Bifunctional Properties of Peroxisome Proliferator–Activated Receptor γ1 in KDR Gene Regulation Mediated via Interaction With Both Sp1 and Sp3

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Vascular endothelial growth factor receptor 2 (KDR) plays a critical role in mediating a variety of vasculogenic and angiogenic processes, including diabetic retinopathy. We previously demonstrated that the promoter activity of the KDR gene in retinal capillary endothelial cells (RCECs) was regulated in part by the relative concentration of positive/negative transcription factors Sp1/Sp3. We also reported that the peroxisome proliferator–activated receptor (PPAR)γ ligand could inhibit intraocular angiogenesis. In the present study, the role of PPARγ1 in KDR gene regulation in RCECs was examined. PPARγ1 protein physically interacted with both Sp1 and Sp3. Transactivation and electrophoretic mobility shift assays clearly demonstrated novel findings that PPARγ1 increased KDR promoter activity by enhancing the interaction between Sp1, but not Sp3, and KDR promoter region without its ligand in RCECs. The ligand-binding site but not the DNA binding site of PPARγ1 enhanced the interaction between Sp1 and KDR promoter region. Conversely, PPARγ1 ligand 15-deoxy Δ (12,14)-prostaglandin J2 dose-dependently suppressed the binding of KDR promoter region with both Sp1 and Sp3, resulting an inhibition of KDR gene expression. In conclusion, PPARγ1 has bifunctional properties in the regulation of KDR gene expression mediated via interaction with both Sp1 and Sp3. *Diabetes* 53:1222-1229, 2004

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15-d PGJ2, 15-deoxy Δ (12,14)-prostaglandin J2; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; PPAR, peroxisome proliferator–activated receptor; RCEC, retinal capillary endothelial cell; VEGF, vascular endothelial growth factor.

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Among many factors promoting angiogenesis, vascular endothelial growth factor (VEGF) is known to be a key regulator of intraocular angiogenic diseases such as diabetic retinopathy, age-related macular degeneration, and retinopathy of prematurity (1). VEGF acts as an angiogenic and permeability factor through the interaction with its receptors, VEGFR1 (flt-1), VEGFR2 (KDR/flk-1), and VEGFR3 (2–5). Most of the angiogenic activity of the VEGFs is attributable to VEGF-A (6), which binds to both VEGFR1 and VEGFR2. In contrast, VEGFR3 binds to VEGF-C and -D, which are primarily thought to be lymphangiogenic mediators (5). All of these receptors possess seven extracellular immunoglobulin-like domains and an intracellular tyrosine kinase region containing a kinase insert (3,5,7). In particular, KDR is thought to be mostly concerned with embryonic, neonatal, and pathological angiogenesis (6). Endothelial and hematopoietic cells share a common progenitor (hemangioblast), which expresses KDR very early during development (8–10). KDR mediates angioblast differentiation (11), whereas flt-1 suppresses hemangioblast commitment (12). Such precursors have been identified in bone marrow, peripheral blood, colonized angiogenic sites, and vascular prostheses in the adult (13,14). These attributes make KDR a therapeutic target to regulate angiogenesis during pathological conditions because of its importance as a mediator of the "angiogenic switch."

We previously demonstrated (15) that specific nuclear protein binding in the KDR to −79/−68 with five critical bases between −74 and −70 is important in mediating transcriptional regulation in bovine retinal capillary endothelial cells (RCECs). Moreover, we have demonstrated that Sp1 binding alters both KDR promoter activity and KDR expression using the 5′-flanking region of human KDR gene reporter assay and Sp1 *cis* element "decoy." Additionally, we have demonstrated that endothelial-selective KDR promoter activity might be partially regulated by alterations in the Sp1-to-Sp3 ratio because Sp1-mediated promoter activation was attenuated by Sp3.

Peroxisome proliferator–activated receptors (PPARs) are members of the steroid receptor superfamily (16,17). Three subtypes of PPARs, α, β (δ), and γ, have been

identified and cloned. Like other members of this superfamily, PPARs mediate transcriptional regulation through their central DNA-binding domain, which recognizes response elements (peroxisome proliferator response elements) in the promoters of specific target genes (18,19). Recent evidences in our laboratory and by others (20,21) reveal that PPARγ ligands are capable of inhibiting angiogenesis both in vitro and in vivo. Using real-time quantitative RT-PCR, it was shown that the PPAR ligand 15-deoxy Δ (12,14)-prostaglandin J2 (15-d PGF2) suppressed KDR gene expression in VEGF-stimulated human umbilical vein endothelial cells grown in three-dimensional collagen gels (20). However, it has not been documented that PPARγ by itself or its ligands actually affect either KDR promoter activity or KDR expression directly. Additionally, there is no consensus peroxisome proliferator response element in the KDR gene promoter at least up to −900 bp. In this study, we evaluated the possibility that PPAR activator–dependent KDR downregulation is mediated by ligand-activated PPAR interaction with other transcriptional factors. We detected a potent and novel inhibitory activity of PPARγ ligands (15-d PGF2 or insulin-sensitizing thiazolidinedione pioglitazone) on KDR gene and protein expression in RCECs mediated by the suppression of both DNA-Sp1 and -Sp3 binding. PPARγ1 was also shown to physically interact with Sp1 and Sp3. The 15-d PGF2 dose-dependently suppressed Sp1- and Sp3-PPARγ interaction. PPARγ1 protein increased KDR promoter activity by the enhancement of the Sp1-KDR promoter region binding in the absence of its ligands. The ligand-binding site (but not the DNA-binding site) of PPARγ1 appeared to be responsible for enhanced Sp1-KDR promoter region binding.

