The role of the amino-terminal domain in the interaction of unliganded peroxisome proliferator-activated receptor γ -2 with nuclear receptor co-repressor

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Abstract

Peroxisome proliferator-activated receptor γ -2 (PPARG2) is a ligand-dependent transcriptional factor involved in the pathogenesis of insulin resistance. In the presence of a ligand, PPARG2 associates with co-activators, while it recruits co-repressors (CoRs) in the absence of a ligand. It has been reported that the interaction of liganded PPARG2 with co-activators is regulated by the amino-terminal A/B domain (NTD) via inter-domain communication. However, the role of the NTD is unknown in the case of the interaction between unliganded PPARG2 and CoRs. To elucidate this, total elimination of the influence of ligands is required, but the endogenous ligands of PPARG2 have not been fully defined. PPARG1-P467L, a naturally occurring mutant of PPARG1, was identified in a patient with severe insulin resistance. Reflecting its very low affinity for various ligands, this mutant does not have transcriptional activity in the PPAR response element, but exhibits dominant negative effects (DNEs) on liganded wild-type PPARG2-mediated transactivation. Using the corresponding PPARG2 mutant, PPARG2-P495L, we evaluated the role of the NTD in the interaction between unliganded PPARG2 and CoRs. Interestingly, the DNE of PPARG2-P495L was increased by the truncation of its NTD. NTD deletion also enhanced the DNE of a chimeric receptor, PT, in which the ligand-binding domain of PPARG2 was replaced with that of thyroid hormone receptor β -1. Moreover, NTD deletion facilitated the *in vitro* binding of nuclear receptor CoR with wild-type PPARG2, mutant P495L, and the PT chimera (PPARG2-THRB). Inter-domain communication in PPARG2 regulates not only ligand-dependent transactivation but also ligand-independent silencing.

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Introduction

Peroxisome proliferator-activated receptor γ -2 (PPARG2) belongs to the nuclear hormone receptor (NHR) superfamily (Desvergne & Wahli 1999, Michalik *et al.* 2006, Lu & Cheng 2010). Through different promoter usage and splicing, the *PPARG* gene generates PPARG1, 2, and 3. PPARG2 is predominantly expressed in adipocytes and macrophages, and has profound effects on insulin sensitivity (Desvergne & Wahli 1999). PPARGs heterodimerize with the retinoid X receptors (RXRs) on short DNA sequences, designated the PPAR-responsive element (PPRE). PPRE consists of a pair of directly repeated half sites (typically AGGTCA) spaced with one random nucleotide (DR1; Desvergne & Wahli 1999). Upon ligand binding, the PPARG2-RXR heterodimer recruits co-activators including members of the p160 protein family, CREB-binding protein/p300, and thyroid hormone receptor (THR)-associated protein 220 (Ge *et al.* 2002), resulting in transactivation (Perissi & Rosenfeld 2005). The function of the ligand-binding domain (LBD) is known to be modulated by the phosphorylation of a serine at codon 112 (S112) in the

amino-terminal A/B domain (NTD) by mitogen-activated protein kinase (MAPK; Hu *et al.* 1996, Adams *et al.* 1997, Camp & Tafuri 1997, Shao *et al.* 1998). Based on functional analyses of S112 phosphorylation, Shao *et al.* (1998) proposed ‘inter-domain communication’, where NTD controls the ligand-binding affinity of the LBD and recruitment of SRC-1, a member of the p160 family.

In the absence of a cognate ligand, THR α -1 (THRA1), β -1 (THRB1), and retinoic acid receptor α (RARA) are known to interact with co-repressors (CoRs) including nuclear receptor CoR (NCoR) and silencing mediator of retinoic acid and THRs (SMRT), resulting in transcriptional silencing. The interaction of CoRs with unliganded PPARG2-LBD has been demonstrated *in vitro* (Zamir *et al.* 1997, Krogsdam *et al.* 2002, Lee *et al.* 2002, Stanley *et al.* 2003) and *in vivo* (Gurnell *et al.* 2000, Wang *et al.* 2004, Yu *et al.* 2005). RNA interference assays against the CoR genes (Guan *et al.* 2005, Yu *et al.* 2005) and chromatin immunoprecipitation assays (ChIP) suggest that PPARG2 associates with CoR *in vivo* and suppresses the basal transcriptional activity of PPARG2-target genes including glycerol kinase (Guan *et al.* 2005), oxidized low-density

lipoprotein receptor 1 (Chui *et al.* 2005), aP2, and PPARG itself (Picard *et al.* 2004). However, there is no evidence that the NTD regulates the interaction of its LBD with CoR in the absence of a ligand.

Unlike THR α s or RARA, studies using conventional reporter assays have failed to demonstrate the silencing function of unliganded PPARG2 (Zhang *et al.* 1999, Chatterjee 2001, Semple *et al.* 2006). In 293EBNA cells without the addition of an exogenous ligand, PPARG did not exhibit silencing function but rather constitutive activation of basal transcription (Barroso *et al.* 1999, Gurnell *et al.* 2003, Agostini *et al.* 2004, Semple *et al.* 2006). Additionally, overexpression of NCoR did not potentiate the suppression by PPARG2 of acyl-CoA oxidase-derived PPRE (Aox-PPRE) in 293T cells (Zamir *et al.* 1997). To detect the silencing effect of wild-type PPARG2 in a conventional reporter assay, it is necessary to maintain the majority of PPARG2 molecules in truly an unliganded condition. Endogenous ligands for PPARG2 are reported to be generated by 3T3-L1 preadipocytes (Kim *et al.* 1998, Camp *et al.* 2001, Madsen *et al.* 2003, Tzamelis *et al.* 2004) and macrophages (Yano *et al.* 2007). Similar substances may also be generated by CV1 cells (DiRenzo *et al.* 1997, Werman *et al.* 1997), which have been often used in the studies of NHRs. Shi *et al.* (2002) reported that, in CV1 cells, 'unliganded' PPARG2 did not suppress ligand-induced transcription by PPARG α (PPARA). These putative ligand(s) for PPARG2 are predicted to dissociate CoR from PPARG2 (Gurnell *et al.* 2000, Wang *et al.* 2004, Yu *et al.* 2005).

