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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.

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Runnig title page

a) PPARγ 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

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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;

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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 (PPARγ) has essential roles in glucose homeostasis and thiazolidinedione PPARγ 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 PPARγ and inhibition of the transcriptional activity of PAX6 that is required for cell-specific activation of the glucagon gene. However, how thiazolidinediones and PPARγ 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 PPARγ was found in the present study to inhibit glucagon gene transcription also after deletion of its DNA-binding domain. Like PPARγ ligands, also retinoid X receptor (RXR) agonists inhibited glucagon gene transcription in a PPARγ-dependent manner. In glutathione *S*-transferase pull-down assays, the ligand-bound PPARγ-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 PPARγ DNA-binding domain. Chromatin immunoprecipitation experiments showed that PPARγ 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 PPARγ-RXR heterodimer physically interacts with promoter-bound PAX6 to inhibit glucagon gene transcription. These data define PAX6 as a novel physical target of PPARγ-RXR.

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Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the superfamily of ligand-regulated nuclear hormone receptors (Desvergene 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) (Desvergene and Wahli, 1999). PPARγ 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 PPARγ-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, PPARγ has been shown to be also capable of repression of gene transcription (Ricote et al., 1998; Schinner et al., 2002)

PPARγ 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 PPARγ are associated with insulin resistance and diabetes mellitus (Semple et al., 2006) suggesting that PPARγ agonists may be useful in the treatment of type 2 diabetes mellitus.

Besides naturally existing ligands like 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a number of synthetic ligands of PPARγ 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 PPARγ leading to a reduction in hepatic glucose output and a decrease in insulin resistance. PPARγ 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).

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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/PPARγ 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 PPARγ responsiveness (Schinner et al., 2002). This sequence motif does not bind PPARγ 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/PPARγ responsiveness (Schinner et al., 2002), suggesting that PPARγ in a ligand-dependent but DNA binding-independent manner inhibits PAX6 transcriptional activity on glucagon gene transcription. However, how PPARγ inhibits PAX6 transcriptional activity in molecular details remained unknown. The results of the present study suggest that thiazolidinediones and PPARγ inhibit glucagon gene transcription through a mechanism that involves a direct interaction of PPARγ with the PAX6 transactivation domain. In contrast to the glucocorticoid receptor, where transrepression is apparently mediated by glucocorticoid receptor

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

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Materials and Methods

Plasmids. The plasmids -350GluLuc, pcDNA3-PPARγ (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-RXRα was generated by *Eco*RI digestion of pSG5-RXRα (Heinlein et al., 1999) and cloning of the insert into *Eco*RI digested pcDNA3. The plasmid pcDNA3-PPARγ was used as PCR template for the generation of all described PPARγ variants. PCR fragments coding for the described PPARγ variants were amplified using primer pairs introducing a 5' *Kpn*I and a 3' *Xba*I 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 µl of GST-PAX6(299-437) or GST beads were added to 250 µl NETN buffer and incubated with 7.5 µl of radioactively labelled *in vitro* transcribed/translated protein at 4 °C overnight. The beads were washed three times with 500 µl ice cold NETN buffer, after the last washing step the supernatant was carefully removed and the pellet was boiled in 30 µl 2x Laemmli sample buffer for 10 min. After SDS-PAGE, the radioactively labelled proteins were visualized using a phosphorimager. Densitometry

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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 µg/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method (Schinner et al., 2002) with 2 µg of reporter gene plasmids and, when indicated, 1 µg 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 µg 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 FluoroCount™ microplate fluorometer (Packard).

Chromatin Immunoprecipitation (ChIP) and quantitive PCR. Two 16-cm dishes of confluent InR1-G9 cells transiently transfected with PPARγ-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 µl 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 µl of equilibrated proteinA/G agarose (Santa Cruz, Heidelberg, Germany). 10 µg of mouse anti-HA antibody (Sigma-Aldrich, Munich, Germany) or IgG were added to 250 µl of the supernatant. The reaction was incubated overnight at 4 °C. 60µl 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

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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^{o}C for 10 minutes, 40 cycles of 94 for 15 seconds, 62^{o}C for 15 seconds, and 72^{o}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 ΔCt 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 µg 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

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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 (Mannhein, Germany) and 9-*cis*-retinoic acid and GW9662 were from Sigma (Taufkirchen, Germany), respectively.

