A Dominant-negative Peroxisome Proliferator-activated Receptor γ (PPARγ) Mutant Is a Constitutive Repressor and Inhibits PPARγ-mediated Adipogenesis*

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The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) promotes adipocyte differentiation, exerts atherogenic and anti-inflammatory effects in monocyte/macrophages, and is believed to mediate the insulin-sensitizing action of antidiabetic thiazolidinedione ligands. As no complete PPARγ antagonists have been described hitherto, we have constructed a dominant-negative mutant receptor to inhibit wild-type PPARγ action. Highly conserved hydrophobic and charged residues (Leu1452 and Glu1453) in helix 12 of the ligand-binding domain were mutated to alanine. This compound PPARγ mutant retains ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator (cAMP-response element-binding protein-binding protein and steroid receptor coactivator-1) recruitment. Unexpectedly, the mutant receptor silences basal gene transcription, recruits corepressors (the silencing mediator of retinoid and thyroid receptors and the nuclear corepressor) more avidly than wild-type PPARγ, and exhibits delayed ligand-dependent corepressor release. It is a powerful dominant-negative inhibitor of cotransfected wild-type receptor action. Furthermore, when expressed in primary human preadipocytes using a recombinant adenovirus, this PPARγ mutant blocks thiazolidinedione-induced differentiation, providing direct evidence that PPARγ mediates adipogenesis. Our observations suggest that, as in other mutant nuclear receptor contexts (acute promyelocytic leukemia, resistance to thyroid hormone), dominant-negative inhibition by PPARγ is linked to aberrant corepressor interaction. Adenoviral expression of this mutant receptor is a valuable means to antagonize PPARγ signaling.

Peroxisome proliferator-activated receptor γ (PPARγ), an orphan member of the nuclear hormone receptor family, was first characterized as a transcription factor that regulates adipocyte-specific gene expression (1) and induces adipocyte differentiation (2), but is now recognized to have a central role in other biological processes. PPARγ mediates inhibition of inflammatory cytokine production (interleukin-6 and tumor necrosis factor α) from monocytes (3), and receptor activation by oxidized low density lipoprotein-derived ligands promotes macrophage foam cell formation (4). PPARγ activation promotes colonic neoplasia (5), but inhibits the growth of breast cancer cells (6). Thiazolidinediones (TZDs), a novel class of anti-diabetic agent that act as insulin sensitizers in vivo, bind PPARγ with high affinity (7), and prostaglandin J2 (8) and fatty acids have been proposed to be natural ligands. PPARγ regulates target gene transcription as a heterodimer with the retinoid X receptor, and this heterodimeric complex has been shown to be activated synergistically by TZDs and RXR-specific ligands (9). However, no complete synthetic or natural PPARγ antagonists have been described hitherto. We have therefore generated a dominant-negative PPARγ mutant to inhibit wild-type receptor action.

In keeping with other members of the nuclear receptor superfamily, PPARγ exhibits a modular structure consisting of a central DNA-binding domain, an amino-terminal activation domain, and a carboxyl-terminal ligand-binding domain (LBD) that encompasses a strong ligand-dependent transactivation (AF-2) function. The extreme C terminus of the PPARγ LBD forms an amphipathic α-helix that can also be delineated in a number of other nuclear receptors. There is striking conservation of hydrophobic (leucine) and negatively charged (glutamic acid) residues within this motif, and mutational analyses in the estrogen (10), thyroid (11, 12), and retinoic acid (13) receptors have shown that they are critical for ligand-dependent transactivation and the recruitment of nuclear receptor coactivators (14). Resistance to Thyroid Hormone is associated with diverse thyroid hormone β (TRβ) receptor mutations that inhibit the action of their wild-type counterparts in a dominant-negative manner (15). We have previously described a natural mutation of the conserved hydrophobic residue (Leu454) in the AF-2 domain of TRβ that exhibits strong dominant-negative activity and is associated with marked refractoriness to thyroid hormone action in vivo (16).
Here, we describe the mutation of homologous hydrophobic and charged residues (L468A and E471A) in PPARγ. The compound mutant receptor exhibits impaired transcriptional activation and coactivator recruitment. In addition, it silences basal transcription by recruitment of corepressors and is a potent dominant-negative inhibitor of wild-type PPARγ action. Adenoviral expression of this mutant receptor in human preadipocytes inhibits thiazolidinedione-induced target gene transcription and cellular differentiation, providing direct evidence for the role of PPARγ in adipogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Full-length human PPARγ1 and PPARγ2 cDNAs were cloned by reverse transcription-polymerase chain reaction from total human preadipocyte RNA and cloned into the pcDNA3 expression vector (Invitrogen). The L468A/E471A PPARγ double mutant was generated by site-directed mutagenesis of the wild-type receptor and verified by sequencing. FLAG epitope-tagged PPARγ was constructed by placing the peptide sequence MDYKDDDKDD in frame upstream of the first methionine of wild-type and mutant PPARγ1. DNA sequences encompassing residues 173–475 of the wild-type and mutant PPARγ1, as well as PPARγ2, were cloned into pSG424 (Amersham Pharmacia Biotech), and AASV (12) to yield Gal4-PPARγ-LBD, GST-PPARγ LBD, and VP16-PPARγ LBD fusions, respectively. WT, L454A-TRβ LBD, and P214A-TRβ LBD expression constructs were generated by cloning EcoRI/EcoRI fragments from corresponding pSG424-TRβ fusions into pCMX (17). Gal4-NCoR contains the nuclear receptor interaction domains (amino acids 2276–2454) of murine NCoR (18) fused in frame to the Gal4 DBD in pSG424, and Gal4-SMRT consists of the 468 C-terminal amino acids of SMRT fused in frame to the Gal4 DBD in pcMX (19). PPARETKLUC (8), UASTKLC (12), pSG5-PPARα and CREBIIKLUC (20); MALTKLUC, RSV-TRβ1, and RSV-RXRα (15); RAR2TKLUC and RSV-RARα (21); MsiaIIα (22); and pCMXNCoRα (18) have been described previously.

