2B, left panel) at concentrations that also activated a PPRE-linked reporter gene (Fig. 2B, right panel). The activation by 9-*cis*-retinoic acid of PPRE-Luc in the presence of PPAR γ but without cotransfection of an RXR expression plasmid indicates that InR1-G9 cells express sufficiently high levels of endogenous RXR, as has been shown previously (Schinner et al., 2002). The maximum inhibition of glucagon gene transcription by the RXR agonist 9-*cis*-retinoic acid in the presence of PPAR γ (by about 40 %) (Fig. 2C, left panel) was somewhat less than the maximum inhibition by the PPAR γ agonist rosiglitazone (inhibition by about 60 %) (Fig. 2C, left panel). Similarly, the activation of PPRE-Luc by 9-*cis*-retinoic acid was lower than that by rosiglitazone (Fig. 2C, right panel). 9-*cis*-retinoic acid acts as an agonist at the RXR and RAR (Boehm et al., 1994; Heyman et al., 1992). Although only RXR, but not RAR, heterodimerizes with PPAR γ (Desvergne and Wahli, 1999), the effect of a specific RXR agonist, BMS 649 (Mukherjee et al., 1997), was studied. Like 9-*cis*-retinoic acid, also BMS 649 inhibited glucagon gene transcription and activated a PPRE-linked reporter gene in the presence of PPAR γ (Fig. 2D). Increasing concentrations of the PPAR γ antagonist GW9662 (Sergeant JM et al., 2004) stimulated glucagon gene transcription (Fig. 2E). These data show that, in addition to the thiazolidinedione PPAR γ agonists, also

RXR agonists inhibit glucagon gene transcription in a PPAR γ -dependent manner, consistent with the view that inhibition of glucagon gene transcription is mediated by a PPAR γ -RXR heterodimer.

A Direct Protein-Protein Interaction Between PPAR γ and the PAX6 Transactivation Domain Depending on the Presence of RXR and the Thiazolidinedione Rosiglitazone Is Revealed by GST

Pull-down Assays. To test whether the inhibition of glucagon gene transcription by thiazolidinediones and PPAR γ may involve a physical interaction between PPAR γ and PAX6 the GST pull-down assay was used. The PAX6 transactivation domain was fused to GST, expressed in *E. coli*, immobilized on glutathione-coated beads, and incubated with PPAR γ and/or RXR α , that had been radioactively labeled with [³⁵S]methionine by *in vitro* transcription/translation. After the binding reaction, the agarose beads were washed and retained proteins were analyzed by SDS polyacrylamide gel electrophoresis. When compared to GST, which served as a negative control, the GST-PAX6 transactivation domain retained the cotranscribed/cotranslated PPAR γ -RXR α heterodimer (Fig. 3A,C,D), whereas PPAR γ alone or RXR α alone were not sufficiently retained (Fig. 3A). The interaction between the PAX6 transactivation domain and the PPAR γ -RXR α heterodimer was further enhanced by the PPAR γ ligand rosiglitazone (Fig. 3B). Also in the presence of rosiglitazone depended the interaction on RXR α as the signal intensity markedly decreased when the PAX6 transactivation domain was incubated with PPAR γ alone (Fig. 3C, compare first lane with third lane). In addition, the PPAR γ antagonist GW9662 reduced the interaction between the PAX6 transactivation domain and PPAR γ (245-475) – RXR α heterodimer (Fig. 3D). In contrast, the ligand-induced interaction did not depend on the PPAR γ DNA-binding domain, since upon cotranscription/cotranslation the PPAR γ (245-475) – RXR α heterodimer bound to the PAX6 transactivation domain (Fig. 3D).

Recruitment of PPAR γ to the Glucagon Gene Promoter Is Revealed by Chromatin

Immunoprecipitation. If the PPAR γ -RXR heterodimer interacts with PAX6 also *in vivo*, PPAR γ should be recruited through PAX6 to the glucagon promoter, in spite of the fact that PPAR γ does not bind to glucagon promoter sequences. This was studied by chromatin immunoprecipitation. After

PPAR γ transfection and rosiglitazone treatment of InR1-G9 cells, chromatin-bound proteins were crosslinked by formaldehyde. After cell lysis and DNA fragmentation by sonication, PPAR γ -bound DNA was immunoprecipitated and amplified by PCR using a primer pair flanking the PAX6 binding site in the glucagon promoter G1 element. When compared to an immunoprecipitation using unspecific immunoglobulins, serving as a negative control, antibodies against PPAR γ precipitated a glucagon promoter fragment that includes the G1 PAX6 binding site (Fig. 4A), indicating recruitment of PPAR γ to the glucagon gene promoter *in vivo*. To investigate whether rosiglitazone enhanced the recruitment of PPAR γ to the glucagon gene promoter *in vivo*, the rat glucagon promoter and the expression for PPAR γ were cotransfected, cells were treated with rosiglitazone and a ChIP assay followed by quantitative PCR was performed. In the presence of the PPAR γ agonist the recruitment of PPAR γ to the glucagon promoter was 5.8-fold enhanced (Fig. 4B). In addition, rosiglitazone enhanced the recruitment of PPAR γ to the genomic (hamster) glucagon promoter 5.9-fold and 1.9-fold, respectively (data not shown), indicating that rosiglitazone stimulated the recruitment of PPAR γ to the glucagon gene promoter *in vivo*.