RESEARCH DESIGN AND METHODS

The 15-d PGF2, Wy14643, and rabbit polyclonal antibody against PPARγ were purchased from Cayman Chemical (Ann Arbor, MI). Pioglitazone was provided by Takeda Chemical Industries (Osaka, Japan). Pioglitazone and 15-d PGF2 were dissolved in DMSO. [α-^{32}P]dCTP and [γ-^{32}P]dATP were purchased from Amersham (Piscataway, NJ). Rabbit polyclonal antibody against Sp1, 2, 3, 4, nuclear factor-κB p65, and PPARγ for supershift assay were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat polyclonal antibody against Sp1 (PEP2), rabbit polyclonal antibodies against Sp3 (D-20), KDR (C-1158), and consensus oligonucleotide of Sp1 were also purchased from Santa Cruz Biotechnology.

Cell cultures. Primary cultures of bovine RCECs were isolated as previously described (15). RCECs were cultured on type I collagen–coated dishes (Iwaki, Chiba, Japan) in endothelial growth medium (Clonetics, San Diego, CA) at 37°C in 5% CO_2, 95% air. RCECs of all experiments were incubated for 6 h in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Life Technologies) before stimulation.

Plasmids. The luciferase reporter vector inserted into the human KDR promoter region used in this work was previously described (17). Plasmid pSV40gal (Promega, Madison, WI) contains the β-galactosidase gene driven by the SV40 promoter and enhancer. For expression plasmid, full-length cDNA fragments of bovine PPARγ2 were isolated from bovine fat cDNA library (22) using a mouse cDNA fragment amplified by RT-PCR as a probe and cloned into the pGEX-3X (Amersham Pharmacia, Piscataway, NJ). Oligonucleotides used for the mouse cDNA probe are the following: mouse PPARγ2, sense 5′-GCGAGGGCGATCTTGACAGGAA-3′ (nt 820–841) and antisense 5′-GTGC AATCAATAGAAGGAACACG-3′ (nt 1,604–1,582).

PPARγ1 cDNA were constructed from PPARγ2 cDNA and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and pGEX-3X. GST-PPARγ1 fusion proteins containing partial deletions of the receptor were constructed using the *Bam*HI restriction enzyme–recognition site and cloned into pGEX-3X. PPARγ2 cDNA was cloned into pcDNA3.1 in reverse (reverse PPARγ2). The human Sp1 and Sp3 cDNA expression vectors used in this work have been described previously (23).

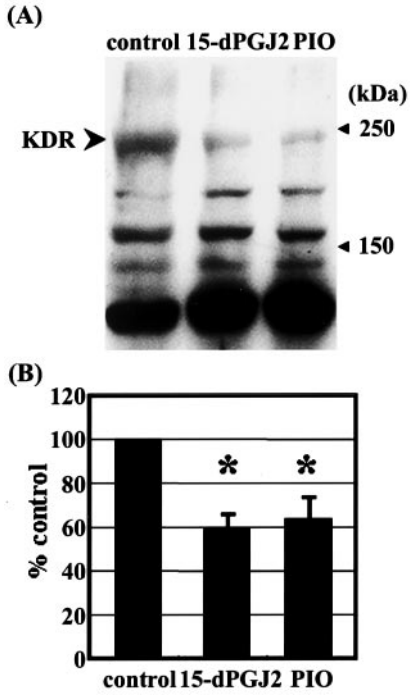

FIG. 1. Suppression of KDR protein expression by PPARγ ligands. Whole-cell lysate extracted from RCECs treated with 10 μmol/l 15-d PGF2 or 20 μmol/l pioglitazone for 24 h were subjected to Western blotting. The lower graph represents KDR protein expression as the percentage of control for three experiments (mean ± SD). *$P < 0.05$ vs. control.

Transfection and luciferase assay. Plasmid DNA was introduced into RCECs with the Lipofect Amine reagent (Invitrogen) as instructed by the manufacturer. The appropriate luciferase reporter construct (1.2 μg) was always cotransfected with 0.8 μg of pSV40gal to normalize the transfection efficiency in the 1.5–3.0 × 10^5 cells used. Cells were harvested 48 h after transfection, and luciferase activity was measured using the luciferase assay system (Promega, San Luis Obispo, CA). Galactosidase activity was assayed as described previously (15). For each transfection, luciferase activity was divided by galactosidase activity to obtain normalized luciferase activity. Results were normalized to the activity of −101/296-luc in the absence of a ligand.

Northern blot analysis. Total RNA samples were isolated from cells using the acid guanidinium thiocyanate-phenol-chloroform–extraction method and subjected to Northern blot analysis as described previously (15). mRNA levels were quantified by densitometry with a Fujix BAS 2500 bioimage analyzer (Fuji, Tokyo, Japan).

MTT cell viability assays. Confluent RCECs were incubated in DMEM with 10% serum for 48 h and then treated with 15-d PGF2 or pioglitazone. After 24 h of stimulation, the MTT assay (Chemicon, Temecula, CA) was performed per the manufacturer's instructions.

Preparation of protein samples and Western blotting. Whole-cell lysates or cytosolic or nuclear extracts were isolated from RCECs as previously described and subjected directly to Western blotting or after immunoprecipitation (15). Protein samples were separated by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes. After blocking with skim milk, the blots were incubated overnight at 4°C with antibodies against Sp3 (1:1,000), PPARγ (1:500), or KDR (1:500). After washing, membranes were incubated with horseradish peroxidase–labeled second antibodies (BioRad, Hercules, CA) (1:3,000) for 1 h at room temperature. Visualization was performed using the Amersham enhanced chemiluminescence detection system per the manufacturer's instructions.

