Dual DNA-binding specificity of peroxisome-proliferator-activated receptor γ controlled by heterodimer formation with retinoid X receptor α

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The peroxisome-proliferator-activated receptor γ (PPARγ) is a member of the steroid/thyroid nuclear receptor superfamily of ligand-activated transcription factors. PPARγ forms a heterodimer with the retinoid X receptor α (RXRα) and binds to a common consensus response element consisting of a direct repeat of two hexanucleotides spaced by one nucleotide (DR1 motif). However, other hexamer configurations for binding of PPARγ have not been considered. By using PCR-mediated random site selection, the DNA sequence preferences for PPARγ binding were examined. In this study, we have demonstrated that PPARγ has dual DNA-binding specificity; binding to both the DR1 motif and a palindromic sequence with three bases as spacers (Pal3 motif). The consensus sequence selected by equimolar amounts of PPARγ and RXRα was a perfect DR1 motif, whereas a relatively large population of Pal3 was observed when a 30-fold molar excess of PPARγ over RXRα was used. Gel-shift analysis revealed that the PPARγ homodimer could bind to Pal3 and that the affinity constant of the PPARγ homodimer for Pal3 was nearly the same as that of the PPARγ/RXRα heterodimer for DR1. The addition of RXRα decreased the binding affinity of PPARγ for Pal3, indicating that the DNA-binding specificity of PPARγ could be altered by heterodimer formation with RXRα.

Key words: direct repeat, hormone response element, nuclear receptor, palindrome, transcription factor.

INTRODUCTION

The superfamily of nuclear hormone receptors can be classified into two subgroups according to their hormone-response elements [1]. The receptors for steroid hormones such as glucocorticoid, mineralcorticoid, progesterone, androgen and oestrogen (ER) recognize palindromic DNA sequences as homodimers. On the contrary, vitamin D receptor (VDR), retinoic acid receptor (RAR), thyroid hormone receptor (TR) and peroxisome-proliferator-activated receptor (PPAR) bind to direct repeats of the hexanucleotide 5′-AGGTCA-3′ motif as heterodimers with retinoid X receptor (RXR). The specificity with respect to the nuclear receptors is dictated by the number of nucleotides between the direct repeats of the two hexanucleotide sequences [2,3].

PPAR, a member of the nuclear hormone-receptor superfamily, is a ligand-dependent transcription factor. Three isoforms of PPAR (α, γ and δ) have been described to date [4]. Among them, PPARγ is expressed predominantly in adipose tissue and is involved in the adipose differentiation process [5,6]. PPAR regulates the transcription of target genes by binding, as a heterodimer with RXR, to *cis*-acting DNA elements called PPAR response elements (PPREs), in the regulatory regions of the genes. The core motif of natural PPREs has been recognized as a direct repeat of two hexanucleotides spaced by one nucleotide (the DR1 motif) [4]. Moreover, it was reported recently that the upstream flanking sequences of the core motif influence the binding of the PPAR/RXR heterodimer. Accordingly, the consensus sequence of the natural PPREs has been identified as 5′-AACTAGGTCAAAGGTCA-3′ [7,8] (with the hexanucleotides underlined). On the other hand, systematic binding studies *in vitro* using direct-repeat elements with various spacers (0–5 bp; DR0–DR5) revealed that the PPAR/RXR heterodimer bound with highest affinity to the DR1 element and to a lesser extent to the DR0 and DR2 elements [9,10]. However, it is still not clear whether other hexamer configurations can constitute recognition sites for the PPAR/RXR heterodimer.

RXR also forms heterodimers with VDR, RAR and TR [1]. Therefore, if these receptors were present at higher concentrations in a cell, the amount of RXR might be reduced by heterodimer formation. In the present study, PCR-mediated random site selection and gel-shift analyses were performed to examine the effect of RXRα on PPARγ–DNA binding. We showed that the presence of RXRα changes the binding specificity of PPARγ. PPARγ alone bound to the Pal3 motif (a palindromic sequence with three bases as spacers) as a homodimer, whereas equimolar amounts of PPARγ and RXRα bound preferentially to the DR1 motif as a heterodimer.

MATERIALS AND METHODS

Expression of recombinant proteins

PPARγ and RXRα were expressed as FLAG-tagged fusion proteins in Sf9 cells using the Bac-to-Bac™ baculovirus expression system (Gibco-BRL). The full-length cDNAs encoding PPARγ and RXRα were inserted into the pFASTBAC1-FLAG donor plasmid. The recombinant plasmids were transformed into competent *Escherichia coli* DH10BAC cells and the

Abbreviations used: DR1 motif, direct repeat of two hexanucleotides spaced by one nucleotide; ER, oestrogen receptor; Pal3 motif, a palindromic sequence with three bases as spacers; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor.