Discussion

Thiazolidinediones are a new class of oral antidiabetic drugs. Through binding to PPAR γ they lower hepatic glucose output and reduce insulin resistance in type 2 diabetes mellitus, emphasizing the pivotal role of PPAR γ in blood glucose control (Semple et al., 2006). As part of their antidiabetic effect, thiazolidinediones have been shown to inhibit glucagon gene transcription by inhibiting the transcriptional activity of PAX6 (Schinner et al., 2002), which is required for α -cell-specific activation of the glucagon gene (Grzeskowiak et al., 2000). The present study now suggests a mechanism through which thiazolidinediones and PPAR γ inhibit PAX6 activity and thus glucagon gene transcription in pancreatic islet α -cells. The results of the present study are consistent with a model in which a ligand-bound PPAR γ -RXR heterodimer physically interacts with promoter-bound PAX6 (Fig. 5). This interaction is independent of the PPAR γ DNA-binding domain. These data thereby define PAX6 as a novel physical target of PPAR γ .

It has been shown previously that PPAR γ inhibits glucagon gene transcription without binding to the glucagon promoter (Schinner et al., 2002). This view is further supported by the finding in the present study that PPAR γ inhibits glucagon gene transcription also after deletion of its DNA-binding domain. The inhibition by PPAR γ does, however, appear to depend on heterodimerization with RXR. PPAR γ and RXR are known to heterodimerize in solution through their ligand-binding domains (Kliwer et al., 1992; Gampe et al., 2000). An intriguing aspect of RXR heterodimers is that some are permissive for activation by RXR ligands, whereas others are not. The PPAR γ -RXR heterodimer falls into the permissive category of RXR heterodimers (Kliwer et al., 1992). Because of the permissive nature of the PPAR γ -RXR heterodimer, RXR agonists have many of the same effects as do PPAR γ agonists and have been shown to possess antidiabetic activity in mouse models of type 2 diabetes mellitus (Mukherjee et al., 1997). The asymmetric interactions between the AF-2 of PPAR γ and helices 7 and 10 of RXR α , as revealed by the crystal structure of the PPAR γ -RXR α ligand-binding domains (Gampe et al., 2000), suggest a structural basis for permissiveness. The net effect of these interactions may be the stabilization of the PPAR γ AF-2 helix in an active conformation even in the absence of a

bound PPAR γ agonist (Gampe et al., 2000). Because in the present study RXR agonists inhibited glucagon gene transcription in a strictly PPAR γ -dependent manner, the results indicate that a PPAR γ -RXR heterodimer confers inhibition of glucagon gene transcription. They thereby suggest that also the inhibition by thiazolidinediones is mediated by PPAR γ -RXR heterodimers.

Several distinct underlying mechanisms for transrepression by nuclear receptors have been described. Nuclear receptors were shown to inhibit AP-1-mediated transcription of target genes by competition for limiting amounts of the coactivators CBP/p300 (Kamei et al., 1996). Alternatively, nuclear receptors may interfere with signalling pathways. For example, glucocorticoids induce the disassembly of c-Jun N-terminal kinase from mitogen-activated protein kinase kinase 7 by promoting its association with the glucocorticoid receptor leading to the inhibition of c-Jun N-terminal kinase-dependent transcription factors and their target genes (Bruna et al., 2003). A third mechanism of transrepression is through direct interaction with transcription factors, as exemplified by the interaction between the glucocorticoid receptor and the p65 subunit of NF- κ B (Garside et al., 2004). Although the data of the present study do not exclude additional mechanisms, they strongly suggest that the mechanism through which PPAR γ inhibits PAX6 activity and thus glucagon gene transcription involves a protein-protein interaction with PAX6. The ligand-bound PPAR γ -RXR heterodimer was found in the GST pull-down assay to bind to the transactivation domain of PAX6. This binding depended on the presence of rosiglitazone and RXR, but was independent of the PPAR γ DNA-binding domain. The requirements for the binding of PPAR γ to PAX6 are thus similar to those for the inhibition of glucagon gene transcription by PPAR γ (Schinner et al., 2002; this study), suggesting that this binding may underlie the repression of transcription. The chromatin immunoprecipitation assay showed that PPAR γ becomes recruited to the PAX6-responsive region of the proximal glucagon promoter, although PPAR γ does not bind to these sequences (Schinner et al., 2002), indicating that PPAR γ associates with PAX6 also *in vivo* and, furthermore, that PPAR γ associates with promoter-bound PAX6. When taken together, the results of the present study thus support a model in which a

ligand-bound PPAR γ -RXR heterodimer physically interacts with promoter-bound PAX6 to inhibit glucagon gene transcription (Fig. 5).

