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Orphan nuclear receptor TR2, a mediator of preadipocyte proliferation, is differentially regulated by RA through exchange of coactivator PCAF with corepressor RIP140 on a platform molecule GRIP1

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ABSTRACT

Orphan nuclear receptor TR2 is a preadipocyte proliferator. Knockdown of *TR2* in 3T3-L1 preadipocytes reduced their proliferation efficiency, whereas specific elevation of TR2 in these cells facilitated their proliferation. All-*trans* retinoic acid (RA) stimulates cellular proliferation in 3T3-L1 preadipocytes by activating *TR2* through an *IR0*-type RA response element, which further activates c-Myc expression. In post-differentiated adipocytes, RA becomes a repressive signal for *TR2* and rapidly down-regulates its expression. The biphasic effect of RA on TR2 expression in 3T3-L1 is mediated by differential RA-dependent coregulator recruitment to the receptor/Glucocorticoid Receptor-Interacting Protein 1 (GRIP1) complex that binds *IR0* on the *TR2* promoter. RA induces the recruitment of histone acetyl transferase-containing/GRIP1/p300/CBP-associated factor (PCAF) complex to the *TR2* promoter in undifferentiated cells, whereas it triggers recruitment of histone deacetylase-containing/GRIP1/receptor-interacting protein 140 (RIP140) complex in differentiated cells. GRIP1 directly interacts with RIP140 through its carboxyl terminal AD2 domain. GRIP1 interacts with PCAF and RIP140 directly and differentially, functioning as a platform molecule to mediate differential RA-induced coregulator recruitment to *TR2* promoter target. This results in a biphasic effect of RA on the expression of TR2 in undifferentiated and differentiated cells, which is required for RA-stimulated preadipocyte proliferation.

INTRODUCTION

The orphan nuclear receptor TR2 is a highly conserved orphan member among the nuclear receptor superfamily. Recent study of *TR2*-knockout animals revealed no apparent developmental or pathological abnormalities in animals housed in laboratory conditions (1). However, results of *in vitro* studies of cultured cells ectopically expressing TR2 suggested potential roles for TR2 in a variety of cellular processes such as cell cycle (2,3), apoptosis (4) and proliferation (5). In terms of gene expression, mouse TR2 was found to be significantly elevated in early embryos (6), proliferating germ cells (7) and P19 embryonal carcinoma stem cells (4,8,9). Interestingly, its expression appeared to be regulated primarily by an alteration in the vitamin A status in animals, as well as by retinoic acid (RA), the principal active ingredient of vitamin A, in cultured cells (4,8,10,11). Using reporters, it was found that the biological activity of TR2 largely overlapped with that of RA receptor (RAR)/retinoid X receptor (RXR) (10,12).

Preliminary studies of TR2 have been conducted mostly in P19 embryonal carcinoma stem cells (4,5) and 3T3-L1 embryonic fibroblasts because of its specifically regulated expression in these systems. These studies revealed a somewhat atypical biphasic pattern of regulation of TR2 expression/activity by RA. Using reporter, gel mobility shift and chromatin immunoprecipitation assays, we located the principal RA-responsive element (RARE) in the *TR2* gene promoter to a region that contained an inverted repeat with no nucleotide in the spacer (*IR0*) (9,13). Following the discovery of a hormone-dependent corepressor receptor-interacting protein 140 (RIP140) (14), the biphasic regulation of this gene by RA was further validated in reporter assays where RA could either

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activate or repress this RARE reporter, depending upon the coexpression of different ligand-dependent coregulators. In the normal COS1 cellular environment where PCAF and other potential coregulators were present, this reporter was activated by RA as predicted (15). However, with the addition of RIP140 into the system, RA activation of this reporter was completely eliminated and even further reduced (13,16).

Using highly purified protein preparations in a surface plasmon resonance-based detection system for protein interactions (BIAcore), we further evaluated the kinetic parameters contributing to the formation of holo-RAR–RXR–PCAF and holo-RAR–RXR–RIP140 complexes (17), In this highly purified system, holo-RAR–RXR appeared to preferentially interact with corepressor RIP140, as compared to PCAF, in an RA-dependent manner. This was primarily attributed to a greater on-rate in the formation of the holo-RAR–RXR–RIP140 complex than the holo-RAR–RXR–PCAF complex (17,18). Based upon these results, a working model was proposed, that the exchange of different types of hormone-dependent coregulators underlined the biphasic regulation of *TR2* by the same hormone, RA. The first goal of this study was to test this model by examining the differential RA-dependent coregulator-recruiting patterns on the endogenous *TR2* gene under physiologically relevant conditions.

