A Carboxyl-terminal Extension of the Zinc Finger Domain Contributes to the Specificity and Polarity of Peroxisome Proliferator-activated Receptor DNA Binding

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Heterodimers of the peroxisome proliferator-activated receptors (PPAR) and the retinoid X receptors (RXR) recognize response elements (PPREs) that exhibit the consensus sequence 5′-A(A/T)CT(A/G)GGNCAAAG(G/T)TACA-3′. The consensus PPRE includes both a 5′-extension and a direct repeat (DR1) comprised of two canonical core recognition sequences (underlined) for nuclear receptor zinc fingers separated by a single nucleotide spacer. The extended binding site recognized by PPARs is very similar to sites that bind monomers of the nuclear receptors Rev-ErbA and ROR suggesting that the latter could bind to PPREs and affect gene transcription. However, Rev-ErbA and ROR bind weakly to naturally occurring PPREs relative to the consensus binding site, and significant effects on PPARα transactivation of a CYP4A6-Z reporter were not observed. In contrast, PPAR/RXR heterodimers bind to a DR2 element containing the conserved 5′-extended sequence that is recognized by dimers of RORα or Rev-ErbA. PPARα/RXRα positively regulate transcription from this element, and co-expression of Rev-ErbA blocks this effect. The nuclear receptors NGFI-B and ROR utilize a carboxyl-terminal extension (CTE) of the zinc finger DNA binding domain in their interactions with the 5′-extension of a single zinc finger-binding site. DNA binding domains (DBD) of PPARs α, δ, and γ that contain the zinc finger motif and a CTE display binding to core recognition sequences that is dependent on the 5′-extended sequence found in PPREs. Unlike DBDs of other nuclear receptors that form heterodimers with RXR, the PPAR-DBDs did not exhibit cooperative binding with the DBD of RXR and exhibit the opposite polarity for binding to the direct repeat motif. In contrast to the corresponding DBD of RXR, the PPAR-DBDs bind as monomers to a single extended binding site as well as to the consensus PPRE. A chimera linking the zinc finger domain of RXRα to the CTE from PPARα bound to a single extended binding site indicating a functional role for the CTE of PPARs in extended binding site recognition.

The peroxisome proliferator activated receptor α (PPARα) mediates the transcriptional regulation of several genes encoding enzymes involved in lipid metabolism in response to peroxisome proliferators and fatty acids. Responsive genes include the microsomal cytochrome P450 fatty acid ω-hydroxylases (1, 2), the peroxisomal fatty acyl-CoA oxidase (3, 4), the peroxisomal bifunctional enzyme (5, 6), and the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthetase (7). PPARα binds to response elements (PPREs) within the 5′-flanking regions of these genes as a heterodimer with the retinoid X receptor, RXR (8–10). PPREs contain a DR1 motif consisting of imperfect direct repeats of the nuclear receptor core recognition sequence (AGGTCA) separated by a single nucleotide (8). The repeated core recognition sequences interact directly with the amino-terminal zinc finger of each nuclear receptor (11). The four nucleotides immediately 5′ of the DR1 motif are also highly conserved among known PPREs and exhibit a consensus of A(A/T)CT (12, 13). These nucleotides are essential for the function of the principal PPRE (Z element) of the gene encoding P450 4A6, a microsomal fatty acid ω-hydroxylase (CYP4A6), and are required for the binding of PPAR/RXR heterodimers to the Z element in vitro (12). The contribution of the extended binding site is less obvious for PPAR/RXR heterodimer binding to elements containing perfect AGGTCA motifs. Rather, it appears to facilitate the binding of PPAR/RXR heterodimers to elements containing divergent core binding sites (12, 14). These imperfect DR1 motifs bind other nuclear receptors poorly. As a result, the deviations of the core sites from the consensus recognition sequence and the presence of a suitable extended binding site sequence may attenuate the extent of interference imposed upon peroxisome proliferator signaling by other signaling pathways that occur through DR1 containing response elements.

The 5′-extensions conserved in PPREs and the adjacent core site of the DR1 are similar to the extended binding sites, (A/T)A(A/T)NT(A/G)GGTCA, utilized by monomers of the nuclear receptors Rev-ErbA (15), BD73 (16, 17), and ROR (18–21). This suggests that the binding mechanism of PPAR to PPREs may resemble that of receptor monomers to extended binding sites. The carboxyl-terminal extension (CTE) of the
zinc finger domains of NGF1-B (22) and ROα (20) appears to recognize 5'-extensions of the core binding site and contribute to the binding of each to a single extended recognition site. In this report, we demonstrate that PPARs contain a functionally similar CTE to that of Rev-Erbα and of ROα that contributes to the recognition of the 5'-upstream sequence conserved in PPREs. An extended DNA binding domain (DBD) of mPPARα containing both the zinc finger motif and the CTE displays monomeric binding that is dependent on the sequence 5' of the upstream core site, as no binding is observed when this upstream sequence deviates substantially from the consensus derived from PPREs. Although the RXR-DBD does not bind to a single extended core site, a chimeric protein linking the RXR zinc finger domain with the CTE of PPARα does bind as a monomer to a single core binding site with a 5' consensus extended sequence. These results indicate that the interaction of the CTE of PPARαs with the 5' sequence flanking the DR1 provides a primary mechanism for PPARα/RXR heterodimers to compete effectively with other receptor dimers for binding to PPREs.