This suggested model contrasts with defined mechanisms of interaction of nuclear receptors with promoter-bound transcription factors. Thus, NF- κ B activity is inhibited by binding of the p65 subunit of NF- κ B to the DNA-binding domain of the glucocorticoid receptor (Nissen et al., 2000; Chung et al., 2000), whereas PPAR γ interacts with PAX6 and inhibits glucagon gene transcription independent of its DNA-binding domain. Furthermore, while PPAR γ acts at PAX6 and the glucagon promoter as a PPAR γ -RXR heterodimer, transrepression by the glucocorticoid receptor is apparently mediated by glucocorticoid receptor monomers (Reichardt et al., 1998). In fact, transactivation-defective mutants of the glucocorticoid receptor, which cannot dimerize or bind DNA, are fully competent in transrepression (Reichardt et al., 1998). Similarly, PPAR γ has been shown to bind the p65 subunit of NF- κ B as a monomer, i.e. in the absence of RXR, and in a ligand-independent manner (Chung et al., 2000). This interaction seems to be stabilized at the mouse inducible nitric oxide synthase promoter in the RAW264.7 macrophage cell line by multiple cofactors (Pascual et al., 2005). This multiplicity of mechanisms indicates that distinct interaction surfaces are used by nuclear receptors depending on both the specific nuclear receptor and, for a given receptor, the target transcription factor. The inhibition by the PPAR γ -RXR heterodimer of transforming growth factor- β 1 gene transcription in the L929 fibroblast cell line, on the other hand, is not through transrepression but seems to be indirectly mediated (Lee et al., 2006). PPAR γ -RXR has been shown to bind a PPRE in the promoter of the gene encoding the 3'-phosphatase PTEN and to induce PTEN expression, which through inhibition of phosphoinositide 3-kinase leads to a diminished phosphorylation and thus inactivation of the transcription factor Zf9 that is required for transforming growth factor- β 1 gene transcription (Lee et al., 2006).

The present study identifies PAX6 as a novel physical target of PPAR γ -RXR. This may have implications beyond the regulation of glucagon gene transcription in pancreatic islet α cells. PPAR γ

and PAX6 are coexpressed also in pancreatic islet β -cells (Dubois et al., 2000; Callaerts et al., 1997; St-Onge et al., 1997). PAX6 expression in the pancreas is initiated in the pancreatic progenitors, concomitant with the onset of hormone expression, after which the expression persists in all endocrine cells throughout development and in the adult pancreas (Callaerts et al., 1997; St-Onge et al., 1997). The phenotype of PAX6 mutants suggests a specific role for this gene in the differentiation of α -cells as well as for the normal numbers and hormone expression of other endocrine cell types including β -cells. In contrast, the activation of PPAR γ has been shown to reduce the proliferation of β -cells (Rosen et al., 2003) in addition to inducing the expression of glucokinase (Kim et al., 2002) and the glucose transporter GLUT2 in β -cells (Kim et al., 2000). An involvement of PAX6 in these actions remains to be examined. A PAX6 – PPAR γ /RXR interaction could have implications also beyond blood glucose control by thiazolidinedione oral antidiabetic drugs in type 2 diabetes mellitus. The transcription factor PAX6 is highly conserved in evolution and is considered to be a master gene for the development of the eye in species from *Drosophila* to man (Callaerts et al., 1997). It plays an important role also in the development of the nose and brain (Callaerts et al., 1997). In addition to the pancreas, PAX6 is accordingly expressed in the eye and nasal epithelium. It is also expressed in the developing and adult brain (Callaerts et al., 1997) where its expression exhibits some temporal and spatial overlap with the expression of PPAR γ (Zhao et al., 2006), raising the possibility of a PPAR γ – PAX6 interaction also in the brain. Noteworthy, the number of PAX6-expressing neurons in the brain is increased by cerebral ischemia, when the activation of PPAR γ is known to promote neuroprotection (Zhao et al., 2006).

Acknowledgments

BMS 649 was kindly provided by Hinrich Gronemeyer, IGBMC, Strasbourg, France. We thank Stephan Herzig (Heidelberg, Germany) for his advice on the chromatin immunoprecipitation assay.