Electrophoretic mobility shift assay. In vitro transcription/translation of human Sp1 and Sp3 cDNA clones were performed using the TNT kit (Promega, San Luis Obispo, CA). The unprogrammed reticlocyte was also simultaneously generated. For the electrophoretic mobility shift assay (EMSA), 3 μg of nuclear protein or each volume of in vitro transcription/translation of human Sp1 or Sp3 was incubated with radioactively labeled oligonucleotide equal to 10^5 cpm in binding buffer (20 mmol/l Tris, pH 7.5, 50

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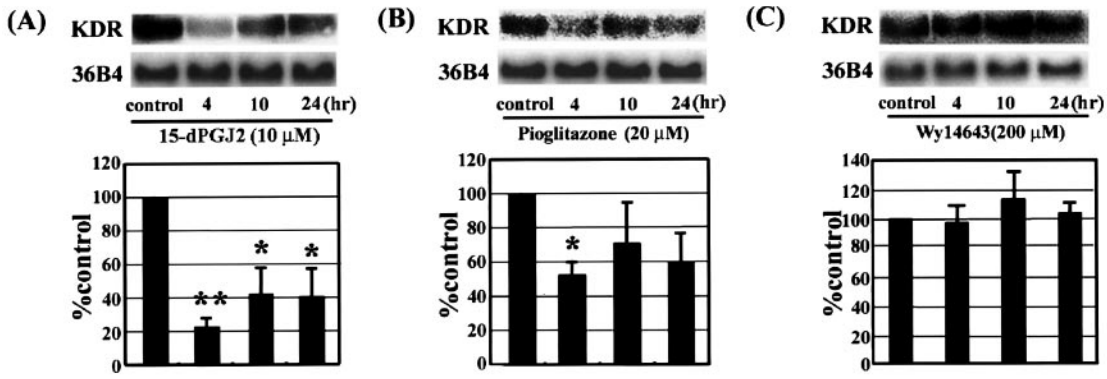

FIG. 2. Suppression of KDR mRNA expression by PPARγ ligands. Total RNAs extracted from RCECs treated with 10 μmol/l 15-d PGF2 (*A*), 20 μmol/l pioglitazone (*B*), or 200 μmol/l Wy14643 (*C*) for the indicated time were subjected to Northern blotting. The lower graph represents KDR mRNA expression normalized to 36B4 (mean ± SD) (n = 4). *P < 0.05, **P < 0.01 compared with the control value.

mmol/l KCl, 1 mmol/l $MgCl_2$, 0.01% Triton X-100, 5% glycerol, and 1 mmol/l DTT) and 2 μg poly(dI/dC) (Boehringer Mannheim, Mannheim, Germany), giving a total volume of 15 μl, for 30 min at room temperature. The sequence of the probe used was the KDR promoter region −85/−56 (15). The protein DNA complex was resolved by native 5% PAGE in 0.5× Tris-borate EDTA buffer. The gel was dried and exposed to imaging plates and analyzed with a Fujix BAS 2500 bioimage analyzer.

Glutathione S-transferase pull-down assay. Full-length glutathione S-transferase (GST)-PPARγ1 fusion protein was synthesized from pGEX-PPARγ1 using the GST Gene Fusion system (Amersham). The proteins were loaded onto glutathione-Sepharose beads, which were washed and resuspended in binding buffer (20 mmol/l HEPES, pH 7.7, 75 mmol/l KCl, 0.1 mmol/l EDTA, 2.5 mmol/l $MgCl_2$, 0.05% Nonidet P-40, 2 mmol/l dithiothreitol, and 10% glycerol) in the presence or absence of 10 μmol/l 15-d PGF2. The beads were incubated with 5 μl of in vitro translated ^{35}S-labeled Sp1 or nuclear extract (400 μl) for 3 h at 4°C in the presence or absence of 1 or 10 μmol/l 15-d PGF2, followed by washing six times with binding buffer in the presence or absence of 1 or 10 μmol/l 15-d PGF2. They were then resuspended in 30 μl of SDS sample buffer and analyzed by SDS-PAGE.

Statistical analysis. The experimental data are expressed as means ± SD. Statistical significance was assumed when was $P < 0.05$ using the Student's t test in normally distributed populations.

RESULTS

PPARγ ligands downregulate KDR protein expression. To examine the effect of PPARγ ligands on KDR protein expression, Western blotting was performed. RCECs were cultured for 24 h with 15-d PGF2 at the concentration of 10 μmol/l or with pioglitazone at a concentration of 20 μmol/l. Both 15-d PGF2 (40%, P = 0.019) and pioglitazone (36%, P = 0.023) significantly reduced KDR protein expression as compared with control (Fig. 1).