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Results

PPARγ 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 PPARγ (Dubois et al., 2000) and PPARγ 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 PPARγ such that activation of a PPRE-linked reporter gene as well as inhibition of glucagon gene transcription by PPARγ agonists requires transfection of a PPARγ expression plasmid (Schinner et al., 2002). Furthermore, in this cell line in the presence of overexpressed PPARγ treatment with 50 μM rosiglitazone decreased the mRNA level of endogenous glucagon by 37 % ± 11.4 ($P < 0.05$; n=4) (data not shown). This cell line therefore allows a direct assessment of the role of PPARγ and PPARγ 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 PPARγ (Schinner et al., 2002). To examine which domain of PPARγ is involved in the repression by PPARγ of glucagon gene transcription, we constructed the PPARγ variant PPARγ–(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 PPARγ, we fused that sequence also to PPARγ full-length (construct PPARγ–(1-475)N). All PPARγ variants were cotransfected with –350GluLuc into InR1-G9 cells. After treatment with rosiglitazone, all PPARγ variants inhibited glucagon gene transcription by 50-70 % (Fig. 1). These data show that the ligand binding domain of PPARγ is sufficient to inhibit glucagon gene transcription. These results

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

RXR Agonists Inhibit Glucagon Gene Transcription in a PPARγ-dependent Manner. PPARγ activates gene transcription by binding as a heterodimer with RXR to PPREs in the promoter region of target genes (Desvergne and Wahli, 1999). PPARγ-RXR is a permissive heterodimer, the transcriptional activity of which can be activated by both, PPARγ agonists and RXR agonists (Kliewer et al., 1992). To examine whether RXR is involved in the repression of glucagon gene transcription by thiazolidinediones and PPARγ, the effect of RXR agonists on glucagon gene transcription was studied. In the absence of PPARγ, the RXR agonist 9-*cis*-retinoic acid at concentrations up to 1 μM had no effect on glucagon gene transcription (Fig. 2B, left panel). However, in the presence of PPARγ, 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 PPARγ 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 PPARγ (by about 40 %) (Fig. 2C, left panel) was somewhat less than the maximum inhibition by the PPARγ 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 PPARγ (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 PPARγ (Fig. 2D). Increasing concentrations of the PPARγ antagonist GW9662 (Sergeant JM et al., 2004) stimulated glucagon gene transcription (Fig. 2E). These data show that, in addition to the thiazolidinedione PPARγ agonists, also

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

A Direct Protein-Protein Interaction Between PPARγ 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 PPARγ may involve a physical interaction between PPARγ 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 PPARγ and/or RXRα, that had been radioactively labeled with [^{35}S]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 PPARγ-RXRα heterodimer (Fig. 3A,C,D), whereas PPARγ alone or RXRα alone were not sufficiently retained (Fig. 3A). The interaction between the PAX6 transactivation domain and the PPARγ-RXRα heterodimer was further enhanced by the PPARγ ligand rosiglitazone (Fig. 3B). Also in the presence of rosiglitazone depended the interaction on RXRα as the signal intensity markedly decreased when the PAX6 transactivation domain was incubated with PPARγ alone (Fig. 3C, compare first lane with third lane). In addition, the PPARγ antagonist GW9662 reduced the interaction between the PAX6 transactivation domain and PPARγ-(245-475) – RXRα heterodimer (Fig. 3D). In contrast, the ligand-induced interaction did not depend on the PPARγ DNA-binding domain, since upon cotranscription/cotranslation the PPARγ-(245-475) – RXRα heterodimer bound to the PAX6 transactivation domain (Fig. 3D).