References

- Boehm MF, Zhang L, Badea BA, White SK, Mais DE, Berger E, Suto CM, Goldman ME and Heyman RA (1994) Synthesis and structure-activity relationships of novel retinoid X receptor-selective retinoids. *J Med Chem* 37:2930-2941.
- Bruna A, Nicolas M, Munoz A, Kyriakis JM, Caelles C (2003) Glucocorticoid receptor-JNK interaction mediates inhibition of the JNK pathway by glucocorticoids. *EMBO J* 22: 6035-6044.
- Callaerts P, Halder G and Gehring WJ (1997) PAX-6 in development and evolution. *Annu Rev Neurosci* 20:483-532.
- Chung SW, Kang BY, Kim SH, Pak YK, Cho D, Trinchieri G, Kim TS (2000) Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-gamma and nuclear factor-kappa B. *J Biol Chem* 275:32681-32687.
- Desvergne B and Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 20:649-688.
- Dubois M, Pattou F, Kerr-Conte J, Gmyr V, Vandewalle B, Desreumaux P, Auwerx J, Schoonjans K and Lefebvre J (2000) Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in normal human pancreatic islet cells. *Diabetologia* 43:1165-1169.
- Fürstenau U, Schwaninger M, Blume R, Kennerknecht I and Knepel W (1997) Characterization of a novel protein kinase C response element in the glucagon gene. *Mol Cell Biol* 17:1805-1816.
- Gampe RT Jr, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliewer SA, Willson TM and Xu HE (2000) Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol Cell* 5:545-555.
- Garside H, Stevens A, Farrow S, Normand C, Houle B, Berry A, Maschera B and Ray D (2004) Glucocorticoid ligands specify different interactions with NF-kappaB by allosteric effects on the glucocorticoid receptor DNA binding domain. *J Biol Chem* 279:50050-50059.

- Grzeskowiak R, Amin J, Oetjen E and Knepel W (2000) Insulin responsiveness of the glucagon gene conferred by interactions between proximal promoter and more distal enhancer-like elements involving the paired-domain transcription factor Pax6. *J Biol Chem* 275:30037-30045.
- Heinlein CA, Ting HJ, Yeh S and Chang C (1999) Identification of ARA70 as a ligand-enhanced coactivator for the peroxisome proliferator-activated receptor gamma. *J Biol Chem* 274:16147-16152.
- Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM and Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68:397-406.
- Hussain MA and Habener JF (1999) Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J Biol Chem* 274:28950-28957.
- Ikedo H, Taketomi S, Sugiyama Y, Shimura Y, Sohada T, Meguro K and Fujita T (1990) Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. *Arzneimittelforschung* 40:156-162.
- Kalderon D, Roberts BL, Richardson WD and Smith AE (1984) A short amino acid sequence able to specify nuclear location. *Cell* 39:499-509.
- Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Glass B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85:403-414.
- Kersten, S, Desvergne B and Wahli W (2000) Roles of PPARs in health and disease. *Nature* 405:421-424.
- Kim HI, Cha JY, Kim SY, Kim JW, Roh KJ, Seong JK, Lee NT, Choi KY, Kim KS and Ahn YH (2002) Peroxisomal proliferator-activated receptor-gamma upregulates glucokinase gene expression in beta-cells. *Diabetes* 51:676-685.
- Kim HI, Kim JW, Kim SH, Cha JY, Kim KS and Ahn YH (2000) Identification and functional characterization of the peroxisomal proliferator response element in rat GLUT2 promoter. *Diabetes* 49:1517-15124.

- Kliwer SA, Umesono K, Mangelsdorf DJ and Evans RM (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* 355:446-449.
- Knepel W, Chafitz J, and Habener JF (1990) Transcriptional activation of the rat glucagon gene by the cyclic AMP-responsive element in pancreatic islet cells. *Mol Cell Biol* 10:6799-6804.
- Lee SJ, Yang EK, Kim SG (2006) Peroxisome Proliferator-Activated Receptor- γ and Retinoic Acid X Receptor α Represses the TGF β 1 Gene via PTEN-Mediated p70 Ribosomal S6 Kinase-1 Inhibition: Role for Zf9 dephosphorylation. *Mol Pharmacol* 70:415-425.
- Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliwer SA (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* 270:12953-12956.
- Mikkola I, Bruun JA, Bjorkoy G, Holm T and Johansen T (1999) Phosphorylation of the transactivation domain of Pax6 by extracellular signal-regulated kinase and p38 mitogen-activated protein kinase. *J Biol Chem* 274:15115-15126.
- Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti JR Jr, Heyman RA (1997) Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* 386:407-410.
- Nissen RM and Yamamoto KR (2000) The glucocorticoid receptor inhibits NF κ B by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* 14:2314-2329.
- Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK and Milburn MV (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* 395:137-143.
- Oetjen E, Diedrich T, Eggers A, Eckert B and Knepel W (1994) Distinct properties of the cAMP-responsive element of the rat insulin I gene. *J Biol Chem* 269:27036-44.
- Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 437:759-763.

- Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P and Schütz G (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 93:531-541.
- Ricote M, Li AC, Willson TM, Kelly CJ and Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391:79-82.
- Rosen ED, Kulkarni RN, Sarraf P, Ozcan U, Okada T, Hsu CH, Eisenman D, Magnuson MA, Gonzalez FJ, Kahn CR and Spiegelman BM (2003) Targeted elimination of peroxisome proliferator-activated receptor gamma in beta cells leads to abnormalities in islet mass without compromising glucose homeostasis. *Mol Cell Biol* 23:7222-7229.
- Schinner S, Dellas C, Schroeder M, Heinlein CA, Chang C, Fischer J and Knebel W (2002) Repression of glucagon gene transcription by peroxisome proliferator-activated receptor gamma through inhibition of Pax6 transcriptional activity. *J Biol Chem* 277:1941-1948.
- Schwaninger M, Blume R, Oetjen E, Lux G and Knebel W (1993) Inhibition of cAMP-responsive element-mediated gene transcription by cyclosporin A and FK506 after membrane depolarization. *J Biol Chem* 268:23111-23115.
- Schwaninger M, Lux G, Blume R, Oetjen E, Hidaka H and Knebel W (1993) Membrane depolarization and calcium influx induce glucagon gene transcription in pancreatic islet cells through the cyclic AMP-responsive element. *J Biol Chem* 268:5168-5177.
- Seargent JM, Yates EA, Gill JH (2004) GW9662, a potent antagonist of PPARgamma, inhibits growth of breast tumour cells and promotes the anticancer effects of the PPARgamma agonist rosiglitazone, independently of PPARgamma activation. *Br J Pharmacol* 143:933-937.
- Semple RK, Chatterjee VK, and O'Rahilly S (2006) PPAR gamma and human metabolic disease. *J Clin Invest* 116:581-589.
- St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouris A and Gruss P (1997) Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 387:406-409.
- Unger RH and Orci L (1981) Glucagon and the A cell: physiology and pathophysiology (first two parts). *N Engl J Med* 304:1518-1524.

Zhao Y, Patzer A, Herdegen T, Gohlke P and Culman J (2006) Activation of cerebral peroxisome proliferator-activated receptors gamma promotes neuroprotection by attenuation of neuronal cyclooxygenase-2 overexpression after focal cerebral ischemia in rats. *FASEB J* 20:1162-1175.

Footnotes

* This work was supported by the Deutsche Forschungsgemeinschaft (SFB402/A3, GRK335).

Please send requests for reprints to Elke Oetjen, MD, Molecular Pharmacology, University of Goettingen, Robert-Koch-Str. 40, 37099 Goettingen, Germany; e-mail: eoetjen@med.uni-goettingen.de

Legends for figures.

Fig. 1. Inhibition of glucagon gene transcription by PPAR γ and a PPAR γ deletion mutant lacking the PPAR γ DNA-binding domain. A luciferase reporter gene under the control of the rat glucagon gene promoter from -350 to +58 (plasmid -350GluLuc) was transiently transfected into InR1-G9 cells together with different expression vectors coding for PPAR γ variants: PPAR γ (1-475), PPAR γ wild-type; PPAR γ (1-475)N, PPAR γ wild-type extended carboxyl-terminally by a nuclear localization signal; PPAR γ (175-475)N, PPAR γ variant lacking the AF-1 and DNA-binding domain and carrying carboxyl-terminally a nuclear localization signal. Rosiglitazone (30 μ M) was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the control (without PPAR γ transfection, no rosiglitazone treatment). Values are means \pm S.E.M. of three independent experiments, each done in duplicate. *Upper panel*, domain structure of PPAR γ . The numbers indicate the amino acids.

Fig. 2. The RXR agonists 9-*cis*-retinoic acid and BMS 649 inhibit glucagon gene transcription in a PPAR γ -dependent manner. A, scheme of the reporter gene constructs -350GluLuc and PPRE-Luc. Control elements in the 5'-flanking region of the glucagon gene are indicated. B, the luciferase reporter gene constructs -350GluLuc (*left panel*) and PPRE-Luc (*right panel*) were transfected into InR1-G9 cells with or without an expression vector encoding PPAR γ . Cells were treated with increasing concentrations of 9-*cis*-retinoic for 24 h before harvest. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective untreated controls. Values are means \pm S.E.M. of three independent experiments, each done in duplicate. C, the luciferase reporter gene constructs -350GluLuc and PPRE-Luc were transfected into InR1-G9 cells with an expression vector encoding PPAR γ . Cells were treated with rosiglitazone (10 μ M) or 9-*cis*-retinoic acid (9-*cis*-RA, 10 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 respective untreated controls. Values are means \pm S.E.M. of three independent experiments, each done in duplicate. D, the constructs -350GluLuc and PPRE-Luc were transfected into InR1-G9 cells together with an expression vector

encoding PPAR γ . Cells were treated with BMS 649 or 9-*cis*-retinoic (9*cis*-RA) for 24 h before harvest. Luciferase activity 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.M. of three experiments, each done in duplicate, *, $P < 0.005$ using Student's *t* test. E, the luciferase reporter gene construct -350GluLuc was cotransfected with PPAR γ and cells were treated with increasing concentrations of the PPAR γ antagonist GW9662 for 24 h. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective untreated controls. Values are means \pm S.E.M. of three independent experiments, each done in duplicate.