PPAR ligands downregulate KDR mRNA. We have previously shown that the 5.7-kb KDR mRNA is expressed constitutively in RCECs (15). Treatment of RCECs with 15-d PGF2 (10 μmol/l) resulted in a decrease in the message level that was evident after 4 h and persisted for up to 24 h. mRNA levels reached 23% (P = 0.004) of 0-h values for KDR after 4 h of treatment (Fig. 2*A*). Both PPARγ and PPARα are expressed in RCECs, as we have previously reported (21). To examine whether the downregulation of KDR by 15-d PGF2 is mediated through PPARγ, we determined the effect of pioglitazone, a PPARγ ligand, and Wy14643, a PPARα ligand, on KDR mRNA expression. Pioglitazone (20 μmol/l) suppressed the KDR mRNA expression after 4 h of treatment (Fig. 2*B*), whereas Wy14643 (200 μmol/l) did not affect the expression of KDR mRNA (Fig. 2*C*). Because PPARγ ligands were reported (24) to have an apoptotic effect in endothelial cells, we also evaluated the viability of RCECs with MTT assay. Treatments of RCECs with 15-d PGF2 (10 μmol/l) or pioglitazone (20 μmol/l) for 24 h did not show any statistically significant changes in cell viability as compared with control (percentage of viable cells: control, 100%; 15-d PGF2, 97 ± 3.3; and pioglitazone, 96 ± 3.5; n = 6) (online appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]).

Effect of PPARγ ligands on KDR gene promoter activity. Using luciferase reporter constructs created from human KDR promoter regions −101/296, both 15-d PGF2 (40% reduction, $P < 0.05$) and pioglitazone (40% reduction, $P < 0.05$) attenuated KDR promoter activity as shown in Fig. 3. In contrast, Wy14643, a PPARα ligand, did not inhibit KDR promoter activity.

PPARγ ligands reduced Sp1/Sp3-KDR promoter binding. To determine whether 15-d PGF2 could affect KDR promoter binding affinity, EMSA was performed using

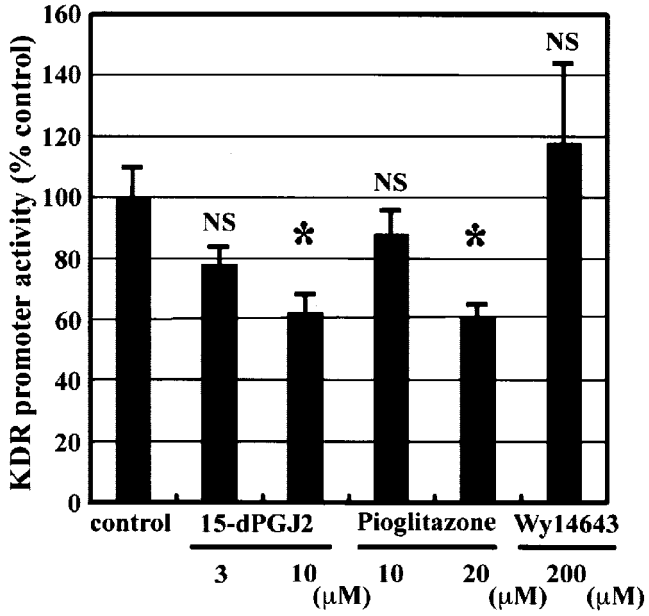

FIG. 3. Transcriptional suppression of KDR gene by PPARγ ligands. RCECs transfected with −101/296-luc construct were treated with the indicated concentrations of 15-d PGF2, pioglitazone, or Wy14643 for 24 h and harvested for measuring luciferase activities. Bar graphs represent the mean ± SD of luciferase activity (n = 3). *P < 0.05 compared with the control value.

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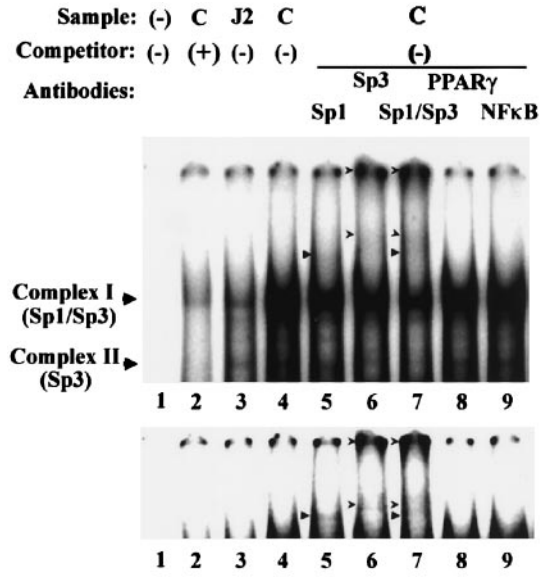

FIG. 4. Sp1/Sp3 interaction with KDR promoter. Radioactively labeled −85/−64 oligonucleotides (the probe) (*lanes 1–9*), or 100× Sp1 consensus oligonucleotide (*lane 2*) were incubated with 3 μg of the nuclear extracts of RCECs treated with or without PPARγ ligand and were sequentially incubated with 1 μl of the indicated antibodies. Protein-DNA complexes are indicated by arrows (complexes I and II). Supershift bands: Sp1 (▶); Sp3 (›).

nuclear extract from RCECs and radiolabeled human KDR promoter region (−85/−64) containing Sp1 and nuclear factor-κB binding sites as a probe (Fig. 4). Specific DNA-protein binding complexes were evident as a major slower migrating band (complex I) and a faster migrating band (complex II) in control (Fig. 4, *lane 4*). These bands were competed by 100-fold molar excess of unlabeled Sp1 consensus oligonucleotides (Fig. 4, *lane 2*). This result suggested that these bands were DNA Sp family–binding complexes. Supershift assay using Sp1 or Sp3 antibodies suggested that both Sp1 and Sp3 comprise complex I, whereas complex II is comprised of only Sp3 (Fig. 4, *lanes 5–7*). In addition, the nuclear factor-κB and PPARγ antibodies could not supershift any bands (Fig. 4, *lanes 8–9*). One-hour treatment with 15-d PGF2 reduced both complex I and II (Fig. 4, *lane 3*). Moreover, incubation with anti-Sp2 and anti-Sp4 antibodies did not affect either complex, and no new bands were observed (data not shown).

