Isolation and Characterization of PBP, a Protein That Interacts with Peroxisome Proliferator-activated Receptor*  

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In an attempt to identify cofactors that could possibly influence the transcriptional activity of peroxisome proliferator-activated receptors (PPARs), we used a yeast two-hybrid system with Gal4-PPARγ as bait to screen a mouse liver cDNA library and have identified steroid receptor coactivator-1 (SRC-1) as a PPAR transcriptional coactivator. We now report the isolation of a cDNA encoding a 165-kDa PPARγ-binding protein, designated PBP which also serves as a coactivator. PBP also binds to PPARα, RARα, RXR, and TRβ1, and this binding is increased in the presence of specific ligands. Deletion of the last 12 amino acids from the carboxyl terminus of PPARγ results in the abolition of interaction between PBP and PPARγ. PBP modestly increased the transcriptional activity of PPARγ, and a truncated form of PBP (amino acids 487–735) acted as a dominant-negative repressor, suggesting that PBP is a genuine coactivator for PPAR. In addition, PBP contains two LXXLL signature motifs considered necessary and sufficient for the binding of several coactivators to nuclear receptors. In situ hybridization and Northern analysis showed that PBP is expressed in many tissues of adult mice, including the germinal epithelium of testis, where it appeared most abundant, and during ontogeny, suggesting a possible role for this cofactor in cellular proliferation and differentiation.

The peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors that regulate the expression of target genes, in particular those associated with lipid metabolism (1, 2). PPARs, which derive the designation by virtue of their ability to mediate predictable pleiotropic effects in response to peroxisome proliferators (1, 3, 4), are members of the nuclear receptor superfamily (5, 6). Three isotypes of PPARs, namely PPARα, PPARδ (also called β or NUC-1), and PPARγ have been identified as products of separate genes from Xenopus, rodents, and humans (1, 7–12). These PPAR isotypes appear to exhibit distinct patterns of tissue distribution and differ considerably in their ligand binding domains, suggesting that they possibly perform different functions in different cell types (7, 13, 14). Indeed, of the three isotypes, PPARα expression is relatively high in hepatocytes, enterocytes, and the proximal tubular epithelium of kidney when compared with other cell types (13, 14), and evidence derived from mice with PPARα gene disruption indicates that this receptor is essential for the pleiotropic responses induced by peroxisome proliferators (15). Several structurally diverse peroxisome proliferators, specific fatty acids, and eicosanoids act as ligands for PPARα (4, 16–19). Although PPARδ isotype is ubiquitously expressed and binds the same ligands as PPARα (18, 19), its functional significance remains largely elusive. PPARγ exists as two isoforms, PPARγ1 and PPARγ2, as a consequence of alternate promoter usage in the gene encoding this receptor (8, 20, 21). While PPARγ1 isoform expression is restricted to liver and few other organs (8, 14), the PPARγ2 isoform, which plays an important role in adipocyte differentiation, is predominantly expressed in adipose tissue (8, 14). Forced expression of the PPARγ1 or PPARγ2 isoforms in fibroblasts has been shown to convert these cells into adipocytes, suggesting that PPARγ exerts a pivotal role in adipocyte development and lipid homeostasis (20, 22). PPARγ is activated by the arachidonic metabolite 15-deoxy-D12,14-prostaglandin-J2, which appears to function as a natural ligand for this receptor, as well as a thiazolidinedione class of antidiabetic drugs (23, 24).

Like other members of the nuclear receptor superfamily, PPARs possess a central DNA-binding domain that recognizes PPAR-response elements (PPREs) in the promoter regions of their target genes (1, 7, 25). PPARα heterodimerizes with RXR (RXR is the receptor for 9-cis-retinoic acid) and the transcriptional regulation of target genes by PPARs is achieved through the binding of these PPAR-RXR heterodimers to PPREs (3, 25, 26). RXR also forms heterodimers with other members of the nuclear receptor superfamily, and these interactions appear to influence the PPAR-regulated transcriptional activation because of the competition among various RXR heterodimerization partners for RXR (27, 28). In addition, tissue and species responses to peroxisome proliferators and other natural PPAR ligands may depend upon pharmacokinetics and or metabolism, the relative abundance of PPAR isotype and its heterodimerization partner RXR, the structural features of PPREs and flanking sequences, and to some extent hormone levels and dietary composition (3, 28, 29).

