Characterization of the Amino-terminal Activation Domain of Peroxisome Proliferator-activated Receptor α

IMPORTANCE OF α-HELICAL STRUCTURE IN THE TRANSACTIVATING FUNCTION

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The transactivating function of the A/B region of mouse peroxisome proliferator-activated receptor α (PPARα; NR1C1) was characterized. The truncated version of PPARα lacking the A/B region had 60–70% lower transactivating function than full-length PPARα in both the presence and absence of the peroxisome proliferator ciprofibrate. When tethered to the yeast Gal4 DNA-binding domain, the A/B region exhibited the significant ligand-independent transactivating function, AF-1 activity. The first 44 amino acid residues were necessary for maximal transactivation, and the minimally essential region was further delimited to amino acids 15–44. This region is highly enriched with acidic residues, but mutational analyses showed that the protein structure, rather than the negative charge itself, was important for the AF-1 activity. An α-helical configuration was predicted for this region, and a CD spectrum analysis of the synthetic peptides showed that mutant sequences with higher AF-1 activity have higher helical contents and vice versa. The most active mutant, in which Met11 was replaced with Leu, was 5-fold more potent than the wild-type A/B region. These findings indicate that the AF-1 region of PPARα is an acidic activation domain and that the helix-forming property is implicated in the transactivating function.

Peroxisome proliferator-activated receptors (PPARs) constitute a subfamily (NR1C, according to the unified nomenclature) of nuclear hormone receptors. Since the first cloning of PPARα (NR1C1) from mouse (2), three isoforms (α, β (or δ), and γ) have been identified in many organisms, including human, rat, and Xenopus (for review, see Ref. 3). Many studies suggest that PPARα regulates the fatty acid metabolism by controlling the expression of the genes involved in fatty acid oxidation as well as lipoprotein subunits, positively or negatively (4). On the other hand, PPARγ (NR1C2) seems to govern more versatile physiological processes such as adipocyte differentiation (4), inflammatory response (5, 6), and maturation of macrophages into foam cells (7, 8). Very recently, evidence showing the involvement of PPARγ (NR1C2) in embryo implantation was presented (9).

PPAR recognizes direct repeat motifs consisting of two AGGTCA or related half-sites separated by a single nucleotide (DR-1) through heterodimerization with another nuclear hormone receptor, the retinoid X receptor (NR2B) (10). PPAR recognizes the 5′-half-site, whereas the retinoid X receptor recognizes the 3′-half-site (11–14). Moreover, for optimal binding, PPAR requires an extended half-site constituted by the AGGTCA hexanucleotide motif and four extra residues on the 5′-side (11, 13, 14). This is in contrast to other nuclear receptors forming heterodimers with the retinoid X receptor, e.g. the retinoic acid receptor (NR1B), thyroid hormone receptor (NR1A), and vitamin D receptor (NR1I1) (15).

Many compounds have been identified as ligands of PPAR (3). The fibrate class of peroxisome proliferators and leukotriene B4 are relatively selective for PPARα, whereas the thiazolidinedione class of anti-diabetic compounds, 15-deoxy-Δ12,14-prostaglandin J2, and anti-inflammatory agents are specific for PPARγ. Unaturated long-chain fatty acids are probably important endogenous ligands common to PPARα and PPARγ.

As in most nuclear receptors, PPAR comprises four discrete functional domains, A/B, C, D, and E, in the order from the amino to the carboxyl terminus (15). In general, the C region is highly conserved among the nuclear receptor superfamily and contains two zinc finger motifs involved in DNA binding. The E region is the next most highly conserved and contains the ligand-binding site and ligand-dependent activation domain (AF-2). The D region is a hinge domain between the C and E regions and contains sequences important for heterodimerization and the AF-2 activity. The A/B region is the most variable among nuclear receptors and, in many cases, has a ligand-independent gene-activating function (AF-1). The AF-1 region acts independently of AF-2 when tethered to heterologous DNA-binding domains and sometimes synergizes with AF-2 through intramolecular interaction. Regulation of the receptor activities by growth factors and other extracellular signals is often mediated through mitogen-activated protein kinase-dependent phosphorylation of specific sites in the AF-1 region.