EXPERIMENTAL PROCEDURES

Expression of Receptor DNA Binding Domains in Escherichia coli—PCR was utilized to generate truncated receptor cDNAs. The upstream oligonucleotide primers contained a codon for the initiator methionine followed by a codon for alanine, GCT, to facilitate expression in E. coli. The lower primers included a stop codon. Both primers contained suitable restriction sites at the 5' ends to simplify construction of expression plasmids. Each of the constructs derived from the mouse PPARα (1), mouse PPARγ (23), human PPARα (Nuc1) (24), human RXRα (25), or human Rev-Erbα (15) cDNAs were placed under the control of the lac promoter in the vector, pSPORT1 (Life Technologies, Inc.), modified to include an Ndel restriction site at the initiation codon located at an optimal distance from the promoter (26). The DNA binding domain (DBD) of RXR was expressed as a fusion protein with glutathione S-transferase using the vector pGEX2T (Amersham Pharmacia Biotech). All PCR amplifications were performed in a Perkin-Elmer 9600 thermal cycler using 30 cycles of denaturation at 90 °C for 15 s and of annealing and extension at 60 °C for 30 s. The segment encoding amino acids 88–210 of PPARα (PPAR-DBD-L) was amplified using primers PPARDBDU, gggggatcc ATG Gct ATT GAG Tgt CGA GTC TG; and RXRDBDU, cgtggatcccat ATG Gct AGC ACG GAC GAG TCC CCC, and was digested with BamHI and SphI to generate the protein domain fused to the GST. The identity and orientation of the resulting product were confirmed by sequencing. After ligation into the vector pGEX2T, the resulting plasmid was transformed into E. coli XL-1 Blue (Stratagene, La Jolla, CA) as described previously (12).

In Vitro Transcription/Translation—cDNAs encoding mouse PPARα (1), mouse PPARγ (23), human RXRα (25), human Rev-Erbα (15), or human ROα (19) were in vitro transcribed and translated in a TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) at 30 °C for 90 min using the manufacturer's protocol.

Transient Transfection Experiments—cDNAs encoding mouse PPARα or human ROα, expressed in cultured cells, were transfected with reporter constructs and analyzed for their ability to activate the thymidine kinase promoter in the thymidine kinase reporter vector pCMV-TPA-Luc (CLONTECH, Palo Alto, CA). The luciferase plasmid pLuc-A46–880 was coexpressed with the Gal (CLONTECH, Palo Alto, CA) have been previously described (30). The expression vector for PPARα/CDE was constructed by inserting the PPARα/CDE region into pCMV5. The luciferase reporter plasmid pLuc-TK-Z and pLuc-TK-RevDR2 were generated by inserting the CYP4A-Z PRE (1) or the RevDR2 element (27, 28) into the luciferase reporter vector harboring the thymidine kinase promoter from herpes simplex virus as described (29). All reporter and expression constructs were introduced into cultured cells by a modified calcium phosphate coprecipitation procedure (10). After a 16-h exposure to the DNA-containing medium, the cells were washed twice with serum-free medium and then placed in medium containing either 10% FCS, 300 μM 2-Deoxy-glucose or the equivalent volume of solvent (Me2SO, 0.25% v/v final concentration). This medium was replaced after 24 h with fresh medium, and after an additional 24 h, the cells were harvested and washed with Dulbecco's phosphate-buffered saline without calcium and magnesium (Irvine Scientific, Santa Ana, CA). Cells were then lysed by suspension in 0.1 M potassium phosphate buffer, pH 7.8, containing 1 mM 2-mercaptoethanol, 150 mM KCl, 0.6% Triton X-100 followed by sonication (2 min on ice) and sonication (2 min on ice). The insoluble material was removed by centrifugation, and luciferase activity was determined using a Monolight 2010 luminescence spectrometer (Analytical Luminescence Laboratory, model SI, Sunnyvale, CA).

Construction of the RXR/PPAR Chimeric DNA Binding Domain—A chimeric containing the zinc finger domain of human RXRα (amino acids 127–200) and the CTE of the zinc finger domain from murine PPARα (amino acids 167–195) was generated by PCR. Two initial amplification reactions were employed and performed as described above. One reaction used RPPU and PPARDBDL3 and the other utilized RPPU and RXRDBDU. The resulting product was digested with NdeI and BamHI and ligated into NdeI-BamHI digested pCMV-TPA-Luc. The products of the two amplification reactions were mixed and used as the template for an amplification reaction containing the outer primers (PPARDBDL3 and RXRDBDU). The resulting product was digested with Ndel and BamHI prior to being ligated into Ndel/BamHI-digested, modified pSPORT. The construct was verified by sequencing the chimera when cloned in E. coli XL-1 Blue (Stratagene, La Jolla, CA) as described (12). 