Barroso *et al.* (1999) and Savage *et al.* (2003) reported two different heterozygous mutations, PPARG1-P467L and V290M, in patients with severe insulin resistance. Both mutants, PPARG1-P467L in particular, have very low affinity for the PPARG ligands and constitutively bind with CoR (Barroso *et al.* 1999, Agostini *et al.* 2004). They are believed to be resistant to the endogenous ligand since they exhibited no transactivation in 293EBNA cells in the absence of an exogenous ligand (Barroso *et al.* 1999, Agostini *et al.* 2004). These mutant PPARG1s also exhibited potent dominant negative effects (DNEs) on the ligand-induced transactivation by wild-type PPARG1 (Barroso *et al.* 1999). These properties are reminiscent of the DNE seen in mutant THR β 1, identified in patients with resistance to thyroid hormones (RTH; Yoh *et al.* 1997, Chatterjee 2001), since mutant THR β 1s lose their T3-binding activity due to amino acid substitutions in their LBD (Refetoff *et al.* 1993) and exhibit DNEs by continuous interaction with CoR (Yoh *et al.* 1997). DNEs against ligand-induced transactivation by wild-type PPARGs have been reported in artificially generated mutant PPARGs that mimic the amino acid substitution of mutant THR β 1s in RTH (Zamir *et al.* 1997, Gurnell *et al.* 2000, Armoni *et al.* 2003, Park *et al.* 2003, Freedman *et al.* 2005).

Using the analogy of inter-domain communication in ligand-dependent transactivation involving PPARG2, we speculate that the NTD may regulate the interaction of CoR with the unliganded LBD. To confirm this, we took advantage of the fact that PPARG1-P467L has very low affinity for a variety of known ligands (Barroso *et al.* 1999, Kallenberger *et al.* 2003, Agostini *et al.* 2004). We examined the effect of NTD truncation on the DNE of PPARG2-P495L, which possesses an amino acid substitution corresponding to PPARG1-P467L (Barroso *et al.* 1999, Gurnell *et al.* 2003, Agostini *et al.* 2004, Semple *et al.* 2006). In addition, we generated a chimeric receptor, PT, by fusing the PPARG2-derived NTD and DNA-binding domain (DBD) to the THR β -LBD in order to evaluate its NTD function. Our study demonstrates that the PPARG2-NTD functions to attenuate the association of CoR with unliganded LBD.

Materials and methods

Plasmid construction

A PT chimera was created by fusing the NTD, DBD, and the A- and T-boxes of mouse PPARG2 (codon 1–232) to the CoR box and LBD of human THR β 1 (codon 192–461) using standard molecular techniques. An expression plasmid for mouse PPARG2-P495L was generated from pSG5-mouse PPARG2 using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) in the same codon as used for human patients (Barroso *et al.* 1999). The construction of mutant THR β 1, pCMX-hTHR β 1-K443E, and tk-TREp-Luc is described elsewhere (Sasaki *et al.* 1995). Using the PCR with the PT chimera, PPARG2-P495L, and the THR β 1-K443E as templates, dNPT, PPARG2-dNP495L, and THR β 1-dNK443E were generated respectively. In dNPT and PPARG2-dNP495L, the N-terminal 127 amino acids were truncated, while, in THR β 1-dNK443E, the N-terminal 94 amino acids of THR β 1-K443E were deleted. The AHT mutation in the CoR box of the THR β 1 hinge domain (codon 263 Ala to Gly, codon 264 His to Gly, and codon 267 Thr to Ala; Horlein *et al.* 1995) was generated with identical codons in the LBD of PT and dNPT to create PT-AHT and dNPT-AHT respectively. The validity of all plasmid constructs was confirmed by sequencing.

Cell culture and transient transfection

CV1 cells were maintained in DMEM supplemented with 10% FCS. Using the calcium phosphate precipitation method (Sasaki *et al.* 1995), 6-well dishes were transfected with 300 ng of the luciferase reporter gene containing three copies of rat Aox-derived PPRE fused to thymidine kinase (tk-AoxPPRE-Luc), 1200 ng of β -galactosidase expression vector (a modified version

of pCH110), and expression plasmids for receptors in the quantities indicated in the figure legends. Empty vector (pCMX or pSG5) was added to adjust the total amount of DNA. The reporter assay with hADRP-4k-Luc was performed using same protocol as tk-AoxPPRE-Luc. The pre-adipocyte cell line 3T3-L1 was maintained in DMEM with 10% FCS. Using 12-well dishes, the cells were transfected by the lipofection method (Sasaki *et al.* 1999) with 300 ng of tk-AoxPPRE-LUC reporter gene, plasmids for receptors, and 500 ng of pRL-TK expression vector (Promega) to standardize transfection efficiency. The luciferase activity was normalized using β -galactosidase activity in CV1 cells or by pRL-TK activity in 3T3L1 cells. To evaluate DNE in CV1 and 3T3L1 cells, wild-type receptor plasmid was co-transfected with twofold molar excess of mutant receptor plasmid. All experiments were performed in duplicate and repeated more than three times.