Protein-Protein Interaction Assays—Bacterially expressed GST fusion proteins were immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) and incubated with in vitro translated 35S-labeled CBP or SRC-1 (23) in 50 mM Tris-HCl, 0.1 M KCl, 0.14 M NaCl, 0.5% Nonidet P-40, and 0.1 mM okadaic acid (pH 8.0) with or without 10 μM BRL49653 for 1 h at room temperature. Following four washes with 20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 (pH 8.0), bound coactivators were resolved by SDS-polyacrylamide gel electrophoresis. Coomasie staining verified equal loading of GST-PPARγ LBD fusion proteins prior to autoradiography.

Hormone and DNA Binding Assays—Hormone binding assays were performed using bacterially expressed GST-PPARγ LBD fusion proteins and the PPARγ-specific radioligand 125I-SB236636 in a modification of a previously described filter binding assay (23). DNA binding was assessed as described previously (15) using in vitro translated WT PPARγ1, L468A/E471A PPARγ1, and human RXRα and oligonucleotide duplexes encoding the acyl-CoA oxidase PPARE for adipocyte P2 (aP2) expression were performed at 24 h, whereas morphologic assessment and glycerol-3-phosphate dehydrogenase activity determination (25) were performed on day 10.

Adenovirus Construction and Expression—Reombinant type 5 adenoviruses (Ad5) expressing GFP (AdGFP) or GFP and full-length L468A/E471A PPARγ1 (Adγw) were generated using an ADENO-QUEST kit (Quantum Biotechnologies, Montreal, Canada). JEG-3 cells cultured in 24-well plates were infected with recombinant virus following calcium phosphate transfection by addition of 1.6 × 107 plaque-forming units/well. Primary human preadipocytes grown in 6- or 96-well plates were infected with 9.6 × 105 or 0.8 × 105 plaque-forming units/well, respectively, 2 days prior to induction of differentiation. Comparable viral infection efficiency was verified by fluorescence microscopy.

RESULTS

The transcriptional activity of WT and mutant L468A/E471A PPARγ was assayed by cotransfection of receptor expression vectors together with a reporter gene (PPARETKLUC) containing three copies of the PPARE from the acyl-CoA oxidase gene linked to the thymidine kinase promoter and luciferase (Fig. 1). Cells transfected with the WT receptor exhibited a strong ligand-dependent transcriptional response following exposure to increasing concentrations of the thiazolidinedione BRL49653. In contrast, in the absence or presence of 10 μM BRL49653 as shown.

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FIG. 4. WT and L468A/E471A PPARγ recruit SMRT in vivo, and ligand-dependent release by L468A/E471A PPARγ is impaired. 293T cells, cultured in 10-cm plates, were transfected with 7.5 μg of expression vectors encoding SMRT and FLAG epitope-tagged WT or L468A/E471A PPARγ. Whole cell lysates were treated with polyclonal anti-SMRT antibody in the absence or presence of 10 μM BRL49653 as shown, and precipitated were Western-blotted and probed with anti-FLAG antibody. The dual band corresponds to phosphorylated and unphosphorylated forms of PPARγ. Cell lysates were also Western-blotted and probed with anti-FLAG and anti-SMRT antibodies to verify comparable transfection efficiencies of PPARγ and SMRT, respectively. IP, immunoprecipitate.

