The peroxisome proliferator-activated receptor γ (PPARγ) is a central regulator of adipogenesis and recruits coactivator proteins in response to ligand. However, the role of another class of nuclear cofactors, the nuclear receptor corepressors, in modulating PPARγ transcriptional activity is less clear. Such corepressors include the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT). Our data suggest that PPARγ recruits SMRT and NCoR in the absence of ligand and that these corepressors are capable of down-regulating PPARγ-mediated transcriptional activity. The addition of the PPARγ ligand pioglitazone results in dissociation of the PPARγ-corepressor complex. To define the role of SMRT and NCoR in PPARγ action, 3T3-L1 cells deficient in SMRT or NCoR were generated by RNA interference. When these cells are exposed to differentiation media, they exhibit increased expression of adipocyte-specific genes and increased production of lipid droplets, as compared with control cells. These data suggest that the nuclear receptor corepressors decrease PPARγ transcriptional activity and repress the adipogenic program in 3T3-L1 cells.

Christine Yu‡§, Kathleen Markan‡, Karla A. Temple‡, Dianne Deplewski§, Matthew J. Brady¶, and Ronald N. Cohen‡

From the §Section of Endocrinology, Department of Medicine and the ¶Section of Pediatric Endocrinology, Department of Pediatrics, The University of Chicago, Chicago, Illinois 60637

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The thyroid hormone receptors (TRs) and retinoic acid receptors (RARs) are nuclear receptors that repress gene transcription in the absence of their respective ligands. This ligand-independent repression is mediated by nuclear receptor corepressors, such as the nuclear receptor corepressor protein (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (1, 2). NCoR and SMRT are recruited by TR and RAR isoforms in the absence of ligand and are released upon ligand binding. NCoR and SMRT, in turn, recruit a complex with histone deacetylase activity to repress transcription of target genes. More recently, NCoR and SMRT have been shown to be recruited by other nuclear receptors, some of which bind nuclear receptor corepressors in the presence of antagonists. However, it has been controversial whether NCoR and SMRT play a significant role in gene regulation by the peroxisome proliferator-activated receptor γ (PPARγ) isoforms (3).

Two types of PPARγ isoforms exist, PPARγ1 and PPARγ2. These receptors differ only in their amino-terminal A/B domain, such that PPARγ2 contains an extra 30 amino acids (4). The function of PPARγ2 has attracted considerable interest because it is specifically expressed in adipocytes and is an essential regulator of adipogenesis. In addition, many important adipocyte-specific genes contain response elements for PPARγ in their promoter regions. Although PPARγ clearly recruits coactivators in response to exogenous ligands, its ability to recruit corepressors is less certain. In contrast to the TR, PPARγ does not appear to be a strong repressor in the absence of its ligand. Such experiments, however, have been limited by the lack of information concerning physiologic endogenous ligands. Early work into PPARγ and corepressor recruitment suggested that PPARγ might not recruit NCoR or SMRT in the presence of DNA response elements (3). Another report suggested that PPARγ could recruit the corepressor SMRT in the presence of signaling by epidermal growth factor (5). Later work suggested that PPARγ can recruit nuclear receptor co-repressors in cells (6, 7), and overexpression of NCoR or SMRT has been shown to repress PPARγ-mediated gene transcription in certain cell types (8). In addition, mutant PPARγ receptors found in patients with PPARγ resistance bind corepressors but release them aberrantly in the presence of exogenous ligands (9). Finally, a recent study suggested that NCoR is required for the NAD-dependent deacetylase Sirt1 to decrease fat accumulation (10).

The 3T3-L1 cell line has been used as a model of adipogenesis because, in the presence of defined hormonal stimulation, 3T3-L1 fibroblasts differentiate into adipocytes. This process has been carefully investigated and is accompanied by an increase in PPARγ expression. In turn, PPARγ2 plays a central role in adipogenesis, by increasing the expression of C/EBPα and other downstream adipocyte-specific genes (11). In the following experiments, we show that PPARγ2 can recruit NCoR and SMRT in 3T3-L1 cells and that these corepressors repress PPARγ-mediated gene transcription. We then generate stable populations of cells deficient in NCoR or SMRT using siRNA technology; 3T3-L1 cells lacking either NCoR or SMRT exhibit enhanced expression of adipocyte-specific genes, suggesting that nuclear receptor corepressors modulate adipogenesis via effects on PPARγ activity.
EXPERIMENTAL PROCEDURES