Gel shift assay

Oligonucleotides containing the Aox-derived PPRE (sense; 5'-aggggaccaggacaaaggtcacgttcggg-3') were labeled with 32 P- γ -ATP using T4 polynucleotide kinase (Toyobo, Osaka, Japan). Receptor proteins were translated using the TNT *in vitro* translation kit (Promega). The translated receptor proteins and 32 P-labeled PPRE oligonucleotides (5×10^4 c.p.m.) were mixed and incubated for 20 min at room temperature. The reaction mixture was loaded onto 5% polyacrylamide gel. The gel was dried and visualized using the BAS-1000 autoradiography system (Fuji, Tokyo, Japan).

GST pulldown assay

Receptor proteins were generated using the TNT *in vitro* translation kit (Promega) in the presence of 35 S-methionine. Bacterially expressed glutathione S-transferase (GST) protein fused to the receptor interacting domain of NCoR (GST-NCoR; Kawai *et al.* 2004) was immobilized on glutathione-Sepharose 4B beads (Amersham Life Science). The beads were washed once with binding buffer (25 mM HEPES, pH 7.6, 2 mM dithiothreitol, 20 mM NaCl, 20% glycerol, 2 mg/ml BSA, and protease inhibitors (0.3 mM phenyl methylsulfonyl fluoride, 1.0 μ g/ml leupeptin, and 2.0 μ g/ml aprotinin)), before being mixed with binding buffer and left overnight at 4 °C. 35 S-methionine-labeled *in vitro* translated receptor proteins were incubated with the GST fusion protein-bound beads for 3 h at 4 °C. After the incubation, the beads were washed twice with 1 ml of binding buffer + 0.1% Triton X-100 and once with 1 ml of 50 mM Tris-HCl (pH 8.0) buffer. The eluted proteins were resolved by SDS-PAGE and visualized by the BAS-1000 autoradiography system (Fuji).

Statistical analysis

Each experiment was performed in duplicate more than three separate times, and each result is expressed as the mean $\pm$ s.d. The statistical significance was determined by the ANOVA and Fisher's protected least significant difference (PLSD) test using StatView 4.0 software (Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was considered significant.

Results

The DNE of PPARG2-P495L is enhanced by the truncation of its NTD

We generated the mutant PPARG2 by deleting its NTD (dNPPARG2; Fig. 1A) and transfecting it into CV1 cells. The expression level of dNPPARG2 was comparable with that of wild-type PPARG2 (Fig. 1B). In the presence of troglitazone, both wild-type PPARG2 and dNPPARG2 stimulated the luciferase reporter gene containing Aox-PPRE fused with the tk promoter (tk-AoxPPRE-Luc; Fig. 1C, lanes 2–5). Although the existence of constitutive activity in the PPARG2-NTD was reported (Werman *et al.* 1997), truncation of the NTD did not affect transactivation (Fig. 1C, lanes 3 and 5). Without the addition of troglitazone, wild-type PPARG2 and dNPPARG2 showed stimulated basal levels of tk-AoxPPRE-Luc activity (Fig. 1C, lanes 2 and 4), suggesting that they had no silencing effect. To exclude the influence of endogenous ligands contained in the CV1 cells (DiRenzo *et al.* 1997, Werman *et al.* 1997), we employed PPARG2-P495L, which has no ligand-binding ability due to its amino acid substitution corresponding to PPARG1-P467L, and compared it with its NTD-truncated mutant, PPARG2-dNP495L (Fig. 1A). The expression levels of PPARG2-P495L and dNP495L were comparable with that of wild-type PPARG2 (Fig. 1B). Both PPARG2-P495L and dNP495L completely lost their ligand-independent (Fig. 1C, lanes 1, 6, and 8) and ligand-dependent transactivation functions (lanes 7 and 9). Co-expression of a twofold excessive amount of PPARG2-P495L repressed the troglitazone-dependent transactivation induced by wild-type PPARG2 (lanes 3 and 10), suggesting the presence of a DNE caused by PPARG2-P495L. Remarkably, the DNE of PPARG2-dNP495L was much more potent than that of PPARG2-P495L (lanes 3, 10, and 11). Likewise, PPARG2-dNP495L exhibited a more rigorous DNE than PPARG2-P495L in 3T3L1 pre-adipocytes (Fig. 1D). Since removal of the NTD potentiates the DNE, the PPARG2-NTD may interfere with the DNE of PPARG2-P495L. We tested the DNE on the activity of Aox-PPRE induced by liganded PPARG1. As shown in Fig. 2A, DNE by PPARG2-dNP495L was stronger than that by P495L (Fig. 2A), although its potency was milder than that on