FIG. 3. a, unliganded repression by L468A/E471A PPARγ is mediated by corepressors (CoR) that interact with TRβ. 293EBNA cells were transfected with 500 ng of UASTKLUC, 100 ng of Bos-β-gal control plasmid, and 50 ng of expression vectors encoding Gal4 DBD (Gal4), Gal4 DBD-L468A/E471A PPARγ LBD (468/471), TRβ LBD (WTTR), or mutant TRβ LBDs defective in coactivator (L454A) or corepressor (P214R) recruitment and cultured in the absence or presence of T3 as shown. b, basal repression by L468A/E471A PPARγ is mediated by NCoR. 293EBNA cells were transfected with reporter, internal control, and Gal4 expression vectors as described for a together with 500 or 1000 ng of NCoR expression vector in the absence or presence of 500 ng of the NCoR-depleting construct mSiah2 as shown. c, L468A/E471A PPARγ demonstrates impaired corepressor release in mammalian two-hybrid assays. 293EBNA cells were transfected with 500 ng of UASTKLUC, 100 ng of Bos-β-gal control plasmid, 50 ng of expression vector encoding the Gal4 DBD fused to the nuclear receptor interaction domains of SMRT (Gal4-SMRT) and NCoR (Gal4-NCoR), and 50 ng of expression vector encoding VP16 or VP16 fused to the LBD of WT PPARγ (VP16 WT) or L468A/E471A PPARγ (VP16 468/471) and cultured in 0, 10, 100, or 1000 nM BRL49653 as shown. US, upstream activating sequence; TK, thymidine kinase.

mutant receptor retained significant ligand binding (WT Kd = 45 ± 12 nM; L468A/E471A Kd = 200 ± 60 nM), suggesting that this did not account for its transcriptional inactivity. Likewise, DNA binding assays performed using WT or mutant PPARγ, retinoid X receptor, and radiolabeled PPARE showed comparable formation of heterodimeric complexes (data not shown).

By analogy with the effect of homologous mutations in TRβ (16), we hypothesized that the interaction of the L468A/E471A PPARγ mutant with transcriptional coactivator proteins might be altered. In a protein-protein interaction assay using bacterially expressed GST-PPARγ LBD fusion proteins, the WT receptor showed strong ligand-dependent recruitment of 35S-labeled CBP and SRC-1 proteins (Fig. 2). In contrast, ligand-dependent coactivator recruitment by the L468A/E471A PPARγ mutant was abolished.

To assess dominant-negative inhibition by the L468A/E471A PPARγ mutant, cells were transfected with the WT receptor plus an equal amount of mutant receptor and increasing concentrations of BRL49653 (Fig. 1). In the presence of the L468A/E471A mutant, reporter gene activation was markedly attenuated (~50% of the WT response) at all ligand concentrations, whereas the transcriptional response to WT plus further WT receptor was unchanged (data not shown).

Cells transfected with empty expression vector (pcDNA3) showed a small but significant response, reflecting transcriptional activation mediated by low levels of endogenous PPARγ (unpublished Western blotting data not shown) in 293 cells (Fig. 1). In comparison, cells transfected with the L468A/E471A mutant exhibited even lower transcriptional activity, presumably reflecting dominant-negative inhibition of endogenous WT receptor (Fig. 1).

Other members of the nuclear receptor family (e.g. TR and RAR) are able to silence basal gene transcription in the absence of ligand by binding corepressor proteins such as NCoR (18) and SMRT (19). Furthermore, corepressor recruitment has been shown to be essential for dominant-negative inhibition by natural TRβ mutants (28). We therefore examined the properties of the unliganded L468A/E471A PPARγ mutant. In comparison to empty pcDNA3 vector, the WT receptor exhibited moderate (~5-fold) constitutive basal activation, whereas the PPARγ mutant showed striking silencing of basal transcription (pcDNA3 = 1.0; L468A/E471 = 0.25) (Fig. 1, inset). To further substantiate that this silencing is PPARγ-mediated, cells were transfected with vectors expressing either the Gal4 DBD alone or linked to the L468A/E471A PPARγ mutant LBD. Again, marked repression of basal transcription was observed (Gal4 = 1.0; Gal4-PPARγ mutant = 0.15) (Fig. 3a), suggesting that PPARγ might interact with corepressors in vivo.

