Identification of ARA70 as a Ligand-enhanced Coactivator for the Peroxisome Proliferator-activated Receptor γ

(Received for publication, February 18, 1999)

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In an effort to understand transcriptional regulation by the peroxisome proliferator-activated receptor γ (PPARγ), we have investigated its potential interaction with coregulators and have identified ARA70 as a ligand-enhanced coactivator. ARA70 was initially described as a coactivator for the androgen receptor (AR) and is expressed in a range of tissues including adipose tissue (Yeh, S., and Chang, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5517–5521). Here we show that ARA70 and PPARγ specifically interact by communoprecipitation and in a mammalian two-hybrid assay. PPARγ and ARA70 interact in the absence of the PPARγ ligand 15-deoxy-Δ12,14-prostaglandin J2, although the addition of exogenous ligand enhances this interaction. Similarly, in transient transfection of DU145 cells, cotransfection of PPARγ and ARA70 induces transcription from reporter constructs driven by either three copies of an isolated PPAR response element or the natural promoter of the adipocyte fatty acid-binding protein 2 in the absence of exogenous 15-deoxy-Δ12,14-prostaglandin J2. However, this PPARγ-ARA70 transactivation is enhanced by the addition of ligand. Thus, ARA70 can function as a ligand-enhanced coactivator of PPARγ. Finally, we show that AR can squelch PPARγ-ARA70 transactivation, which suggests that cross-talk may occur between PPARγ- and AR-mediated responses in adipocytes.

The peroxisome proliferator-activated receptors (PPARs) are structurally similar members of the nuclear hormone receptor superfamily that become transcriptionally active in response to a diverse group of chemical compounds, including fibrate hyperlipidemic drugs, thiazolidinedione anti-diabetic drugs, arachidonic acid derivatives, fatty acids, and peroxisome proliferating chemicals (reviewed in Ref. 2). These receptors are considered to play key roles in lipid metabolism and storage (3). The three mammalian PPAR members (α, γ, and δ) are each encoded by a separate gene and show a distinct but overlapping tissue distribution (4). PPARγ is highly expressed in the liver, kidney, and adrenal gland and has been shown to regulate expression of genes involved in hepatic lipid metabolism, including the P450 fatty acid ω-hydroxylase gene and genes involved in the peroxisomal β-oxidation pathway (5, 6). PPARδ (also referred to as NUC1 or FAAR) is expressed ubiquitously and has been reported to repress transcription by PPARs and the thyroid hormone receptor (7). PPARγ exists as two isoforms encoded by a single gene, PPARγ1 and PPARγ2 (8). PPARγ2 is transcribed from an alternative promoter and is 30 amino acids longer than PPARγ1. Although PPARγ2 is the predominant isoform in adipocytes (8), PPARγ1 is also expressed in a number of other tissues including the adrenal gland and spleen (4). Consistent with its adipocyte localization, PPARγ is involved in the regulation of adipocyte-specific genes, including the adipocyte fatty acid binding protein 2 (ap2) (8). PPARγ is also implicated in adipogenesis (9). Stable transfection of PPARγ1 or PPARγ2 into fibroblasts induces them to differentiate into adipocytes (3, 8). Ligands identified for PPARγ include the insulin-sensitizing thiazolidinedione antidiabetic drugs and the naturally occurring prostaglandin derivative, 15-deoxy-Δ12,14-prostaglandin J2 (15dJ2), each of which can promote adipocyte differentiation (10–12). In addition to its role in adipocyte differentiation and function, PPARγ has recently been implicated in a number of pathological conditions including atherosclerosis and colorectal cancer (13–15).

The PPAR isoforms regulate target gene transcription through binding to PPAR response elements (PPREs), a DR1-type hormone response element. This binding is enhanced by heterodimerization to the retinoid X receptor (RXR) (16, 17). The PPAR-RXR heterodimer is responsive to both PPAR ligands and the RXR ligand 9-cis-retinoic acid (17, 18). Functional PPREs have been identified in the promoters of a number of genes including acyl-CoA oxidase (6), phosphoenolpyruvate carboxykinase (19), CYP4A6 (5), lipoprotein lipase (20), and ap2 (8).