3T3-L1 preadipocytes differentiate into adipocytes that store energy in the form of triglycerides. RA was shown to repress adipocyte differentiation and/or stimulate proliferation in preadipocytes (19–22). Unlike other cell differentiation models, 3T3-L1 was usually induced by a differentiation cocktail that included insulin, triiodothyronine, dexamethasone and isobutylmethylxanthine but not RA. In fact, RA and bone morphogenetic protein-2 could cooperate to repress adipocyte differentiation, stimulate proliferation and induce osteoblast differentiation (21). Interestingly, *TR2* was found to be regulated by RA also in a biphasic pattern in this culture. Further, RIP140 corepressor was not detected in the undifferentiated preadipocytes but was significantly elevated during adipogenesis (23). It was hypothesized that the elevation of corepressor RIP140 in differentiated 3T3-L1 would potentially create a repressive cellular environment for RA-target genes such as *TR2*. Therefore, this culture model provided an appropriate system to test our working model of differential recruitment of various hormone-dependent coregulators in a physiological context, as well as the physiological function of TR2 in RA-signaling pathway in terms of cell proliferation prior to adipocyte differentiation.

Several categories of coregulatory proteins are known for nuclear receptors and other transcription factors, including members of the p160 family (steroid receptor coactivator-1 [SRC-1], transcriptional intermediary factor-2 [TIF2/Glucocorticoid Receptor-Interacting Protein 1 (GRIP1), SRC-2] and SRC-3), coactivators that encode or recruit histone acetyl transferases (HATs) (e.g. p300/CBP, PCAF and GCN5) and histone methyltransferase (e.g. CARM1 and PRMT1) and corepressors, some of which encode or recruit histone deacetylases (HDACs). It is generally believed that the coregulatory complexes are highly dynamic and the compositions of coregulatory complexes change as genes are subjected to different regulatory events. The general principle of complex formation describes the recruitment of corepressors by aporeceptors and the recruitment of coactivators by holoreceptors (24,25), which holds true for typical hormone-activation of target genes. However, for the increasingly known hormonal repression of certain direct target genes, it remains unclear whether and how different hormone-dependent coregulators exchange on the target genes in the continuous presence of the hormone. It is crucial to elucidate the underlying molecular mechanisms of this phenomenon in a physiologically relevant context.

In this study, we first observed the functional role for TR2 in mediating 3T3-L1 preadipocyte proliferation and addressed the biphasic effect of RA on TR2 expression in the 3T3-L1 culture. This occurs because of the RA-dependent replacement of coactivator PCAF with corepressor RIP140 on a platform molecule GRIP1. We then showed that GRIP1 itself provided no activating or repressive activity; rather it served as a component of a platform complex by directly and differentially interacting with the different RA-dependent coregulators. The results of these studies consistently suggested a potential role for TR2 in mediating the physiological activity of vitamin A, or more specifically, RA. Previously, this theory has been viewed as speculative, largely because of the lack of studies to demonstrate its relationship with RA signaling in a physiologically relevant system. For this purpose, our results elucidated cell proliferation as the most likely target of TR2 in the 3T3-L1 system, which appeared to be closely modulated by the biphasic regulation of *TR2* by RA in this commonly used cell culture model.

MATERIALS AND METHODS

Construction of expression vector and TR2-inducible 3T3-L1 clones

Mammalian expression vector of RAR, RXR, PCAF, GRIP1, RIP140 and IR0-tk-luciferase reporter, and GST constructs of RIP140 has been described earlier (13,16). GRIP1 constructs in pSG5.HA expression vector were a generous gift from Michael R. Stallcup (26). For isolation of TR2-inducible clones, 3T3-L1 cells transfected with pTet-On (Clontech) and pTRE-TR2 were selected by treatment with 500 μg/ml of G418 for 14 days. TR2 was induced by adding tetracycline (1 μg/ml). A 1 μM working concentration of all-*trans* RA was used in all experiments (21) inasmuch as low concentrations (1 pM to 10 nM) are known to have opposite effects on adipogenesis (27).

Cell culture and cell proliferation assay

3T3-L1 cells were maintained and differentiated by differentiation cocktail that included insulin, triiodothyronine, dexamethasone and isobutylmethylxanthine as described earlier (28). For cell proliferation, a thymidine

incorporation assay was performed. Twenty-four hours after TR2 silencing or induction, cells were synchronized in DMEM supplemented with 1% calf serum for 6 h. Cells were grown in regular growth media for an additional 2 h prior to addition of 1 μCi ^{3}H-thymidine for 6 h. Cells were washed twice with PBS, once with ice-cold 10% TCA and two more times with PBS. Finally, cells were lysed in 500 μl of 2N NaOH for over 6 h and samples were analyzed by a scintillation counter. Qualitative estimation of triglycerides was performed by oil-red-O staining. Quantitative determination of triglycerides was performed by alkaline hydrolysis and measurement of glycerol released by the Free Glycerol Determination Kit (Sigma).