Interaction between PPARγ1 and Sp1/Sp3 proteins in vitro. We next explored whether PPARγ1 and Sp1 could directly interact. Figure 5*A* shows the results of immunoprecipitation using nuclear extract. PPARγ1 was coprecipitated with anti-Sp1 antibody in the absence of 15-d PGF2, suggesting a physical interaction between PPARγ1 and Sp1. This interaction was reduced in the presence of 15-d PGF2 (Fig. 5*A*). The immobilized PPARγ1-GST fusion protein bound in vitro translated Sp1 protein (Fig. 5*C*). The 15-d PGF2 dose-dependently suppressed the physical interaction between PPARγ1 and Sp1 (Fig. 5*C*) (60% reduction with 10 μmol/l 15-d PGF2; $P < 0.05$). Virtually identical results were obtained using Sp3 (Fig. 5*B* and *D*).

PPARγ1 overexpression enhances KDR promoter activity. Using luciferase reporter constructs created from human KDR promoter regions −101/296, we transfected either PPARγ1 expression vector, reverse PPARγ2 expression vector, or empty vector into RCECs and examined the effect of PPARγ1 on KDR promoter activity. PPARγ1 overexpression enhanced KDR promoter activity (36% increase; $P = 0.026$), whereas reverse PPARγ2 overexpression did not (Fig. 6).

PPARγ1 protein enhances complex I formation. To determine whether PPARγ1 by itself could affect Sp family KDR promoter region binding affinity, EMSA was performed using nuclear extract from RCECs with or without bacterially expressed GST-PPARγ1 fusion protein. The GST-PPARγ1 protein dose-dependently enhanced complex I formation (Fig. 7*A*).

PPARγ1 enhances Sp1- but not Sp3-binding to the KDR promoter. To determine which Sp family protein (Sp1 or Sp3) KDR promoter region binding complex was enhanced by the GST-PPARγ1 fusion protein, we performed EMSA using either in vitro translated Sp1 or Sp3 with GST-PPARγ1 fusion protein (Fig. 7*B–D*). Both in vitro translated Sp1 and Sp3 could bind the KDR promoter region (−81/−64). Intensity of the retarded band on EMSA was directly proportional to the concentration of Sp1 in

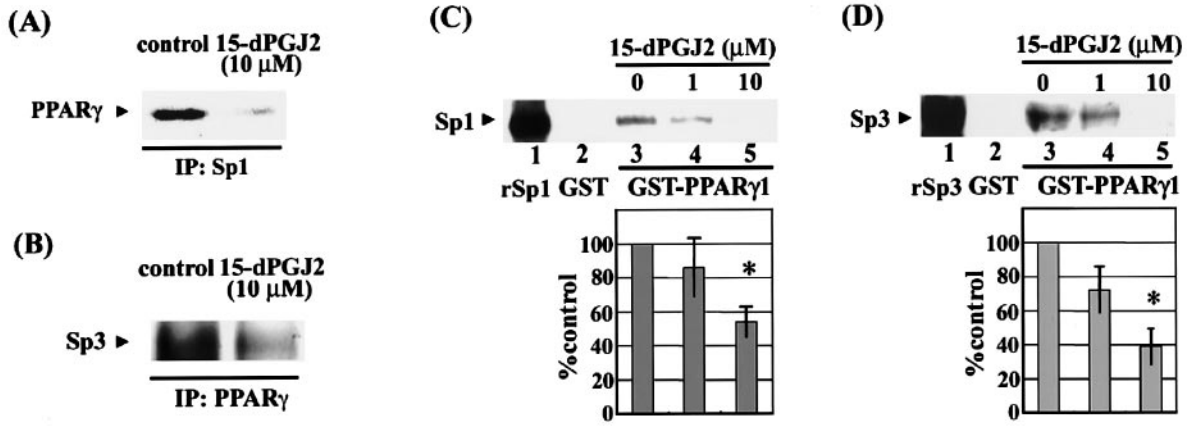

FIG. 5. Direct interaction of PPARγ1 and Sp1 or Sp3. *A* and *B*: Nuclear extracts from RCECs untreated or treated with 15-d PGF2 for 1 h were incubated with the indicated antibody (1 μg). Proteins were precipitated and analyzed by Western blotting. *C* and *D*: ^{35}S-labeled Sp1 proteins (5 μl) (*C*) or nuclear extracts (400 μl) (*D*) were incubated with GST or GST-PPARγ immobilized on glutathione-Sepharose beads. The proteins were subjected to SDS-PAGE followed by autoradiography (*C*) or Western blotting using anti-Sp3 antibody (*D*). *Lane 1*, 10% volume of the total mixture; *lane 2*, proteins bound to GST; *lanes 3–5*, proteins bound to GST-PPARγ with the indicated concentration of 15-d PGF2. Bar graphs represent the mean ± SD as a percentage of control ($n = 3$). *$P < 0.05$ compared with control.

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the presence of a small amount of reticulocyte lysate solution (Fig. 7*B*, *lanes 1–4*). Sp1-DNA binding was enhanced by GST-PPARγ1 fusion protein (Fig. 7*B*, *lanes 1–4*, *6–9*). In fact, GST PPARγ1 could enhance Sp1-KDR promoter region binding dose dependently (Fig. 7*C*, *lanes 1–3*), but could not enhance Sp3-KDR promoter region binding (Fig. 7*D*, *lanes 1–4*).