Plasmids—Plasmids for Gal4-TR, Gal4-RAR, upstream activating sequence-luciferase (UAS-Luc), VP16-NCoR, VP16-SMRT, Gal4-NCoR, and Gal4-SMRT-luciferase have previously been described (12–14). Gal4-PPARγ was constructed by placing the coding sequence for the PPARγ ligand-binding domain downstream of the Gal4 DNA-binding domain in the vector pECE. VP16-PPARγ2 was constructed by placing the entire coding sequence of PPARγ2 downstream of VP16 in the vector pVP16 (Clontech). PP-RE-Luc was made by placing two copies of the modified DR3 element AGGACAAAGGTCAGTTTCAAGAGAAATCAGGAACACTCTTCCTTTTTTTGGAAA. The negative control siRNA construct used was provided by Ambion. Cell Culture and Transfection—3T3-L1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin, streptomycin, glutamine, and 10% calf serum. The cells were transfected with Lipofectamine Plus (Invitrogen) per the manufacturer’s instructions with the following exceptions. For mammalian two-hybrid assays, the cells were transfected on day 1, and 3 h after transfection (in serum-free medium), the cells were washed with PBS and placed in fresh serum-free media, with or without varying concentrations of pioglitazone (a generous gift from Takeda Pharmaceuticals); luciferase activity was assessed 20–24 h later. For NCoR or SMRT overexpression studies, the cells were transfected on day 1, and 3 h after transfection, the cells were washed with PBS and changed to full medium. On day 2, the cells were washed with PBS again and changed to serum-free medium. Luciferase activity was measured on day 3. All of the transient transfections were performed in triplicate and repeated at least three times. For stable transfections, 6 μg of the indicated siRNA construct in pSilencer-Hygro were transfected in cell culture dishes and placed in fresh medium 3 h later. The following day, the cells were washed with PBS and placed in full medium supplemented with hygromycin. The cells were placed in fresh hygromycin-containing medium every 3–4 days. When the cells were about 75% confluent, they were split into new culture dishes with fresh hygromycin.

3T3-L1 Cell Differentiation—The 3T3-L1 cells were allowed to grow until confluence and then refed with fresh medium for 2 more days. The cells were plated in medium containing 10% fetal calf serum, insulin, dexamethasone, and isobutylmethylxanthine for 3 days, as previously described (16). After 3 days, the cells were plated in medium with fresh 10% fetal calf serum and insulin for 2 days. After this truncated differentiation protocol, the cells were either harvested for Western blot experiments or allowed to differentiate further and then fixed and stained with Oil Red O.

Western Blots—After 3T3-L1 cell differentiation, the cells were prepared for either whole cell extracts (for PPARγ, C/EBPα, NCoR, and SMRT) or cytoplasmic extracts (for adiponectin, perilipin, and protein phosphatase 1), which were then analyzed by SDS-PAGE, transferred to nitrocellulose, and blotted with specific primary antibodies. The antibodies used were as follows: anti-NCoR (a generous gift from Tony Hollenberg, M.D., Boston, MA), anti-SMRT (generated from the peptide sequence LIKMEKERNAR), anti-PPARγ (Santa Cruz Biotechnologies), anti-C/EBPα (Santa Cruz Biotechnologies), anti-protein phosphatase 1 (Santa Cruz Biotechnologies), anti-adiponectin (Chemicon), and anti-perilipin (Research Diagnostics, Inc.). For the anti-SMRT blot, whole cell extracts were first immunoprecipitated with the anti-SMRT antibody, then run on SDS-PAGE, and blotted with the anti-SMRT antibody.

Oil Red O Staining—The Oil Red O stock solution was prepared by mixing 0.5 g of Oil Red O in 100 ml of isopropanol. Differentiated 3T3-L1 cells were fixed with 10% formaldehyde for 10 min at room temperature and then washed with PBS. The cells were incubated with an Oil Red O dilution (6 ml of Oil Red O stock, 4 ml of distilled water) for 30–60 min with gentle agitation, followed by further washing with PBS.