Transcriptional regulation of nuclear hormone receptors involves the participation of basal transcription factors, including TATA-binding protein and TFIIIB, and other cofactors, known as nuclear transcriptional coactivators or corepressors, that bridge the association between nuclear receptors and the basal transcription machinery (30, 31). The cofactors identified in recent years include, CBP/p300 (32, 33), SRC-1/NCoA1 (34, 35), TIF-2/GRIP-1/NCoA2 (36, 37), p/CIP (37), N-CoR (38),…
SUGI/TRIP1 (39), SMRT (40), and RIP140 (41) among others. Of these CBP/p300, as well as SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2, pCIP, and RIP140, function as nuclear receptor coactivators, whereas NCoR and SMRT function as transcriptional corepressors. In an effort to understand possible tissue- and species-specific differences in the transcriptional activity of PPAR isotypes, we initiated studies to identify cofactors that influence PPAR transcriptional activity. Using the PPARγ ligand binding domain as the bait in a yeast two-hybrid system to screen a mouse liver cDNA library, we previously identified Src-1 as a PPAR coactivator (42). Here we report the cloning and characterization of PBP, a new PPAR-binding protein. In addition, we show that PBP binds to TRβ1, RARα, and RXRα. Functional studies reveal that PBP modestly increases the transcriptional activity of PPARγ and a truncated PBP (amino acids 487–735), which contains putative PPAR binding region (amino acids 626–686), acts in a dominant-negative fashion causing a decrease in the transcriptional activity of PPARγ.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening—To isolate cDNAs encoding proteins that specifically interact with PPARγ, yeast two-hybrid screening was used as described elsewhere (42). Briefly, this screening system employed GAL4-PPARγ (expressing GAL4-DNA-binding domain and mPPARγ-ligand binding domain fusion protein), which was cotransformed into yeast with a second vector that expressed fusion proteins between GAL4-activating domain and mouse liver cDNA. Of the 13 clones that exhibited positive interaction with PPARγ, two were identified previously as mSRC-1 (42). Of the remaining 11 clones, 2 revealed overlapping cDNA sequences. As these positive clones contained only partial cDNA sequences, we used RACE PCR to obtain the remaining 5'- and 3'-end sequences. Briefly, for 5'-RACE PCR, the first amplification was performed using the adapter primer 1 and the gene-specific primer (5'-CAATAGAGACAGTGGGTTG-3') for 20 cycles. Each cycle consisted of 20 s at 94 °C, 30 s at 60 °C, and 4 min at 68 °C; 1 µl of the PCR product was used as the template for the second amplification with the adapter primer 2 and the nested gene-specific primer (5'-GCGCTGTATGGTGTCCTTC-3') for 20 cycles, essentially using the same conditions as those used for the first amplification. The PCR products were cloned into pGem-T (Promega), and three independent clones were sequenced. For 3'-RACE PCR, the sequences of the gene-specific and nested gene-specific primers were 5'-CATCCTCTCAGAATCAACATGGCAG-3' and 5'-CCAAAGGGAA-ATCTCAGATGAGG-3', respectively. These PCR amplifications were performed using the mouse liver marathon ready cDNA (CLONTECH) and rTth DNA polymerase. The full-length cDNA we cloned has been designated PBP to reflect its ability to bind PPARs.