The ligand-binding domain of TR mediates interaction with NCoR or SMRT when unliganded, and the addition of T3 promotes corepressor dissociation and coactivator recruitment (18,
When cotransfected with the Gal4-PPARγ mutant, the unliganded TR LBD relieved transcriptional silencing by the mutant receptor, and this effect was reversed by the addition of T3 (Fig. 3a). In contrast, coexpression of a mutant (P214R) TR LBD with impaired corepressor binding (29) did not affect basal repression by the Gal4-PPARγ mutant (Fig. 3a). Cotransfection of a mutant (L454A) TR LBD that exhibits impaired hormone-dependent corepressor release (30) also relieved silencing by the Gal-PPARγ mutant, but this persisted following the addition of T3 (Fig. 3a).

To specifically address the role of individual corepressors in mediating silencing by the Gal4-PPARγ mutant, we examined the effects of mSiah2, a novel protein that has been shown to target the corepressor NCoR for proteasomal degradation (22). Cotransfected mSiah2 significantly impaired transcriptional silencing by the Gal4-PPARγ mutant (Fig. 3b). Furthermore, coexpression of NCoR was able both to enhance basal repression by the mutant receptor and to restore silencing in the presence of mSiah2 in a dose-dependent manner (Fig. 3b).

To examine the association of WT and mutant PPARγ with corepressor in vivo, cells were cotransfected with an SMRT expression vector together with FLAG epitope-tagged full-length WT or L468A/E471A PPARγ (Fig. 4). In the absence of ligand, both WT and mutant PPARγ bound SMRT, with greater quantitative binding by the mutant receptor. The addition of 10 μM BRL49653 ligand resulted in complete dissociation of SMRT from the WT receptor, whereas the L468A/E471A mutant retained significant corepressor binding, suggesting that ligand-dependent release of corepressor from the PPARγ mutant might be impaired. We tested this hypothesis in a mammalian two-hybrid assay using the Gal4-NCoR (residues 2276–2454) or Gal4-SMRT (residues 982–1448) fusion together with the VP16 construct with WT or mutant PPARγ LBD. In the ab-
sence of ligand, both WT and mutant PPARγ were recruited comparably to Gal4-SMRT, whereas interaction of the L468A/E471A mutant with the Gal4-NCoR fusion was slightly enhanced. In contrast to a dose-dependent dissociation of the WT receptor from corepressors following the addition of ligand, the enhanced. In contrast to a dose-dependent dissociation of the WT E471A mutant with the Gal4-NCoR fusion was slightly en-

comparably to Gal4-SMRT, whereas interaction of the L468A/E471A mutant from both SMRT and NCoR was markedly impaired (Fig. 3c).

To introduce the L468A/E471A PPARγ mutant into primary cells and tissues, we have constructed a recombinant adenovirus expressing the mutant receptor as well as GFP. In the first instance, we tested the ability of PPARγ mutant adenovirus (Adγm) to inhibit the action of transfected nuclear receptors. Mutant adenoviral infection of cells blocked ligand-dependent transactivation by both human PPARγ and PPARγ2 isoforms, whereas receptor-mediated activation was unaffected in cells infected with control adenovirus expressing GFP alone (AdGFP) (Fig. 5, a and b). Furthermore, the mutant receptor adenovirus only partially inhibited PPARα-mediated signaling (Fig. 5c) and was unable to block ligand-dependent transacti-

vation by human RARα (Fig. 5d), human RXRα (Fig. 5e), or human TRβ (Fig. 5f).

PPARγ plays a central role in murine preadipocyte differen-
tiation (2). We have shown previously that thiazolidinediones promote the differentiation of cultured human preadipocytes (25) and therefore tested the effect of the PPARγ mutant adenovirus compared with uninfected or GFP virus-infected cells. Thus, the L468A/E471A PPARγ mutant is capable of blocking responses mediated by endogenous wild-type PPARγ.