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(A) DR1 motif

TACA AGGTCA A AGTTCA	CACA AGGTCA A AGGTCA CA	TATA AGGTCA A AGGTCA CC
CAAA GGGTCA A AGGTCA GACA	ACAG GGGTCA A AGGTCA CTG	CCCA AGGTCA A AGGTCA TGCA
CTAA AGGTCA A AGGTCA ACGA	GGAG GGGTCA A AGGTCA TGGG	TGAG GGGTCA A AGGTCA TAAG
TCTG AGGTCA A AGGTCA CTAG	GTTA AGGTCA A AGGTCA CCA	AAAG AGGTCA A AGGTCA CC
TCAA AGGTCA A AGGTCA AGAG	CGGG AGGTCA A AGGTCA TAGA	G GGGTCA A AGGTCA TGCG
TCGT AGGTGA A AGGTCA	CCAA AGGTCA A AGGTCA AAGG	ATAG GGGTCA A AGGTCA TCTT
CAAA AGGTCA A AGGTCA AAGT	GGTG AGTTCA A AGGTCA	TTAG GGGTCA A AGGTCA TGA
AGTG GGGTCA A AGGTCA CAGA	G GGGTCA A AGGTCA TGGG	GCTT GGGTCA A AGGTCA
CG GGGTCA A AGGTCA ACCT	TAG GGGTCA A AGGTCA CGCT	GAAA GGGTCA A AGGTCA A
CCAG GGGTCA A AGGTCA ACCC	TAAG GGGTCA A GGGTCA	CATA AGGTCA A AGGTCA CACA
G GGGTCA A AGGTCA C	ACGG GGGTCA A AGGTCA CCGA	G GGGTCA A AGGTCA CATA
GAAA AGGTCA A AGGTCA	CTAG GGGTCA A AGGTCA	CTTG GGGTCA A AGGTCA CAC
AGGTCA A AGTTCA TACT	TTTG GGGTCA A AGGTCA TCTA	CAG GGGTCA A AGGTCA AGTT
CGAG GGGTCA A AGGTGA	GGAG GGGTCA A AGGTCA	CCAC AGGTCA A AGGTCA CC
GGTG GGGTCA A AGGTCA TGCG	ACAG AGGTCA A AGGTCA CC	GGGTCA A AGGTCA ACAA
TCAA AGGTCA A AGGTCA ACCA	CATG GGGTCA A AGGTCA TG	ATTG GGGTCA A AGGTCG GATT
CTAG GGGTCA A AGGTCA CCGG	ACGC GGGTCA A AGGTCA AA	TCAA AGGTCA A AGGTTA TCTT
GCTG GGGTCA A AGGTCA ACTG	TGTG GGGTCA A AGGTCA CACG	TCGG AGGTCA A AGGTCA
AATG GGGTCA A AGGTCG CGCG	AGCG GGGTCA A AGGTCA ACTT	GGGT AGGTCA A AGGTCA
TTGG AGGTCA A AGGTCA TAGT	G AGGTCA A AGGTCA TCCG	ATAG GGGTCA A AGGTCA TCTA
AACG AGGTCA A AGGTCA AATA	ACGG GGGTCA A AGGTCA	TTTG GGGTCA A AGGTCA CGAT
GCAA AGGTCA A AGGTCA TCGG	CCAA AGGTCA A AGGTCA TGG	GGAG GGGTCA A AGGTCA AATT
ACAG AGGTCA A AGGTCA TTCA	G GGGTCA A AGGTCA AGTG	TTCG GGGTCA A AGGTTA TCAG
TGAA AGGTGA A AGGTCA	GTGA AGGTCA A AGGTCA CGTC	TGTA GGGTCA A AGGTCA

Position	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10
G	13	14	9	45	41	72	71	0	2	0	0	1	72	70	0	1	2	2	15	13	16
A	14	13	31	20	31	0	0	0	0	72	72	71	0	0	0	0	70	16	17	8	14
T	18	15	17	3	0	0	1	72	0	0	0	0	0	2	72	2	0	20	3	13	12
C	16	21	7	2	0	0	0	0	70	0	0	0	0	0	0	69	0	20	20	15	2
	N	N	A	G	G	G	G	T	C	A	A	A	G	G	T	C	A	A	G	N	G
				A	A													T	A		A
																		C	C		T