RT-PCR and RNA interference

To monitor the expression of *TR2*–GRIP1–PCAF–RIP140 at the mRNA level, total RNA was isolated from 3T3-L1 cells using a TRIzol kit (Invitrogen) and RT-PCR was conducted as described earlier (16). Primers specific to *TR2* (14), GRIP1 (sense, 5′-AACCA ACACCTTCGGCAGAG-3′ and antisense, 5′-TTCATG CTGCTCAAGCCAC-3′), PCAF (sense, 5′-CACTTGG AGAATGTGTCAAGAG-3′ and antisense, 5′-GATGGT GAAGACCGAGCGAA-3′) and RIP140 (sense, 5′-AAC ACGAGCTATAAGAATCA-3′ and antisense, 5′-CAAC TGAAGAAGAGTGAC-3′) were used. Actin-specific primers (29) were included as internal controls in each RT-PCR. 3T3-L1 cells were transfected with scrambled RNA (Dharmacon) or *TR2*-specific siRNA (5′-GCACCA GAUAAGGCUGAAUTT-3′/5′-TTCGUGGUCCUAU UCGACUUA-3′) using DharmaFECT 3 (Dharmacon) for 72 h. Total RNA or proteins were analyzed by RT-PCR or western blot.

Immunoprecipitation and western blot analyses

Immunoprecipitation and western blot assays were performed as described earlier (15). ±RA-induced endogenous complexes on RAR–RXR in preadipocytes/differentiated 3T3-L1 were precipitated with anti-RAR (Santa Cruz Biotechnology). For experiments requiring double immunoprecipitation, RAR immunoprecipitates on protein G beads were washed two times with pre-elution buffer, followed by elution/solubilization in 100 μl of low pH buffer (100 mM glycine, pH 2.5) with immediate neutralization in collection buffer (1/20 volume phosphate, pH 8.0). The supernatant was diluted with 4 volumes of 10 mM phosphate, pH 6.8 and subjected to a second round of immunoprecipitation using anti-GRIP1 (Upstate Biotechnology). Beads were washed and resuspended in SDS loading buffer followed by western blot analyses probed with anti-GRIP1, anti-PCAF (Santa Cruz Biotechnology) and anti-RIP140 (30). Endogenous expression of RAR, GRIP1, PCAF and RIP140 was monitored by western blot analysis of cell lysates and constituted the input. TR2 expression was probed with anti-TR2 (Santa Cruz Biotechnology).

GST pull-down assays

Various GST–RIP140 constructs [full-length (amino acids 1–1161), N-terminal (amino acids 1–496), central (amino acids 333–1006), C-terminal (amino acids 977–1161)] were expressed and purified in *E. coli* and bound to GST beads as described earlier (16). Washed beads were incubated with *in vitro*-transcribed and translated (Promega) [^{35}S] Met-labeled GRIP1 constructs [full-length (amino acids 1–1462), N-terminal (amino acids 5–479), extended N-terminal (amino acids 5–765), central (amino acids 563–1121), C-terminal (amino acids 1122–1462)]. After extensively washing with the binding buffer, specifically bound proteins were resolved by SDS-PAGE and detected with a Phosphorimager. Equal volumes subjected to SDS-PAGE and stained with Coomassie blue constituted the GST input. Also, each sample of *in vitro* transcription-coupled translation was subjected to SDS-PAGE and detected with a Phosphorimager and constituted TNT input (30%).

HAT and HDAC enzymatic assays

Deacetylase and acetylase activity was measured in the samples using a kit (Upstate Biotechnology) according to the manufacturer's instructions with some adaptations. For HAT assays, RA-induced RAR-associated complexes were precipitated with RAR antibody and incubated with HAT assay buffer 5×, 10 μg of histone (Sigma), 3.2 μCi of [^{3}H]-acetyl CoA and an appropriate volume of deionized water. After mixing the contents, the tubes were incubated at 30°C for 3–4 h on a rotating platform. The reaction mixture was centrifuged and the supernatant was resolved on a 20% SDS-polyacrylamide gel by electrophoresis. Gels were soaked in 1 M sodium salicylate (enhancer), dried and exposed to films. The dried gels were resuspended in water and stained with Coomassie blue to determine histone (substrate) input. The pellets were probed for RAR and constituted the RAR-complex (enzyme) input. For the HDAC activity, precipitated RAR complexes were incubated with 50 000 C.P.M (counts per minute) of a biotin-conjugated [^{3}H]-acetyl histone-H4 peptide bound to a streptavidin-agarose slurry. The mix was incubated at 37°C on a rotating platform for 6 h. The reaction was stopped by addition of a quenching solution and the beads were collected by centrifugation. The released [^{3}H]-acetate in the supernatant was monitored by scintillation counter.