Recruitment of PPARγ to the Glucagon Gene Promoter Is Revealed by Chromatin Immunoprecipitation. If the PPARγ-RXR heterodimer interacts with PAX6 also *in vivo*, PPARγ should be recruited through PAX6 to the glucagon promoter, in spite of the fact that PPARγ does not bind to glucagon promoter sequences. This was studied by chromatin immunoprecipitation. After

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PPARγ transfection and rosiglitazone treatment of InR1-G9 cells, chromatin-bound proteins were crosslinked by formaldehyde. After cell lysis and DNA fragmentation by sonication, PPARγ-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 PPARγ precipitated a glucagon promoter fragment that includes the G1 PAX6 binding site (Fig. 4A), indicating recruitment of PPARγ to the glucagon gene promoter *in vivo*. To investigate whether rosiglitazone enhanced the recruitment of PPARγ to the glucagon gene promoter *in vivo*, the rat glucagon promoter and the expression for PPARγ were cotransfected, cells were treated with rosiglitazone and a ChIP assay followed by quantitative PCR was performed. In the presence of the PPARγ agonist the recruitment of PPARγ to the glucagon promoter was 5.8-fold enhanced (Fig. 4B). In addition, rosiglitazone enhanced the recruitment of PPARγ to the genomic (hamster) glucagon promoter 5.9-fold and 1.9-fold, respectively (data not shown), indicating that rosiglitazone stimulated the recruitment of PPARγ to the glucagon gene promoter *in vivo*.

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Discussion

Thiazolidinediones are a new class of oral antidiabetic drugs. Through binding to PPARγ they lower hepatic glucose output und reduce insulin resistance in type 2 diabetes mellitus, emphasizing the pivotal role of PPARγ 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 PPARγ 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 PPARγ-RXR heterodimer physically interacts with promoter-bound PAX6 (Fig. 5). This interaction is independent of the PPARγ DNA-binding domain. These data thereby define PAX6 as a novel physical target of PPARγ.

It has been shown previously that PPARγ 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 PPARγ inhibits glucagon gene transcription also after deletion of its DNA-binding domain. The inhibition by PPARγ does, however, appear to depend on heterodimerization with RXR. PPARγ and RXR are known to heterodimerize in solution through their ligand-binding domains (Kliewer 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 PPARγ-RXR heterodimer falls into the permissive category of RXR heterodimers (Kliewer et al., 1992). Because of the permissive nature of the PPARγ-RXR heterodimer, RXR agonists have many of the same effects as do PPARγ 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 PPARγ and helices 7 and 10 of RXRα, as revealed by the crystal structure of the PPARγ-RXRα 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 PPARγ AF-2 helix in an active conformation even in the absence of a

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bound PPARγ agonist (Gampe et al., 2000). Because in the present study RXR agonists inhibited glucagon gene transcription in a strictly PPARγ-dependent manner, the results indicate that a PPARγ-RXR heterodimer confers inhibition of glucagon gene transcription. They thereby suggest that also the inhibition by thiazolidinediones is mediated by PPARγ-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-κB (Garside et al., 2004). Although the data of the present study do not exclude additional mechanisms, they strongly suggest that the mechanism through which PPARγ inhibits PAX6 activity and thus glucagon gene transcription involves a protein-protein interaction with PAX6. The ligand-bound PPARγ-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 PPARγ DNA-binding domain. The requirements for the binding of PPARγ to PAX6 are thus similar to those for the inhibition of glucagon gene transcription by PPARγ (Schinner et al., 2002; this study), suggesting that this binding may underlie the repression of transcription. The chromatin immunoprecipitation assay showed that PPARγ becomes recruited to the PAX6-responsive region of the proximal glucagon promoter, although PPARγ does not bind to these sequences (Schinner et al., 2002), indicating that PPARγ associates with PAX6 also *in vivo* and, furthermore, that PPARγ associates with promoter-bound PAX6. When taken together, the results of the present study thus support a model in which a

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ligand-bound PPARγ-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-κB activity is inhibited by binding of the p65 subunit of NF-κB to the DNA-binding domain of the glucocorticoid receptor (Nissen et al., 2000; Chung et al., 2000), whereas PPARγ interacts with PAX6 and inhibits glucagon gene transcription independent of its DNA-binding domain. Furthermore, while PPARγ acts at PAX6 and the glucagon promoter as a PPARγ-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, PPARγ has been shown to bind the p65 subunit of NF-κB 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 PPARγ-RXR heterodimer of transforming growth factor-β1 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). PPARγ-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-β1 gene transcription (Lee et al., 2006).

