Acetylation modification regulates the functions of histone and nonhistone proteins, including transcriptional activity, protein interaction, and subcellular localization. Although many nuclear receptors have been shown to be modified by acetylation, whether retinoid X receptors (RXRs) are acetylated and how the acetylation is regulated remains unknown. Here, we provide the first evidence of RXR acetylation by p300 on lysine 145. Acetylation of RXR by p300 facilitated its DNA binding and subsequently increased its transcriptional activity. Furthermore, we discovered that TR3, an orphan receptor, exerted a negative regulation on p300-induced RXR acetylation. TR3 significantly reduced the p300-induced RXR acetylation and transcriptional activity, and such inhibition required the interaction of TR3 with RXR. Binding of TR3 to RXR resulted in the sequestration of RXR from p300. 9-cis retinoic acid, a ligand for RXR, enhanced the association of RXR with TR3, rather than acetylation of RXR by p300. Biological function analysis revealed that the mitogenic activity of RXR stimulated by p300 was acetylation dependent and could be repressed by TR3. Upon the treatment of 9-cis retinoic acid, RXR was translocated with TR3 from the nucleus to the mitochondria, and apoptosis was induced. Taken together, our data demonstrate the distinct regulatory mechanisms of p300 and TR3 on RXR acetylation and reveal a previously unrecognized role for orphan receptor in the transcriptional control of retinoid receptors. (Molecular Endocrinology 21: 2877–2889, 2007)
corticoid receptor, progesterone receptor, hepatocyte nuclear factor, and steroidogenic factor 1, is conserved among different species, including vertebrates, arthropods, and nematodes. Other types of motif for acetylation have also been identified, such as GIK and SK (9). p300 is a transcriptional coactivator that possesses an intrinsic histone acetyltransferase activity. It contributes to the formation of a protein activation complex that bridges various factors to the general transcription machinery. p300 has been shown to acetylate a growing number of nonhistone proteins, notably transcription factors such as p53, E2F1, high-mobility group protein isoform I and Y, hepatocyte nuclear factor 4, HIV Tat, and nuclear receptors such as androgen receptor (16, 17), and estrogen receptor-α (18, 19).

In the present study, we demonstrated for the first time that RXRs, including RXRα and RXRγ, are subjected to p300 acetylation. Such acetylation promotes RXRα binding to RXRE, thereby increasing the transcriptional activity of RXRα. We further revealed that TR3 has a significant inhibitory effect on p300-induced RXRα acetylation. TR3 competes with p300 in RXRα heterodimerization, translocation, and mitochondrial targeting. Taken together, our results demonstrated that the orphan receptor TR3 plays an important role in p300-induced RXRα acetylation by functioning as a negative regulator.

**RESULTS**

**p300 Induces Acetylation of RXRα on K145**

Retinoid X receptors (RXRs) consist of three different isoforms, RXRα, RXRβ, and RXRγ. To investigate whether RXRs can be acetylated by p300, green fluorescent protein (GFP)-tagged isoforms of RXRs were transiently transfected into 293T cells. Lysates were prepared from these transfected cells, and GFP-tagged proteins were immunoprecipitated and blotted with an acetylation-specific antibody. As shown in Fig. 1A, none of RXRα, RXRβ, and RXRγ could be detected by the acetylation-specific antibody when they were transfected into cells alone. However, when p300 was cotransfected, RXRα and RXRγ, but not RXRβ, became detectable by the same antibody, indicating that RXRα and RXRγ were acetylated in the presence of p300.

We chose RXRα for further study because its acetylation level was higher than RXRγ (Fig. 1A). The acetylation level of RXRα seemed to be enhanced by the increase of transfected p300 (Fig. 1B). However, cotransfection of a p300 mutant (p300DY) deficient in acetylase activity (20) resulted in no acetylation of RXRα (Fig. 1B), suggesting that the acetylase activity of p300 is directly responsible for the acetylation of RXRα. The acetylated RXRα could also be clearly detected in HeLa and MCF-7 cells (Fig. 1C, left panel, black arrow), both of which are known to stably express p300 (Fig. 1C). When small interfering RNA (siRNA)-p300 was introduced into these two cell lines to inhibit the expression of endogenous p300, the acetylation level of RXRα greatly decreased (Fig. 1C, right panel, black arrow). Together, these results suggest that p300 is essential and necessary to induce the acetylation of RXRα.