For PPAR, AF-1 activities were found in the A/B region of the γ-isofrom (16, 17). Mitogen-activated protein kinase-dependent phosphorylation in the A/B region has also been demonstrated and is implicated in the actions of insulin and growth factors on adipocyte differentiation and other PPARγ functions (16, 18–23). The AF-1 activation domain, however, has not yet been mapped precisely in the A/B region. In this study, we identified and characterized the AF-1 domain in the A/B region of PPARα. This domain is enriched with acidic amino acids. Mu-
Acidic Activation Domain of PPARα AF-1

EXPERIMENTAL PROCEDURES

Plasmids—The expression vector of full-length PPARα was pNCM-VPPARα (11), constructed by replacing the lacZ gene in the cytomegalovirus promoter-driven vector pCMVβ (24) with mouse PPARα cDNA. A deletion construct lacking the A/B region was created by inserting a double-strand linker composed of 5′-TGTCGATATCTGCGGGA-CAGG-3′ and 5′-CTTCTGCCCAATATTGGACATG-3′ between the BstXI site encompassing the initiation codon and the StuI site in the early portion of the C domain-encoding region. The resulting plasmid, pNCMVPPARαΔA/B, codes for a protein sequence in which the initiation methionine is directly linked to the first cysteine residue (Cys250) of the C domain.

For constructing the fusion of yeast Gal4-14BD and the A/B domain of PPARα, the cDNA sequence of the corresponding region was amplified by polymerase chain reaction using an upstream primer (5′-CCAGATATC-TAATTGAGACAGAAGCCCG-3′) and a downstream primer (5′-CGTCTACCTAGTGTCCAGGCACTGCC-3′). The amplified fragment was digested with EcoRI and BclI, yielding two fragments of 76 and 227 base pairs corresponding to the presence of an internal EcoRI site. The longer fragment was first inserted between the EcoRI and BamHI sites of the plasmid pCMX-Gal4-N, which encodes a yeast Gal4-14BD (amino acids 1–147) and is driven by the cytomegalovirus promoter. The shorter fragment was then inserted at the EcoRI site, and a clone containing the fragment in the correct orientation was verified by nucleotide sequencing.

The A/B region-coding sequences of mouse PPAR-1 and PPAR-2 were also amplified by polymerase chain reaction and inserted in pCMX-Gal4-N. The cDNA sequence of the PPAR-1 A/B region was created from the PPAR-2 sequence, taking advantage of PPAR-2, which has 30 extra amino acid residues at the N terminus compared with the PPAR-1 sequence, and also, the sequence C-terminal to the second methionine (Met250) is identical to that of PPAR-1. Thus, the region from the NcoI site encompassing the Met253 codon to the position corresponding to the end of the PPAR-2 A/B domain was cleaved and inserted in pCMX-Gal4-N.

For assaying the transcriptional activation by PPAR constructs containing the C region, a reporter vector, pAOXPPREluc (11), containing the PPAR-binding site and the basal promoter derived from the rat acyl-CoA oxidase gene was used (25). For assaying the activation by Gal4 fusion constructs, tk-GALp2x3-luc, containing three copies of the Gal4-binding sites (upstream activation site (UAS)) and the herpes simplex virus thymidine kinase promoter, was mostly used. tk-GALp2x1-luc, containing one copy of the Gal4-binding site, was also used in some experiments. This construct was accidentally produced by recombination during amplification in bacteria.

Construction of Mutants—Deletion constructs of the PPARα A/B region were produced by cutting pCMX-Gal4-PPARαA/B with restriction enzymes having recognition sites in the A/B region-coding sequence. These enzymes were EcoRI (comprising the codons for Glu86 and Phe97), SacI (Ser96), SacI (Thr97), and PvuII (Ser96). DNA sequence coding for the portion C-terminal to amino acid residue 15 was created by polymerase chain reaction amplification.

Site-directed mutagenesis was carried out using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. All mutations were confirmed by nucleotide sequencing.