(B) Pal3 motif

AACT AGGTCA TGG TGACCC ACTT	TAGT AGGTCA GCG TGACCT CAAT
ATTT GGGTCA CTG TGACCT ACT	TAT AGGTCA CGG TGACCC AGTA
ACT GGGTCA CGA TGACCT AGTT	ATGT GGGTCA CGG TCACCC AGAT
AACT AGGTCA TGG TGACCC ATTT	AAGT AGGTCA CAG TGCCCT ACT
AACT AGGTCA TCG TGACCC AGT	AAAT GGGTCA CCA TGACCT AGTT
CAGT AGGTCA CGG TTACCT ACTT	AACT AGGTCA TGG TGCCCC ACTT
AGT AGGGCA CTG TGACCT ACTT	ATGT AGGTAA CAG TGGCCT ACTT
ATAT AGGTCA GAG TGACCC AGTT	AATT GGGTCA CAC TCACCT ACTT
AGT AGGGCA CTG TGACCT ACTT	AAGA AGGTGA CGT TCACCC ACTT
CAGC AGCTGA ATC TACCCT T	CAGT AGGTCA AAG TCACCT ACAT
AAGT AGGGCA CCA TGACCC AAAT	AACT GGGTCA CTC TGACCT ATAT
AAGT AGGTGA GTG TGACCC AATT	AACT AGGTCA CCG TGACCC TAGT
AAGT GGGTGA ACG TCACCT ACTT	ATGT AGGTCA ACG TAACCT ACTA
TAGT AGGTCA CGT TGACCT ACAT	T AGGTCA CAT TGACCT ACAT

Position	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10	+11
G	0	0	15	0	7	28	27	3	4	0	3	9	19	0	20	1	0	0	0	0	6	1	0
A	18	21	3	1	21	0	0	0	1	28	4	6	3	0	2	24	0	0	0	25	4	7	2
T	2	6	2	26	0	0	0	25	0	0	4	6	3	28	1	0	0	0	17	2	2	19	22
C	3	0	7	1	0	0	1	0	23	0	17	7	3	0	5	3	28	28	11	1	15	0	0
	A	A	G	T	A	G	G	T	C	A	C	N	G	T	G	A	C	C	T	A	C	T	T
																			C				

Figure 1 Sequence alignment of recovered putative PPARγ-binding sites

(**A**) The core DR1 sequences obtained by random binding-site selection, using 30 ng each of PPARγ and RXRα, are highlighted. The flanking sequences as well as the internal core sequence (shaded section) of DR1 were aligned from positions -10 to $+10$. (**B**) The core Pal3 sequences obtained by random binding-site selection using 30 ng of PPARγ and 1 ng of RXRα are highlighted. The flanking sequences as well as internal core sequence (shaded section) of Pal3 were aligned from positions -11 to $+11$.

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transformation mixture was spread evenly over the surface of Luria–Bertani agar plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), Bluo-gal (100 μg/ml) and isopropyl β-D-thiogalactoside (40 μg/ml). Plates were incubated at 37 °C for selection of recombinant bacmid. The recombinant bacmid DNAs were prepared by a modified alkaline lysis method.

Sf9 cells, a clonal isolate of the Sf21 cell line established from *Spodoptera frugiperda*, were seeded at 9×10^5 cells/35 mm well in 2 ml of Grace's Insect Medium (Gibco-BRL). For each transfection, 2.5 μl of bacmid DNA and 6 μl of CELLFECTIN reagent (Gibco-BRL) were each diluted with 100 μl of the medium, and then these two solutions were combined and incubated at room temperature for 30 min to allow lipid–DNA complexes to form, and the mixtures were overlaid on to the cells. After the cells were incubated at 27 °C for 5 h, the medium was removed and replaced with 2 ml of Grace's Insect Medium containing 10 % fetal bovine serum. After 3 days of transfection the viral supernatant was harvested and used to infect fresh insect cells for viral amplification. The supernatant was collected at 48 h post-infection, and re-infected into Sf9 cells at a multiplicity of infection of 5–10.

After 3–4 days of infection the Sf9 cells were harvested and suspended in 0.1 M HM buffer (25 mM Hepes/KOH, pH 7.9/12.5 mM $MgCl_2$/1 mM dithiothreitol/20 % glycerol/0.1 M KCl). The mixture was then sonicated for 2 min on ice with an ultrasonicator and centrifuged at 1000 ***g*** for 5 min at 4 °C. The supernatant was collected and the production of recombinant proteins was examined by Western-blot assay (results not shown).

Protein purification

The FLAG-tagged PPARγ and RXRα were affinity purified with M2 agarose (Sigma) from 24 ml of culture. The anti-FLAG M2 affinity gel suspension (0.4 ml) was washed with 0.1 M HM buffer three times and incubated with the cytosolic fraction containing the FLAG fusion proteins overnight at 4 °C. Thereafter, the mixture was washed with TBS (10 mM Tris/HCl, pH 7.5/150 mM NaCl) three times and the FLAG fusion proteins were eluted with 1–5 molar column equivalents of FLAG peptide (Sigma) in TBS.