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

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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 PPARγ 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 – PPARγ/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 PPARγ (Zhao et al., 2006), raising the possibility of a PPARγ – 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 PPARγ is known to promote neuroprotection (Zhao et al., 2006).

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Footnotes

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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

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Fig. 1. Inhibition of glucagon gene transcription by PPARγ and a PPARγ deletion mutant lacking the PPARγ 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 PPARγ variants: PPARγ-(1-475), PPARγ wild-type; PPARγ-(1-475)N, PPARγ wild-type extended carboxyl-terminally by a nuclear localization signal; PPARγ-(175-475)N, PPARγ variant lacking the AF-1 and DNA-binding domain and carrying carboxyl-terminally a nuclear localization signal. Rosiglitazone (30 μM) was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the control (without PPARγ transfection, no rosiglitazone treatment). Values are means ± S.E.M. of three independent experiments, each done in duplicate. *Upper panel*, domain structure of PPARγ. The numbers indicate the amino acids.

Fig. 2. The RXR agonists 9-*cis*-retinoic acid and BMS 649 inhibit glucagon gene transcription in a PPARγ-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 PPARγ. 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 ± 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 PPARγ. Cells were treated with rosiglitazone (10 μM) or 9-*cis*-retinoic acid (*9cis-RA*, 10 μM) 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 ± 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 PPARγ. Cells were treated with BMS 649 or 9-*cis*-retinoic (*9cis-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 ± 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 PPARγ and cells where treated with increasing concentrations of the PPARγ 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 ± S.E.M. of three independent experiments, each done in duplicate.

Fig. 3. Protein-protein interaction between PPARγ-RXR and the transactivation domain of PAX6 in GST pull-down assays. A, *in vitro* transcribed/translated and [^{35}S]methionine-labeled PPARγ, RXRα, and PPARγ/RXRα 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 PPARγ-RXRα plus GST-PAX6-TAD group. Values are means ± S.E.M. of four independent experiments. PPARγ and RXRα are of similar size and are not clearly separated on the gel used. B, PPARγ and RXRα were cotranscribed/cotranslated and labeled with [^{35}S]methionine *in vitro*. PPARγ/RXRα 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 μM). 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 ± S.E.M. of five experiments. Lanes "1" and "2"show 5% input. C, *in vitro* transcribed/translated and [^{35}S]methionine-labeled PPARγ and PPARγ/RXRα were incubated with GST-PAX6-TAD immobilized on glutathione agarose beads. GST immobilized on glutathione

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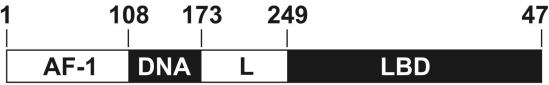
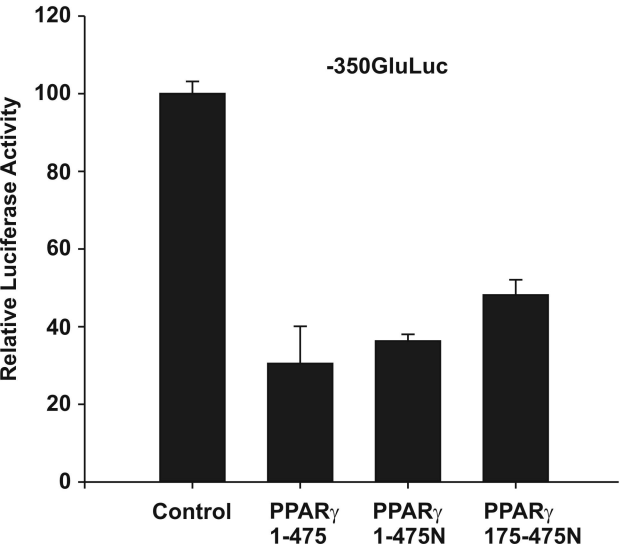
agarose beads was used as a control. The incubations were performed in the presence or absence of rosiglitazone (50 μM). The resulting complexes were resolved by SDS-PAGE and visualized by phosphorimaging. The 5 % input of PPARγ/RXRα (lane "1") and PPARγ (lanes "2" and "3") is shown. D, PPARγ–(245–475), containing only the ligand-binding domain, and RXRα were cotranscribed/cotranslated and labeled with [^{35}S]methionine *in vitro*. PPARγ–(245–475)/RXRα were incubated in the presence of rosiglitazone (50 μM) or GW9662 (50μM) 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*, RXRα; *lower band*, PPARγ-(245-475).