The COOH-terminus of PPARγ1 enhances Sp1-KDR promoter region (−85/−64) binding. To delineate the PPARγ1 domains required for enhancement of Sp1-KDR promoter region binding, we constructed GST-PPARγ1 fusion proteins containing partial deletions of the receptor (Fig. 8*A*) and used them in EMSA (Fig. 8*B*). GST fusion protein comprising either the COOH-terminus of PPARγ1 (454–1,329; ligand-binding domain L) or the whole PPARγ1 molecule (D/L) enhanced Sp1-KDR promoter region binding. In contrast, the effect of the NH_2-terminus of PPARγ1 (1–453; DNA-binding domain D) on Sp1-KDR promoter region binding was not apparent. To determine which domains of PPARγ1 interact with Sp1, we performed pull-down assays with nuclear extract and GST fusion proteins containing PPARγ1 or partial PPARγ1 deletions. Immobilized GST fusion proteins comprising the COOH-terminus of PPARγ1 (L) bound to Sp1. In contrast, Sp1 scarcely interacted with the NH_2-terminus of PPARγ1 (D) or GST alone (Fig. 8*C*). The 15-d PGF2 inhibited the interaction between Sp1 and L but not between D.

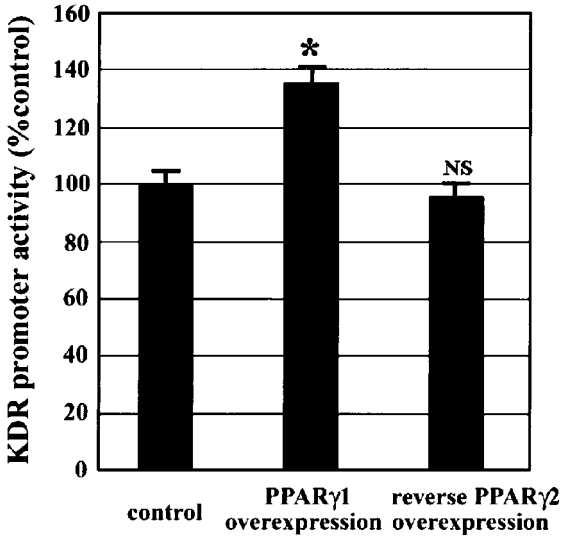

FIG. 6. PPARγ1 overexpression enhances KDR promoter activity. RCECs cotransfected with −101/296-luc and either PPARγ1 (1 μg), reverse PPARγ2 expression vector (1 μg), or empty vector (1 μg) were incubated for 24 h and harvested for measuring luciferase activity. The bar graph represents the means ± SD of luciferase activity (n = 3). *P < 0.05 compared with control.

DISCUSSION

In the present study, we demonstrated that PPARγ ligands reduced the expression of KDR in cultured RCECs through the suppression of both Sp1- and Sp3-DNA binding activity. Additionally, we demonstrated that Sp1-KDR promoter region (−85 to −64) binding was enhanced by PPARγ1 itself, whereas Sp3-KDR promoter binding was not. Moreover, we confirmed that this property is distributed over the COOH-terminal region of PPARγ1, which contains a ligand-binding domain.

Mechanisms for antiangiogenetic property of PPARγ ligands. We previously demonstrated (21) that PPARγ ligands could inhibit intraocular angiogenesis, whereas the detailed mechanisms of this effect have not been clearly determined. Recent studies (25,26) revealed that PPARγ ligands upregulated VEGF gene expression in several cell types including smooth muscle cells and monocytes/macrophages. We also examined the effect of PPARγ ligands on VEGF gene expression in RCECs. Treatment of RCECs with pioglitazone (20 μmol/l) resulted in an increase of VEGF mRNA that was evident after 10 h, whereas 15-d PGF2 (10 μmol/l) did not affect up to 24 h (data not shown). Although VEGF is known to play a prominent role in the regulation of ocular and tumor angiogenesis, it is possible that decreased KDR expression might be one of the key effects of PPARγ ligands as an antiangiogenic agent because KDR predominantly mediates the angiogenic effect of VEGF.

Possible mechanisms of KDR gene suppression by PPAR ligands

PPARγ1 enhances Sp1-KDR promoter region binding. PPARγ1 protein might have bifunctional properties in KDR gene regulation. PPARγ1 by itself can enhance Sp1-DNA binding in the absence of its ligands, whereas PPARγ1 suppresses Sp1-DNA binding in the presence of its ligands. The interaction between PPARγ1 and either Sp1 or Sp3 might be mediated by Sp family DNA binding since 15-d PGF2, which could suppress the interaction between PPARγ1 and Sp1/Sp3, also inhibited both Sp1- and Sp3-DNA binding (Figs. 4 and 5*A–D*). In vitro translated Sp1-KDR promoter region binding was specifically enhanced by PPARγ1 by itself (Fig. 7*B*). This result was in agreement with other studies (27,28), in which nuclear receptors including retinoic acid receptor and vitamin D3 receptor enhanced Sp1 binding to its cognate DNA sequence. In addition, PPARγ1 might effectively associate with Sp1 and this interaction could promote Sp1-DNA binding formation. In contrast, PPARγ1 could not enhance Sp3-KDR promoter region binding. Although this differentiation between Sp1 and Sp3 is not explained from the present study, PPARγ1 might promote KDR gene expression in RCECs by alterations in the Sp1-to-Sp3 ratio (15).