Random binding-site selection

The sequences of the oligonucleotides used for the binding-site selection were as follows (*Bam*HI and *Sal*I restriction sites are underlined): random oligonucleotide, 5′-AGAGCCACTTC-CTCAAC<u>GGATCC</u>GTCN$_{27}$ATA<u>GTCGAC</u>ACTGTCAGTC-GTCTGAC-3′; primer 1, 5′-AGAGCCACTTCCTCAAC-<u>GGATCC</u>-3′; primer 2, 5′-GTCAGACGACTGACAGT-<u>GTCGAC</u>-3′.

The random oligonucleotide was rendered double stranded by PCR using Ampli*Taq* DNA polymerase (Perkin-Elmer) and primers 1 and 2. Enrichment for binding sites was performed by the filter-binding method [11]. The binding mixture contained purified protein (30 ng each of PPARγ and RXRα or 30 ng of PPARγ and 1 ng of RXRα) expressed by baculovirus, 5 μg/ml BSA, 0.3 μg of double-stranded random oligonucleotide, 125 mM EDTA and 3.5 μl of 10 × binding buffer (100 mM Tris/HCl, pH 7.5/50 % glycerol/10 mM dithiothreitol/10 mM EDTA). Each mixture was incubated for 30 min at room temperature. Thereafter, this solution was passed slowly through a presoaked nitrocellulose filter. The filter was washed three times with 3 ml of 1 × binding buffer before the bound oligonucleotides were eluted with 100 μl of elution buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 20 mM NaCl and 0.1 % SDS. The eluate was phenolized and then amplified by PCR using primers 1 and 2. The amplified products were put back into a binding reaction and this procedure repeated. After five rounds of enrichment, the selected oligonucleotides were digested with *Bam*HI and *Sal*I, labelled with [α-^{32}P]dCTP using Klenow DNA polymerase, and used as a probe for gel-shift analysis. The labelled amplified products were incubated with PPARγ and RXRα, and protein–DNA complexes were separated on a native polyacrylamide gel. The bound DNA was eluted with a solution of 0.5 M ammonium acetate, 1 mM EDTA, 0.1 % SDS, 10 % methanol and 50 μg/ml proteinase K. The eluate was phenolized and then amplified by PCR using primers 1 and 2. The amplified products were digested with *Bam*HI and *Sal*I and then subcloned into pBluescript KS. The clones obtained were sequenced using an ABI Prism 310 (Perkin-Elmer).

Gel-shift analysis

The sequences of the oligonucleotides for gel-shift analyses were as follows (only upper strands are shown; bases in lower case indicate the linker sequence for a *Xba*I site): Pal3, 5′-ctagTTACTAACTAGGTCACCGTGACCTAGTTCAGATC-3′; DR1, 5′-ctagTCTAGGGGTCAAAGGTCACCGGTC-3′; Pal3 fl (wild-type), 5′-ctagTTACTAAGTAGGTCACCGTGA-CCTACTTCAGATC-3′; Pal3 mut (flanking sequences mutated), 5′-ctagTTACTTGAGAGGTCACCGTGACCTCTCA-CAGATC-3′.

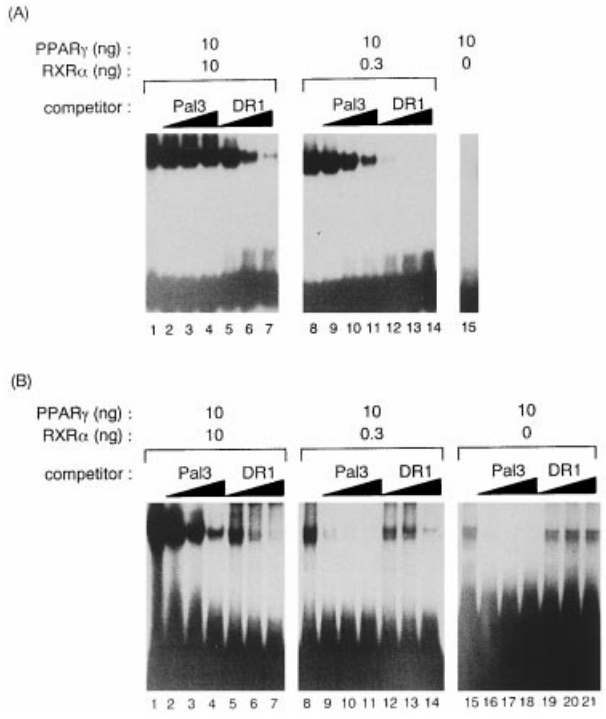

Figure 2 Effect of RXRα on DNA-binding specificity of PPARγ

Double-stranded oligonucleotide, DR1 (**A**) or Pal3 (**B**), was used as a probe for the binding analysis. The probe was incubated with PPARγ and/or RXRα. Molar excesses of 10-fold (lanes 2, 5, 9, 12 in **A**, lanes 2, 5, 9, 12, 16, 19 in **B**), 30-fold (lanes 3, 6, 10, 13 in **A**, lanes 3, 6, 10, 13, 17, 20 in **B**) or 100-fold (lanes 4, 7, 11, 14 in **A**, lanes 4, 7, 11, 14, 18, 21 in **B**) of unlabelled oligonucleotides were used for the competition analyses.