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The binding of each of the receptors to the CYP4A6-Zs, ACO-A, and ARE7 PPREs (shown in Table I) was characterized relative to the extent of binding observed for each receptor with the DR1P oligonucleotide. The values observed for the other oligonucleotides are expressed as a percentage relative to the binding obtained for that receptor with DR1P, Fig. 1. PPAR/ RXR heterodimers display highly similar binding intensities for the naturally occurring PPREs, although the ARE7 element displays preferential complex formation with PPARγ relative to PPARα. In contrast, these elements are bound relatively weakly by Rev-ErbA and RORα1 compared with their binding to DR1P, with the exception of the moderate binding displayed by RORα1 for the Zs element. The similar mobilities of the complexes formed with these PPREs, relative to the complexes formed with the probe corresponding to a single extended consensus site, SHSP, suggest that Rev-ErbA and RORα1 bind as monomers. This binding was not altered by the inclusion of RXRα (not shown). The availability of specific antibodies for Rev-ErbA and PPARα enabled verification of the identity of the protein comprising the complexes obtained with these lysates (Fig. 1, A and C). As expected, inclusion of antibody for Rev-ErbA efficiently shifts the mobility of the Rev-ErbA-containing complexes, and the addition of PPARα antibody quantitatively abolishes PPARα/RXR complex formation indicating that the complexes observed result from the expression of these receptors.

Transfection experiments employing RK-13 cells were used to examine the functional significance of Rev-ErbA and ROR binding to PPREs and the potential negative impact that competition for binding might have on PPAR-mediated signaling. The PPRE luciferase reporter utilized the TK promoter and the CYP4A6-Z PPRE. In order to assess whether RORα and Rev-ErbA were functionally expressed under these conditions, a second reporter construct, Luc-TK-RevDR2, was employed. This reporter construct harbors a direct repeat of the monomer-binding site that forms a DR2 motif with a 5′-extended binding site (Table I). This response element has been shown by others to mediate repression by Rev-ErbA (27) and stimulation by RORα (28). In addition, cotransfection of Rev-ErbA with RORα effectively blocks the stimulation by RORα (19). These results were confirmed in the present studies (Fig. 2A) providing evidence that both receptors are expressed. Cotransfection of PPARα (50 ng) and the expression vectors for Rev-ErbA (1 μg) or RORα1 (1 μg), using the Luc-TK-Z reporter, was also examined. In contrast to the results obtained with the TK-RevDR2 reporter, Rev-ErbA did not have a significant effect on luciferase expression from the Luc-TK-Z reporter (Fig. 2B), and RORα1 effected a very weak positive response (Fig. 2C) compared with the effect of PPARα in the presence of the ligand WY-14,643. Interestingly, PPARα also exhibited a strong stimulation of transactivation from the Luc-TK-RevDR2 reporter (Fig. 2, B and C). In contrast to the results seen for Luc-TK-Z, coexpression of Rev-ErbA blocked the transactivation elicited by PPARα on the RevDR2 element (Fig. 2B). However, cotransfection of RORα did not significantly change the level of transactivation through the RevDR2 element exhibited by PPARα (Fig. 2C). Similar results were also observed in JEG-3 cells (data not shown). The inability of Rev-ErbA and RORα1 to activate transcription through the CYP4A6-Z PPRE is consistent with the relatively weak binding exhibited by these receptors for this PPRE when normalized to the binding of each to DR1P. These results suggest that Rev-ErbA and RORα1 are not likely to interfere with PPAR/RXR transactivation of this element.

The observation that PPARα can positively regulate the expression of the RevDR2 reporter led us to examine the binding of the other PPAR isoforms to the RevDR2 element. As shown in Fig. 3A, PPARα exhibits synergistic binding to the RevDR2 element with RXR. This synergistic binding is also evident for PPARγ and Nuc1. Although no homodimer is evident for RXR in binding to this DR2 element, it can form a homodimeric complex when greater amounts of in vitro transcribed/translated lysate were used (data not shown). As expected, inclusion of
of antibody for PPARα quantitatively abolishes PPARα/RXR complex formation indicating that the complex contains PPAR. PPAR binding to this DR2 is relatively weak compared with that seen with the DR1P element (13%) when quantitated using a PhosphorImager and was normalized to the binding observed with DR1P. When supershift assays were performed, 1 μl of antibody was included in the incubation. The sequences of the oligonucleotide probes are shown in Table I. A, EMSA and supershift assays were performed with lysate containing in vitro transcribed/translated PPARα. The mobilities of PPARα complex (monomer), antibody-supershifted complex (supershift), and free probe are indicated. B, EMSA was performed with lysate containing in vitro transcribed/translated RORα1. C, EMSA and supershift assays were performed with a mixture of lysates containing in vitro transcribed/translated PPARα and RORα. The mobilities of PPAR/RXR complex (P/R) and free probe are indicated. D, EMSA was performed with a mixture of lysates containing in vitro transcribed/translated PPARγ and RORα.