Sequence analysis of RXRα revealed four consensus acetylation motifs located at approximately 144–145 (GK), 212–213 (GK), 362–363 (SK), and 387–388 (SK), respectively (Fig. 2A). To determine which site is responsible for RXRα acetylation, different deletion mutants of RXRα were constructed (Fig. 2A) and cotransfected with p300 into 293T cells for acetylation assays. Wild-type RXRα and deletion mutants of RXRα/D2 and RXRα/D3, but not RXRα/D1 mutant, were found to be acetylated (Fig. 2B), indicating that the region around amino acids 133–156 is responsible for RXRα acetylation. Because that region contains only a candidate acetylation motif (Lys145), we then constructed a new point mutant of RXRα (K145R) in which Lys145 was replaced with Arg. As expected, the K145R mutation significantly abolished the acetylation of RXRα induced by p300 in 293T cells (Fig. 2C). These results clearly demonstrate Lys145 as the p300-induced acetylation site on RXRα.

**Acetylation of RXRα Increases Its DNA-Binding and Transcriptional Activity**

Acetylation of nuclear receptors is often closely related to the regulation of their DNA-binding and transcriptional activity. To determine the correlation between RXRα acetylation and its transcriptional activity, we assessed the activity of a luciferase reporter gene linked with RARE in 293T cells. With the presence of endogenous RXRα, p300 could enhance the reporter activity in a concentration-dependent manner (Fig. 3A), whereas the acetyltransferase-dead mutant of p300 (p300DY) failed to do so (Fig. 3B). When RXRα mutant K145R was used for cotransfection, p300 had no effect on the reporter activity (Fig. 3C). Taken together, these results clearly demonstrated a positive regulation of p300-induced acetylation on the transcriptional activity of RXRα.

We next analyzed the subcellular localization of RXRα and p300 by confocal microscopy. When expressed alone in 293T cells, p300 displayed as microspeckles in the nucleus, whereas RXRα and its mutant K145R mainly located in the nucleus with diffused pattern (Fig. 3D, first panel). Once coexpressed, p300 and RXRα showed a colocalization pattern in about 50% cells by forming obvious speckles in the nucleus (Fig. 3D, second panel). By contrast, such
speckles could only be seen in less than 10% of cells that were transfected with either p300DY/RXRα (Fig. 3D, third panel) or K145R/p300 (Fig. 3D, last panel). We suspected that the formation of RXRα/H9251 with p300 into speckles might be related to its DNA-binding property. To verify this possibility, EMSA was performed with the use of RXRE as a probe for incubation with nuclear proteins. As shown in Fig. 3E, nuclear proteins extracted from GFP-RXRα/H9251-transfected 293T cells formed a complex with RXRE, and p300 enhanced this complex formation obviously (Fig. 3E, black arrow). The complex band was up-shifted only when the nuclear proteins were preincubated with anti-GFP antibody (aGFP) (Fig. 3E, white arrow), indicating that the complex is composed of homodimer of RXRα/H9251. Chromatin immunoprecipitation (ChIP) assay further demonstrated that p300 could enhance the binding of RXRα to the RARβ promoter (Fig. 3F). However, the K145R mutant of RXRα did not bind to RXRE, even in the presence of p300 (Fig. 3E), and p300 also failed to promote its binding to RARβ promoter (Fig. 3F). Together, these data suggested that RXRα acetylation by p300 results in its binding to DNA, which is correlated well with the activation of its transcriptional activity.

**TR3 Binds to RXRα and Attenuates Its Acetylation by p300**

The orphan receptor TR3 has been shown to inhibit p300-induced acetylation of p53 (7). Because TR3 heterodimerizes with RXRα in vivo (3, 21, 22), we suspected that TR3 may also be involved in regulation of RXRα acetylation. When TR3 was introduced into 293T cells that had been transfected with RXRα and p300, we found that the p300-induced RXRα acetylation became significantly attenuated (Fig. 4A). A similar result was also observed in HeLa cells that were transfected with TR3 alone (Fig. 4B, black arrow). We further investigated the inhibitory effect of endogenous TR3 on RXRα acetylation. When siRNA-TR3 was introduced into HeLa cells to inhibit endogenous TR3 expression, more acetylated RXRα could be detected (Fig. 4C, black arrow). Quantitative analysis by densitometry further confirmed the effect of TR3 on regulating RXRα acetylation (Fig. 4, B and C).
TR3 Competes with p300 for RXRα Binding