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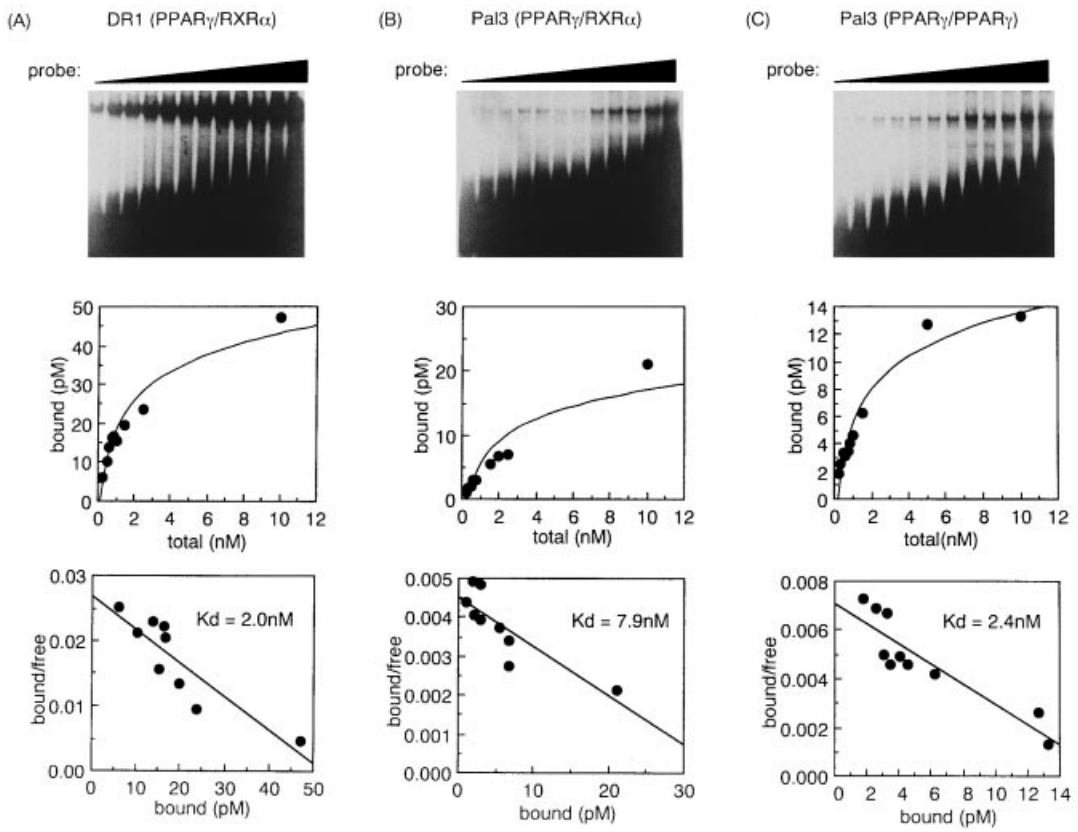

Figure 3 DNA-binding specificities of the PPARγ homodimer and the PPARγ/RXRα heterodimer

DNA-binding activities of the PPARγ/RXRα heterodimer with DR1 (**A**), the PPARγ/RXRα heterodimer with Pal3 (**B**) and the PPARγ homodimer with Pal3 (**C**) were analysed as a function of DNA concentration. (Top panels) Autoradiographic images identifying positions of bound and free probe. From left to right, each panel shows multiple lanes corresponding to binding reactions that contained a constant amount of purified proteins and increasing amounts of radiolabelled probes. (Middle panels) The radioactivities associated with free oligonucleotide and with receptor–oligonucleotide complexes were quantified directly using a Fuji Imaging Analyser. The amount of bound probe was plotted as a function of total input. (Bottom panels) Scatchard plots of saturation curves and derived dissociation constants are shown.

A protein fraction containing 30 μg of BSA, purified PPARγ and/or RXRα was mixed with the same volume of 20 mM Tris/HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 20 mM EDTA and 0.2 ng of labelled probe. The binding reaction was performed at room temperature for 30 min. Each reaction mixture was loaded on to a non-denaturing polyacrylamide gel, electrophoresed at 150 V for 1.5 h, fixed with 10% methanol/10% acetic acid and autoradiographed.