Figure 1 The effect of NTD truncation on the DNE of PPARG2-P495L. (A) A schematic representation of wild-type PPARG2, dNPPARG2, PPARG2-P495L, and PPARG2-dNP495L. P495L, PPARG2-P495L; dNP495L, PPARG2-dNP495L; NTD, amino-terminal domain; DBD, DNA-binding domain; hinge, hinge domain; LBD, ligand-binding domain; AF-2, activation function 2. The arrows indicate the substitution of proline at codon 495 with leucine in PPARG2-P495L and dNP495L. The numbers below the schema represent the position in the amino acid sequence. The NTD encompassing the initiation methionine and histidine at codon 127 was truncated in dNPPARG2 and PPARG2-dNP495L. (B) The expression levels of PPARG2-P495L (arrow), dNPPARG2, and PPARG2-dNP495L (arrowhead) were comparable with that of wild-type PPARG2 (arrow). CV1 cells in 10 cm dish were transfected with equal amount (10 µg/dish) of the expression plasmid for wild-type or mutant PPARG2s. Whole cell extracts were fractionated by SDS-PAGE and subjected to western blot with anti-PPARG2 antibody against the hinge region of PPARG2. The band in the lane of PPARG2-dNP495L that exhibits retarded mobility close to the position of wild-type PPARG2 (arrow) appears to be non-specific. (C) The DNEs of PPARG2-P495L and dNP495L on the troglitazone-dependent transactivation driven by the wild-type PPARG2s. CV1 cells were transfected with 15 ng of wild-type or mutant PPARG2s together with 300 ng of tk-AoxPPRE-Luc in the absence (open bars) or presence (solid bars) of 5 µM troglitazone (lanes 1–11). Tro, troglitazone (5 µM). The transactivation function of wild-type PPARG2 was inhibited by the co-expression of a twofold excessive amount (30 ng) of PPARG2-P495L or dNP495L (lanes 10 and 11). * $P < 0.05$. (D) 3T3-L1 cells were transfected with 15 ng of wild-type PPARG2 and 300 ng of tk-AoxPPRE-Luc in the absence (open bar) or presence (solid bars) of 5 µM troglitazone. The transactivation function of wild-type PPARG2 was inhibited by the co-expression of a twofold excessive amount (30 ng) of PPARG2-P495L or dNP495L. * $P < 0.05$.

PPARG2-mediated activity (Fig. 1C). A functional PPRE has been reported in the promoters of the human and mouse adipose differentiation-related protein (*ADRP*) genes (Tachibana *et al.* 2005). We examined the DNE on the troglitazone-induced activity of the reporter gene, hADRP-4K-Luc, where the human ADRP promoter containing -2981/+1066 was fused with luciferase gene. Ligand-induced activity of the ADRP-derived PPRE by dNPPARG2 (lanes 4 and 5) was similar to that by wild-type PPARG2 (lanes 2 and 3), suggesting that deletion of NTD does not affect the transactivation function of the *ADRP* gene. While co-expression of twofold excessive amount of P495L did not repress the troglitazone-dependent transactivation induced by

wild-type PPARG2, same amount of PPARG2-dNP495L exhibited mild but significant DNE (Fig. 2B), suggesting that NTD truncation potentiates DNE on ligand-induced transactivation via ADRP-derived PPRE.

The DNE of mutant THR1-K443E is not potentiated by the truncation of its NTD

To compare the effect of the NTD truncation of PPARG2 with that of THR1, we generated THR1-dNK443E (Fig. 3A), in which the NTD was deleted from a mutant THR1-K443E that had been identified in a patient with RTH (Sasaki *et al.* 1995). The expression levels of THR1-K443E and THR1-dNK443E were

Figure 2 NTD truncation of PPARG2 enhances its DNE on the PPARG1-mediated activity of Aox-PPRE and the transactivation of the human ADRP promoter driven by PPARG2. (A) Truncation of NTD from PPARG2-P495L potentiates its DNE on the transactivation driven by liganded PPARG1. CV1 cells were transfected with 15 ng of wild-type mouse PPARG1 together with 300 ng of tk-AoxPPRE-Luc in the absence (open bars) or presence (solid bars) of 5 μ M troglitazone. Tro, troglitazone (5 μ M). The transactivation function of wild-type PPARG1 (lane 3) was inhibited by the co-expression of a twofold excessive amount (30 ng) of PPARG2-P495L or dNP495L (lanes 4 and 5). * P <0.05. (B) Twofold molar excess of PPARG2-dNP495L but not P495L exhibits DNE on the activity of the human ADRP promoter induced by liganded wild-type PPARG2. CV1 cells were transfected with 15 ng of wild-type PPARG2 together with 300 ng of hADRP-4K-Luc in the absence (open bars) or presence (solid bars) of 5 μ M troglitazone. Tro, troglitazone (5 μ M). Ligand-induced activity of the ADRP-PPRE by dNPARG2 was similar to that by wild-type PPARG2. The transactivation function of wild-type PPARG2 (lanes 2 and 3) was inhibited by the co-expression of a twofold excessive amount (30 ng) of PPARG2-dNP495L (lane 7) but not P495L (lane 6). * P <0.05.

comparable with that of wild-type THR1 (Fig. 3B). The luciferase-based reporter gene, which had a typical T3-responsive element (TRE) fused to the tk promoter (tk-TREpal-Luc), was transfected into CV1 cells together with the wild-type and mutant THR1 expression plasmids. Unliganded THR1 alone significantly repressed the basal transcriptional activity (Fig. 3C), suggesting transcriptional silencing. As we reported previously (Sasaki *et al.* 1995), the co-expression of THR1-K443E reduced the T3-dependent transactivation of tk-TREpal-Luc stimulated by wild-type THR1, indicating that THR1-K443E has a DNE on wild-type THR1. Contrary to the results with PPARG2-P495L (Fig. 1C, lanes 3, 10, and 11), the DNE induced by THR1-dNK443E was comparable with that of full-length THR1-K443E, suggesting that NTD truncation does not affect the DNE.