RESULTS

SMRT and NCoR share a similar overall structure and contain amino-terminal repressing domains and carboxyl-terminal interacting domains (ID). The interactions between nuclear receptors and corepressors are dependent on these ID, which contain so-called CoRNR box sequences of the form (Val/Leu-X-X-Lys/Lys-Trp, where X represents any amino acid (17–19). SMRT contains two interacting domains, whereas NCoR additionally contains a third domain that is proximal to the other two (Fig. 1A). To determine whether PPARγ recruits SMRT and NCoR in 3T3-L1 cells, we initially used a mammalian two-hybrid assay. The ligand-binding domains of PPARγ, TRα, and RARα were placed downstream of the Gal4 DNA-binding domain. These Gal4 constructs were then cotransfected with VP16-corepressor constructs and a UAS-Luc reporter vector to assess interactions in 3T3-L1 cells. Consistent with our prior data (12, 13), TRα interacts more strongly with NCoR than SMRT, and RARα interacts more weakly than TRβ with NCoR (Fig. 1B). Surprisingly, however, Gal4-PPARγ was able to recruit both NCoR and SMRT to a similar degree as RARα in 3T3-L1 cells. To determine whether full-length PPARγ2 could also recruit corepressors, full-length PPARγ2 was placed downstream of VP16 and cotransfected with Gal4-SMRT or Gal4-NCoR. As shown in Fig. 1C, full-length PPARγ2 interacted with both SMRT and NCoR in 3T3-L1 cells, although the interaction with SMRT was much stronger. To determine which SMRT ID was responsible for this strong interaction, the CoRNR box sequences in either SMRT ID1 or ID2 were mutated. It has been shown that mutation of the initial isoleucine or leucine to alanine abolishes interaction between the corepressor ID and nuclear receptors (12, 17). Therefore, the initial CoRNR box amino acids in SMRT ID1 and ID2 were individually altered to alanine in Gal4-SMRT to create Gal4-SMRTID1 and Gal4-SMRTID2. As shown in Fig. 1D, VP16-PPARγ2 interacts strongly with Gal4-SMRTID2, but interactions with Gal4-SMRTID1 are abolished. These data suggest that SMRT ID1 is required for full interactions with PPARγ2, consistent with prior data using isolated IDs in heterologous 293 cells (9). To confirm that this interaction occurred with wild-type protein in 3T3-L1 cells, the cells were allowed to differentiate for 3 days with insulin, dexamethasone, and isobutylmethylxanthine to increase endogenous PPARγ levels. Then whole cell extracts from these cells were immunoprecipitated with either an anti-SMRT, anti-NCoR, or control antibody and then subjected to Western blot using an anti-PPARγ antibody. As shown in Fig. 2A, the cell fraction containing NCoR and SMRT in differentiated 3T3-L1 cells also contains PPARγ, suggesting that endogenous PPARγ recruits corepressors in vivo. To determine whether the interaction of PPARγ and corepressors in 3T3-L1 cells is functional, a PPRE-Luciferase construct was transfected into 3T3-L1 cells in the presence or absence of overexpressed PPARγ2, because PPARγ2 is not expressed at high levels in undifferentiated cells. As expected, cotransfected PPARγ2 resulted in an increase in luciferase activity, in part because of the ligand-independent activation function in its amino terminus (Fig. 2B). Overexpression of pSG5-SMRT resulted in a modest decrease in luciferase activity in the absence of overexpressed PPARγ2. In contrast, in the presence of PPARγ2, transfection of NCoR or SMRT resulted in a strong decrease in luciferase activity (Fig. 2B), suggesting that PPARγ2 recruits corepressors to modulate its activity in 3T3-L1 cells.

PPARγ has been identified as a key regulator of adipogenesis. We reasoned that if corepressors repress PPARγ-mediated gene transcription, a decrease in corepressor levels might enhance the expression of adipocyte-specific genes. Therefore, we used the pSilencer vector-based siRNA system (Ambion) to stably transfet siRNA constructs for NCoR or SMRT (or a negative control) in 3T3-L1 cells. Stable transfection has been used by other groups to identify factors important in the dif-
To identify functional NCoR or SMRT siRNA constructs, multiple potential sequences were tested for their ability to decrease overexpressed NCoR or SMRT by transient transfection (data not shown). The functional siRNA constructs identified were then stably expressed in 3T3-L1 cells by transfection followed by selection with hygromycin. Cells stably expressing NCoR or SMRT siRNA constructs (or a negative control siRNA construct) were then analyzed by Western blot. As shown in Fig. 3A, cells containing the stably expressed NCoR siRNA specifically downregulated NCoR expression, without decreasing SMRT levels. In contrast, the SMRT siRNA construct specifically downregulated SMRT levels and led to a mild increase in NCoR levels. Neither population of cells had altered levels of other measured proteins, including CBP and C/EBPα. In the undifferentiated state, neither population of cells significantly expressed such adipocyte-specific proteins as adiponectin or perilipin (data not shown), suggesting that the absence of corepressors proteins is not sufficient in itself for adipocyte differentiation.