The PPRE in the gene encoding the rat bifunctional enzyme (peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) contains two DR1 motifs separated by two nucleotides (30). Results from other laboratories indicate that mutations that disrupt the DR1 motifs but preserve the DR2 motif greatly diminish the responsiveness of the PPRE and the binding of PPAR/RXR (30, 31). In contrast to the Rev-DR2 element, the 5′-extended sequence is not preserved in this DR2 (Table I). This suggested that binding of PPAR/RXR to the Rev-DR2 may reflect the contribution of the 5′-extended binding site. We therefore examined the binding of PPAR/RXR to a DR2 element of the rat bifunctional enzyme in which the 5′-extended binding site is disrupted (HDDR2B, Table I) as described by Bardot et

| Oligo Probe                      | SHSP | DR1P | Zs | ACO-A | ARE1 |
|----------------------------------|------|------|----|-------|------|
| Control lysate                   | +    | +    | +  | +     | +    |
| Rev-ErbA                          | +    | +    | +  | +     | +    |
| anti-Rev-ErbA                     | +    | +    | +  | +     | +    |
| % DR1P                           | 0%   | 0%   | 0% | 0%    | 0%   |

| Oligo Probe                      | SHSP | DR1P | Zs | ACO-A | ARE1 |
|----------------------------------|------|------|----|-------|------|
| Control lysate                   | +    | +    | +  | +     | +    |
| mPPARγ-RXRα                      | +    | +    | +  | +     | +    |
| preimmune                        | +    | +    | +  | +     | +    |
| anti-PPARα                       | +    | +    | +  | +     | +    |
| % DR1P                           | 0%   | 0%   | 0% | 0%    | 0%   |

Fig. 1. Comparison of PPRE binding by in vitro translated full-length Rev-ErbA, RORα1, PPARα/RXR, and PPARγ/RXR. Each EMSA was performed with 10 fmol of the indicated double-stranded oligonucleotide and lysates containing in vitro transcribed/translated proteins (5 μl). The control lysate was generated in the absence of cDNA. The relative binding to PPREs by in vitro transcribed/translated proteins was determined using a PhosphorImager and was normalized to the binding observed with DR1P. When supershift assays were performed, 1 μl of antibody was included in the incubation. The sequences of the oligonucleotide probes are shown in Table I. A, EMSA and supershift assays were performed with lysate containing in vitro transcribed/translated Rev-ErbA. The mobilities of Rev-ErbA complex (monomer), antibody-supershifted complex (supershift), and free probe are indicated. B, EMSA was performed with lysate containing in vitro transcribed/translated RORα1. C, EMSA and supershift assays were performed with a mixture of lysates containing in vitro transcribed/translated PPARα and RORα. The mobilities of PPAR/RXR complex (P/R) and free probe are indicated. D, EMSA was performed with a mixture of lysates containing in vitro transcribed/translated PPARγ and RORα.
Fig. 2. Cotransfection of Rev-ErbA or RORα does not affect PPARα-activated expression of the TK-CYP4A6-Z reporter construct. Each of the reporter constructs (1 µg), pLuc-TK-RevDR2 (filled bars), pLuc-CYP4A6-Z (cross-hatched bars), or pLuc-TK (open bars) was cotransfected with 50 ng of either pCMV-mPPARα (PPAR), and/or 1 µg of either pCMV-Rev-ErbA (Rev), and/or 1 µg of pCMX-RORα1 (ROR) into RK13 cells. The total DNA for each transfection was normalized with the respective vector control (pCMV or pCMX). After incubation with DNA for 16 h, the cells were washed and either solvent or peroxisome proliferator (WY-14,643) was added to fresh medium for an additional incubation of 48 h. Only the data obtained from the WY-14,643 treatment is shown. The bar graphs represent the mean values plus standard deviation obtained from three independent transfections and have been normalized to the data obtained with individual vector in the presence or absence of the RXR/MBP fusion protein (Fig. 4B, Control). Expression of the PPARα-DBD encompassing amino acids 88–195 resulted in a protein that bound strongly to the Z element. This binding was seen in the presence or absence of the RXR/MBP fusion protein (Fig. 4B, DDB) indicating that the DBD of PPAR can bind without RXR.

al. (31). As shown in Fig. 3B, the binding of PPAR/RXR is not evident for HDDR2B. These changes also disrupt the binding of Rev-ErbA and significantly diminish the binding of RORα1 when compared with the RevDR2 element where they bind as dimers.

The DBD Domain of PPARα Binds to DNA Independently of RXR—The binding of Rev-ErbA and RORα1 to the extended binding site in natural PPREs, albeit weak, suggests that PPARα may utilize a similar binding mechanism. As PPARs do not display detectable binding to PPREs in the absence of RXR, we took advantage of the domain structure of nuclear receptors to express the DBD of PPAR in order to examine its binding to extended binding sites. The design of this construct was similar to that of the DBD of RXRα1 which had been characterized previously (11). In addition, we examined constructs containing additional domains in order to assess their effects on the capacity of the DBD to bind to PPREs (Fig. 4A).