Truncation of the NTD in PPARG2s, but not THR1-K443E, facilitates the *in vitro* interaction between its LBD and NCoR

The enhancement of the DNE by the NTD truncation in PPARG2-P495L led us to investigate whether this domain modulates the interaction of its LBD with CoR. We performed an *in vitro* binding assay using bacterially

expressed NCoR fused to GST (GST-NCoR) and 35 S-radiolabeled receptors. Deletion of the NTD from PPARG2-P495L drastically increased its binding affinity to NCoR (Fig. 4A, lanes 8 and 9). We tested another mutant PPARG2, PPARG2-V318M. This mutant PPARG2 possesses an amino acid substitution corresponding to PPARG1-V290M (Barroso *et al.* 1999), LBD of which has the structure different from that of PPARG1-P467L (Kallenberger *et al.* 2003). Although PPARG2-V318M may have some affinity for PPARG ligands (Agostini *et al.* 2004), *in vitro* experiments allowed us to evaluate the NCoR interaction in the ligand-free condition. As shown in Fig. 4B, the NTD-deleted mutant, PPARG2-dNV318M, also exhibited increased affinity for NCoR (lanes 5 and 6). The *in vitro* translated products of THR1 and THR1-K443E produced two major bands due to the two initiation methionines in their NTDs (Fig. 4C, lanes 1 and 2), while THR1-dNK443E appeared as a single band (lane 3). Although we utilized an excessive amount of GST-NCoR and adjusted the amounts of

Figure 3 The effect of NTD truncation on the DNE of THR1-K443E. (A) A schematic representation of wild-type THR1, THR1-K443E, and dNK443E. K443E, THR1-K443E; dNK443E, THR1-dNK443E. The arrows indicate the substitution of lysine at codon 443 with glutamine in THR1-K443E and dNK443E. The numbers below the schema represent the position in the amino acid sequence. The NTD encompassing initiation methionine and cysteine at codon 88 was truncated in THR1-dNK443E. (B) The expression levels of THR1-K443E (arrow) and dNK443E (solid arrowhead) CV1 cells in 10 cm dish were transfected with the equal amount (10 μ g/dish) of expression plasmids for wild-type or mutant THR1s. Whole cell extracts were fractionated by SDS-PAGE and subjected to western blot with anti-THR1 antibody against the LBD of THR1. The band in the lane of wild-type THR1 and THR1-K443E that exhibits faster mobility (open arrowhead) appears to be the translation variant from downstream methionine. (C) In the presence (solid bars) and absence (open bars) of 100 nM T3, wild-type THR1 (15 ng) and tk-TREpal-Luc (300 ng/dish) were co-transfected into CV1 cells along with a twofold excessive amount (30 ng/dish) of THR1-K443E or dNK443E. * P <0.05.

Figure 4 The effect of PPARG2-NTD truncation on the interaction between LBD and NCoR *in vitro*. (A) The wild-type PPARG2 (arrow), PPARG2-P495L (arrow), and PPARG2-dNP495L (solid arrowhead) were labeled with ³⁵S-methionine and incubated with GST protein that was fused to the receptor interacting domain of NCoR (GST-NCOR; lanes 7–9). P495L, PPARG2-P495L; dNP495L, PPARG2-dNP495L. The binding fraction was analyzed by SDS-PAGE. (B) PPARG2-V318M (arrow) and PPARG2-dNV318M (solid arrowhead) were labeled with ³⁵S-methionine and incubated with GST protein that was fused to the receptor interacting domain of NCoR (GST-NCOR; lanes 5 and 6). V318M, PPARG2-V318M; dNV318M, PPARG2-dNV318M. The binding fraction was analyzed by SDS-PAGE. (C) ³⁵S-labeled wild-type THR1, K443E, and dNK443E were incubated with GST-NCOR (lanes 7–9). The affinities of wild-type THR1 (arrows), THR1-K443E (arrows), and dNK443E (solid arrowhead) for GST-NCOR were comparable. The band in the lane of THR1-K443E that exhibits faster mobility (open arrowhead) appears to be the translation variant from downstream methionine. K443E, THR1-K443E; dNK443E, THR1-dNK443E. (D) The wild-type PPARG2 (arrow) and dNPPARG2 (arrowhead) were labeled with ³⁵S-methionine and incubated with GST-NCOR. The binding fraction was analyzed by SDS-PAGE.

radiolabeled receptors in the input lanes (lanes 1–3), the signals from discrete bands were not affected by truncation of the NTD (lanes 8 and 9). Because there is the possibility that structure of CoR-binding surface may be altered in mutant PPARG2s, we tested the wild-type PPARG2 and dNPPARG2. As shown in Fig. 4D (lanes 5 and 6), the interaction of wild-type PPARG2 with NCoR was again enhanced by the truncation of its NTD. Together, it is considered that the NTD in PPARG2s, but not THR1-K443E, is able to prevent the interaction of NCoR with its LBD.

The construction of the chimeric receptor, PT, in which PPARG2-derived NTD and DBDs were fused to a THR1-derived LBD

To exclude the influence of endogenous ligand in CV1 and 3T3L1 cells, we further attempted to generate a chimeric receptor, in which the PPARG2-LBD was replaced with a THR1-derived LBD. The PPAR-RXR

heterodimer binds to a DR1-type PPRE with PPAR on the 5' end and RXR on the 3' end (Desvergne & Wahli 1999). The inverted polarity is dictated by the A- and T-boxes in the PPARG2-DBD. Based on these considerations, we constructed a chimeric receptor designated PT, which possesses A- and T-boxes derived from PPARG2 (Fig. 5A). A gel shift assay revealed that the PT chimera, as well as PPARG2, formed heterodimers with RXR on PPRE (Fig. 5B). As expected, deletion of the A- or T-box abolished heterodimer formation with RXR (data not shown). As shown in Fig. 4C, the PT chimera activated tk-AoxPPRE-Luc in the presence of 100 nM T3 to a level similar to that induced by wild-type PPARG2 in the presence of 5 μM troglitazone. In contrast with wild-type PPARG2, the PT chimera significantly repressed basal transcriptional activity (Fig. 5C, inset), suggesting that PT possesses a silencing function in the absence of T3. The PT chimera did not stimulate tk-TREpal-Luc, presumably due to the specificity of the PPARG2-derived DBD (Fig. 5D).