Transfection and reporter assays

Transfection and specific reporter assays conducted in COS1 cells were as described (13,31).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were conducted as described (32) with slight modifications to examine RA-induced complexes on endogenous *TR2* promoter (*IRO*) in preadipocyte and differentiated 3T3-L1. Antibodies against GRIP1, HDAC3, acetylated histone H3, RNA-Pol II, PCAF from Upstate Biotechnology and RIP140 (30) were used. For repeated ChIP assays, chromatin DNA precipitated with anti-GRIP1 was eluted, and subjected to repeated immunoprecipitation with the second antibody followed by classical ChIP protocol. Five percent of total DNA was used in the input. Collected DNA was amplified with

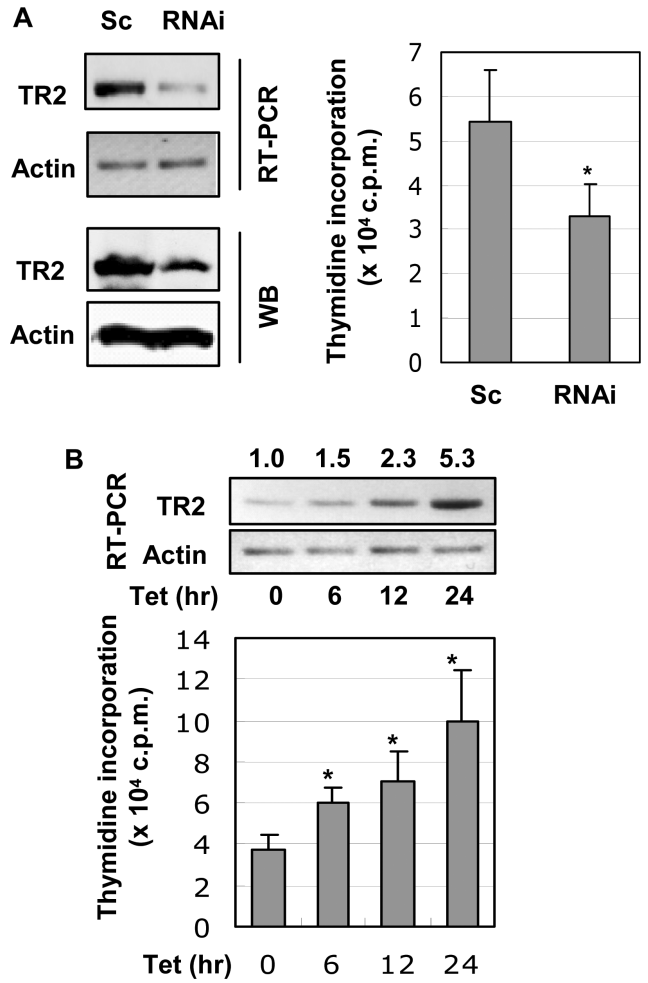

Figure 1. TR2 and preadipocyte proliferation. (A) TR2 was silenced by RNAi in preadipocytes (left panel) and cell proliferation was monitored by thymidine incorporation (right panel). (B) Tet-inducible TR2 expressing (top panel) 3T3-L1 clones were monitored for thymidine incorporation (bottom panel) as cells were induced.

primer sets specific to the *IRO*-containing promoter region of the endogenous *TR2* gene (sense, 5′-CCGCCGCTGGACTCCGGGGCCCTC3′ and antisense, 5′-CGCTGGGCTGGAGAGAGCAGAGGC3′) (13).

RESULTS

TR2 function in 3T3-L1 preadipocyte proliferation

To determine the functional role for TR2 in proliferating 3T3-L1 preadipocytes, we first employed gain- and loss-of-function strategies using tetracycline-inducible expression and RNA interference (RNAi)-mediated silencing in undifferentiated 3T3-L1 preadipocytes. The results of loss-of-function studies are shown in Figure 1A. Approximately 80% knockdown of *TR2* mRNA expression and 60% knockdown of TR2 protein levels (left panel) reduced the rate of thymidine incorporation in these cultures by approximately 50% (right panel). For gain-of-function experiments, several stable tetracycline-inducible TR2-expressing clones were examined (Figure 1B). The rate of thymidine incorporation into these cultures was increased on average by approximately 3-fold after 24 h of *TR2* induction. These results showed a causal effect of TR2 on the increased preadipocyte proliferation in the 3T3-L1 model.