Fig. 3. Protein-protein interaction between PPAR γ -RXR and the transactivation domain of PAX6 in GST pull-down assays. A, *in vitro* transcribed/translated and [35 S]methionine-labeled PPAR γ , RXR α , and PPAR γ /RXR α were incubated with GST-PAX6-TAD immobilized on glutathione agarose beads. GST immobilized on glutathione agarose beads was used as a control. The resulting complexes were resolved by SDS-PAGE and visualized by phosphorimaging (*top*). The bands were quantified by densitometry. Optical density is expressed as percentage of the optical density measured in the PPAR γ -RXR α plus GST-PAX6-TAD group. Values are means \pm S.E.M. of four independent experiments. PPAR γ and RXR α are of similar size and are not clearly separated on the gel used. B, PPAR γ and RXR α were cotranscribed/cotranslated and labeled with [35 S]methionine *in vitro*. PPAR γ /RXR α was incubated with GST-PAX6-TAD immobilized on glutathione agarose beads. GST immobilized on glutathione agarose beads was used as a control. The incubations were performed in the presence or absence of rosiglitazone (50 μ M). The resulting complexes were resolved by SDS-PAGE and visualized by phosphorimaging (*top*). The bands were quantified by densitometry. Optical density is expressed as percentage of the optical density measured in the GST-PAX6-TAD group (no rosiglitazone). Values are means \pm S.E.M. of five experiments. Lanes "1" and "2" show 5% input. C, *in vitro* transcribed/translated and [35 S]methionine-labeled PPAR γ and PPAR γ /RXR α were incubated with GST-PAX6-TAD immobilized on glutathione agarose beads. GST immobilized on glutathione

agarose beads was used as a control. The incubations were performed in the presence or absence of rosiglitazone (50 μ M). The resulting complexes were resolved by SDS-PAGE and visualized by phosphorimaging. The 5 % input of PPAR γ /RXR α (lane "1") and PPAR γ (lanes "2" and "3") is shown. D, PPAR γ -(245–475), containing only the ligand-binding domain, and RXR α were cotranscribed/cotranslated and labeled with [³⁵S]methionine *in vitro*. PPAR γ -(245–475)/RXR α were incubated in the presence of rosiglitazone (50 μ M) or GW9662 (50 μ M) with GST-PAX6-TAD immobilized on glutathione agarose beads and GST-covered beads for control. The resulting complexes were resolved by SDS-PAGE and visualized by phosphorimaging. 1, 5% input. *Upper band, RXR α ; lower band, PPAR γ -(245-475).*

Fig. 4. Recruitment of PPAR γ to the PAX6-binding proximal glucagon promoter as revealed by chromatin immunoprecipitation. A, InR1-G9 cells were transfected with PPAR γ and treated with rosiglitazone (50 μ M, for 24 h). After cell lysis and DNA fragmentation by sonication, PPAR γ -bound DNA was immunoprecipitated and amplified by PCR using a primer pair flanking the PAX6 binding site in the glucagon promoter G1 element. Chromatin was precipitated with unspecific IgG antibody for control. A positive control for the fragment size is also shown. B, The effect of rosiglitazone on the ChIP of PPAR γ /PAX6 was quantified using quantitative PCR. InR1-G9 cells were transfected with -350GluLuc and PPAR γ and treated as described above. ChIP signal activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the untreated control. Values are means \pm S.D. of two independent experiments.

Fig. 5. Model for the mechanism of rosiglitazone-induced repression of the glucagon gene. See text for details.