Figure 5 Construction and functional evaluation of PT chimera.

(A) A schematic representation of PT chimera. The N-terminal half (codon 1–231) of wild-type PPARG2, which contains NTD, DBD, and the A- and T-boxes, was fused to the C-terminal half of wild-type THR1, which harbors the CoR box and LBD (codon 197–461). PPARG2, wild-type PPARG2; THR1, wild-type THR1. (B) The affinity of the PT-RXR heterodimer for Aox-PPRE is similar to that of wild-type PPARG2. ³²P-labeled PPRE derived from the Aox promoter was incubated with *in vitro* translated PT or wild-type PPARG2. In all lanes, *in vitro* translated mouse RXRB was added. The arrow and NS indicate the specific and the non-specific binding respectively. (C) T3-dependent transactivation by the PT is comparable to that by wild-type PPARG2 in the presence of troglitazone. The luciferase activity of tk-AoxPPRE-Luc was co-transfected with wild-type PPARG2 or PT in the presence of 5 μM troglitazone or 100 nM T3 respectively. In the absence of T3, PT repressed the basal reporter activity (silencing), while wild-type PPARG2 enhanced it (inset). (D) The PT is not able to stimulate the TRE. TREpal-tk-Luc was co-transfected with wild-type THR1 or the PT in the presence (solid bars) or absence (open bars) of 100 nM T3. **P* < 0.05.

The truncation of NTD potentiates the DNE of the PT chimera receptor via interaction with NCoR

We tested whether the PT chimera in the absence of T3 also interferes with the troglitazone-induced transactivation driven by wild-type PPARG2. In CV1 cells, transactivation of tk-AoxPPRE-Luc driven by liganded wild-type PPARG2 was inhibited by the co-expression of the PT chimera in the absence of T3 (Fig. 6B, lanes 3 and 4). The CoR box in the THR1 hinge region (Horlein *et al.* 1995) is necessary for interaction with CoRs (Marimuthu *et al.* 2002). To investigate whether the DNE of the PT chimera is mediated through its association with CoRs, we introduced the AHT mutation (Horlein *et al.* 1995) into the CoR box of the PT chimera (Fig. 6A). As illustrated in Fig. 6B (lanes 4 and 6), the DNE of PT-AHT was abrogated, indicating that the repression by the PT chimera was mediated through its interaction with CoRs. Subsequently, we constructed dNPT, in which the NTD was deleted from the PT chimera (Fig. 6A). While the T3-dependent transactivation by dNPT was similar to that of the PT

chimera (Fig. 6C), the DNE of dNPT was more potent than that of the full-length PT chimera (Fig. 6B, lanes 4 and 5). The introduction of the AHT mutation into the CoR box of dNPT again reduced its DNE (Fig. 6B, lanes 5 and 7). The expression levels of dNPT, PT-AHT, and dNPT-AHT were comparable with that of the PT chimera (Fig. 6D). Similar results were obtained in 3T3L1 pre-adipocytes (Fig. 6E). As shown in Fig. 6F, the *in vitro* interaction with NCoR was enhanced by the truncation of the NTD in the PT chimera. These results indicate that PPARG2-NTD inhibits the binding of CoRs with the LBD derived from PPARG2 as well as that derived from THR1, resulting in transcriptional upregulation.

Discussion

It was reported that the association of CoRs requires the CoR box, which is typically found in the hinge region of THR1 and RARA (Horlein *et al.* 1995). However, later analyses revealed that the amino acid residues directly

Figure 6 The DNE of the PT and its truncation mutant, dNPT. (A) A schematic representation of the PT chimera, dNPT, PT-AHT, and dNPT-AHT. The NTD encompassing the initiation methionine and histidine at codon 127 derived from wild-type PPARG2 was truncated in dNPT and dNPT-AHT. The arrows indicate the position of the AHT residues at codon 223, 224, and 227 that were mutated to GGA in the CoR box derived from THR1. (B) CV1 cells were transfected with 15 ng of the wild-type PPARG2s together with 300 ng of tk-AoxPPRE-Luc in the absence (open bars) or presence (solid bars) of 5 μ M troglitazone (lanes 1–3). The transactivation function of wild-type PPARG2 (15 ng) was inhibited by the co-expression of a twofold molar excessive amount (30 ng) of the PT chimera or dNPT (lanes 4 and 5 respectively). PPARG2, wild-type PPARG2. (C) In the presence (solid bars) or absence (open bars) of T3, 300 ng of tk-AoxPPRE-Luc was co-transfected into CV1 cells along with 15 ng of the PT chimera or dNPT. The T3-dependent transactivation of tk-AoxPPRE-Luc by the PT chimera is comparable with that of dNPT. (D) The expression levels of dNPT (arrowhead), PT-AHT (arrow), and dNPT-AHT (arrowhead) were comparable with that of the PT chimera (arrow). CV1 cells in 10 cm dish were transfected with the equal amount of expression plasmids (10 μ g/dish) for wild-type or mutant THR1s. Whole cell extracts were fractionated by SDS-PAGE and subjected to western blot with anti-THR1 antibody against the hinge region of PPARG2. (E) 3T3-L1 cells were transfected with 15 ng of wild-type PPARG2 and 300 ng of tk-AoxPPRE-Luc in the absence (open bars) or presence (solid bars) of 5 μ M troglitazone. The transactivation function of wild-type PPARG2 was inhibited by the co-expression of a twofold excessive amount (30 ng) of PT chimera or dNPT. * $P < 0.05$. (F) The PT (arrow) and dNPT (arrowhead) were labeled with 35 S-methionine and incubated with GST-NCoR. The binding fraction was analyzed by SDS-PAGE and visualized by autoradiography.