Biphasic regulation of *TR2* in the 3T3-L1 differentiation model and differential expression of coregulators

To decipher events upstream of *TR2* gene expression in preadipocytes, we found that RA increased its transcriptional efficiency and, as expected, increased cell proliferation (Figure 2A). Very interestingly, RA exerted a biphasic effect on the expression of *TR2* in 3T3-L1 in relationship to its differentiation (Figure 2B). *TR2* was induced by RA in undifferentiated cells but repressed by RA in differentiated cells. This was probably due to the different types of coregulators expressed in 3T3-L1, before and after differentiation, might be different. The expression patterns of several endogenous coregulators for RAR–RXR and the effects of RA on the expression of these endogenous components in 3T3-L1 cells before and after differentiation were then examined as shown in Figure 2B. Interestingly, the expression levels of the endogenous coactivators PCAF and GRIP1 were maintained at relatively constant levels during the course of RA induction in cells before and after differentiation. In contrast, RIP140 was not detectable during the entire course of RA treatment in the undifferentiated cells and was significantly elevated in differentiated cells. RA seemed to have little effect on the expression of endogenous RIP140 in these differentiated cells. According to our working model, the significantly elevated expression of RIP140 in differentiated cells would switch the cellular environment from a condition suitable for hormonal activation to that for hormonal repression. This would explain the biphasic effects of RA on the expression of TR2, i.e. a rapid induction of *TR2* by RA in undifferentiated cells followed by the second phase of RA action in differentiated cells—a rapid repression of *TR2* by RA.

Different RA-dependent receptor–coregulator complexes in 3T3-L1 before and after differentiation

To determine if RA induced the formation of different types of RA-dependent receptor–coregulator complexes in undifferentiated versus differentiated cells, sequential co-immunoprecipitation (IP) procedures were carried out to examine several key coregulatory components that could be recruited by holo-RAR–RXR. While a number of coactivators have been reported for RAR–RXR, we focused on three components that are most relevant to this system: the coactivators GRIP1–PCAF and corepressor RIP140. Anti-RAR was used for the first IP, which apparently co-precipitated GRIP1 in both undifferentiated and differentiated cells as shown on the western blot probed with anti-GRIP1 (Figure 2C). RA enhanced the formation of receptor–GRIP1 complex equally well in cells both before and after differentiation (Figure 2C).