Transcriptional activation or repression by nuclear hormone receptors can be augmented by transcriptional coactivators and corepressors, which can serve as a bridge between the nuclear receptor and the basal transcriptional machinery. A number of coactivators, including ARA70 (1), RIP140 (21), CBP/p300 (22, 23), TIF2 (24), SRC-1 (25), and ARA54 (26) have recently been identified as interacting with one or more nuclear hormone receptors. These interactions are typically dependent on the presence of ligand. Recently a number of cofactors for the PPAR isoforms have been identified. SRC-1, PBP, CBP, and PGC-1 (27–30) have been shown to interact with PPARγ, and p300 has been shown to be a coactivator for PPARα (31). The rat dUTPase is a potential corepressor for all the PPAR isoforms.

* This work was supported by National Institutes of Health Grants CA55638, CA68568, and CA77532. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; mPPAR, mouse PPAR; DBD, DNA binding domain; 15dJ2, 15-deoxy-Δ12,14 prostaglandin J2; AR, androgen receptor; hAR, human AR; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator 1; ap2, adipocyte fatty acid-binding protein 2; DHTR, dihydrotestosterone; CAT, chloroamphenicol acetyltransferase; BES, 2-(bis(2-hydroxyethyl))amino)ethanesulfonic acid; TK, thymidine kinase; CBP, CAMP response element-binding protein-binding protein; PBP, PPAR-binding protein.
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(32). To further understand transcriptional regulation by PPARγ, we have investigated the potential interaction between PPARγ and ARA70. ARA70 was originally described as a coactivator of the androgen receptor and shows a broad tissue distribution of expression, including adipose tissue (1). Here we present evidence to demonstrate that ARA70 can induce PPARγ target genes through a PPARγ-ARA70 complex. We also show that PPARγ- and AR-mediated pathways may be linked through their common use of ARA70. Unlike previously described receptor-coactivator interactions, ARA70 can confer transcriptional activity to PPARγ in the absence of ligand, although the presence of ligand enhances PPARγ-ARA70 transactivation.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid expressing the GALA-DNA binding domain (DBD) and the mPPARγ ligand binding domain (pGAL4DBD-mPPARγ) was constructed by inserting the mouse PPARγ ligand binding domain (from amino acids 162–475), isolated as a ScaI/BamHI fragment from pGBTM-mPPARγ in-frame into pCMX4a4-D4. The plasmid expressing VP16-ARA70 was constructed by inserting a fragment of ARA70 cDNA encoding amino acids 1–401 into the VP16 activation domain plasmid pCMX-VP16. The site-directed mutagenesis was generated using the following four primers: ‘‘-CGCGAATTCGTCGACCCAA-GCTCTACTCGGCAGCGGGCCAGTTCAATTG-3’’; ‘‘-GGTCT-3’’; ‘‘-GCTCTACTCGGCAGCGGGCCAGTTCAATTG-3’’; ‘‘-TACCCTTACATGGGTC-3’’. Mutagenesis was carried out on the ARA70 cDNA fragment encoding amino acids 1–401 by polymerase chain reaction (33). The mutated fragment was then reinserted in-frame into pCMX-VP16. The construction of pSG5-PPAR was done by inserting the Asp718-SauII fragment from pGBTM-PPARγ into pSG5.