Fig. 4. Recruitment of PPARγ to the PAX6-binding proximal glucagon promoter as revealed by chromatin immunoprecipitation. A, InR1-G9 cells were transfected with PPARγ and treated with rosiglitazone (50 μM, for 24 h). After cell lysis and DNA fragmentation by sonication, PPARγ-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 PPARγ/PAX6 was quantified using quantitative PCR. InR1-G9 cells were transfected with -350GluLuc and PPARγ 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 ± 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


1
108
173
249
475
AF-1
DNA
L
LBD


-350GluLuc
Relative Luciferase Activity
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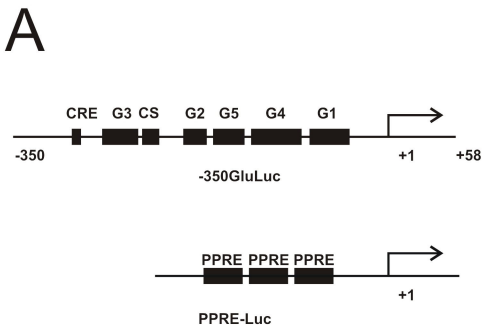
100
80
60
40
20
0
Control
PPARγ 1-475
PPARγ 1-475N
PPARγ 175-475N

Fig. 2


A
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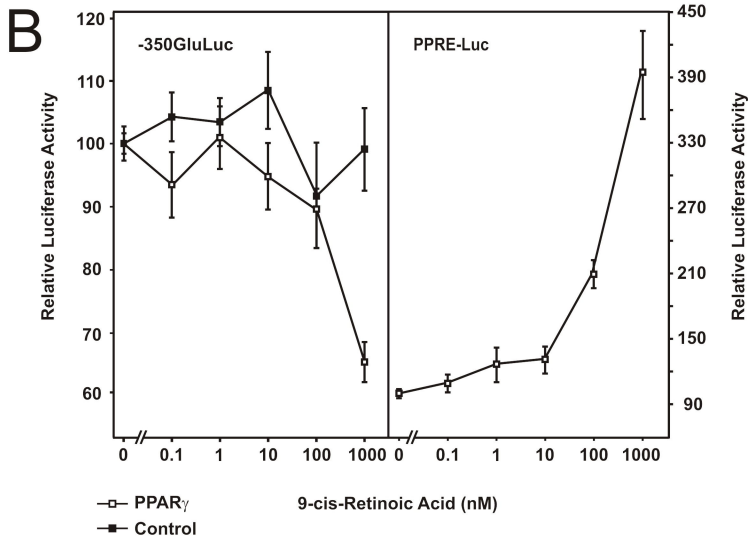
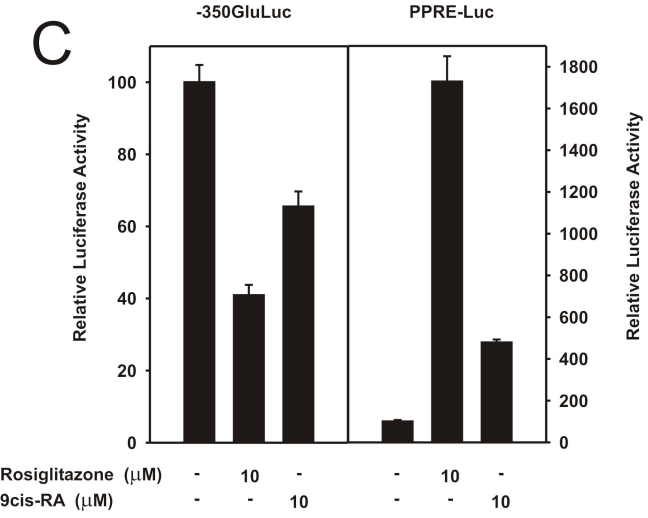
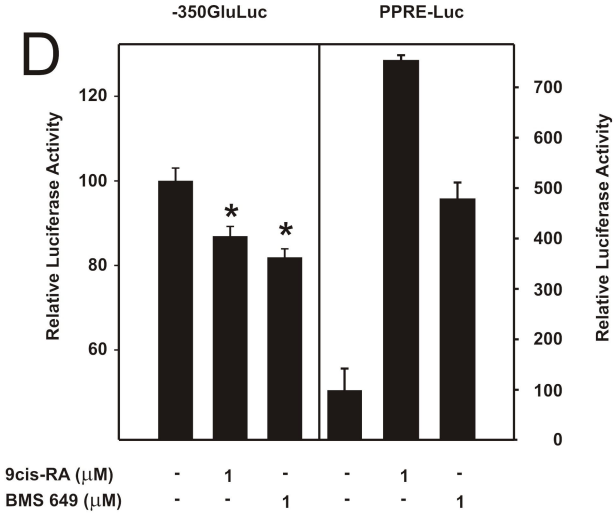
-350
+1
+58
-350GluLuc
PPRE PPRE PPRE
+1
PPRE-Luc