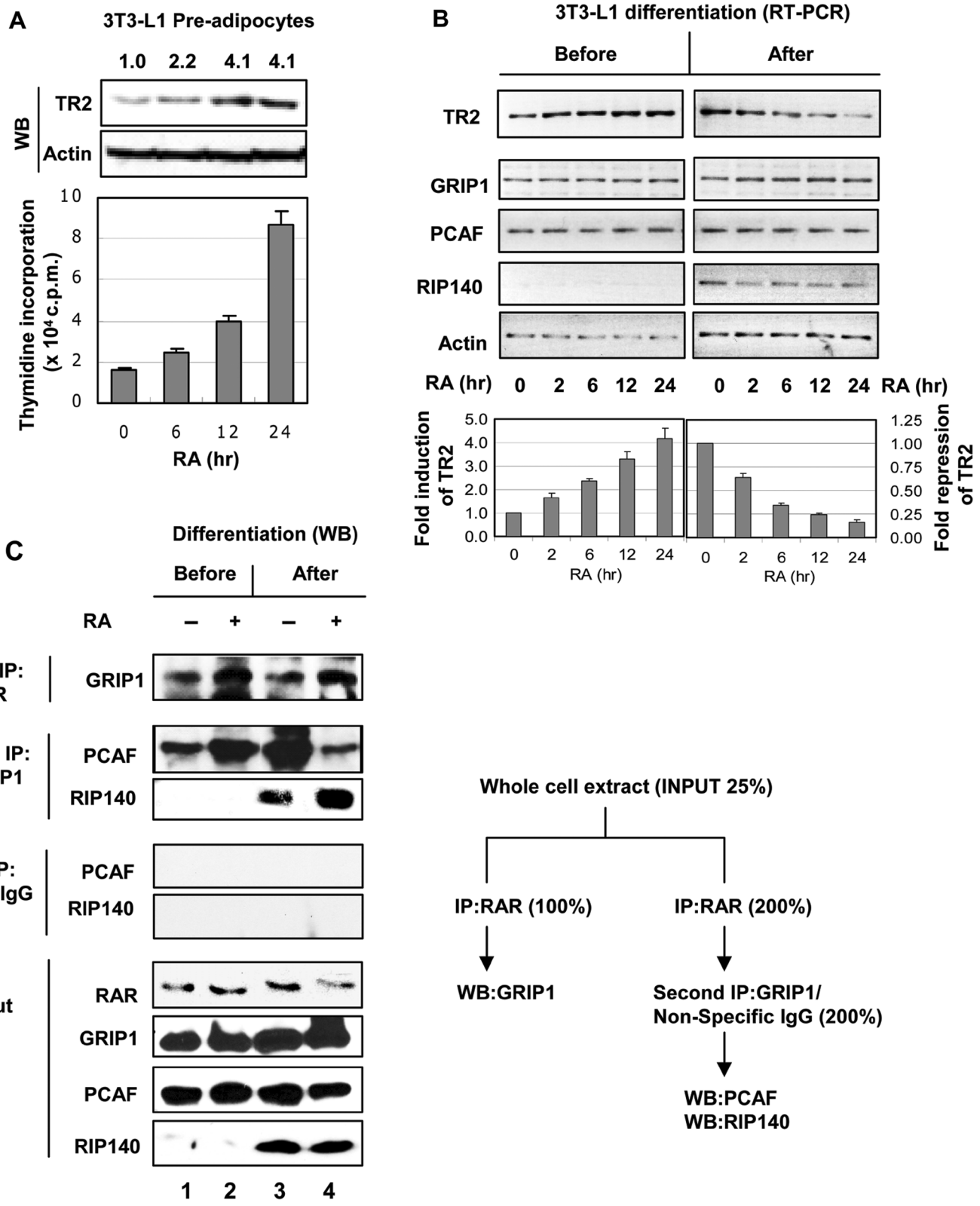

Figure 2. Biphasic regulation of TR2 by RA and the formation of different endogenous coregulator complexes in 3T3-L1. (**A**) At various time points of RA treatment, TR2 protein levels were monitored by western blot (top panel) and preadipocyte proliferation confirmed by thymidine incorporation (bottom panel). (**B**) RT-PCR analyses of RA-induced TR2, but not coregulators, in preadipocytes and differentiated cells. (**C**) Formation of different coregulatory complexes in preadipocytes/differentiated 3T3L1. The first immunoprecipitation was conducted using anti-RAR and receptor-associated GRIP1 was monitored by western blot analysis. The RAR-associated complexes were subjected to a second round of immunoprecipitation using anti-GRIP1, followed by western blot analysis of PCAF and RIP140.

The anti-RAR immunoprecipitated complex was subsequently precipitated with anti-GRIP1, followed by western blot analyses using either anti-PCAF or anti-RIP140. In undifferentiated cells, PCAF formed complexes with RAR–RXR–GRIP1 even without additional RA, contributing to the constitutive, basal level of expression of TR2 in undifferentiated cells. As predicted, RA enhanced the level of PCAF recruitment to the RAR–RXR–GRIP1 complex. Interestingly, while the level of PCAF expression remained constant in both undifferentiated and differentiated cells as shown in Figure 2B and C, PCAF apparently was dissociated from the RAR–RXR–GRIP1 complex upon treatment with RA in the differentiated cells. More importantly, in the differentiated cells RIP140 was significantly recruited to the RAR–RXR–GRIP1 complex. The formation of RAR–RXR–GRIP1–RIP140 complex was further enhanced by RA treatment of differentiated cells despite the constant expression of PCAF. As predicted, RIP140 was not detected on the RAR–RXR–GRIP1 complex in undifferentiated cells because RIP140 was not expressed in these cells (Figure 2B and C). These results demonstrated that RA induced the formation of different receptor–coregulator complexes in undifferentiated versus differentiated cells.

It was interesting to note that while both PCAF and RIP140 were present in differentiated cells, the formation of RIP140-containing complex was significantly favored over the formation of PCAF-containing complex by the receptors, which was in agreement with our previous kinetic studies that detected a higher binding affinity of holo-receptors toward RIP140 than PCAF (17). This result suggested that the cellular environment could be altered from a condition for hormone activation to that for hormone repression by simply altering the expression of a potent hormone-dependent corepressor RIP140. To test this hypothesis, RIP140 was ectopically expressed in the undifferentiated cells. In these engineered RIP140-expressing undifferentiated cells, *TR2* gene could no longer be activated by RA, rather it was significantly repressed by RA (Figure 3A, right). As a control, PCAF was also overexpressed in the undifferentiated cells, where *TR2* was effectively activated by RA (Figure 3A, left).

The role of GRIP1 has been proposed as a coactivator (33–35). However in the 3T3-L1 system, GRIP1 could be associated with either the coactivator or the corepressor complex (Figure 2C). To clarify the role of GRIP1, it was overexpressed in the undifferentiated and differentiated cells (Figure 3B). Differential regulation of *TR2* by RA in these two cell populations was not altered by additional GRIP1, indicating that GRIP1 was not a deciding factor for the activity of the coregulatory complexes in regulating the expression of TR2 in this particular culture system. Instead, GRIP1 probably just served as a platform molecule for facilitating the formation of ligand-induced regulatory complexes. This result supported the notion that the expression of a potent corepressor RIP140 rendered hormone target genes responding to hormone in a negative fashion.