Cloning of PBP—The yeast expression vector GAL4-PPARγ, GST-PPARγ, GST-RXRα, PCMV-PPARγ, PPRE-TK-LUC, and GAL-TK-LUC have been described elsewhere (42). The vectors for in vitro transcription and translation of RARα and TRβ1 were provided by Dr. L. Madison (Northwestern University Medical School). The construction of pSV-sport-PPARα for in vitro transcription was described previously (13, 43). GAL-PPARα-L2 was constructed by inserting PCR-amplified cDNA fragment encoding amino acids 174–463 of mPPARα into EcoRI/Sal1 site of pGHT (CLONTECH). This GAL-PPARα-L2 construct does not include the last 12 amino acids at carboxyl terminus of the mPPARα ligand binding domain (amino acids 174–475). PCMV-PPARβ was generated by inserting the full-length coding region of RBP cDNA into NotI site of PCMV-FLAG-2 (Eastman Kodak Co.). PCMV-PBP, for in vitro transcription, was constructed by inserting the full-length PBP cDNA into the BamHI/Sal1 site of PCMV. To construct SK-BPT ( truncated PBP consisting of residues 487–735), GST-BPT-P and PCMV-BPT-P, the partial PBP cDNA fragment encoding amino acids 487–735 was released from PGADH10-PBT clone isolated by a yeast two-hybrid system and subcloned into BamHI site of pBluescript SK, NotI site of the modified PCMVP1-A22, which contains a nuclear targeting signal peptide PKKKRKV, and BamHI site of pGEX-5X-2.

Quantitative β-Galactosidase Assays—For quantitative characterization of the interaction of PPARα with PBP, appropriate plasmids were cotransformed into yeast HFTc, plated on selective media (containing PPARγ ligand BRL49653 at a concentration of 10–5 M, or no ligand), and the plates were incubated for 4 days at 30 °C. For each assay, five colonies were suspended in 150 µl of buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 35 mM 2-mercaptoethanol). The cell suspension (10 µl) was diluted in 190 µl of buffer Z, and the A550 was measured to estimate cell density. The remaining cell suspension was pelleted by centrifugation, and β-galactosidase activity was determined by a chemiluminescent reporter protocol (Galacto-light kit, Toyo, Bedford, MA).

Interaction of PBP with PPARγ, RXRα, PPARα, RARα, and TRβ1 in Vitro—PPAR in PCMX was transcribed and translated using rabbit reticulocyte lysate (Promega) and labeled with [35S]methionine. GST, GST-mPPARγ, and GST-RXRα in PEGX-3X were produced in Escherichia coli DH5α and bound to glutathione-Sepharose beads according to manufacturer's instructions (Pharmacia). A 10 µl aliquot of PPAR-GBP, mPPARγ-GBP, and loaded on glutathione-Sepharose beads was incubated with 5 µl of [35S]methionine labeled full-length PBP protein for 2 h in 600 µl of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). BRL49653 (at a final concentration of 1 × 10–5 M) or 9-cis-retinoic acid (at 1 × 10–6 M) was at the beginning of incubation when required. Bound proteins were washed three times with binding buffer, eluted by boiling for 2 min in 20 µl of SDS sample buffer, analyzed by SDS-PAGE, and autoradiographed.

To assay the binding of PPARα, RARα, and TRβ1 to PBP, a truncated fragment of PBP (from amino acids 487–735) which contains a putative PPAR-binding domain was generated in E. coli, using the expression plasmid GST-PBP, and allowed to interact with [35S]methionine-labeled PPARγ, RARα, or TRβ1 produced in vitro using rabbit reticulocyte translation system. The binding was assayed in the presence or absence of specific ligands: Wy-14463 (1 × 10–4 M) for PPARγ, 9-cis-retinoic acid (1 × 10–6 M) for RARα, and T3 (1 × 10–8 M) for TRβ1. Bound proteins were washed three times with binding buffer, NETN, eluted, and subjected to SDS-PAGE as described above.

Cell Culture and Transfection—NIH3T3 or CV-1 cells (1 × 104) were plated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum or fetal calf serum in six-well plates, respectively, and cultured for 24 h before transfection. Cells were transfected for 5 h with 1.25 µg of luciferase reporter plasmid DNA, 0.75 µg of appropriate expression plasmid DNA, and 0.5 µg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,trimethylammonium methyleste- mediated transfection method (Boehringer Mannheim). Cell extracts were prepared 36 h after transfection and assayed for luciferase and β-galactosidase activities (Tropix, Bedford, MA).