Three truncated forms of mPPARα were expressed in E. coli, and the DNA binding properties of each as well as of the full-length mPPARα and RXRα were analyzed by EMSA. As shown previously (12), the full-length mPPARα does not bind alone (Fig. 4B, PPAR) but does bind cooperatively with a full-length RXRα/maltose-binding protein (MBP) fusion (Fig. 4B, PPAR + RXR) to the CYP4A6-Z PPRE and the A PPRE from the rat fatty acyl-CoA oxidase (ACO) gene. The RXR/MBP fusion protein does not bind to the Z PPRE but displays some binding to the ACO PPRE that is not apparent in the presence of PPARα. Lysates produced from the E. coli host did not exhibit detectable complex formation with the probe oligonucleotides (Fig. 4B, Control). Expression of the PPARα-DBD encompassing amino acids 88–195 resulted in a protein that bound strongly to the Z element. This binding was seen in the presence or absence of the RXR/MBP fusion protein (Fig. 4B, DDB) indicating that the DBD of PPAR can bind without RXR.

Inclusion of the amino-terminal domain with the DBD of PPARα led to a loss of binding to the CYP4A6-Z element in the presence or absence of the RXR/MBP fusion protein (Fig. 4B, ABC). Immunoblotting of the ABC polypeptide using an antibody specific for PPARα confirmed that this construct was expressed at concentrations similar to that seen for the full-length receptor in cell lysates (data not shown). The negative impact of the amino-terminal region on the interaction of the DBD with the PPRE was confirmed by examining the effect of deleting amino acids 1–88 from the full-length receptor on its capacity to bind to the PPRE in the absence of RXR. The resulting protein, CDEF, bound to the Z element in the absence of RXR (Fig. 4B, CDEF). A second complex of faster mobility is also present in the reactions containing the CDEF protein. The mobility of this second complex is very similar to that of the DBD construct suggesting that the second complex is comprised of a proteolytic degradation product of the CDEF polypeptide. The inability of the ABC construct or the full-length mPPARα alone to bind PPREs, when contrasted with the ability of the CDEF and DBD constructs to bind DNA in the absence of RXR, suggests that the amino-terminal 88 amino acids may interfere with response element recognition by PPAR.

The binding of the CDEF construct was greatly increased in the presence of the RXR/MBP fusion protein. The resulting complex exhibits a much slower mobility that is consistent with the formation of a heterodimer between CDEF and RXR. These results are concordant with previous indications that residues in the DEF region are required for heterodimerization with
as the extent of activation with added peroxisome proliferator, WY14,643-independent activation displayed by CDEF, as well as the extent of activation with added peroxisome proliferator, is nearly identical to that of the full-length mPPARα. These results suggest that the CDEF polypeptide retains the sequence information required for heterodimer formation, ligand binding, response element recognition, and gene transactivation. This implies that the amino-terminal 88 amino acids do not make necessary or significant contributions to these functions under these conditions.

The PPAR-DBD Does Not Exhibit Cooperative Binding with the DBD of RXR—Cooperative binding has been reported for RAR/RXR-DBD heterodimers to DR5 containing elements and for TR/RXR-DBD heterodimers to DR4 containing elements (34, 35). In order to determine if a portion of the heterodimerization interface is included in the PPAR-DBD polypeptide, mixing EMSA experiments were performed with the DBD of RXR expressed as a fusion protein with glutathione S-transferase (GST) that displays a mobility distinct from the PPAR-DBD fusion protein (Fig. 6). As shown in Fig. 6, lysates prepared from the E. coli host do not exhibit complex formation (Fig. 6, control). In contrast, lysates containing the PPAR-DBD display complex formation with the consensus PPRE oligonucleotide DR1P (Table I). Lysates expressing the RXX-DBD/GST fusion protein produce a slow mobility complex that is consistent with previous studies that have shown homodimeric binding to DR1 elements by an RXR-DBD complex (Fig. 6). This binding suggests that the PPAR-DBD and RXR-DBD complex is not monomeric and dependent on an upstream extension of the core site. We therefore examined the binding of a panel of mPPARα polypeptides to the CYP4A6-Z element and oligonucleotides detailed in Table I that contain consensus single core sites combined with either a consensus (SHSP) or mutated (SHSM) 5′-extended binding site. Control lysates did not result in detectable binding.

**FIG. 5.** Amino-terminal truncated PPARα can transactivate the CYP4A6–880 reporter construct in COS-1 cells. The reporter construct pLuc-4A6–880 (1 μg) was cotransfected with 50 ng of either pCMV-PPARα-G (G), pCMV-PPARα (W), pCMV-PPARα-CDEF (CDEF), or pCMV (CMV) into COS-1 cells. After incubation with DNA for 16 h, the cells were washed and then either solvent (Me2SO, open bars) or peroxisome proliferator (WY-14,643, filled bars) was added to fresh medium for an additional incubation of 48 h. The bar graphs represent the mean values plus standard deviation obtained from three independent transfections that were normalized to the data obtained with pLuc-4A6–880 and pCMV-PPARα-G in the presence of peroxisome proliferator.