Fig. 1

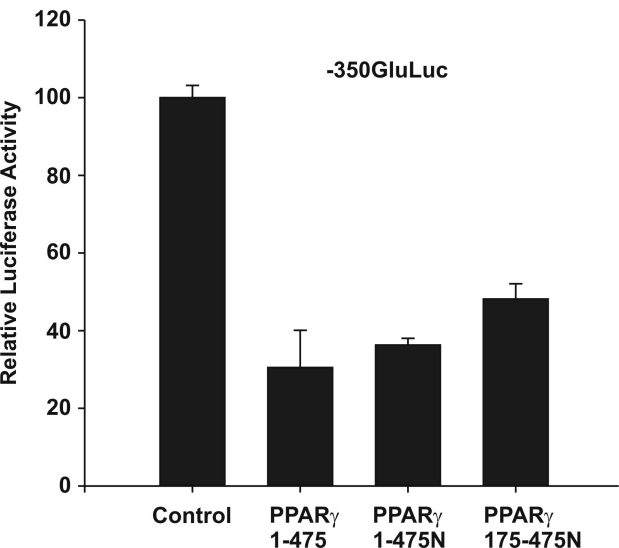
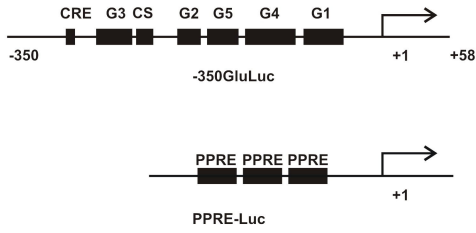
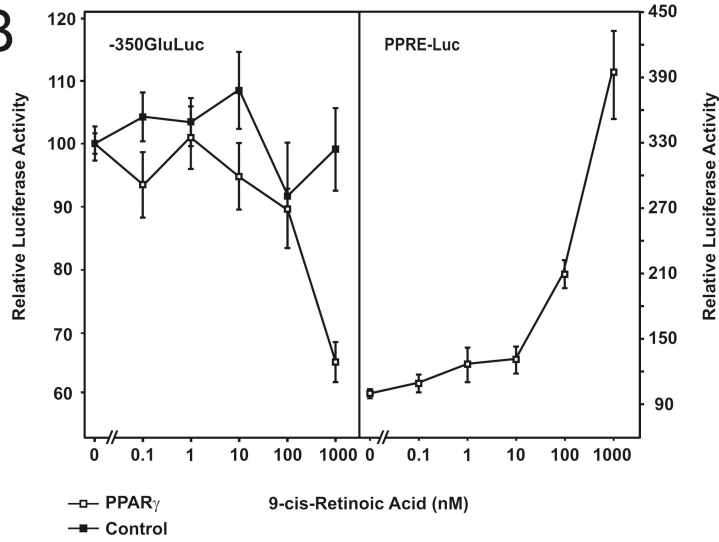