Northern Blot Analysis and in Situ Hybridization—Fifty nanograms of PBP cDNA were random-primer and used as a probe to hybridize mouse multiple tissue Northern blot (CLONTECH). For in situ hybridization, mouse embryos from embryonic (E)9.5 days and E13.5 days and adult mouse tissues were immersed in 4% paraformaldehyde for 16–20 h at 4 °C and processed as described previously (44). Both sense and antisense PBP riboprobe were generated with [α-35S]UTP (Amer- sham Corp.) with T3 and T7 polymerases from pSKPBPT. Hybridization, washing, and examination of results were as described elsewhere (44).

RESULTS

Cloning of PBP—To identify novel factors involved in PPAR signaling, we employed a yeast two-hybrid assay that would detect proteins interacting with PPARγ. Of the 13 positive clones that interacted with PPARγ, two represented the overlapping sequences of mSRC-1 (42). Cloning and characterization of the full-length mSRC-1 cDNA showed that SRC-1 acted as a PPARγ coactivator (42). In this report, we describe the characterization of two other overlapping clones, 759 and 2116 bp in size, respectively, that represented a second cDNA. Since these two clones did not contain the full-length cDNA, we used RACE PCR to obtain the remaining 5'- and 3'-end sequences. The nucleotide sequence of putative full-length composite cDNA is shown in Fig. 1. The cDNA, 5676 bp in length, contains a short 5'- (120 bp)- and a long 3'- (996 bp)-untranslated region and an open reading frame of 4680 which encodes a peptide of 1560 amino acids with the predicted molecular mass of the protein of 165 kDa. This protein was designated PBP (PPAR-binding protein) to signify its ability to bind PPARs. The start of the coding sequence was defined by the first ATG downstream of an in-frame stop codon at position 66. The
sequences (GTAAGATGAGCTCC) surrounding ATG essentially conform to a consensus sequence for the translation initiation site (45). The two partial cDNAs isolated by the two-hybrid system represented amino acid residues 487–739, and 626–1297 of PBP, respectively. Comparison of the deduced primary structure of the PBP protein with sequences in the data base revealed that the central domain of the full-length PBP cDNA shows 90% similarity to TRIP2, a 250-bp cDNA fragment isolated from human HeLa cDNA library by the two-hybrid system using TRb1 as a bait (46). Information on the full-length cDNA sequence of this TRIP-2, and its role in receptor signaling is not available. TRIP-2 cDNA fragment corresponds with the 607–686-amino acid stretch of the full-length mouse PBP. PBP contains two LXXLL (where L is leucine and X is any amino acid) motifs which are at 589–593, and 630–634 amino acids, respectively. LXXLL has been recently identified as a signature motif in transcriptional coactivators that mediates binding to nuclear receptors (37, 47). A third motif in reverse orientation (LLXXL) is present at 4–8 amino acids (Fig. 1).

**Interaction of PBP with PPARg in Yeast**—The influence of PPARg ligand BRL49653 on the interaction between PPARg and PBP was examined in yeast. PGADH10-PBP1, which was isolated the by two-hybrid system and expressed as the fusion protein between the GAL4 activation domain and truncated PBP (from 487 to 739 amino acids) in yeast, was co-transformed with GAL-PPARg into yeast HF7C, and the β-galactosidase activity measured as an indication of the relative strength of the interaction in the presence or absence of ligand. The interaction between PPARg and PBP resulted in a 48-fold increase in the β-galactosidase activity and this interaction is enhanced approximately 2-fold in the presence of ligand (Fig. 2). Thus, the ligand can moderately increase the affinity of the interaction.