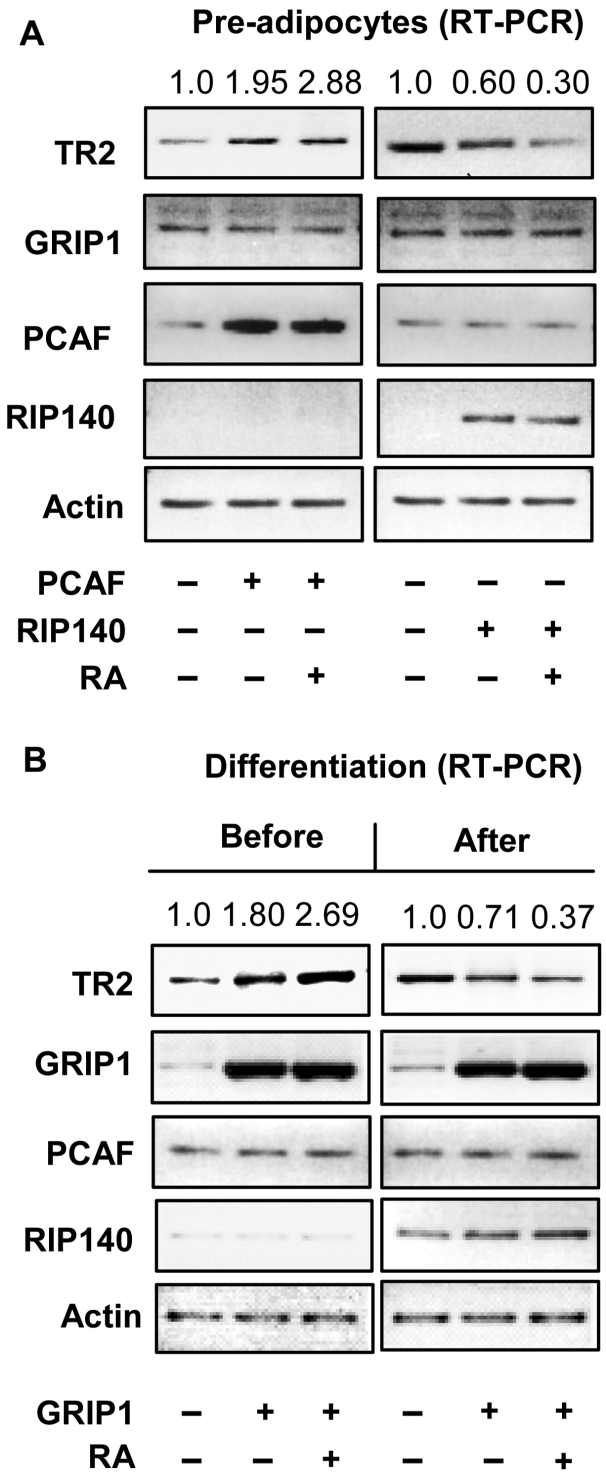

Figure 3. Differential regulation of TR2 by RA in the presence of different coregulators in 3T3-L1. (**A**) 3T3-L1 preadipocytes were transiently transfected with PCAF (left) or RIP140 (right) with or without RA. The endogenous TR2 mRNA expression in preadipocytes was evaluated by RT-PCR (top panel). The expression of GRIP1, PCAF and RIP140 and internal control actin, was monitored (bottom four panels). (**B**) The endogenous TR2 mRNA expression in undifferentiated (left) and differentiated (right) cells transfected with GRIP1 was analyzed by RT-PCR.

GRIP1 directly interacts with RIP140 through the AD2 domain of GRIP1 and the amino terminal domain of RIP140

The p160 family coregulators can directly interact with PCAF. The GRIP1–PCAF coactivator complex is frequently detected on hormone-activated gene promoters (35,36). Our finding that RIP140 was also co-precipitated with receptor–GRIP1 complex (Figure 2C) would suggest that GRIP1 could probably interact with RIP140. To test this possibility, GST pull-down assays were conducted (Figure 4). GRIP1 indeed directly interacted with RIP140 through its carboxyl terminal domain containing the activating domain 2 (AD2) (Figure 4A, lower right panel). Further tests using dissected RIP140 fragments revealed that RIP140 directly interacted with GRIP1 through its amino terminal domain (Figure 4B, lower panel). The observation that GRIP1 directly interacted with RIP140, together with the reported phenomenon that GRIP1 directly interacted with PCAF, further supported the notion that GRIP1 probably serves as a platform molecule by facilitating the exchange of coregulators on the receptor complexes.