Commmunoprecipitation—Recombinant mPPARγ, RXRα, and ARA70 were expressed by the TNT Coupled Reticulocyte Lysate System (Promega) incorporating [35S]methionine according to the manufacturer’s instructions. Fifteen μl of labeled receptors were mixed with 50 μl of ARA70 and incubated with 15–20 μl of antibody to PPARγ (Santa Cruz), RXRα (Santa Cruz), or with the AR antibody NH27 (34). Proteins and antibody were incubated in 70 μl of HC400 (20 mM Heps-KOH, pH 7.9, 400 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.1 mg/ml bovine serum albumin) for 1 h at 4 °C before adding 10 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech). DHT was added to the AR + ARA70 reaction at a final concentration of 100 nM. The reaction was then incubated while rocking overnight at 4 °C. Immunoprecipitated complexes were collected by centrifugation at 2000 rpm at 4 °C for 1 min. Immunoprecipitated complexes were washed three times with HC400, four times with 10 mM KPO4, pH 8.0, 0.1 % X1, and then mixed with SDS sample buffer, boiled, and separated by 8% SDS-polyacrylamide gel electrophoresis.

Cell Culture and Transfection—Human prostate DU145 cells were grown in Dulbecco’s minimal essential medium containing 5% fetal calf serum at 37 °C. The cells were transfected by modified BES-calcium phosphate procedure (25). Cells were plated 4 × 10^5/60-mm Petri dish 1 day before transfection. Transfection medium contained a constant amount of reporter plasmid and indicated amounts of pSG5-mPPARγ, pSG5-RXRα, and pSG5-hARα70 using pSG5 as a carrier to provide equal amounts of transfected DNA. One hour before transfection, the medium was changed to Dulbecco’s minimal essential medium with 5% charcoal-stripped fetal calf serum, and the medium was changed again 20 h post-transfection and treated with steroid hormone or 15dJ2 for another 14–16 h. Cell extracts were prepared and assayed for CAT or luciferase activity (Promega) and normalized against β-galactosidase or Renilla luciferase activity as indicated. All data were the average results from three to six independent experiments.

RESULTS

Interaction of ARA70 with PPARγ—To investigate the potential interaction between ARA70 and PPARγ, we first analyzed their interaction in a mammalian two-hybrid assay in DU145 cells. DU145 cells were used because they do not express AR, and the subline in our hands shows a low level of endogenous ARA70 activity to coactivate AR-mediated transcription. The ligand binding domain of AR, PPARγ, and RXR were fused to the GAL4 DNA binding domain (GAL4DBD). These were cotransfected with a vector expressing the VP16 activation domain alone or linked with ARA70. In agreement with our previous results (1), coexpression of GAL4DBD-AR with VP16-ARA70 induced CAT activity by 11-fold in the presence of DHT (Fig. 1A, lanes 7 and 8). ARA70 is also able to interact with RXR in the presence of its ligand, 9-cis-retinoic acid. In contrast, GAL4DBD-PPARγ cotransfected with VP16-ARA70, did not require the presence of ligand to induce CAT activity. In this case, CAT activity was induced 36-fold in the absence of the PPARγ ligand 15dJ2 (10, 11) and 52-fold in the presence of exogenous ligand (Fig. 1A, lanes 4 and 10). It has previously been reported that SRC-1 is a ligand-dependent cofactor of PPARγ (28). Here we also find that VP16-SRC1 interacts with GAL4DBD-PPARγ to induce luciferase activity. A slight interaction between SRC-1 and PPARγ is observed in the absence of ligand, but this is significantly weaker than the ligand-independent activation seen with ARA70 (Fig. 1B, lanes 2 and 3). In the presence of 15dJ2, the interaction between PPARγ and ARA70 is more than twice that of PPARγ and SRC-1 (Fig. 1B, lanes 5 and 6).

Interaction between PPARγ and RXR with ARA70 was further confirmed by communoprecipitation. The receptors and ARA70 were expressed in vitro using rabbit reticulocyte lysate and communoprecipitated using antibodies specific for AR, RXR, or PPARγ as indicated (Fig. 1C). Although AR communoprecipitated ARA70 only in the presence of DHT, PPARγ and RXR were able to bind ARA70 even in the absence of their respective ligands.