Because TR3 interacts with RXRα, it may compete with p300 in the RXRα binding to attenuate the p300-induced acetylation of RXRα. Coimmunoprecipitation/Western blotting confirmed that either TR3 or p300 was able to interact with RXRα independently, whereas RXRα point mutant K145R almost failed to bind with p300, although it still interacted with TR3 (Fig. 5A). As we expected, increasing the amount of TR3 enhanced the TR3-RXRα interaction and decreased the p300-RXRα interaction, and vice versa (Fig. 5B), indicating that TR3 and p300 bind to RXRα in a competition manner. We further transfected the HA-TR3, HA-p300, and Myc-RXR into 293T cells and detected higher level of TR3 than p300 in RXRα immunoprecipitates (Fig. 5C, right panel, lane 3), although HA-TR3 and HA-p300 were expressed at similar levels (Fig. 5C, left panel, lane 3). This result further suggested that RXRα has a higher ability to interact with TR3 than p300 when both proteins coexist.

Confocal microscopic analysis revealed that transfection of TR3 inhibited the formation of RXRα-p300 speckles in 293T cells, with the percentage of cells containing p300/RXRα speckles dramatically dropped from 46.8 to 6.1%. Although TR3 colocalized with RXRα in a diffused pattern, p300 existed as microspeckles (Fig. 5D). We further found that the TR3/ΔM mutant, but not the TR3/ΔN mutant, could interfere with the formation of RXRα/p300 speckles (Fig. 5D). The percentage of cells with speckles was much higher in TR3/ΔM-transfected cells (39.3%) than in TR3/ΔN-transfected cells (11.0%). Therefore, the reduced colocalization of RXRα with p300 is most likely due to its sequestration by TR3.

9-cis Retinoic Acid Facilitates Translocation of Endogenous RXRα with TR3

Because 9-cis retinoic acid is a well known ligand for RXRα (23, 24), we went on to investigate how it regulates the physical interactions of RXRα with p300 and TR3 as well as the acetylation of RXRα by p300. Coimmunoprecipitation/Western blotting revealed that 9-cis retinoic acid enhanced the interaction of endogenous RXRα with TR3 and simultaneously decreased the interaction of RXRα with p300 (Fig. 6A). The increased TR3/RXRα association consequently resulted in the translocation of both proteins from the nucleus to the mitochondria, which was seen in more than 65% of HeLa cells (Fig. 6B). On the other hand, the acetylation level of RXRα in HeLa cells was reduced to some extent upon 9-cis retinoic acid treatment (Fig. 6C, black arrow). This is most likely due to the attenuation of p300/RXRα association (Fig. 6A), because the expression level of endogenous RXRα and p300 was not affected by 9-cis retinoic acid (Fig. 6C, top panel), although the acetylation of RXRα was inhibited by 9-cis retinoic acid (Fig. 6C, bottom panel). A similar result was also observed in MCF-7 cells (data not shown).
shown). As a consequence of the reduced acetylation, the transcriptional activity of RXRα in HeLa cells remained to be induced by 9-cis retinoic acid (Fig. 6D, black bars) and slightly repressed by the transfection with an increased amount of TR3 (Fig. 6D). Together, these results demonstrate that 9-cis retinoic acid facilitates RXRα translocation with TR3 rather than its acetylation by p300 in HeLa cells.

p300 and TR3 Have Distinct Cellular Functions through Regulation of RXRα Acetylation

Finally, we investigated the biological significance of p300 positive regulation and TR3 negative regulation on RXRα acetylation. p300 has been shown to promote apoptosis as well as cell proliferation, which appears to be highly context dependent (25, 26). We
Fig. 4. Attenuation of p300-Induced RXRα Acetylation by TR3