**FIG. 4.** DNA binding of PPARα truncations to the ACO-A and CYP4A6-Z PPREs, A, schematic illustration of the PPAR truncations expressed in E. coli. The letters refer to functional domains of the nuclear receptors as discussed (50). The amino acid positions defining the constructs are indicated at the top. B, the left panel displays EMSA results demonstrating the cooperative binding of PPARα and the RXR/MBP fusion protein to the CYP4A6-Z and ACO-A PPREs. The mobilities of the free probe, PPARα/RXR heterodimeric complexes (P/R), and a homodimeric complex of RXR/MBP (RXR) with the ACO probe are indicated on the left. The right panel shows the results obtained for the truncations of PPARα with the Z PPRE in the presence or absence of the RXR/MBP fusion protein. The mobilities of complexes containing the truncated proteins are indicated on the right. EMSA was performed with 10 fmol of each labeled, double-stranded oligonucleotide, together with bacterial lysates containing expressed PPARα (6 μg), PPAR-ABC (6 μg), PPAR-CDEF (6 μg), PPAR-DBD (2 μg), and/or partially purified RXR/MBP fusion protein (RXR, 0.02 μg). Control assays with bacterial lysates without expressed receptor (6 μg) were also performed (Control).
The PPAR-DBD and RXR-DBD do not form heterodimers. EMSA was performed with 10 fmol of labeled double-stranded DR1P oligonucleotide, together with bacterial lysates containing expressed PPAR-DBD (3 μg) and/or partially purified RXR-DBD/GST fusion protein (0.3 μg) in 8% polyacrylamide gels. Control assays with bacterial lysates without expressed receptor (3 μg) were also performed (Control). The mobilities of complexes corresponding to the PPAR-DBD bound probe (DBD), the RXR-DBD-bound probe (RXR), and the free probe (probe) are indicated.

Fig. 7. The PPAR-DBD does not form homodimers and requires a 5′-extended core site for binding. A, a schematic representation of each construct is shown. B, EMSA was performed with 20 fmol of each double-stranded oligonucleotide and bacterial lysates (6 μg) containing expressed PPAR-DBD-L, PPAR-DBD, PPAR-DBD-S, or RXR-DBD/GST fusion protein using 4 and 8% polyacrylamide gels, respectively. Control assays with bacterial lysates without expressed receptor (6 μg) were also performed (Control). Complementary oligonucleotide pairs containing dual or single consensus core sites (AGGTCA) and 5′-flanking sequences corresponding to the PPRE consensus 5′-extended binding site (DR1P, SHSP) or divergent from the PPRE consensus 5′ site (DR1M, SHSM) were used as probes. The mobilities of free probe (probe) and protein-probe complex (Complex) are indicated. C, EMSA was performed with 20 fmol of 32P-labeled double-stranded SHSP oligonucleotide and bacterial lysates containing expressed PPAR-DBD (DBD) and/or a longer PPAR-DBD-L (DBDlong) protein. Control lysates without expressed receptor were also tested (Control). The same amounts of the bacterial lysates containing the DBD or DBD-L proteins used for the individual reactions were combined in the mixed reaction (DBDmix). The mobilities of the complexes containing DBD-L and DBD are indicated.

PPAR-DBD-L and the PPAR-DBD (DBDmix) display two complexes corresponding in mobility to those seen with the individual proteins (Fig. 7C). The absence of an intermediate sized complex or obvious alterations in the ratios of the individual complexes when the lysates are mixed supports the conclusion that complex formation results from the binding of a single DBD to the oligonucleotide probe.

Binding of the PPAR-DBD constructs to a single core site with a 5′-extension is similar to that reported for RORγ (20) and NGFI-B (22) that have been shown to utilize the CTE region for binding. The panel of PPAR-DBD constructs was made to determine the minimal length of the CTE domain required for binding, and these constructs correspond closely to those used to analyze the binding of RORα1 to a single half-site with 5′ A/T-rich sequence (20). The binding patterns of PPAR-DBD-L and PPAR-DBD-S are similar to that obtained with PPAR-DBD. However, complex formation by PPAR-DBD-S is significantly diminished relative to the other two constructs (Fig. 7B). No binding was evident when PPAR-DBD-VS was used (data not shown). The EMSA results obtained with the E. coli-expressed PPAR DBD panel (Fig. 7B) mirror the data obtained with similar deletions to RORα1 in terms of loss of
The DBDs of PPARα, PPARγ, and Rev-ErbA Bind as Monomers to the Extended Binding Site—Additional EMSA were conducted to determine whether the DBDs of other forms of PPAR and the DBD of Rev-ErbA would also bind as monomers and display binding sensitivity to the 5′-extended sequence. The DBDs of hNuc1 (human PPARα), mPPARγ, and Rev-ErbA were each expressed in E. coli and assayed for binding to the single core site oligonucleotides flanked by either the consensus (SHSP) or divergent (SHSM) 5′-extended sequence (Fig. 8). As expected, the Rev-ErbB DBD binds the SHSP oligonucleotide but not the SHSM oligonucleotide. This result was also obtained with the PPAR-DBDs indicating that the 5′-extended sequence in PPREs is a fundamental determinant of binding that is shared by the PPAR isoforms and the closely related Rev-ErbB DBD.