DNA Transfection—The HeLa human cervical cancer cell line was used in most experiments, whereas the HepG2 human hepatoma cell line and the CV-1 monkey kidney cell line were used in some cases. Cells were cultured in Dulbecco’s modified Eagle’s medium or Ham’s F-12 medium containing 10% fetal bovine serum.

Transfection was carried out by the calcium phosphate method (26) with slight modifications. For transfection, 1 × 10⁵ cells were seeded in 60-mm dishes and cultured overnight. The following day, each dish received DNA/calcium phosphate precipitates containing 1 μg of the expression vector of a PPAR construct, 4 μg of an appropriate luciferase reporter plasmid, and 4 μg of a β-galactosidase expression vector (pCMVβ) as a reference. After 4 h, the precipitates were removed, and the cells were cultured for 40 h in the presence or absence of 0.5 μm ciprofibrate, a peroxisome proliferator. Cells were lysed, and luciferase and β-galactosidase activities were measured as described previously (11). Luciferase activity was normalized for transfection efficiency based on the β-galactosidase activity. Three or more independent assays were carried out for most series of experiments, and the results are expressed as mean relative values ± S.D.

Synthesis of Peptides and CD Spectrum Measurement—To analyze the secondary structure of the N-terminal region of PPARα, a peptide corresponding to amino acids 21–39 (SPLSEEFLQEMGNIEISIQ) was synthesized on an automated solid-phase peptide synthesizer (Shimadzu PSRM-8) using Tenta Gel TRG-AM resin and Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry. Three extra amino acids (GGY-amide) were attached to the C terminus to make the spectrophotometric determination of peptide concentration feasible without affecting the CD signal (27). Mutant peptides containing amino acid substitutions were also synthesized. Peptides were purified by reverse-phase high performance liquid chromatography on a C18 column, and their respective molecular masses were confirmed by mass spectrometry on a time-of-flight mass spectrometer (Shimadzu/Kratos Kompact MALDI II). The CD spectra of these peptides were recorded on a Jasco J-720 CD spectrometer at pH 6.8 in the presence of 0–40% trifluoroethanol (TFE).

RESULTS

Effect of Deletion of the A/B Region from Full-length PPARα—We first examined whether the transcriptional activating function is affected when the A/B region is deleted from PPARα. When the full-length PPARα expression vector was transfected together with the luciferase reporter vector carrying a single copy of the peroxisome proliferator response element of rat acyl-CoA oxidase, the luciferase expression was enhanced 15-fold as compared with the value obtained with an empty expression vector, pCMVNot (Fig. 1, bars 1 and 5). The luciferase expression was further stimulated nearly 4-fold in the presence of ciprofibrate, a peroxisome proliferator (Fig. 1, bar 6). When PPARα with the A/B region deleted was expressed, the luciferase activity decreased by 60–70% in either the presence or absence of ciprofibrate (Fig. 1, bars 3 and 4). Thus, deletion of the A/B region from PPARα decreased the transcriptional activating function—3–fold without affecting inducibility by ciprofibrate.

AF-1 Activity of the A/B Region—We next investigated whether the PPARα A/B region has a ligand-independent activating function (AF-1). For this purpose, we constructed plasmids in which the coding sequence of the A/B region as well as...
full-length PPARα was tethered to the Gal4-BD-coding sequence. When the fusion construct of full-length PPARα was cotransfected with a luciferase reporter vector containing three copies of Gal4-UAS (tk-GALpx3-luc), strong transcriptional activation dependent on ciprofibrate was observed (Fig. 2A). The scale of ligand dependence was much larger than that obtained with unfused PPARα on the reporter gene driven by the peroxisome proliferator-response element (compare Figs. 1 and 2A). On the other hand, the fusion construct of the A/B region with the A/B regions of mouse PPAR isoforms. Cells were cultured in the absence of ciprofibrate. Other experimental conditions were as given in the legend to Fig. 1.

We compared the AF-1 activities of the PPARα, PPARγ1, PPARγ2, and PPARδ isoforms, all of mouse origin (Fig. 2B). Under the assay conditions, the AF-1 activity of PPARα was significantly higher than that of other isoforms. The A/B region of PPARγ2 exhibited a small AF-1 activity, but the activity was negligible for PPARγ1 and PPARδ. Much higher AF-1 activity was reported for PPARγ2 compared with PPARγ1 (16).