A, TR3 antagonized the acetylation of RXRα induced by p300. Myc-TR3, together with GFP-RXRα and HA-p300, were transfected into 293T cells. The levels of RXRα acetylation were determined as described as Fig. 1B. B, TR3 inhibited acetylation of endogenous RXRα. HeLa cells were transfected with Myc-TR3 and the levels of RXRα acetylation (black arrow) were determined as described in Fig. 1C. White arrow indicates a heavy chain. C, Effect of siRNA-TR3 on RXRα acetylation. HeLa cells were transfected with siRNA-TR3, and the levels of RXRα acetylation (black arrow) were determined as described as Fig. 1C. The levels of RXRα acetylation regulated by TR3 were quantified by densitometry in B and C. D, Interaction of RXRα with TR3 and its deletion mutants. Schematic diagrams of deletion mutants of TR3 are shown at the top. 293T cells were transfected with GFP-RXRα and Flag-TR3 or its different deletion mutants as indicated. Cell lysates were immunoprecipitated with anti-GFP antibody. The immunoprecipitates and cell lysates were then analyzed by Western blotting separately using anti-Flag-antibody for TR3 and its deletion mutants and anti-GFP antibody for RXRα. E, Effect of TR3 deletion mutants on acetylation of RXRα by p300. Flag-TR3 and its deletion mutants, together with GFP-RXRα and HA-p300, were transfected into 293T cells, and the levels of RXRα acetylation were determined as described in Fig. 1B.
Fig. 5. TR3 Competed with p300 Binding to RXRα

A. Interaction of RXRα with TR3 or p300. 293T cells were transfected with HA-TR3, HA-p300, and Myc-RXRα or its point mutant K145R as indicated. Cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitates and cell lysates were then analyzed by Western blotting separately using anti-HA antibody for TR3, anti-HA antibody for p300, and anti-Myc antibody for RXRα. B. TR3 and p300 bound to RXRα competitively. Increasing amounts of TR3 or p300 together with RXRα were transfected into 293T cells as indicated. Cell lysates were immunoprecipitated with anti-Myc antibody to pull down RXRα. The immunoprecipitates and cell lysates were then analyzed by Western blotting separately using anti-HA antibody for p300 and anti-GFP antibody for TR3. C. The relative binding ability of p300 and TR3 to RXRα. 293T cells were transfected with Myc-RXRα, HA-TR3, and HA-p300. Western blot against the common HA tag was used to compare the expression levels of TR3 and p300 (left panel, indicated by arrow). Binding ability of TR3 and p300 to RXRα is shown by using anti-HA antibody in RXRα immunoprecipitates (right panel, indicated by arrow). D. Effect of TR3 and its deletion mutants on p300-RXRα colocalization. Flag-TR3 or its deletion mutants together with Myc-RXRα and HA-p300 were transfected into 293T cells as indicated. The transfected cells were immunostained by anti-Flag antibody followed by Alexa fluor 350-conjugated secondary antibody to detect TR3 and its point mutants, by anti-HA antibody followed by Texas Red-conjugated secondary antibody to detect p300, or by anti-Myc antibody followed by FITC-conjugated secondary antibody to detect RXRα. Stained cells were visualized with the confocal microscope. The same data are plotted to indicate the percentage of cells with speckle RXRα (bottom panel).
thus examined HeLa cells expressing p300, RXRα, and its point mutant for cell proliferation by using a bromodeoxyuridine (BrdU) assay. As shown in Fig. 7A, RXRα did not show any effect on BrdU incorporation (first panel, green curve) compared with control. However, cotransfection of p300 with RXRα displayed an obvious increase in BrdU incorporation (first panel, blue curve). By contrast, p300DY failed to enhance BrdU incorporation even in the presence of RXRα (first panel, black curve), strongly suggesting that p300 functions as a mitogenic factor to stimulate cell growth, probably through inducing RXRα acetylation. We therefore used the RXRα point mutant to further characterize this possibility. Compared with RXRα, no increased BrdU incorporation was observed in cells transfected with K145R in the absence or presence of p300 (second panel, purple curve). These data thus indicate an important role of p300 acetylation on RXRα in cell proliferation by promoting mitogenic activity in HeLa cells.

On the other hand, we found that TR3 could attenuate the p300-induced cell proliferation via RXRα in...
HeLa cells, as a clear left-shifted curve was seen (third panel, yellow curve). Moreover, like TR3, TR3/ΔN that bound to RXRα (Fig. 4D) and blocked p300 acetylation (Fig. 4E) also diminished the p300 effect on stimulating cell growth (third panel, gray curve), whereas TR3/ΔM that did not interact with RXRα (Fig. 4D) failed to antagonize p300-induced mitogenic activity (third panel, pink curve). Based on these results and the fact that TR3 heterodimerization with RXRα facilitated their shuttling to the mitochondria in response to 9-cis retinoic acid (Fig. 6B), it is possible that TR3 attenuation on RXRα acetylation might correlate with apoptosis induction, because mitochondrial targeting of TR3 is critical for triggering apoptosis in cancer cells (3, 5, 6), just as, for example, when TR3/RXRα-cotransfected HeLa cells were treated with 9-cis retinoic acid, more apoptotic cells were seen and the apoptotic rate reached to 24.5% (Fig. 7B). Similar results were also observed in TR3ΔN/RXRα transfected cells (25.7% apoptotic cells) and K145R/TR3-transfected cells (18.4% apoptotic cells), but not in TR3ΔM/RXRα-transfected cells (less than 5%). We therefore concluded that p300 positively regulates RXRα acetylation to facilitate cell proliferation and TR3 negatively regulates RXRα acetylation for apoptosis induction.