The enhanced effect of PPARγ1 is located within its COOH-terminal ligand-binding domain. Our results demonstrated that the ligand-binding site of PPARγ1, which is thought to be essential for its interaction with Sp1, could enhance Sp1-DNA binding. This suggests that the ligand-binding domain of PPARγ1 cannot interact with Sp1 while occupied by its ligands. Ligand binding leads to a significant conformational change within the ligand-binding domain, which results in the creation of a recognition surface for transcription cofactors (29,30). A chain of these events within the ligand-binding domain might suppress the interaction between PPARγ1 and Sp1. Reduced interaction between PPARγ1 and Sp1 also inhibited Sp1-DNA binding.

PPARγ1 might indirectly enhance Sp1-DNA binding. We could not detect a supershifted complex using a PPARγ-specific antibody on nuclear extracts (Fig. 4), suggesting that PPARγ1 is neither directly bound to this region of the KDR promoter nor tightly bound to either

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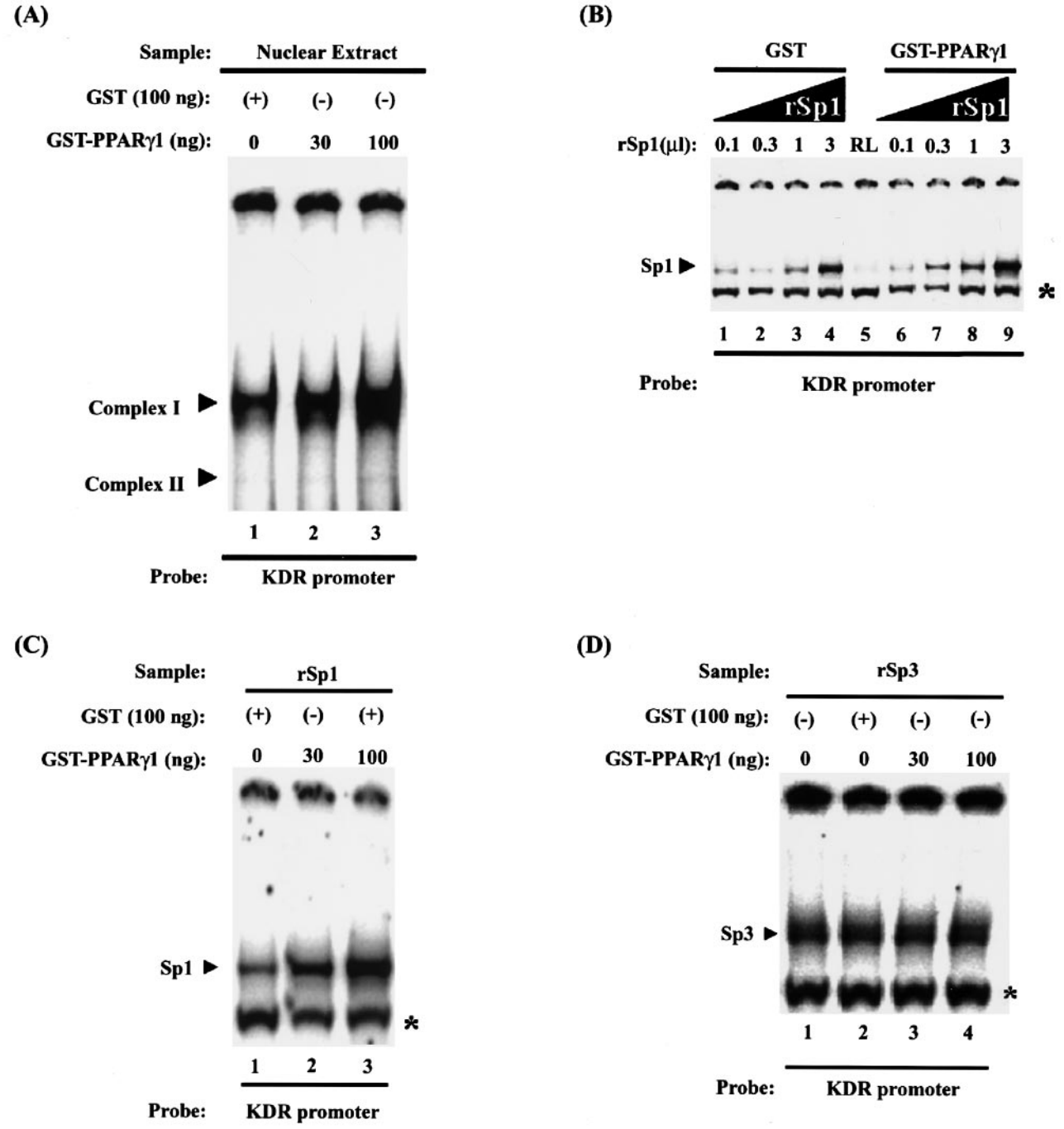

FIG. 7. PPARγ1 protein directly enhances Sp1- but not Sp3-DNA binding. *A*: The probe was incubated with 3 μg of the nuclear extracts of RCECs in the presence of either GST (*lane 1*) or PPARγ1 protein (*lanes 2–3*). Protein-DNA complexes are indicated by arrowheads (complexes I and II). *B*: The probe was incubated with rSp1 protein (recombinant Sp1: generated by in vitro translation reaction) in the absence (*lane 5*) or presence of either GST (100 ng) (*lanes 1–4*) or GST-PPARγ1 protein (100 ng) (*lanes 6–9*). Amounts of Sp1 per lane are indicated. RL, unprogrammed reticlocyte lysate solution (3 μl). *C* and *D*: The probe was incubated with either rSp1 or rSp3 protein (3 μl) in the absence (−) or in the presence of GST or GST-PPARγ1. The asterisk (*) indicates nonspecific proteins.