The extreme carboxyl-terminal region of the ligand binding domain conserved among the nuclear receptors appears essential for the ligand-dependent transcriptional activation. Deletion of this region reduces the transcriptional activation, but does not affect the ligand binding activity. To ascertain whether this region is important to the binding of mPPAR with PBP, we used GAL-PPARgD12, which lacks the last 12 amino acids at the carboxyl terminus of the PPARg ligand binding domain, to cotransform with PGADH10-PBP1 into yeast HF7C. As shown in Fig. 2, the presence of PBP did not affect the interaction of PBP with the truncated PPARg.
increase the \( \beta \)-galactosidase activity over the control in the presence or absence of ligand, suggesting that the mutant PPAR\( \gamma \) is unable to bind with PBP.

**In Vitro Binding of PBP to Different Nuclear Receptors**—To determine whether PBP directly interacts with PPAR\( \gamma \), in vitro binding was assayed with bacterially generated fusion protein of GST with PPAR\( \gamma \) (GST-PPAR\( \gamma \)) and in vitro translated PBP. A matrix-bound GST-PPAR\( \gamma \), but not GST alone, retained radiolabeled PBP in the presence or absence of PPAR\( \gamma \) ligand (Fig. 3A). The presence of BRL49653, a PPAR\( \gamma \) ligand in the assay mixture, increased the physical interaction (Fig. 3). We also examined the ability of PBP to bind with PPAR heterodimerization partner, RXR. As shown in Fig. 3A (lanes 3 and 5), a matrix bound fusion protein of GST-RXR, but not GST alone, retained \( [\text{S}^{35}] \)methionine-labeled PBP, and the presence of ligand, 9-cis-retinoic acid enhanced this interaction.

To ascertain if PBP interacts with some other nuclear receptors, we used a truncated PBP (from 487 to 739 amino acids, designated PBPT) that was capable of binding to PPAR\( \gamma \). A fusion protein of GST with PBPT was bacterially produced and used for binding assays with \( [\text{S}^{35}] \)methionine-labeled in vitro translated PPAR\( \alpha \), RAR\( \alpha \), and TR\( \beta \). All three receptors bind to PBP, and the interaction is stronger in the presence of respective ligands (Fig. 3B). It appears that PPAR\( \alpha \) and RAR\( \alpha \) interaction with PBP is more prominent in the presence of their respective ligands.
PPAR-binding Protein

FIG. 4. PBP moderately enhances mPPARγ-mediated transactivation of reporter expression in NIH 3T3 cells. NIH 3T3 cells were cotransfected with 1.5 μg of reporter construct PPRE-TK-LUC, 0.25 μg of PCMV-mPPARγ, 0.5 μg of PCMV-PBP, and 0.5 μg of PCMVβ in the absence (−) or presence (+) of 10−8 M BRL49653. Transfection without PCMV-PBP was compensated by adding the same amount of PCMV-FLAG2 DNA. The activity obtained on transfection of the PPRE-TK-LUC without exogenous PBP in the absence of ligand was taken as 1. Results are the mean of four independent transfections and normalized to the internal controls of β-galactosidase expression.

FIG. 5. Repression of PPARγ-mediated transactivation by truncated PBP. PCMV-PPARγ was cotransfected, as in legend for Fig. 4, with PCMV-PBP along with PCMV-PBPT (amino acids 487–739), and PCMVβ into CV-1 cells in the presence or absence of BRL49653 (10−8 M). For control transfections PCMV-FLAG2 plasmid was used instead of PCMV-PBPT. Luciferase activity is presented as percent where induced mPPARγ activity in the presence of BRL49653 is arbitrarily set at 100%.

FIG. 6. Northern blot analysis of PBP mRNA. A mouse multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A) RNA for each tissue was probed with 32P-labeled PBP cDNA. The PBP hybridized blots were exposed to film at −80°C with intensifier screens for 24 h. The transcript size of PBP165 is 8 kb in all tissues examined. An additional 2.7-kb transcript is present in testis.

FIG. 7. In situ hybridization with sense-strand probe of PBP (data not shown).