**Fig. 7.** Different Functions of p300 and TR3 in Activating Mitogenic Activity and Inducing Apoptosis

A, Effects of RXRα, p300, and TR3 on the mitogenic activity of HeLa cells. Different expression vectors, including RXRα, p300, TR3, and their related mutants, were transfected into HeLa cells as indicated. After transfection, the cells were maintained in BrdU-containing medium for 2 h. The mitogenic activity was then identified by flow cytometry as described in Materials and Methods. B, TR3 and RXRα induced apoptosis in HeLa cells. HeLa cells were transfected with GFP-TR3 and Myc-RXRα or their mutants as indicated and then treated with 9-cis retinoic acid (10⁻⁶ mol/liter) for 48 h. The nuclear morphology stained by 4',6-diamidino-2-phenylindole was visualized under fluorescent microscope, and apoptotic cells were scored by examination of 500 transfected cells.
DISCUSSION

Posttranscriptional modification of nuclear receptors integrates a variety of signaling pathways (8). Although the exact number and variety of nuclear receptors that are acetylated in the cells is still largely unknown, it is clear that far more candidates are modified by this mechanism than initially appreciated. In the current study, we provide the first evidence that RXRs, including RXRα and RXRγ, are acetylation targets of p300. Acetylation by p300 enhanced the DNA binding and activated the transcriptional activity of RXRα, thereby promoting cell proliferation. Furthermore, we found that TR3, an orphan receptor, functions as a negative regulator to attenuate RXRα acetylation by competition with p300 for RXRα binding. As a result, p300-mediated DNA binding of RXRα was repressed and RXRα turned to form a complex with TR3, which would translocate from the nucleus to the mitochondria to facilitate apoptosis induction in response to 9-cis retinoic acid. These findings allowed us to propose a novel model in which RXRα acetylation is positively regulated by p300 and negatively regulated by TR3 (Fig. 8).

RXRα processed four candidate acetylation sites, and one of them, the lysine residue 145, could be acetylated by p300. Mutation of Lys145 to Arg greatly abrogated RXRα acetylation. As a result of acetylation, the transcriptional activity of RXRα was increased in a p300-dependent manner. Because K145 locates in the first zinc finger of the DNA-binding domain of RXRα (27), p300-induced RXRα acetylation might recruit RXRα to its response element. Indeed, in the presence of p300, much more RXRα was found to bind to its response element RXRE, whereas RXRα/K145R that could not be acetylated by p300 failed to do so. These results demonstrated a good consistency between structural characteristics of RXRα and biological function of p300 on regulation of RXRα acetylation.

So far, little is known about how the acetylation of nuclear receptors is regulated. We unexpectedly found that the orphan receptor TR3 could act as a negative factor to regulate the p300-induced acetylation of RXRα. TR3 seems to achieve its inhibitory effect on RXRα acetylation by competing with p300 for RXRα binding, because the interaction of RXRα was a prerequisite for TR3 to inhibit p300-induced RXRα acetylation. When RXRs, TR3, and p300 coexisted in cells, RXRα preferred to interact with TR3 rather than p300. 9-cis retinoic acid further enhanced the TR3/RXRα binding but decreased the p300/RXRα interaction. Therefore, TR3 regulation seems to be highly contextual and may be dependent on the relative expression levels of TR3, RXRα, and p300 in the cell. The cellular level of TR3 and p300 may act as a signal switch leading to diverse pathways: cell growth inhibition or cell proliferation. For example, a high level of TR3 may serve as a potent inhibitor of certain transcription factors by competing with p300 or disturbing p300 histone acetyltransferase activity directly. To address this possibility, more detailed analysis of other transcription factors should be carried out in future.