B
-350GluLuc
PPRE-Luc
Relative Luciferase Activity
120
110
100
90
80
70
60
450
390
330
270
210
150
90
0
0.1
1
10
100
1000
PPARγ
Control
9-cis-Retinoic Acid (nM)


C
-350GluLuc
PPRE-Luc
Relative Luciferase Activity
100
80
60
40
20
0
1800
1600
1400
1200
1000
800
600
400
200
0
Rosiglitazone (μM) - 10 - - 10 -
9cis-RA (μM) - - 10 - - 10


D
-350GluLuc
PPRE-Luc
Relative Luciferase Activity
120
100
80
60
700
600
500
400
300
200
100
0
*
*
9cis-RA (μM) - 1 - - 1 -
BMS 649 (μM) - - 1 - - 1

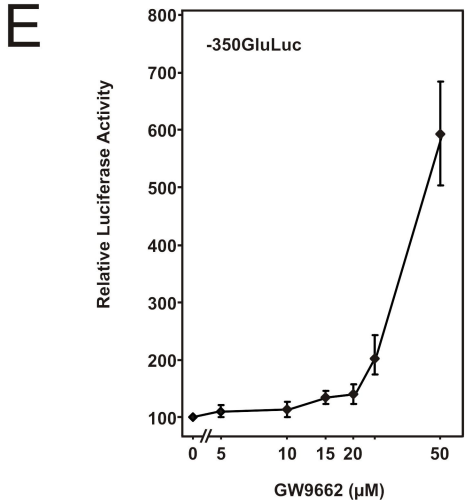

E
-350GluLuc
Relative Luciferase Activity
800
700
600
500
400
300
200
100
0
5
10
15
20
50
GW9662 (μM)