**DISCUSSION**

The carboxyl terminus of a number of nuclear receptors, including PPARγ, contains a C-terminal amphipathic α-helix that is required for ligand-dependent transcriptional activation (AF-2) function (10, 11, 13). We have mutated conserved hydrophobic (Leu468) and negatively charged (Glu471) residues in the putative AF-2 domain of PPARγ to alanine. Functional studies indicate that PPARγ transcriptional activation by this comp-

ound mutant is severely impaired even in the presence of saturating concentrations of thiazolidinedione ligand sufficient to overcome its modestly reduced ligand binding affinity (Fig. 1). Protein-protein interaction assays indicate negligible ligand-
dependent recruitment of CBP and SRC-1 coactivators by the PPARγ mutant, accounting for its functional impairment. The crystal structure of the PPARγ LBD in complex with the interaction domain of the coactivator SRC-1 (31) reveals that Leu468 and Glu471 are both situated at the receptor coactivator interface. The side chain of Glu471 is oriented such that it makes several hydrogen bonds with the backbone amino groups of the coactivator helix, with the negative charge of the carboxyl complementing the positive end of the helix dipole. Leu468 is situated at the bottom of the hydrophobic cleft into which the coactivator helix binds and as a result is completely buried between hydrophobic residues on helix 3 and others on the coactivator helix. It is clear that the impairment of coactivator binding seen in functional studies with this mutant is entirely consistent with the role of these residues in the structure of the receptor-coactivator complex.

The L468A/E471A PPARγ mutant was also able to inhibit the action of its WT counterpart in a dominant-negative man-

ner (Fig. 1). Mutations in the C-terminal AF-2 domain of other
nuclear receptors also generate mutant proteins with strong dominant-negative activity; for example, this region is deleted in the oncogene v-erbA, a potent inhibitor of TR andRAR action (32). We (16) and others (33) have described powerful dominant-negative amphipathic α-helix TRβ mutants in the syndrome of resistance to thyroid hormone. A subset of nuclear receptors including TR and RAR have been shown to repress basal transcription in the absence of ligand by recruitment of corepressor proteins such as NCoR (18) and SMRT (19). However, the role of corepressors in repression remains unclear. Whereas PPARγ can interact weakly with NCoR and SMRT in vitro, the WT receptor exhibits negligible transcriptional repression in vivo (24) (Fig. 1, inset), although mitogen-activated protein kinase-dependent phosphorylation has been shown to inhibit AF-2 function via SMRT recruitment (34). In contrast, our observations indicate that the L468A/E471A PPARγ mutaton is a potent transcriptional repressor. Repression is exhibited by both the full-length mutant receptor (Fig. 1) as well as a Gal4-PPARγ LBD fusion (Fig. 3a), indicating that its silencing function is independent of N-terminal domain phosphorylation. Coexpression of the unliganded TRβ LBD attenuates repression by the PPARγ mutant, suggesting that this function is mediated by shared cellular factors (35), and a TRβ LBD mutant (P214R) that is defective for corepressor binding fails to inhibit repression. Evidence that NCoR mediates silencing by the PPARγ mutant is provided by the observation that coexpression of mSiah2, which targets NCoR for proteasomal degradation (22), also attenuates repression (Fig. 3b). Co-immunoprecipitation experiments (Fig. 4) demonstrate that the L468A/E471A PPARγ mutant interacts with SMRT in vivo, suggesting that this corepressor may also mediate transcriptional silencing. In addition to enhanced corepressor binding, the L468A/E471A PPARγ mutant also exhibits impaired ligand-dependent corepressor release (Fig. 3c), indicating a role for helix 12 of PPARγ in corepressor dissociation as has been documented with other nuclear receptors (30, 33, 36). Our observation that the dominant-negative PPARγ mutant is a powerful repressor is consonant with the properties of other nuclear receptors, including TRβ mutants in Resistance to Thyroid Hormone (28), PMLRAR in acute promyelocytic leukemia (37), and v-erbA (38). Furthermore, corepressor recruitment has been shown to be required for dominant-negative inhibition (28). Our finding that the PPARγ mutant is a strong repressor raises the question as to why WT PPARγ appears to lack silencing activity. The crystal structure of the apo-PPARγ LBD indicates that it is possible for helix 12 to adopt the same conformation as the liganded receptor-coactivator complex. It is therefore likely that, in the absence of ligand, PPARγ is still able to recruit coactivator, albeit less efficiently than the holoreceptor. Abolishment of such coactivator binding may also mediate transcriptional silencing. In addition factors (35), and a TRβ LBD mutant (P214R) that is defective for corepressor binding fails to inhibit repression. Evidence that NCoR mediates silencing by the PPARγ mutant is provided by the observation that coexpression of mSiah2, which targets NCoR for proteasomal degradation (22), also attenuates repression (Fig. 3b). Co-immunoprecipitation experiments (Fig. 4) demonstrate that the L468A/E471A PPARγ mutant interacts with SMRT in vivo, suggesting that this corepressor may also mediate transcriptional silencing. 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