Previously, we found that RXR-selective retinoids SR11246 and SR11345 can induce the transcriptional activity of RXRα (28). However, we showed here that 9-cis retinoic acid, another well-known ligand for RXRα, promoted RXRα/TR3 translocation, rather than RXRα transcriptional activity. Such an effect of 9-cis retinoic acid appears to be contradictory to its known transcriptional activation of RXRα. In fact, our previous study found that RXRα undergoes nucleocytoplasmic shuttling (3). Like other nuclear receptors, it is not always statically associated with chromatin (29–31). Thus, it is likely that the fate of cellular RXRα may depend on distinct stimuli and cell contexts. For example, p300 facilitates RXRα DNA binding through acetylation, whereas 9-cis retinoic acid enhances the interaction of TR3 with RXRα and induces TR3/RXRα translocation. The difference between current (using 9-cis retinoic acid) and previous (using RXR-selective retinoids SR11246 and SR11345) results can be explained by the possibility that whether transcriptional activity or translocation of RXRα will be activated may depend on the natural properties of the compound. In support of this viewpoint, a published report revealed that the nonsteroidal antiinflammatory drug R-etodolac binds RXRα and acts as a RXRα antagonist to inhibit its transactivation, an event that is asso-
associated with its tumor-selective induction of apoptosis in animals (32). Our current observation that RXRα exhibits nucleocytoplasmic shuttling by itself and acts as a carrier for TR3 translocation from the nucleus to the mitochondria in response to 9-cis retinoic acid (3) also provides collateral evidence. Accordingly, RXRα nucleocytoplasmic localization appears to be one of the major factors determining 9-cis retinoic acid sensitivity.

Although increasing evidence supports the view that p300 can be under aberrant control in tumor cells (33), the importance of p300 in malignancy remains to be elucidated. RXRs usually form heterodimers with many members of the nuclear receptors (1, 2) and therefore play an essential role in regulation of multiple nuclear hormone-signaling pathways through their unique and potent dimerization capacity. Intriguingly, RXRα alone did not impair the BrdU incorporation rate, whereas coexpression of p300 increased the BrdU incorporation rate effectively, indicating that p300 might enhance the mitogenic ability by RXRα acetylation in HeLa cells. The fact that p300DI lost its ability to affect cell proliferation even in the presence of RXRα further verified that p300-stimulated cell proliferation was correlated with its ability to acetylate RXRα. More importantly, overexpression of TR3 diminished p300-induced cell proliferation by interacting with RXRα and cooperated with RXRα for apoptosis induction by translocating to the mitochondria in HeLa cells. There is no doubt that gaining further insights into the significance of this modification and translocation will be a benefit in facilitating the design of new approaches toward controlling malignancy of cancers.

In summary, different p300/RXRα and TR3/RXRα heterodimers in cells may exist in a dynamic equilibrium depending on their cellular environment. Abundant p300 may induce a dimerization interface switch that promotes RXRα DNA binding to ensure p300/RXRα colocalization and efficient transcriptional regulation, which finally contributes to cell proliferation. In contrast, TR3 may sequestrate RXRα from p300 and preferentially heterodimerize with RXRα through their mutual interaction. The TR3/RXRα heterodimer then translocates to the mitochondria to induce cell apoptosis. Our study reveals an unexpected role of TR3 in the cross-talk between orphan receptor and retinoid receptors and suggests that competition of TR3 and p300 for the regulation of RXRα acetylation might control a dynamic process between cell proliferation and cell apoptosis.

**MATERIALS AND METHODS**

**Cell Culture and Transient Transfection**

The cell lines cervical cancer HeLa, human breast cancer MCF-7, and human embryonic kidney (HEK) 293T were obtained from American Type Culture Collection (Rockville, MD). All the cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU penicillin, and 100 μg/ml streptomycin. Transfection was performed using a calcium phosphate precipitation method for 293T cells as described previously (22) and liposomal transfection reagent (Fugene 6; Roche Molecular Biochemicals, Indianapolis, IN) for HeLa and MCF-7 cells according to the manufacturer’s instructions.