Fig. 3A

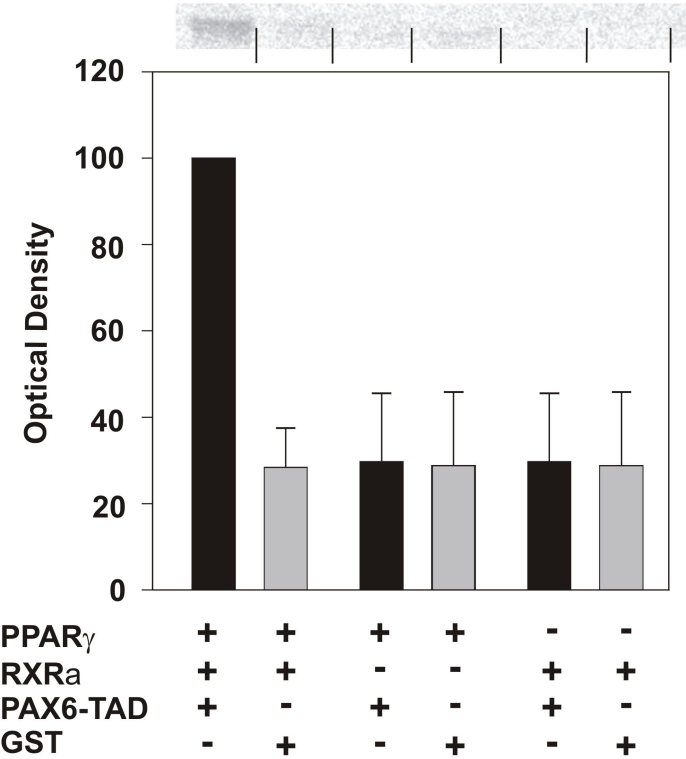


Fig. 3B

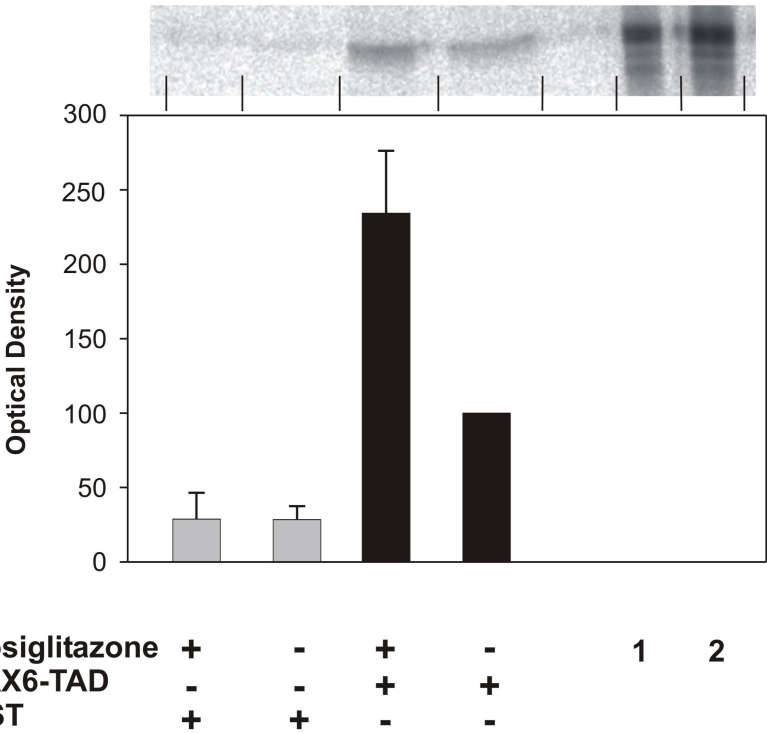
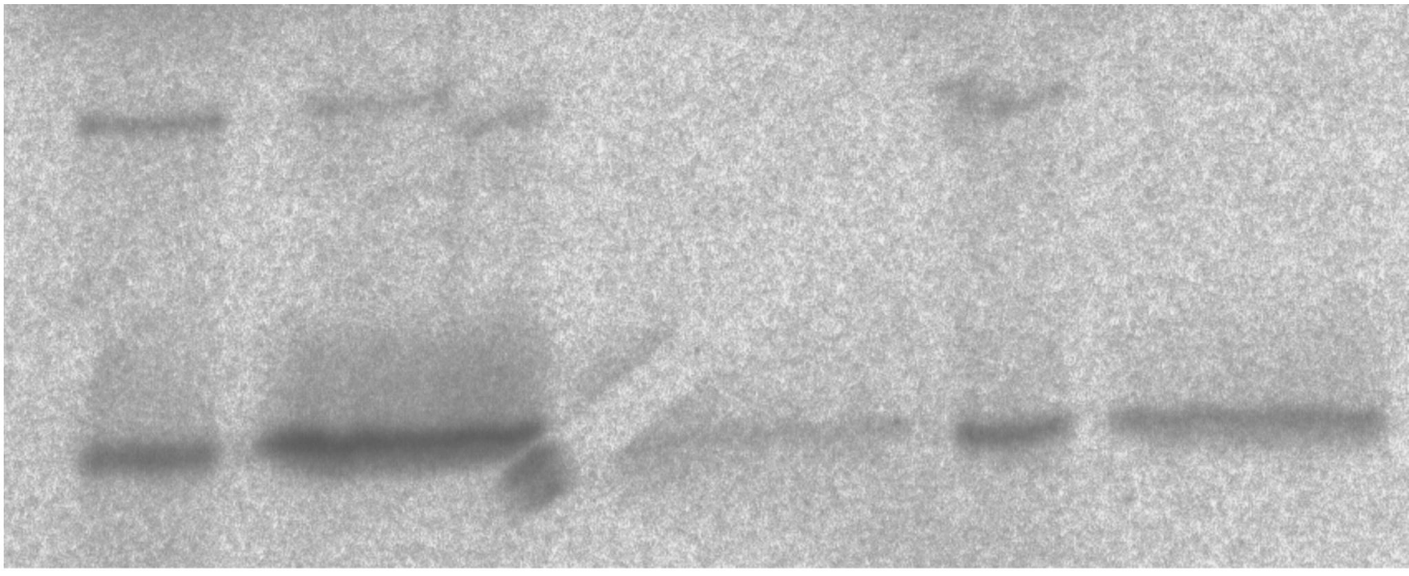