The PPAR CTE Confers Monomeric Binding to the Zinc Fingerm Domain of RXR—The requirement for the 5′-extended binding site seen for the PPAR-DBD is similar to that seen for other nuclear receptors that bind to a single extended core binding site as monomers. These receptors interact with the 5′-extended sequence utilizing a carboxyl-terminal extension (CTE) of the zinc finger domain. The loosely defined CTE region includes the T box, defined by a portion of the RXR-DBD that is required for DNA binding and homodimerization (11), and the A box that has been shown to be required for the binding activity of NGFI-B to a single extended binding site (22). The amino acid sequence of the region corresponding to the CTE of the PPARs is highly conserved among the isoforms and is similar to the CTEs of Rev-ErbB and RORα that bind to single extended core sites as monomers (Fig. 9).

Although the RXR-DBD does not bind as a monomer, a chimera containing the RXRβ zinc finger domain and the CTE domain of NGFI-B was expressed in E. coli as a Trp-E fusion protein was shown to bind to a single extended site as a monomer (22). In order to assess the ability of the PPARα CTE to confer monomeric binding and discrimination of the 5′-extended binding site to the RXR-DBD, a similar chimera containing the PPARα CTE domain and the RXRα zinc finger domain was expressed in E. coli and examined by EMSA (Fig. 10). This chimera displays a clear preference for the consensus 5′-extended sequence as evidenced by the strong binding observed with the SHSP and DR1P oligonucleotides. Weak binding of the chimera to both the SHSP and DR1M oligonucleotides is detectable indicating that the binding of the chimera is less dependent than the PPAR-DBD on the 5′-extended site. As seen with the NGFI-B chimera, the CTE domain of PPARα is sufficient to confer binding of the RXRα zinc finger domain to single core sites flanked by the appropriate 5′-extended sequence. The mobility of the complexes formed with the chimeric protein relative to that of the RXR-DBD/GST fusion protein and the PPAR-DBD suggests that the chimera binds to both the SHSP and DR1P oligonucleotides as a monomer. Thus, the CTE of PPAR appears to prevent the binding of a second chimeric DBD to the DR1P probe.

DISCUSSION

This study has characterized the binding of the DBD of PPARs with PPREs. The PPAR-DBD binds as a monomer to the 5′ core recognition site of the PPRE, and this binding is dependent on a conserved upstream sequence. Our results also indicate that PPARs employ an extended DBD consisting of the zinc finger domain and a CTE to recognize the extended core recognition site in PPREs.

PPARs belong to a group of nuclear receptors that bind to DNA as heterodimers with RXR. Domain mapping experiments have demonstrated that the DBDs of other members of this group such as RAR, TR, and the vitamin D receptor heterodimerize with the DBD of RXR on their cognate binding sites (34, 38–40). Expression of similarly truncated PPARs results in DBDs that display monomeric binding to a single core site without cooperative heterodimeric binding with the RXR-DBD on elements that contain direct repeats of the core sequence (A/G)GGTCA. This result would imply that there is no significant contribution of the DBDs to PPAR/RXR heterodimer formation and that such protein-protein interactions require the participation of additional domains that reside elsewhere, such as the dimerization interface found in the ligand binding domain of this group of receptors (32, 33).

The binding of the PPAR-DBD to the single core site requires the 5′-extended binding site. This finding supports our observation that PPREs are tripartite elements composed of two core sites and an AT-rich 5′-extension similar to that seen for sites that bind monomers of orphan nuclear receptors, such as Rev-ErbA and RORα (12). A CTE of the zinc finger domain has been shown previously to mediate the binding of monomers of NGFI-B to a similar but distinct extended binding site (22). The CTE of PPARs exhibits a high degree of sequence similarity to the CTEs of RORα and Rev-ErbA that bind to single core recognition sites with similar 5′-extensions. As shown here, substitution of the CTE domain in the RXR-DBD with that of PPARα generates a chimera that binds strongly to a single core recognition sequence with the 5′-extended binding site. Thus, it is likely that the CTE of PPARs interact with the 5′-extension of the DR1 motif.
The homology evident in the CTE region and the similarity of the binding sites of PPAR, Rev-ErbA, and RORα raise the possibility of interaction between these receptors and PPAR-mediated transcriptional activation. Interestingly, Rev-ErbA (45) and RORα (46) are induced during adipocyte differentiation (45), a process that can be triggered by the ectopic expression of PPARγ2 (47). The similarity in the binding sequences for these receptors could allow Rev-ErbA or RORα to modulate PPAR signaling by competing for binding to PPREs (27). Rev-ErbA binds weakly to the CYP4A6-Zs PPRE as a monomer, and binding to the ACO-A and ARE-7 PPREs could not be readily detected. The binding of RORα appeared to be stronger and could be detected for both the CYP4A6-Zs and ACO-A PPREs. However, cotransfection studies using a 20-fold excess of expression vector for either Rev-ErbA or RORα did not significantly affect transcription of the Luc-TK-Z reporter plasmid in the presence or absence of PPARα. These results suggest that very high ratios of Rev-ErbA or RORα to PPAR are likely to be required to provoke significant competition for binding and diminution of PPAR-mediated transcription.