**DISCUSSION**

In a previous study, using the yeast two-hybrid system, we isolated and characterized mouse SRC-1 as a PPAR coactivator (42). The data presented in this report demonstrate that PPAR is capable of interacting with factor(s) other than SRC-1. Our data suggest that PBP, a 165-kDa protein that interacts with PPAR, serves as a coactivator. We also show that PBP binds to PPARα, RXRα, RARα, and TRβ1 and this binding is increased in the presence of their respective ligands. It is pertinent to note that the ligands for PPARα and RARα were effective in enhancing the interaction between PBP-PPARα and PBP-RARα, respectively. On the other hand, there was only a modest increase in the interaction between PBP and other receptors, namely TRβ1, PPARγ, and RXRα, in the presence of their respective ligands. The significance of the differential influence of ligands on the interaction remains to be explored. Further studies are also needed to determine whether PBP is capable of interacting with steroid receptors, such as estrogen, growth hormone, and progesterone receptors. Overexpression...
of PBP exerted only a modest influence on the transcriptional activity of the PPAR, implying that this protein does not appear to be a rate-limiting factor. This is in contrast to the coactivator activity of CBP/p300 and SRC-1, which either singly or together are able to markedly increase the transcriptional activation by several nuclear receptors including PPAR (31, 48–52). Nonetheless, we have shown that the truncated form of PBP (amino acids 487–735), which contains the putative receptor-binding domain, acts as a dominant-negative repressor, suggesting that it is a genuine coactivator for PPAR-mediated gene expression.

Northern blot analysis reveals that the gene encoding PBP is widely expressed, but at different levels in various tissues of the adult, the most prominent being the testis. The distribution of PBP in the adult tissues, in general, parallels the expression of PPARs (14). The abundance of expression in the testis suggests a possible role for PBP in cellular division and differentiation. In situ hybridization data on the developing mouse embryo revealed widespread expression of PBP, suggesting that this gene may play an important role in development and differentiation, which is consistent with the function of coactivators. PBP is detectable as early as E9.5 with strong expression in neural epithelium, primitive gut, and branchial arches, suggesting its possible biological involvement in the genesis of their derivatives. The in situ hybridization data of E13.5-day embryo, which reveals expression in tongue, lower jaw, and other organs further supports this assumption.

The finding that PBP failed to interact with PPAR γ that had the deletion of the last 12 amino acids at the extreme carboxyl terminus of the ligand binding domain is of interest, in that this domain may be critical for the interaction of coactivators. For example, RIP140 and TIF1 are incapable of binding to nuclear receptors that lack this domain (36, 41). This region is important for the transcriptional activation function of the nuclear receptors and indispensable for the hormone binding and heterodimerization function (27). Two studies published recently point out that a sequence motif LXXLL is necessary and sufficient for the binding of some cofactors to nuclear receptors (37, 47). PBP contains two copies of this motif that are located at residue 589–593 and residue 630–634, respectively. Based on the sequence data of the overlapping regions of two partial PBP cDNA clones isolated using PPAR as a bait, we suggest a single LXXLL motif at residue 630–634 is sufficient for the binding of the PBP to PPAR, and other receptors analyzed for binding in this study. Nonetheless, detailed studies on the mutational analysis of this motif, as well as detailed studies on the mapping of binding sites, are needed to ascertain other regions in PBP protein that might play a role in protein–protein interactions.

There is increasing evidence for the participation of multiple molecular partners in determining the transcriptional outcome of nuclear receptors in response to ligands (30, 31, 34, 37). We have shown that both SRC-1 and PBP act as coactivators for PPAR target gene expression. We also ascertained that SRC-1 and PBP do not interact with each other.2 Since SRC-1 interacts with CBP/p300 to augment transcription of nuclear receptors (31), it remains to establish whether PBP is capable of binding or interacting with CBP/p300. As the transcriptional activity of the nuclear receptors appears to vary, depending on the cell type and the nature of the response elements in the target gene promoter, there is a need to fully dissect the role and availability of different combinations of cofactors and corepressors in the cell-specific target gene expression. In an earlier study we demonstrated that deoxyuridine triphosphatase serves as a corepressor of PPAR target gene transcription (43), suggesting a role for both coactivators and corepressors in the PPAR-mediated transcription. In summary, the availability of different cofactors to a specific gene promoter may determine the specificity of gene expression.

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