300
250
200
150
100
50
0
Optical Density
Rosiglitazone + - + - 1 2
PAX6-TAD - - + +
GST + + - -

Fig. 3C

Rosiglitazone	+	+	+	+	-	-	**1**	**2**	**3**
PPARγ	+	+	+	+	+	+			
RXRa	+	+	-	-	-	-			
PAX6-TAD	+	-	+	-	+	-			
GST	-	+	-	+	-	+			

Fig. 3D



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

Fig. 4

A


Positive Control
IgG Control
Anti-PPARγ

B

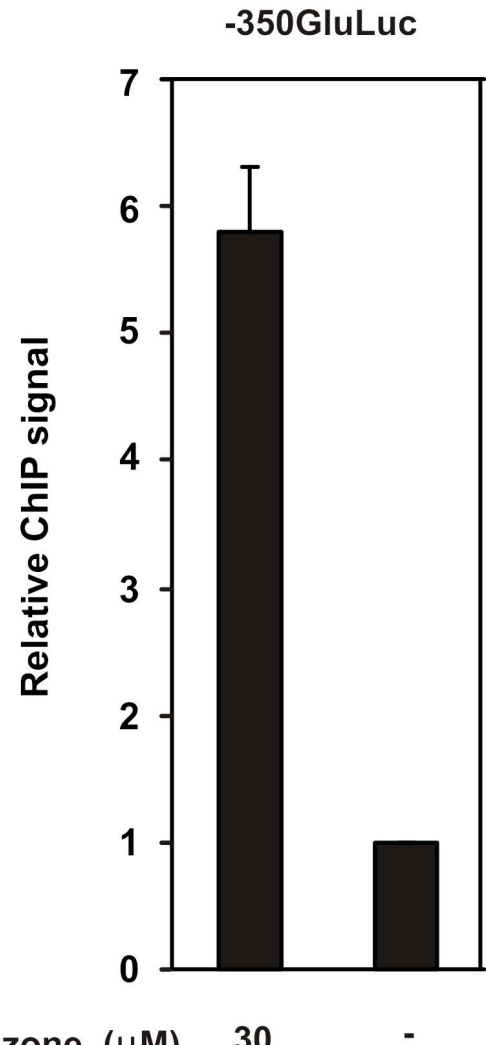

-350GluLuc
Relative ChIP signal
0
1
2
3
4
5
6
7
Rosiglitazone (μM)
30
-

Fig. 5