Dimers of Rev-ErbA have been reported to act as repressors through DR2 elements formed by the overlap of two monomeric binding sites such as the RevDR2 (27) response element employed in this study. The RevDR2 response element contains a direct repeat of the core recognition sequence separated by 2 nucleotides with a 5'-extended binding site that resembles the recognition site for Rev-ErbA. The requirement for dimer binding may account in part for the absence of repression on the pLuc-TK-Z reporter. Interestingly, PPARα/RXR heterodimers activate transcription through this same DR2 response element that mediates Rev-ErbA repression and RORα stimulation. Naturally occurring PPREs do not generally exhibit a DR2 motif with the exception of the PPRE of the rat bifunctional enzyme that contains two DR1 motifs separated by two nucleotides (30). As reported by others, PPAR/RXR heterodimers bind to each of the DR1 motifs but not to the DR2 motif (30, 31).

In contrast, both binding and transactivation are evident for the RevDR2 element. This may reflect conservation of the extended binding site utilized by PPARα in the RevDR2 element that is not evident in the DR2 motif found in the PPRE of the gene for the rat bifunctional enzyme. Although Rev-ErbA or ROR may not have a significant impact on PPAR/RXR-regulated pathways mediated by PPREs, the potential exists for cross-talk with PPAR/RXR on DR2 response elements that exhibit the consensus 5'-extended sequence.

Demonstration that the PPARα CDEF construct is sufficient for RXR heterodimer formation, DNA binding, and ligand-activated transcription suggests that it is functionally equivalent to the full-length receptor, and this supports the use of such constructs for ligand binding EMSA assays (48). The binding of the CDEF construct to a PPRE in the absence of RXR suggests that the amino-terminal 88 amino acids of mPPARα interfere with recognition of the extended binding site and thus limits the capacity of PPARα to function as a monomeric receptor. This could also account for the absence of complex formation by the ABC construct. It is possible that the masking of the DBD by the amino terminus prevents PPAR alone from interfering with monomeric receptor-mediated processes. Amino-terminal masking of DNA binding would represent an interesting evolutionary change that allowed regulatory specialization/divergence of PPAR away from the closely related monomeric receptors and exploitation of compound hybrid response elements as a heterodimer with RXR. Clearly, full utilization of the hybrid site by the heterodimer affords binding discrimination of PPAR/RXR from other receptors that are

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**FIG. 10.** The CTE of PPAR confers monomeric binding to the RXR zinc finger domain. EMSA was performed with 10 fmol of each double-stranded oligonucleotide and included bacterial lysates containing either the PPAR-DBD (1 μg), or the chimeric protein (chimera) (1 μg), or RXR-DBD/GST fusion protein (RXRDBD) (100 ng). Control assays using bacterial lysates without expressed receptor (Control) (1 μg) were also performed. The identity of the probe is indicated at the top. The mobilities of complexes and free probe (Probe) are indicated.

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The dependence of PPAR-DBD binding on the 5'-extension of the core site further supports our earlier conclusion that PPARs bind to the 5'-extended core-binding site, which would place the RXR component of the heterodimer on the 3' core site of the DR1 (12). Additional evidence for this binding orientation was recently published for PPARγ/RXR heterodimers (36) and is consistent with recent studies that indicate that RXR binds to the 3' core site of PPREs (14, 37). This polarity could underlie the lack of cooperativity in binding between the DBDs of PPAR and RXR. The DBD of RXR exhibits the opposite polarity when it binds cooperatively with the DBDs of TR and RAR to DR4 and DR5 motifs, respectively. The crystal structure of the heterodimer formed by the DBDs of RXR and TR bound to a DR4 element has been determined (41). The dimerization interface of RXR bound to the 5' site is formed in part by residues in the D box region of the carboxyl-terminal zinc finger. The D box also forms part of the dimerization interface in homodimers of the glucocorticoid DBD bound to palindromic binding sites (42). As shown here, the PPAR-DBD did not form dimers with the DBD of RXR on the consensus PPRE. This could reflect the unusual D box of the PPARs that includes only 3 amino acids rather than the 5 seen in other nuclear receptors.

Alterations of the DBD of RXR by the exchange of its CTE for that of PPAR prevented the dimerization of the chimera on the DR1 repeat. The loss of binding to the 3' core site could reflect steric restrictions imposed by the single nucleotide separating the two core sites. It has been suggested that the optimal spacing of direct repeats may reflect in part limitations imposed by a requirement to accommodate the CTE region (43). The structure of the TR and RXR-DBD dimer reveals that the CTE domain of TR forms a helix with specific DNA contacts 5' of the core site where the receptor is bound (41). This is consistent with the participation of this domain in DNA sequence recognition. TR has also been shown to bind as a monomer to a single half-site containing an extended 5'-binding site. Footprinting analysis indicated that the TR CTE contacts the first 2 nucleotides immediately 5' of the core site (44). In the structure of the heterodimer of the RXR and TR DBDs bound to the DR4 element (41), these CTE contact residues reside in the 4 nucleotide spacer separating the core sites of the DR4 motif and may define the minimum length for productive interaction with CTEs (43).
soydependent on either of the overlapping monomeric and DR1 sites comprising PPREs.

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