**Combinonprecipitation and Western Blotting**

Cells were transfected with various plasmids as required and incubated for 36 h before analysis. Cell lysate preparation, immunoprecipitation, and Western blotting were performed as described previously (7). Briefly, cell lysates were incubated with the appropriate antibody for 1 h and subsequently incubated with protein A-Sepharose beads for 1 h. The protein-antibody complexes that were recovered on beads were subjected to Western blot analysis after separation by SDS-PAGE. The immunoreactive products were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**In Vivo Acetylation Assay**

Cell lysates were extracted in cell lysis buffer [20 mmol/liter HEPES (pH7.5), 0.1 mol/liter KCl, 0.4 mmol/liter EDTA, 0.2% Nonidet P-40, 10 mmol/liter β-mercaptoethanol, 0.1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg pepstatin/ml, 1 μg NaVO₄/ml] and immunoprecipitated with anti-GFP antibody or anti-RXRα antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were then separated by SDS-PAGE and blotted with specific anti-acetyl-lysine antibody (Upstate Biotechnology, Lake Placid, NY).

**Immunofluorescent Staining and Microscopic Observation**

Cells seeded on coverglass overnight were fixed in 4% paraformaldehyde. To stain exogenous proteins including Myc-RXRα, HA-p300, and Flag-TR3 proteins, cells were incubated with different primary antibodies, including anti-Myc, anti-HA, or anti-Flag antibody, followed by the corresponding secondary antibodies. For observation of endogenous RXRα, TR3, or heat-shock protein 60 (Hsp60), cells were incubated with anti-RXRα, anti-TR3, or anti-Hsp60 (Santa Cruz) antibody, respectively, followed by fluorescein isothiocyanate (FITC)-Texas red- or Alexa fluor 350-conjugated secondary antibody. Nucleus was stained by 4',6-diamidino-2-phenylindole (DAPI) (Roche). Stained cells were visualized with confocal microscope (Leica TCS SP2 SE).

**Luciferase Assay**

Cells were transfected with various plasmids, including luciferase-linked reporter gene (Luc-RARE) (34), β-galactosidase (β-gal) expression vector, and the vector expressing RXRα, TR3, or p300 as required. After 36 h after transfection, luciferase activity was normalized for transfection efficiency using corresponding β-gal activity. The ratios of luciferase/β-gal activity were used as indicators for activity of RARE. All transfections were performed in duplicate, and the data are presented as means ± SD of at least three separate experiments.

**EMSA**

EMSA was done using a LightShift chemiluminescent EMSA kit (Pierce Chemical Co., Rockford, IL) with biotin-labeled oligonucleotide, corresponding to cellular retinol binding protein type II (CRBPII) RXRE (AGCTTCCAGGTCAGAGGTCA-GAGAGC; Invitrogen, Carlsbad, CA). After binding reaction, samples were loaded onto 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer and electrophoresed at 100 V at 4°C.
for 2 h. Biotin-labeled, double-stranded DNA was electrophoretically transferred to positively charged nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Little Chalfont, UK). After cross-linking the transferred DNA to membrane, biotin-labeled DNA was integrated with streptavidin-horseradish peroxidase conjugate. Finally, chemiluminescence was detected using enhanced chemiluminescence. For the supershift experiment, 5.0 μg nuclear extract was incubated with 200 ng anti-GFP antibody for 2 h (Santa Cruz) before incubation with the biotin-labeled probe.

**ChIP Assay**

Cells were cross-linked with 0.75% formaldehyde for 10 min, and then the cross-linking was stopped by adding glycine. Cells were collected in PBS and resuspended in FA lysis buffer [50 mM HEPES-KOH (pH 8.0), 140 mM NaCl, and 1% Triton X-100] with 1 μl protease inhibitor cocktail (Roche) and then sonicated. After centrifugation, 10% of the total supernatant was saved as total input control. The remaining supernatant was processed as follows: After immunoprecipitation, 30 μl protein A/G-Sepharose was added, and cells were incubated for another 2 h at 4 C. Sepharose beads were washed sequentially in low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl] three times and in high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 500 mM NaCl] one time, and beads were then extracted with 200 μl elution buffer (1% SDS, 0.1% Triton X-100, 20 mM Tris-HCl (pH 8.0)). Two microliters of RNase A (0.5 mg/ml) were added and then heated at 65 C for 5 h to reverse the formaldehyde cross-linking. DNA fragments were purified with a DNA purification kit. For quantitative PCR, the primer sequence (RARα promoter) was as follows: sense 5'–CTCCTCGCCGTGCTATTATTA–3' and antisense 5'–CTGCTCTGAACAGCTCACT–3'.

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