interacting with CoRs are distributed widely in THR-LBD, although the CoR box is necessary for maintaining the global structure of the THR-LBD for CoR interaction (Marimuthu *et al.* 2002). This implies that the CoR box is not necessarily the determinant of CoR interaction. X-ray crystal analysis showed that CoRs interact with the PPARG-LBD (Xu *et al.* 2002, Agostini *et al.* 2004), the interaction surface of which is very similar to that of the THR-LBD (Kallenberger *et al.* 2003). PPARG has high homology ($\sim 71\%$) in the amino acid sequence of its LBD with the PPARG-LBD (Xu *et al.* 2002, Agostini *et al.* 2004). Direct binding of CoRs with wild-type PPARG2 was demonstrated by GST pull-down assay (Zamir *et al.* 1997, Krogsdam *et al.* 2002,

Stanley *et al.* 2003), co-immunoprecipitation (Gurnell *et al.* 2000, Yu *et al.* 2005), gel shift assay (Lee *et al.* 2002), and an *in vitro* pulldown assay with RXR-PPARG2 heterodimers on PPREs (Krogsdam *et al.* 2002). Using a ChIP assay, Guan *et al.* (2005) showed that, in adipocytes without the addition of an exogenous ligand, PPARG2 molecules associated with CoRs are present in PPREs in the promoter region of the glycerol kinase gene (Guan *et al.* 2005). Similar results were reported in other PPARG-target genes including oxidized low-density lipoprotein receptor 1 (Chui *et al.* 2005), aP2, and PPARG (Picard *et al.* 2004). These findings suggest that unliganded PPARG2 recruits CoRs, resulting in transcriptional silencing.

It should be noted, however, that the NCoR recruitment may depend on the sequence of PPREs (Guan *et al.* 2005). This could explain why twofold excessive amount of PPARG2-P495L failed to exhibit DNE on the ADRP promoter activity (Fig. 2B).

PPARG-LBD possesses a very large ligand-binding pocket (1300 Å³; Desvergne & Wahli 1999) and can accommodate a variety of ligands (Michalik *et al.* 2006, Semple *et al.* 2006). It is currently difficult to completely eliminate the endogenous ligands produced by cells (DiRenzo *et al.* 1997, Werman *et al.* 1997, Kim *et al.* 1998, Camp *et al.* 2001, Madsen *et al.* 2003, Tzamelis *et al.* 2004, Yano *et al.* 2007). The existence of endogenous ligands may mask the silencing by unliganded PPARG2. This could explain why conventional reporter assays have failed to detect the silencing effect by PPARG2 despite the absence of exogenous ligands (Zamir *et al.* 1997, Barroso *et al.* 1999, Zhang *et al.* 1999, Chatterjee 2001, Gurnell *et al.* 2003, Agostini *et al.* 2004, Semple *et al.* 2006). For the creation of ligand-free conditions *in vivo*, we took advantage of the fact that PPARG1-P467L is resistant to various ligands (Barroso *et al.* 1999, Agostini *et al.* 2004) and generated a corresponding mutant, PPARG2-P495L. Our analyses with a conventional luciferase reporter assay demonstrated that NTD truncation potentiated the DNE of PPARG2-P495L on the ligand-induced transactivation induced by wild-type PPARG2 (Fig. 1C and D). Potentiation of DNE by NTD truncation was also observed in the PPARG1-mediated activation of Aox-PPRE (Fig. 2A) and in the transactivation of the human ADRP promoter stimulated by PPARG2 (Fig. 2B). Moreover, in the cell-free conditions with GST pulldown assay, truncation of NTD enhances NCoR binding with not only PPARG2-P495L (Fig. 4A) and V318M (Fig. 4B) but also wild-type PPARG2 (Fig. 4D). These results indicate that the NTD controls the association of CoR with unliganded PPARG2-LBD via inter-domain communication, which was originally proposed in the context of ligand-dependent transactivation by PPARG2 (Shao *et al.* 1998). It is necessary in future to examine whether NTD modulates the effect of ligand to dissociate NCoR from LBD.

We created a chimera, PT, by replacing PPARG-LBD with THR-B-LBD. This chimeric receptor has many advantages for analyzing the function of unliganded PPARG2. First, the ligand-binding pocket of THR-B-LBD (600 Å³) is much smaller than that of PPARGs (Desvergne & Wahli 1999) and is specific for T3. The concentration of T3 in culture medium is easy to measure, and the method for its removal is established (Samuels *et al.* 1979). Second, the interaction of THR-B-LBD with CoR has been characterized in detail (Horlein *et al.* 1995, Yang *et al.* 1999, Marimuthu *et al.* 2002). Accumulated information from the natural THR-B1 mutants in RTH is also available (Refetoff *et al.* 1993). The functional relevance in the context of