For Scatchard analyses, the gel-shift assays were performed as a function of DNA concentration by using a constant amount of protein. Following electrophoresis, gels were fixed, dried and exposed to an imaging plate. The radioactivity associated with free oligonucleotide and with receptor–oligonucleotide complexes was then quantified directly using an image analyser (BAS 2000, Fuji).

RESULTS

Identification of PPARγ-binding sequences

To clarify the effect of RXRα on the DNA-binding specificity of PPARγ, we employed the method of PCR-mediated random site selection. The PCR-amplified DNAs recovered after each round of selection were subjected to gel-shift analysis, and the enrichment of binding sites was detected (results not shown). DNAs recovered after five rounds of selection were used for gel-shift analysis, and the shifted DNA fragments were cloned into a plasmid vector and individual sequences were determined.

All oligonucleotides selected with equimolar amounts (30 ng each) of PPARγ and RXRα, 72 in total, contained the DR1 motif, but with a PPARγ/RXRα molar ratio of 30:1 the selected sequences were divided into three groups: six clones containing the DR1 motif, 28 clones containing the Pal3 motif and 25 clones containing other motifs. Other motifs were DR0 (seven clones), DR2 (three clones), DR3, Pal0, Pal4 (two clones each), DR4, DR9, Pal1, Pal10 (one clone each) and half sites (with only one hexanucleotide; five clones). Thus only 10% of the selected sequences contained the DR1 motif. These results suggest that the DNA-binding specificity was altered according to the ratio of PPARγ to RXRα.

Next, we aligned the sequences of the DR1 and Pal3 motifs recovered mainly by the random site selection under the two different sets of conditions (Figure 1). When equimolar PPARγ and RXRα were used, a clear DR1 sequence was obtained, with the A between the hexanucleotides as a consensus spacer sequence for DR1, as described previously [7,8]. However, the 5′-flanking nucleotides of DR1 were not AACT, but NNAR (Figure 1A). The 5′- and 3′-flanking nucleotides of Pal3 obtained by the 30:1 molar ratio of PPARγ/RXRα were conserved as AAGT and ACTT, respectively (underlined below). Accordingly, the con-

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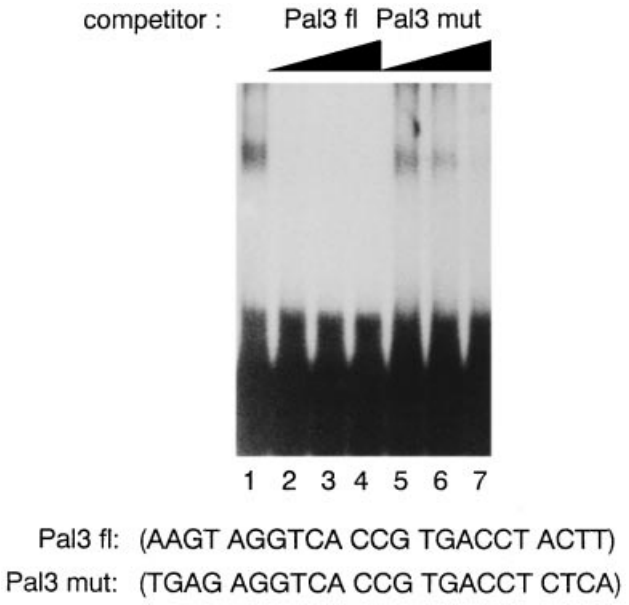

Figure 4 Analysis of the importance of the flanking nucleotides of Pal3 for binding affinity of the PPARγ homodimer

Double-stranded oligonucleotide, Pal3 fl, was used as a probe for the binding analysis. The probe was incubated with PPARγ. Molar excesses, 10-fold (lanes 2 and 5), 30-fold (lanes 3 and 6) or 100-fold (lanes 4 and 7), of unlabelled oligonucleotides were used for competition analysis.

sensus sequence was identified as AAGTAGGTCACNGTGACCYACTT for the Pal3 motif (Figure 1B).