The 3T3-L1 cells were then differentiated in the presence of insulin, dexamethasone, and isobutylmethylxanthine for 3 days and then insulin alone for 2 days. After this truncated differentiation protocol, Western blots were used to analyze protein levels. In cells lacking either NCoR or SMRT, there was increased expression of adiponectin, perilipin, and C/EBPα (Fig. 3B), suggesting that these cells exhibited increased expression of adipocyte-specific genes. In contrast, there was no significant difference in the expression of protein phosphatase 1, a protein that is expressed in both preadipocytes and adipocytes (21). After 2 further days of differentiation, the cells were stained with Oil Red O to identify lipid droplets. Cells deficient in NCoR or SMRT exhibited increased cellular staining with Oil Red O (Fig. 3C), consistent with the Western blot data suggesting an increased expression of adipocyte proteins.

To determine the effect of ligand on the PPARγ-corepressor interactions, Gal4-SMRT and Gal4-NCoR were cotransfected with VP16-PPARγ2 and a UAS-Luc construct in 3T3-L1 cells, in the presence or absence of varying concentrations of pioglitazone. Pioglitazone is a thiazolidinedione compound used in the treatment of type 2 diabetes mellitus, and thiazolidinediones have been shown to be high affinity ligands for PPARγ (22). As shown in Fig. 4A, pioglitazone causes a strong decrease in corepressor-PPARγ2 interactions in this two-hybrid assay. To investigate the effects of pioglitazone on endogenous PPARγ-corepressor interactions, 3T3-L1 cells were first differentiated with insulin, dexamethasone, and isobutylmethylxanthine for
3 days to increase PPARγ levels (as in Fig. 2A) and then treated with serum-free medium with or without 10 μM pioglitazone. Whole cell extracts were immunoprecipitated with anti-NCoR or anti-SMRT antibodies and then analyzed by Western blot using an anti-PPARγ antibody. B, 3T3-L1 cells were transfected with a PPRE-Luciferase construct; 330 ng of pSG5 or pSG5-PPARγ; and 1 μg of either pSG5-empty vector, pSG5-NCoR, or pSG5-SMRT. The data are expressed as relative luciferase activity (± S.E.), where 1.0 is defined as the luciferase activity in the presence of pSG5 alone.

3T3-L1 cells lacking corepressors have enhanced expression of adipocyte proteins. A, 3T3-L1 cells were transfected with siRNA constructs for NCoR, SMRT, or a nonspecific sequence. Stable transfectants were selected with hygromycin. Western blots of whole cell extracts were subjected to Western blot analysis with antibodies to NCoR, C/EBPα, CBP, or PPARγ. For SMRT, whole cell extracts were immunoprecipitated with anti-SMRT antibodies, separated by SDS-PAGE, and analyzed by Western blot using an anti-SMRT antibody. B, 3T3-L1 cells lacking NCoR or SMRT (or control cells stably transfected with a nonspecific siRNA construct) were differentiated with insulin, dexamethasone, or isobutylmethylxanthine for 3 days and with insulin for 2 further days. Whole cell extracts (for C/EBPα or cytoplasmic extracts (for adiponectin, perilipin, or protein phosphatase 1 (PP1)) were obtained and subjected to Western blot analysis using the indicated antibodies. C, 3T3-L1 cells lacking NCoR or SMRT (or control cells) were differentiated as in B, then allowed to accumulate further lipid droplets, and stained with Oil Red O. A red stain indicates the presence of lipid droplets. Staining was also significantly enhanced in these cells compared with control cells (data not shown).

DISCUSSION

PPARγ is known to be a vital transcription factor in the regulation of adipocyte differentiation (11). Obesity is a significant problem in the industrialized world, and the increasing incidence of obesity is expected to result in an increased prevalence of the metabolic syndrome and its consequences, including type 2 diabetes mellitus and cardiovascular disease (24). Thiazolidinediones medications that activate PPARγ and enhance adipogenesis increase insulin sensitivity and are used in the treatment of type 2 diabetes mellitus, although the exact mechanisms underlying this process remain unclear (22). A fat-specific knock-out of PPARγ results in decreased numbers of adipocytes, decreased levels of adipokines, and increased hepatic gluconeogenesis (25, 26). Recent evidence suggests that PPARγ has other important actions in addition to its effects on adipogenesis. For example, a muscle-specific deletion of PPARγ results in glucose intolerance and insulin resistance (27). Recent data also suggest a role for PPARγ in macrophage function and atherosclerosis (28). However, the actions of PPARγ have been most widely studied in adipogenesis, and adipocyte differentiation presents a unique model system in which to study PPARγ action (Fig. 5).