Sp1 or Sp3, whereas Sp1 and Sp3 are bound to the promoter. Based on our data, we suggest that PPARγ1 might act to promote Sp1-DNA association with other cofactors or through other mechanisms. One of these possible mechanisms may be the phosphorylation of Sp1 by PPARγ1 (31,32). However, we could not demonstrate that PPARγ1 by itself promoted phosphorylation of Sp1 (data not shown).

Recently, the inhibitory effect of PPARγ1 on Sp1-DNA complex by its ligands in other systems has been reported (33,34); however, PPARγ1 itself also suppressed the Sp1-DNA complex in vascular smooth muscle cells. In addition, interaction between PPARγ1 and Sp1 was enhanced by incubation with troglitazone belonging to thiazolidinediones. The reason for the discrepancy with our results is unclear. Several studies (35) showed that PPARγ ligands have ligand type-specific effects. We confirmed that pioglitazone, belonging to thiazolidinediones, also suppressed the interaction between PPARγ1 and Sp1/Sp3 in RCECs (online appendix Fig. 2). This discrepancy might

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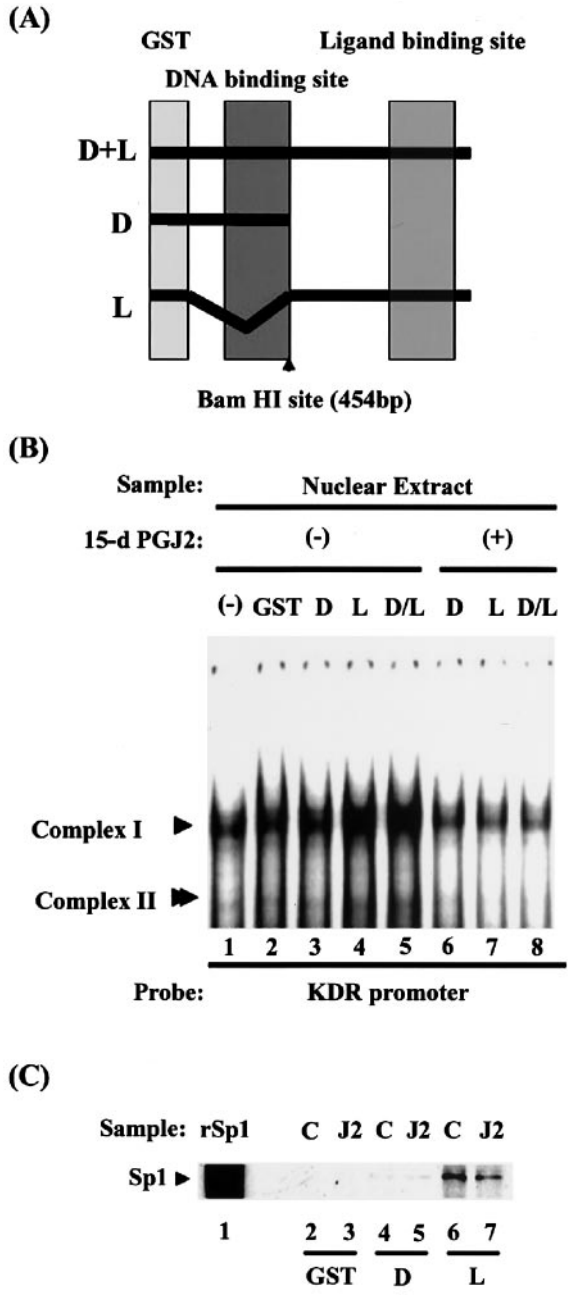

FIG. 8. PPARγ1 domains involved in the enhancement of Sp1-DNA binding and in physical interaction with Sp1 and Sp3. *A*: The restriction enzyme–recognition site used for construction is indicated. *B*: The probe was incubated with 3 μg of nuclear extracts in the absence (*lanes 1–5*) or presence (*lanes 6–8*) of 15-d PGF2 (10 μmol/l). EMSA binding reactions contained either 100 ng of GST-PPARγ1 (D/l), an aliquot of deletion mutant (D, L), GST, or no fusion protein (−). *C*: Nuclear extracts (400 μl) were incubated with GST or constructs immobilized on glutathione-Sepharose beads. *Lane 1*, 10% volume of the total mixture; *lanes 2–3*, proteins bound to GST; *lanes 4–5*, proteins bound to the NH_2-terminus of GST-PPARγ1; *lanes 6–7*, proteins bound to the COOH-terminus of GST-PPARγ1 in the absence or presence of 15-d PGF2 (10 μmol/l).

not be explained from PPARγ ligand type-specific effects. Potential explanations might include differential expression of cofactors by the different cell types evaluated.

Although more precise investigation concerning the interaction between nuclear factors is necessary, PPARγ1 enhances KDR gene expression at the transcriptional level via the enhancement of Sp1-KDR promoter binding in the absence of its ligands in RCECs. PPARγ ligands, however, suppress KDR gene expression via a decreased interaction between PPARγ and Sp1/Sp3. PPARγ1 appears to have bifunctional properties in the regulation of KDR gene expression mediated via interaction with both Sp1 and Sp3 in RCECs.

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