Differences in the DNA-binding properties between the PPARγ homodimer and the PPARγ/RXRα heterodimer

To ascertain whether the DNA-binding specificity was altered according to the ratio of PPARγ and RXRα, the gel-shift analyses were performed with radiolabelled DR1 or Pal3 oligonucleotides as a probe (Figure 2). As expected, the PPARγ/RXRα heterodimer could form a complex with DR1 at the 1:1 molar ratio of PPARγ/RXRα (Figure 2A, lane 1). On the other hand, the PPARγ homodimer could not bind to DR1 (Figure 2A, lane 15). Accordingly, the DR1–protein complex was thought mainly to be the result of the formation of the PPARγ/RXRα heterodimer with the molar ratio of 30:1 (Figure 2A, lane 8). Unlabelled DR1 oligonucleotide successfully competed with the PPARγ/RXRα complex on the probe DR1 (Figure 2A, lanes 5–7 and 12–14), but the Pal3 oligonucleotide scarcely or weakly inhibited binding to the DR1 (Figure 2A, lanes 2–4 and 9–11). These results suggest that the PPARγ/RXRα heterodimer binds to the DR1 element much more efficiently than to the Pal3 element. Therefore, on the basis of the competition experiments, at the PPARγ/RXRα molar ratio of 1:1, the Pal3–protein complex was thought mainly to be the result of the formation of the PPARγ/RXRα heterodimer (Figure 2B, lanes 1–7). On the contrary, a prominent Pal3–protein complex was also formed at the 30:1 molar ratio of PPARγ/RXRα (Figure 2B, lane 8), and this binding was more strongly inhibited by Pal3 than DR1 (Figure 2B, lanes 9–14). Moreover, the PPARγ homodimer bound specifically to Pal3 (Figure 2B, lanes 15–21), suggesting that a 30-fold molar excess of PPARγ over RXRα allowed PPARγ to bind preferentially to Pal3 as a homodimer.

In order to substantiate the difference in binding properties seen in the gel-shift assay, we also determined the dissociation constants (K_d). The saturation curves and the derived Scatchard plots are shown in Figure 3. The PPARγ/RXRα heterodimer bound to DR1 with a K_d of 2.0 nM (Figure 3A) and to Pal3 with a K_d of 7.9 nM (Figure 3B). However, the dissociation constant of the PPARγ homodimer for Pal3 (2.4 nM) was nearly the same as that of the PPARγ/RXRα heterodimer for DR1. Thus the PPARγ homodimer binds to Pal3 with higher affinity than the PPARγ/RXRα heterodimer binds to Pal3.

As shown in Figure 1(B), four bases each of the 5′- and 3′-flanking nucleotides, besides the Pal3 motif, were also conserved. Therefore we next tested whether the flanking nucleotides of Pal3 are crucial for the binding of the PPARγ homodimer. Competition experiments were performed with the radiolabelled Pal3 fl (wild-type) oligonucleotide as a probe and either Pal3 fl or Pal3 mut (flanking sequences mutated) as a competitor. As shown in Figure 4, the amount of oligonucleotide required for inhibiting complex formation with the radiolabelled probe was higher for Pal3 mut than for Pal3 fl, indicating that the flanking regions increase the binding affinity of PPARγ.

DISCUSSION

It has been well established that the PPAR/RXR heterodimer binds to a common consensus response element (PPRE), which consists of a direct repeat of two hexanucleotides spaced by one nucleotide (DR1) [4]. The four nucleotides immediately 5′ of the DR1 motif are also highly conserved among known PPREs and exhibit a consensus of AACT. However, the contribution of the extended binding site is not obvious for PPAR/RXR heterodimer binding to elements containing a perfect DR1 motif, but rather it appears to facilitate the binding of the heterodimer to elements containing imperfect DR1 motifs [7,8]. Therefore, since the consensus sequence selected by equimolar PPARγ and RXRα was a perfect DR1 motif, the 5′-flanking nucleotide might not be similar to that of the native PPREs.

In this study, when PPARγ was in excess of RXRα, it was found that PPARγ bound significantly to Pal3. Steroid-hormone nuclear receptors, such as glucocorticoid receptor, mineralcorticoid receptor, progesterone receptor, androgen receptor and ER, generally recognize palindromic DNA sequences as homodimers [1]. PPARγ also could bind with high affinity to Pal3 as a homodimer. It seems that the binding affinity of the PPARγ homodimer to Pal3 is almost the same as that of the PPARγ/RXRα heterodimer to DR1. The addition of RXRα, however, decreased the binding affinity of PPARγ for Pal3. These results suggest that the PPARγ homodimer and the PPARγ/RXRα heterodimer have different DNA-binding specificities; the former binds preferentially to Pal3 and the latter to DR1.

Unlike the case of PPARγ, it was reported previously that the consensus sequence selected by the VDR/RXR heterodimer is essentially the same as that by the VDR homodimer [12]. The DNA-binding domain containing two zinc fingers is the most conserved domain among nuclear receptors. The amino acids between the first and second cysteines of the second zinc finger, the so-called D-box, are involved in contacts between dimerizing receptors [13]. Almost all nuclear receptors in the superfamily have five amino acids between the first and the second cysteines in the D-box, while PPARs have a unique D-box in which only three amino acids exist between the cysteines [4]. As a

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consequence, it is likely that the unique D-box might be responsible for the dual DNA-binding specificity of PPARγ.