Site-directed Mutation of ARA70 Attenuates Its Interaction with PPARγ and RXR—A number of nuclear receptor coactivators contain a LXXLL motif (or NR box) that is considered to play an important role in nuclear receptor-coactivator interaction (35, 36). ARA70 contains one NR box (LXXLL) at amino acids 92–96 (Fig. 2A). To investigate whether the ARA70 NR box influences the PPARγ-ARA70 and RXR-ARA70 interactions, we mutated this region. Mutation of the leucine doublet to alamines (LXXLL → LXXAA) of ARA70 (mTARA70) was tested in a mammalian two-hybrid assay in DU145 cells. The VP16-mTARA70 showed reduced transactivation of both GAL4DBD-RXR and GAL4DBD-PPARγ (Fig. 2B). In the presence of the appropriate ligand, the VP16-mTARA70 significantly reduced the transactivation of both receptors compared with the wild type ARA70. In the case of GAL4DBD-PPARγ, the mutant ARA70 also reduced transactivation in the absence of ligand (Fig. 2B, lanes 3 and 6). This demonstrates the importance of the ARA70 NR box in the interaction of ARA70 with both PPARγ and RXR.

ARA70 Enhances the Transcriptional Activity of PPARγ—To investigate the functional relevance of the interaction between PPARγ and ARA70, we coexpressed PPARγ and ARA70 in DU145 cells. We examined PPARγ-mediated transcriptional activity on two luciferase reporter genes, one driven by three copies of a PPRE linked to a TK promoter (Fig. 3) and another containing 5.4 kilobases of the ap2 gene promoter (Fig. 4). The addition of ARA70 increases the PPARγ-mediated transcription of both reporter constructs. The transfection of ARA70 with PPARγ alone or with PPARγ and RXR did not show significant differences, probably because of abundant endogenous RXR expression in DU145 cells. RXR, like PPAR, binds to a DR-1-type response element and therefore in theory could activate our reporter constructs. Transfection of RXR with ARA70 or ARA70 alone did not result in reporter gene expression, showing that both reporter genes are activated through PPARγ. In agreement with previous reports (17, 37), we show that transcriptional activation occurs in response to either 15dJ2 or 9-cis-retinoic acid and that maximal transcription by PPARγ-RXR occurs in the presence of both ligands (Fig. 3). In
the presence of both ligands, PPARγ-RXR-ARA70 elicits a stronger transcriptional response than PPARγ-SRC-1 (data not shown).

AR Can Squelch ARA70 from PPARγ—In a previous report (1), we demonstrated that ARA70 and AR physically interact and that ARA70 can function as an androgen-dependent coactivator for AR. We were interested to know if AR and PPARγ could compete for ARA70. Cotransfection of PPARγ, RXR, and AR does not significantly influence PPARγRXR-ARA70 transcription (Fig. 5). However, AR is able to significantly reduce PPARγRXR-ARA70 transcriptional activity even in the presence of 15dJ2 and 9-cis-retinoic acid (Fig. 5, lanes 15 and 16). Potentially, cross-talk between AR and PPARγ could occur through their functional association with ARA70.

DISCUSSION

Previously, we have demonstrated that ARA70 is a relatively specific transcriptional coactivator of the androgen receptor that shows only a very marginal induction of transcriptional activity by the estrogen, glucocorticoid, and progesterone receptors in DU145 cells (1). Here we show that ARA70 is also a
coactivator of PPAR$\gamma$. Unlike the AR-ARA70 interaction, which requires the presence of androgen, the PPAR$\gamma$-RXR-ARA70 interaction occurs in the absence of exogenous ligand as determined by a mammalian two-hybrid assay and by coimmunoprecipitation. Cotransfection of PPAR$\gamma$ and ARA70 also results in low level constitutive expression from an ap2 promoter and an isolated PPRE-driven construct. However, in both the mammalian two-hybrid system and the transfection studies, the transcriptional activation of the reporter genes could be further enhanced by the addition of the appropriate ligands, demonstrating that the binding of ligand is still necessary for maximal transcriptional response. Interestingly, in the presence of ligand, ARA70 mediates a greater increase in transcriptional activity than SRC-1, suggesting that in adipose tissue it may be the more important coactivator of PPAR$\gamma$. Recently, CBP and PBP have been identified as cofactors of PPAR$\gamma$ (27, 30). CBP and PBP demonstrate ligand-independent physical interaction with PPAR$\gamma$ but require ligand to show a transcriptional effect. This suggests that although unliganded PPAR$\gamma$ may be permissive to some cofactor interactions, the nature of its interaction with ARA70 is different in that it allows constitutive transcription.