Fig. 2

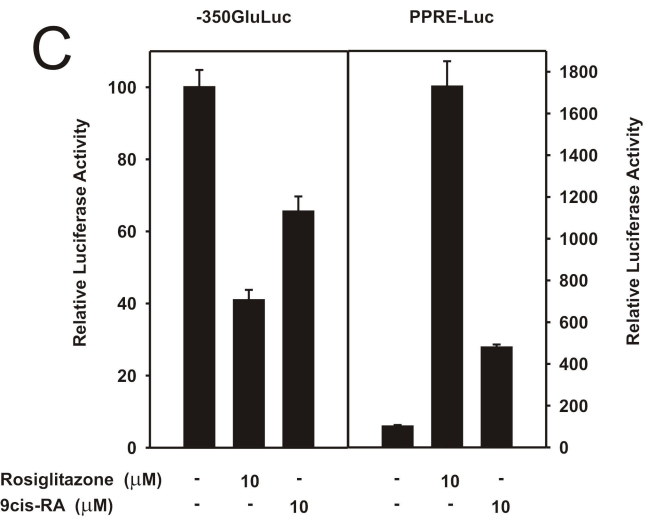
A



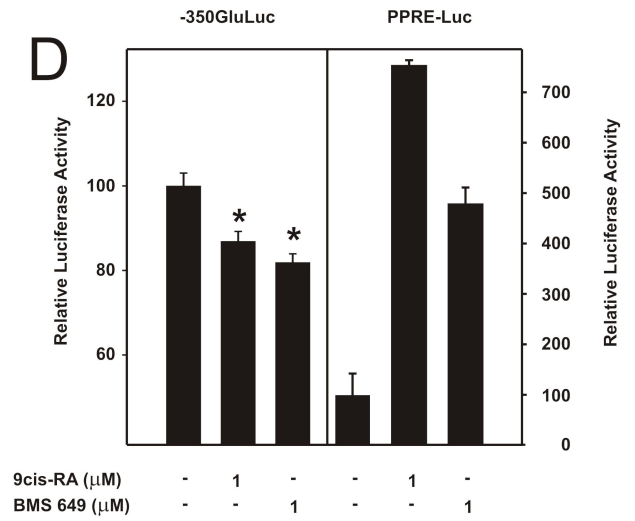
B



C



D



E

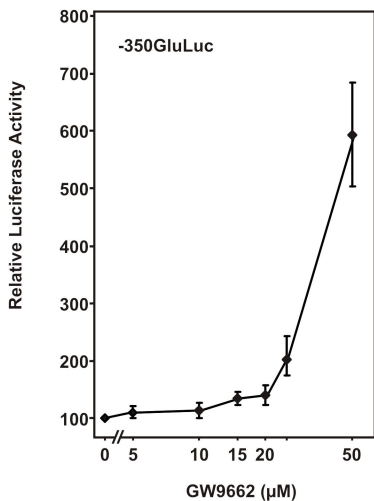


Fig. 3A

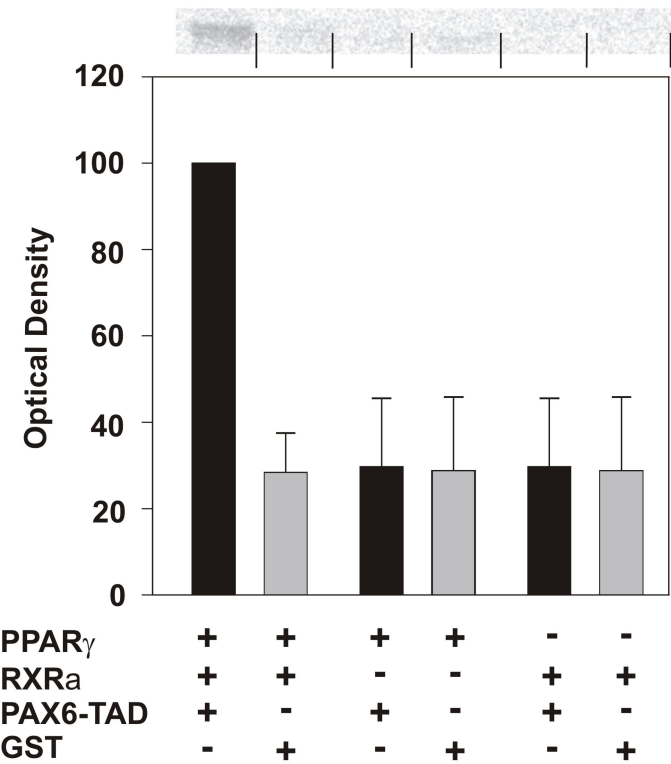
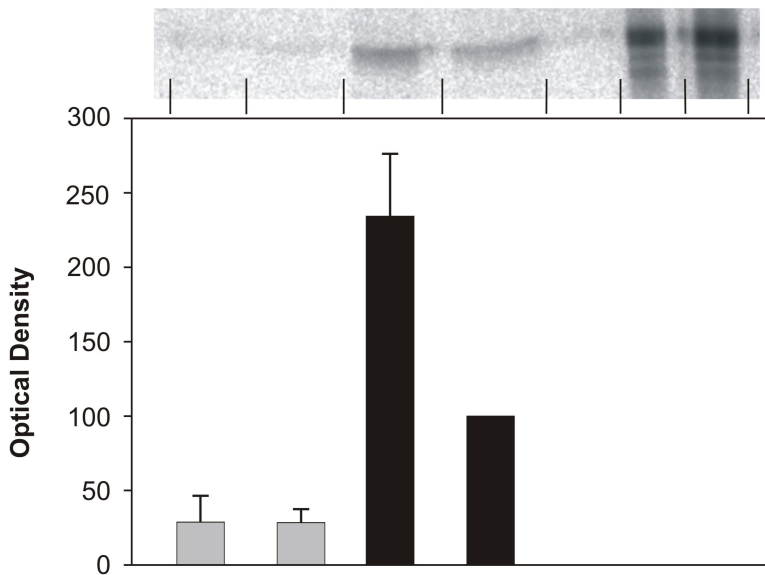


Fig. 3B



Rosiglitazone
PAX6-TAD
GST

+ **-**
- **-**
+ **+**

+ **-**
+ **+**
- **-**

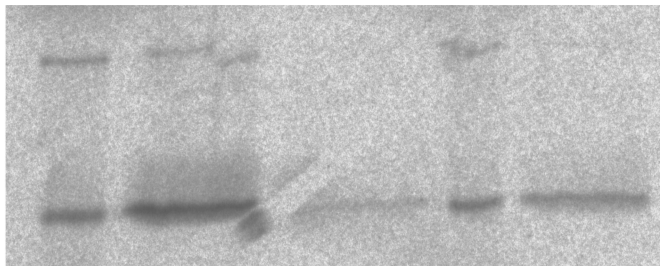
1 **2**

Fig. 3C



Rosiglitazone	+	+	+	+	-	-	1	2	3
PPARγ	+	+	+	+	+	+			
RXRα	+	+	-	-	-	-			
PAX6-TAD	+	-	+	-	+	-			
GST	-	+	-	+	-	+			

Fig. 3D



GW9662	1	-	-	1	+
Rosiglitazone		+	+		-
PPARγ 245-475		+	+		+
RXRα		+	+		+
PAX6-TAD		+	-		+
GST		-	+		-

Fig. 4

A



B

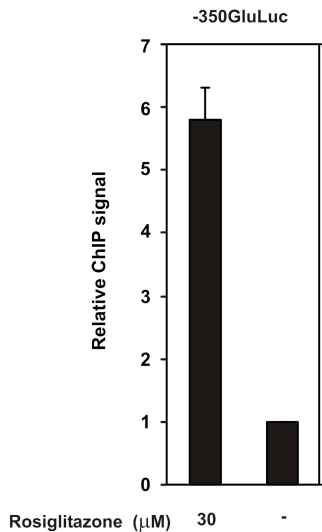


Fig. 5