Although much is known concerning the ability of PPARγ to recruit coactivators to stimulate the expression of target genes,
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modulate the transcriptional activity of PPARγ has been controversial. Partly, this has been due to the fact that PPARγ is not considered to be a strong repressor, and PPARγ binds corepressors only weakly in electrophoretic mobility shift assay (3). Importantly, however, immunoprecipitation studies have shown a significant interaction in cells (6, 7). Increasing evidence also suggests that other nuclear receptors that are not strong repressors in themselves are capable of recruiting corepressors to modify their transcriptional activity. For example, HNF4α, an orphan nuclear receptor, recruits SMRT, which then blocks coactivator recruitment (29). In fact, the ability of nuclear receptors to recruit corepressors and coactivators has been shown to be a more dynamic process than previously recognized (30), and the distinct cellular environment plays a significant role in tissue-specific gene regulation (31). In addition, recent evidence suggests that mutant PPARγ receptors capable of causing PPARγ resistance recruit corepressors well and exhibit dominant-negative activity toward wild-type alleles (9).

Although the nuclear receptor corepressors NCoR and SMRT have been implicated in the pathogenesis of resistance to thyroid hormone, hypothyroidism, and certain types of leukemias (32), the specific functions of NCoR and SMRT in cells have been difficult to ascertain for a variety of reasons. For example, knock-out of NCoR has been found to be lethal in the embryonic period (33), limiting the ability to determine which functions of corepressors might be NCoR-specific. In vitro work has suggested that NCoR and SMRT serve a variety of repressing functions and are recruited by nuclear receptors in the absence of ligand or in the presence of certain antagonists. However, overexpression studies of nuclear cofactors have not always been able to fully identify the functions of these proteins. For example, initial experiments suggested significant overlap between p160 coactivators, but in vivo knock-out studies have shown that their functions are nonoverlapping (34). The use of siRNA allows for the decreased expression of cofactors in cells to allow for a better understanding of their functional roles. We therefore took advantage of these techniques in 3T3-L1 cells to identify the roles of NCoR and SMRT in adipogenesis. Our data suggest that decreased expression of either NCoR or SMRT is able to increase the expression of adipocyte-specific genes and thus enhance adipogenesis.

Although there are multiple reasons why an alteration in corepressor levels could affect adipocyte differentiation, our data also suggest that these effects likely depend on the transcriptional activity of PPARγ. This is true for a number of reasons. First of all, multiple experiments have shown that PPARγ is a central regulator of adipogenesis, and alterations in PPARγ activity profoundly affect adipogenesis (35–37). Second, it has been shown that mutant PPARγ receptors that recruit corepressors aberrantly can block adipogenesis (7, 9, 15). Interestingly, heterozygous knock-out of PPARγ paradoxically leads to enhanced insulin sensitivity as opposed to insulin resistance, suggesting that PPARγ could have ligand-independent effects mediated by corepressors (38). Finally, our data suggest that wild-type PPARγ is capable of recruiting both SMRT and NCoR in 3T3-L1 cells and that these nuclear receptor corepressors repress PPARγ-mediated transcriptional activity. In addition, deficiency in SMRT or NCoR enhances adipogenesis, either in the presence of a standard differentiation mixture or one that requires a thiazolidinedione, a PPARγ-dependent process. Thus, our data suggest that a decrease in NCoR or SMRT levels enhances PPARγ activity and increases the expression of adipocyte-specific genes. Interestingly, a recent report suggested that the NAD-dependent deacetylase Sirt1 also represses PPARγ activity and re-

![Fig. 4. Pioglitazone decreases corepressor interaction with PPARγ.](image)

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less is known concerning the ability of PPARγ to interact with the nuclear receptor corepressor family members, NCoR and SMRT. In fact, the ability of these nuclear corepressors to
duces fat accumulation (10). In that paper, the Sir1 effect was dependent on NCoR expression, and NCoR was shown to be recruited to the PPARγ-responsive aP2 promoter by chromatin immunoprecipitation experiments (10). The current data are in agreement with the data of Picard et al. (10) and suggest that alterations in NCoR and SMRT by themselves can directly modulate adipogenesis.

Our work shows that PPARγ recruits NCoR and SMRT and that these nuclear receptor corepressors repress the ability of PPARγ to stimulate adipogenesis. This represents an alternative or even complementary pathway to the ability of thiazolidinedione compounds (such as pioglitazone) to increase PPARγ transcriptional activity and adipogenesis (Fig. 4) and suggests that alteration in corepressor activity might allow for modulation of adipocyte differentiation and possibly insulin sensitivity in vivo. In sum, modification in NCoR or SMRT expression and/or function provides a mechanism for altering the adipogenic program, which has been shown to be important in the pathogenesis of obesity and type 2 diabetes mellitus.

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