Ligand-independent activation by PPAR$\gamma$ in the presence of a cofactor has also been observed for SRC-1 (28). It is possible that these effects are due to the presence of an endogenous ligand. However, transfection of PPAR$\gamma$ and SRC-1 in our system does not lead to transcription above background, suggesting that ARA70 alone induces a low level of PPAR$\gamma$-mediated transcription.
case of PPARγ, the mutant ARA70 showed a decreased interaction in the presence and absence of 15dJ2. This suggests that ligand-dependent and -independent interactions between PPARγ and ARA70 both occur through the same ARA70 domain. However, more detailed interaction data between PPARγ and ARA70 is needed to confirm this possibility.

The heterodimeric partner of PPARγ, RXR, also interacts with ARA70 but in a ligand-dependent manner. PPARγ-RXR-mediated transcription can be activated by the addition of RXR ligands (Fig. 3 and Ref. 28). It is therefore possible that in vivo PPARγ and RXR may independently recruit ARA70 to contribute to transactivation by the heterodimer, as has been reported for SRC-1 (28). Whether ARA70 can enhance transactivation by RXR-RAR heterodimers is currently under investigation.

The relative specificity of ARA70 could allow cross-talk between PPARγ and AR in tissues where they are coexpressed with ARA70, as is the case in adipocytes. We have shown that when PPAR and AR are cotransfected with ARA70, DHT treatment reduces the expression of a PPARγ reporter gene. This may indicate that liganded AR is able to compete with PPARγ for ARA70 to differentially regulate their respective target genes. PPARγ and AR are both known to mediate adipose tissue metabolism and have both been implicated in relation to insulin sensitivity. It has been demonstrated that activators of PPARs, and particularly of PPARγ, promote adipocyte differentiation in preadipocytes (46) and in the multipotential cell line C3H10T1/2 (2). Stable transfection of PPARγ can induce differentiation of fibroblastic NIH 3T3 cells to adipocytes in the presence of PPARγ ligands (9, 11). PPARγ is also known to transcriptionally activate a number of adipocyte-expressed genes including lipoprotein lipase (20) and ap2 (8). Testosterone affects the regulation of lipolysis by increasing β-adrenoceptors and regulating adenylyl cyclase activity (47). Testosterone in some studies downregulates lipoprotein lipase (48–50). The differential expression of lipoprotein lipase may contribute to gender-specific regional fat distribution (47, 48).

The thiazolidinedione class of drugs increase insulin sensitivity in patients with noninsulin-dependent diabetes and are known to be PPARγ ligands (11, 12), although the mechanism of this affect is unknown. Aberrant levels of testosterone have been associated with insulin resistance. Female rats treated with testosterone or human females with polycystic ovary syndrome (which results in higher levels of circulating androgens) show an increased incidence of insulin resistance (51, 52). In males, low levels of androgens have been correlated with insulin resistance. Castrated male rats or hypogonadal men have increased insulin resistance, which can be improved by low level testosterone treatment (49, 50). That PPARs and AR share a coactivator suggests that cross-talk between the two receptor-mediated pathways may occur. However, further investigation is necessary to establish the degree of this possible interaction in vivo.

Acknowledgments—SRC-1 was a gift from Dr. B. W. O'Malley (Baylor College of Medicine, TX) and the ap2(5-4)-luciferase reporter plasmid was a gift from Dr. B. Zhang (Merck, NJ). We also thank Karen Wolf for the critical reading of the manuscript.

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