Localization of the AF-1 Activity in the N-terminal 44 Residues—We tried to narrow down the PPARα AF-1 region in the Gal4-BD fusion context (Fig. 3). When the A/B region sequence was deleted stepwise from the C-terminal side starting at amino acid 101, the construct containing amino acids 1–44 exhibited almost full AF-1 activity, whereas containing amino acids 1–27 did not. On the other hand, deletion of 14 residues from the N-terminal side decreased the reporter expression by 70%, and deletion of the first 25 residues completely removed the activity. Hence, AF-1 activity is assigned to the N-terminal 44-amino acid sequence, in particular to residues 15–44.

We confirmed for representative constructs that the fusion proteins were correctly expressed in the transfected cells. Proteins of the expected sizes were detected by Western blotting with anti-Gal4-BD antibody for Gal4-BD and Gal4 fusion constructs with the PPARα A/B region (amino acids 1–27 and 1–55) (Fig. 4, lanes 1–4). The expression levels were comparable except for the wild-type PPARα A/B region fusion construct, which exhibited significantly lower expression reproducibly in repeated experiments.

PPARα AF-1 Is an Acidic Activation Domain—The amino acid sequence of the A/B region of PPARα is shown in Fig. 5A. This region consists of 101 amino acid residues and contains 18 acidic residues, but no basic residues. The region of amino acids 15–44 that was minimally essential for transactivation was particularly enriched with acidic residues in which 10 out of 30 residues were acidic. Thus, the AF-1 region of PPARα seemed to be a typical acidic activation domain.

To examine the roles of acidic residues, we performed point mutation studies on the Gal4-BD-PPARα/A/B background. We changed doublets of acidic residues (Asp17-Asp18, Glu25-Glu26, and Glu43-Glu44) to asparagine doublets (mutants dm1–3) (Fig. 5A) and also made combinations of these mutations. Thus, up to six negative charges were removed from the original 10. We did not use glutamine for glutamic acid to avoid the possible creation of a glutamine-rich activation domain. Series of asparagine substitutions were successfully employed for a viral acidic activator, VP16 (28).

Individual doublet mutations of the acidic residues had only a marginal effect on AF-1 activity (Fig. 6A). Combinations of two doublet mutations exhibited a significant decrease in AF-1 activity compared with the AF-1 activity of the wild-type A/B region, particularly the combination of dm1 and dm2. The effect of dm3 seemed smaller than those of dm1 and dm2. The combination of the three doublet mutations resulted in only a slightly larger decrease in the activity compared with the dm1/dm2 combination, and even this mutant still retained a significant level of transcriptional activation as compared with Gal4-BD (2.6-fold). We confirmed these results with the (UAS)1 reporter and also with two other cell lines, HepG2 and CV-1 (data not shown). The results support the importance of acidic residues for AF-1 activity, but the effects of mutations were unexpectedly small if the negative charges themselves are important.

Implication of Protein Structure in the AF-1 Activity—The secondary structure of the PPARα A/B region predicted by the Chou-Fasman method (29) is shown in Fig. 5B. Strikingly, almost the whole region minimally essential for AF-1 activity is far overscaled by guest on July 18, 2018http://www.jbc.org/Downloaded from
was deduced to take the α-helical configuration. In helical wheel analysis (30) of region acid 15–44, most of the acidic amino acids were found on one side, whereas the hydrophobic residues were on the other (Fig. 5C). Thus, this region seemed to form an amphiphilic α-helix. Doublet mutations dm1 and dm2 were likely to break the helical structure locally, and the combination of dm1 and dm2 expanded the unstructured region additively (Fig. 5B). On the other hand, mutation dm3, which was less effective in decreasing the AF-1 activity in the transfection assay, did not seem to affect the configuration of the main part of the AF-1 domain, either by itself or in combination with other mutations. These predictions led us to assume that the protein structure, rather than the negative charges, in this region was important.