Previous studies have demonstrated that ER can also bind to Pal3 [1]. Adipose tissue is a major site of oestrogen metabolism [14], and is the predominant site of oestrogen production [15]. In addition to PPARγ [5,6], ERα [16–18] and the novel ERβ [19,20] are expressed in adipocytes. Accordingly, competition for binding and functional interference between PPARγ and ER may occur in adipocytes.

In conclusion, we employed random binding-site selection and gel-shift analysis to clarify the binding specificity of PPARγ. It has been demonstrated that PPARγ can bind with high affinity to Pal3 as well as to the DR1 motif. However, it is necessary to note that this phenomenon is likely to occur only when the amounts of RXRα are limiting. Since RXRα also forms a heterodimer with VDR, RAR and TR [1], it is quite possible that free RXRα is scarcely present in cells as a result of deprivation caused by the other nuclear receptors. In such cases, the PPARγ homodimer could bind to the Pal3 motif and regulate the transcription of target genes containing Pal3 but not DR1.

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REFERENCES

1 Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995) The nuclear receptor superfamily: the second decade. Cell **83**, 835–839

2 Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell **65**, 1255–1266

3 Naar, A. M., Boutin, J. M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K. and Rosenfeld, M. G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell **65**, 1267–1279

4 Lemberger, T., Desvergne, B. and Wahli, W. (1996) Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu. Rev. Cell Dev. Biol. **12**, 335–363

5 Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell **79**, 1147–1156

6 Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I. and Spiegelman, B. M. (1994) mPPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. **8**, 1224–1234

7 Palmer, C. N. A., Hsu, M.-H., Griffin, K. J. and Johnson, E. F. (1995) Novel sequence determinants in peroxisome proliferator signaling. J. Biol. Chem. **270**, 16114–16121

8 IJpenberg, A., Jeannin, E., Wahli, W. and Desvergne, B. (1997) Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor heterodimer binding to DNA. J. Biol. Chem. **272**, 20108–20117

9 Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. and Evans, R. M. (1992) Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature (London) **358**, 771–774

10 Issemann, I., Prince, R. A., Tugwood, J. D. and Green, S. (1993) The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. Biochimie **75**, 251–256

11 Inoue, S., Kondo, S., Hashimoto, M., Kondo, T. and Muramatsu, M. (1991) Isolation of estrogen receptor-binding sites in human genomic DNA. Nucleic Acids Res. **19**, 4091–4096

12 Nishikawa, J., Kitaura, M., Matsumoto, M., Imagawa, M. and Nishihara, T. (1994) Difference and similarity of DNA sequence recognized by VDR homodimer and VDR/RXR heterodimer. Nucleic Acids Res. **22**, 2902–2907

13 Rastinejad, F., Perlmann, T., Evans, R. M. and Sigler, P. B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature (London) **375**, 203–211

14 Deslypere, J. P., Verdonck, L. and Vermeulen, A. (1985) Fat tissue: a steroid reservoir and site of steroid metabolism. J. Clin. Endocrinol. Metab. **61**, 564–570

15 Hankinson, S. E., Willett, W. C., Manson, J. E., Hunter, D. J., Colditz, G. A., Stampfer, M. J., Longcope, C. and Speizer, F. E. (1995) Alcohol, height, and adiposity in relation to estrogen and prolactin levels in postmenopausal women. J. Natl. Cancer Inst. **87**, 1297–1302

16 Pedersen, S. B., Hansen, P. S., Lund, S., Andersen, P. H., Odgaard, A. and Richelsen, B. (1996) Identification of oestrogen receptors and oestrogen receptor mRNA in human adipose tissue. Eur. J. Clin. Invest. **26**, 262–269

17 Mizutani, T., Nishikawa, Y., Adachi, H., Enomoto, T., Ikegami, H., Kurachi, H., Nomura, T. and Miyake, A. (1994) Identification of estrogen receptor in human adipose tissue and adipocytes. J. Clin. Endocrinol. Metab. **78**, 950–954

18 Price, T. M. and O'Brien, S. N. (1993) Determination of estrogen receptor messenger ribonucleic acid (mRNA) and cytochrome P450 aromatase mRNA levels in adipocytes and adipose stromal cells by competitive polymerase chain reaction amplification. J. Clin. Endocrinol. Metab. **77**, 1041–1045

19 Mosselman, S., Polman, J. and Dijkema, R. (1996) ER β: identification and characterization of a novel human estrogen receptor. FEBS Lett. **392**, 49–53

20 Crandall, D. L., Busler, D. E., Novak, T. J., Weber, R. V. and Kral, J. G. (1998) Identification of estrogen receptor β RNA in human breast and abdominal subcutaneous adipose tissue. Biochem. Biophys. Res. Commun. **248**, 523–526

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