DNEs is confirmed in mutant PPARGs harboring artificial amino acid substitutions corresponding to those of mutant THR-B1s *in vitro* (Zamir *et al.* 1997, Gurnell *et al.* 2000, Chatterjee 2001, Nugent *et al.* 2001, Park *et al.* 2003) and *in vivo* (Freedman *et al.* 2005). Finally, THR-LBD is flexible with regard to the orientation of its DBD in a variety of TREs (Kurokawa *et al.* 1993). This feature is critical for mimicking the inverted polarity of the PPARG2-RXR heterodimer in the PPRE (Desvergne & Wahli 1999). Using the PT chimera, we observed that NTD truncation enhanced the DNE on the ligand-dependent transactivation induced by wild-type PPARG2. This suggests that the PPARG2-NTD functions to regulate the association of CoR with unliganded LBD. The data from the AHT mutation in CoR box (Horlein *et al.* 1995) of the PT chimera indicate that DNEs are mediated through CoR recruitment but not the RXR squelching proposed previously (Juge-Aubry *et al.* 1995, Hunter *et al.* 1996, Winrow *et al.* 1996, Lu & Cheng 2010).

PPARG2-NTD may have multiple functions including direct interactions with p300 (Gelman *et al.* 1999) and Tip60 (van Beekum *et al.* 2008) and modification by small ubiquitin-related modifier-1 (Ohshima *et al.* 2004, Yamashita *et al.* 2004). Consistently, a microarray analysis using a chimera between PPARG2 and PPARδ (PPARD) revealed that the PPARG2-NTD is the major determinant for the PPARG2-specific gene expression profile (Hummasti & Tontonoz 2006). However, Bugge *et al.* (2009) recently reported that, of the 257 genes induced by rosiglitazone, only 25 genes exhibited the reduced transcriptional activity in the cell expressing truncated PPARG2 lacking NTD. We speculate that the NTD-dependent function of unliganded PPARG2 to regulate the CoR association may be another determinant for the PPARG2-specific gene expression profile. An attempt to confirm the *in vivo* function of NTD in unliganded PPARG2 is currently underway in our laboratory. Our study indicates that the inter-domain communication between NTDs and LBDs, originally proposed in the context of ligand-dependent transactivation (Shao *et al.* 1998), also plays a role in CoR association and the silencing function mediated by unliganded PPARG2. Similar modification of LBD–CoR interactions by the NTD has been suggested in other NHRs (Kumar & Thompson 2003, Pippal & Fuller 2008). For example, insulin-induced phosphorylation in the PPAR-NTD enhances the dissociation of CoRs from its LBD (Juge-Aubry *et al.* 1999). Direct interaction between NTDs and LBDs was reported in estrogen receptor (Metivier *et al.* 2000, Metivier *et al.* 2001) and androgen receptor (AR; Cheng *et al.* 2002, He & Wilson 2002, Liao *et al.* 2003). In AR, the interaction between NTD and LBD, referred to as ‘N/C interaction’ (He & Wilson 2002), was interfered by the overexpression of NCoR or SMRT (Liao *et al.* 2003). It is also necessary

to confirm the inter-domain communication of unliganded PPARG2 using the chromatinized PPRES.

Hollenberg *et al.* (1996) reported that pituitary-specific THR β 2 does not have a silencing function in the absence of T3. One may assume common features between the THR β 2-NTD and PPARG2-NTD in the mechanism of loss of silencing function. However, in contrast to PPARG2 (Fig. 4D), the truncation of the NTD from THR β 2 did not affect its physical interaction with CoRs *in vitro* (Yang *et al.* 1999). Thus, the mechanism underlying the CoR interaction of THR β 2 is different. According to Yang *et al.* (1999), CoRs interact with not only THR β 2-LBD but also its NTD, and CoR-NTD contact neutralizes the repressive function of CoRs. On the other hand, the same authors reported that, in wild-type THR β 1, NTD truncation did not affect its silencing function or *in vitro* binding with CoR (Yang *et al.* 1999). This is in agreement with our finding that deletion of the NTD from THR β 1-K443E did not affect its interaction with NCoR (Fig. 4C) or its DNE on the transactivation induced by T3-bound THR β 1 (Fig. 3C).

Given that the transcriptional silencing by unliganded PPARG2 is controlled by its NTD, genetic modification of this domain may alter its insulin sensitivity *in vivo*. In the PPARG2-NTD, the serine residue at codon 112 is phosphorylated by MAPK (Hu *et al.* 1996, Adams *et al.* 1997, Camp & Tafuri 1997). Rangwala *et al.* (2003) generated knock-in mice, in which the serine at codon 112 was substituted for phosphorylation-resistant alanine (S112A), and noted an increase in insulin sensitivity. Of note, thiazolidinedione-sensitive genes such as phosphoenolpyruvate carboxykinase (*PEPCK*) were not significantly activated in S112A mice. Rangwala *et al.* (2003) suggested that the property of S112A-PPARG2 in the reduction of insulin resistance may be qualitatively different from that of liganded PPARG2. In agreement with this, Armoni *et al.* (2003) demonstrated *in vitro* that S112A substitution diminishes the silencing activity caused by unliganded PPARG2. Polymorphism in the NTD of the *PPARG2* gene from CCA (proline) at codon 12 to GCA (alanine) (P12A) is also associated with higher insulin sensitivity (Deeb *et al.* 1998). However, it was shown that transactivation by PPARG2-P12A in the presence of a cognate ligand is similar or reduced compared to that of the wild-type receptor (Deeb *et al.* 1998, Masugi *et al.* 2000). Further studies are required to clarify whether these genetic modifications in the NTD may affect the insulin sensitivity through the modulation of the interaction between CoRs and unliganded PPARG2.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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