To examine this possibility, we searched for other mutations that were expected to break the α-helix based on the Chou-Fasman prediction. We found that Met31 contributes to a great extent to the helix-forming probability. Substitution of Met 31 with Gly or Ala was predicted to change the configuration between residues 31 and 38 from α-helix to β-sheet (Fig. 5B, bottom). Accordingly, we made fusion proteins of M31G and M31A mutant versions of the PPARα A/B region and Gal4-BD. The AF-1 activity was mostly lost in both mutants, with the effect of M31G being more severe than that of M31A (18.6 and 26.9% active as compared with the wild type, respectively) (Fig. 6B). We also investigated the effects of combinations of the M31G or M31A mutation with dm1, dm2, or dm3. The dm series of mutations resulted in no further decrease in the AF-1 activity over the M31G or M31A mutation (data not shown). We confirmed that the M31G, M31A, and dm3 fusion proteins were expressed at comparable levels in the cells upon transfection of the respective expression vectors as compared with Gal4-BD and two deletion mutants (Fig. 4).

We next examined whether these mutations indeed broke the α-helical structure by CD spectrum analysis of the synthetic peptides corresponding to the wild-type and mutant AF-1 sequences. We first predicted the α-helical contents of a series of peptides using the AGADIR algorithm (31) to select the peptide sequences to be synthesized. The peptide sequence 21–39 attached with the GGY tripeptide at the C terminus was expected to have an α-helical content of 4.05% at pH 7.0 (Fig. 7A), whereas M31G and M31A exhibited α-helical contents of 1.00 and 1.73%, respectively. The relative order in helical contents of these peptides did not change when calculation was performed on longer peptide sequences, and the highest values were obtained with the above segment. The E25N/E26N double mutant (dm2) was also expected to have a significantly lower α-helical content. Conversely, we found that the M31L and E26R mutants have the highest predicted helical contents...
among the possible mutants at residues 31 and 26, respectively. The E26R/M31L double mutant was expected to have an even higher helical content.

Based on these predictions, we synthesized the above peptides and measured the CD spectra in the presence and absence of TFE (Fig. 7B). Because the absolute ellipticity values at 222 nm (\([\theta]_{222}\)) of the peptides in the absence of TFE were too small to be compared, increasing concentrations of TFE, which is known to stabilize native-like helical structures by strengthening peptide hydrogen bonds, were added to evaluate the helix-forming properties of these peptides. The difference in \([\theta]_{222}\) among peptides was expanded at higher TFE concentrations. The E25N/E26N double mutant (dm2) responded to TFE in an unusual manner, exhibiting almost no spectral change around 222 nm, even when the TFE concentration was raised to 40%. This mutant peptide might have an abnormal secondary structure in solution. The three other mutant peptides were also analyzed, and the calculated \(\alpha\)-helical contents at 30% TFE are shown in Fig. 7C. As expected, M31G and M31A had lower \(\alpha\)-helical contents and M31L had a higher \(\alpha\)-helical content compared with the wild type. On the other hand, mutation E26R did not increase the \(\alpha\)-helical content, contrary to the prediction using the AGADIR algorithm. Thus, mutant E26R had a slightly lower \(\alpha\)-helical content compared with the wild type, whereas the value of E26R/M31L was intermediate between those of the wild type and M31L.

Based on these results, we produced mutant Gal4-BD-PPAR\(\alpha\)A/B fusion constructs carrying the mutations M31L, E26R, and E26R/M31L. In the transfection assay, M31L had a strikingly higher AF-1 activity than the wild type (Fig. 6B). In
contrast, E26R had a slightly lower AF-1 activity than the wild type, whereas E26R/M31L had an activity intermediate between those of M31L and the wild type. Thus, change in the charge by +2 in mutant E26R did not drastically affect the transcriptional activating function, as in the case of mutant dm2. We plotted the AF-1 activities of wild-type and mutant constructs against the α-helical contents of corresponding peptides (Fig. 7C). AF-1 activity correlated well with the helical content following an exponential function, suggesting the importance of the α-helix-forming property of this region.

**Effects of M31G and M31L Mutations in the Whole PPAR Sequence—**We introduced the mutations into the whole PPARα protein sequence. When the expression vectors of mutant PPARs were cotransfected with the reporter vector driven by a peroxisome proliferator response element, M31G exhibited a lower transactivation than wild-type PPARα in both the presence and absence of the peroxisome proliferator (Fig. 1). The M31L mutation yielded a slight increase in activity compared with the wild type, and the difference between the activities of M31L and M31G was quite significant. The effects of these mutations in the intact PPARα context were smaller than those in the Gal4 fusion context, possibly due to the structural constraint. The activities of the wild type and the two mutants, however, were in the same order as those of the Gal4 fusion constructs of the A/B region mutants.

**DISCUSSION**

These results indicate that a ligand-independent transactivating function is located in the A/B region of mouse PPARα. The sequence important for the activity is restricted to region 1–44, particularly amino acids 15–44. After completion of this study, the AF-1 activity of human PPARα and its regulation through phosphorylation were reported (32), but the minimally essential region was not specified.

The main part of the AF-1 region is highly enriched with acidic residues and devoid of any basic residue. Thus, this region seems to be a typical acidic activation domain. The results of mutation studies, however, raised a question concerning the importance of the negative charge itself. Rather, these results together with the CD spectrum analysis of synthetic peptides suggest the importance of the α-helix-forming properties of this region, i.e. the mutations resulting in a higher helical content gave higher AF-1 activity and vice versa. Helical wheel analysis suggests that this region takes an amphiphilic α-helix. These characteristics are consistent with reported results on acidic activation domains of other proteins: the importance of the negative charge itself was not supported by mutation studies on the viral activator VP16 (33). Involvement of the α-helical structure induced by the interaction with one of the TATA-binding protein associated factors, TAFp31, was shown by an NMR study on VP16 (34). The importance of the amphiphilic α-helix was also shown in a crystallographic study on the complex of the acidic activation domain of p53 and the attenuator protein MDM2 (35). The TFE-induced formation of the α-helix in the PPARα AF-1 region possibly mimics the structural change induced by a protein-protein interaction.

Comparison of several acidic activation domains as well as mutation analysis led to a proposal regarding the importance of the FXXxF motif (34), where Δ denotes a hydrophobic amino acid. In PPARs, sequences 27–31 correspond with this motif at Phe27 and Met31, but not at Glu30. The importance of the primary structure including several aromatic and hydrophobic residues was proposed based on sequence comparison among the AF-1 regions of nuclear receptors (36, 37). The AF-1 region of PPARs contains only one aromatic residue (Phe27) and has no significant similarity to the proposed consensus sequence. Thus, the AF-1 region of PPARα seems to share the proposed characteristics only imperfectly with other acidic activation domains.

For PPAR isoforms, phosphorylation at the mitogen-activated protein kinase sites in the A/B regions has been highlighted in relation to AF-1 activity. PPARα was shown to be a phosphoprotein (38), and very recently, the phosphorylation sites were mapped at Ser112 and Ser211 (32). It has also been shown that phosphorylation is promoted by insulin, potentiating the AF-1 activity. The second phosphorylation site (Ser211) is located within the essential region of AF-1 characterized in this study. How phosphorylation at these sites affects the protein structure of the AF-1 region of PPARα would be an interesting issue. On the other hand, phosphorylation of Ser212 of PPARγ1 or Ser112 of PPARγ2 by the mitogen-activated protein kinase family has been reported by many researchers (16, 18–23). Most of them (18–22) argued that phosphorylation of the PPARγ A/B region suppresses the transcriptional activating function of the receptor and leads to decreased differentiation of preadipocytes, a major physiological consequence of PPARγ activation. Others (16, 22), however, presented contradictory results, claiming potentiation of the activating function of PPARγ as well as promotion of adipogenesis by phosphorylation. Ligand-independent AF-1 activities were shown in the A/B regions of PPARγ1 and PPARγ2 (16), the latter being markedly more active. On the other hand, Shao et al. (22) documented an interdomain communication between the A/B and ligand-binding domains of PPARγ, resulting in modulation of the subjects of future studies.

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