Enzymatic activities of receptor–GRIP1–PCAF and receptor–GRIP1–RIP140 complexes

The receptor–GRIP1–PCAF complex was predicted to possess HAT activity encoded by the PCAF molecule, whereas the receptor–GRIP1–RIP140 complex was predicted to possess HDAC activity due to the recruitment of HDAC3/4 by RIP140 (16,37). This prediction was tested using *in vitro* enzyme assays of immunoprecipitated receptor–coregulator complexes. The results of *in vitro* assays for HAT and HDAC conducted using core histone proteins/[^{3}H]-acetyl histone H4 peptide as substrates are shown in Figure 5A and B, respectively. Only the receptor complex containing both GRIP1 and PCAF encoded significant HAT activity (Figure 5A, top panel). GRIP1 had neither HAT nor HDAC activity of its own. The receptor–RIP140 complex encoded the predicted HDAC activity. This was fully retained in the receptor–RIP140–GRIP1 complex (Figure 5B). The biological effects of these coregulators on RA regulation of *TR2* promoter were further verified using a *TR2* reporter (Figure 5C). Addition of GRIP1 or PCAF slightly enhanced RA activation of this reporter, and coexpression of GRIP1 and PCAF further enhanced RA activation of the reporter. In contrast, addition of RIP140 completely eliminated RA activation, and even further reduced the reporter activity as compared to the uninduced control. Coexpression of GRIP1–RIP140 further repressed the reporter activity, which could not be rescued by PCAF. These results validated the enzymatic basis for the coactivator complex GRIP1–PCAF and corepressor complex GRIP1–RIP140.

Dynamics of RA-induced coregulator recruitment to the endogenous *TR2* promoter in cells before and after differentiation

To monitor the dynamics of differential RA-induced coregulator recruitment to the endogenous *TR2* promoter,

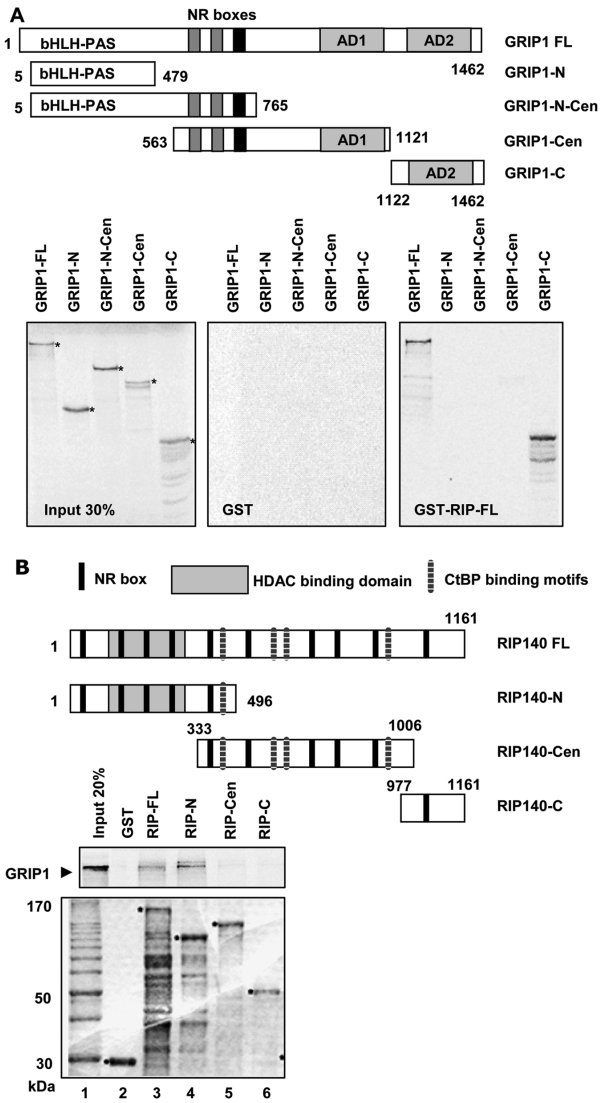

Figure 4. GST pull-down assays to determine the interaction domains of GRIP1 and RIP140. (**A**) Interaction domain of GRIP1. Recombinant GST–RIP140 protein was incubated with ^{35}S-labeled GRIP1 full-length or fragments of GRIP1, including an amino terminal (amino acid 5-479), an extended amino terminal (amino acid 5-765), a central domain (amino acid 563-1121) and a C-terminal domain (amino acid 1122-1462) (Upper panel). Interacting proteins were resolved by SDS-PAGE and analyzed by autoradiography (Lower right panel). Protein sample input (30%) and negative control are shown in the lower left and lower central panels, respectively. (**B**) Interaction domain of RIP140. GST–RIP full-length (amino acid 1-1161), RIP N-terminal (amino acid 1-496), RIP central (amino acid 333-1006) and RIP C-terminal (amino acid 977-1161) fragments (upper panel) were tested against ^{35}S-labeled GRIP1, and analyzed by autoradiography (lower top panel). GST-protein inputs are shown (lower bottom panel).

and the status of its chromatin histone modification in cells before and after differentiation, ChIP was conducted as shown in Figure 6A. To examine this in a physiological setting, only the endogenous proteins were analyzed. In undifferentiated cells, GRIP1 was found to be first recruited to the endogenous *TR2* promoter (already significant at the first time point: 6 h after RA treatment),

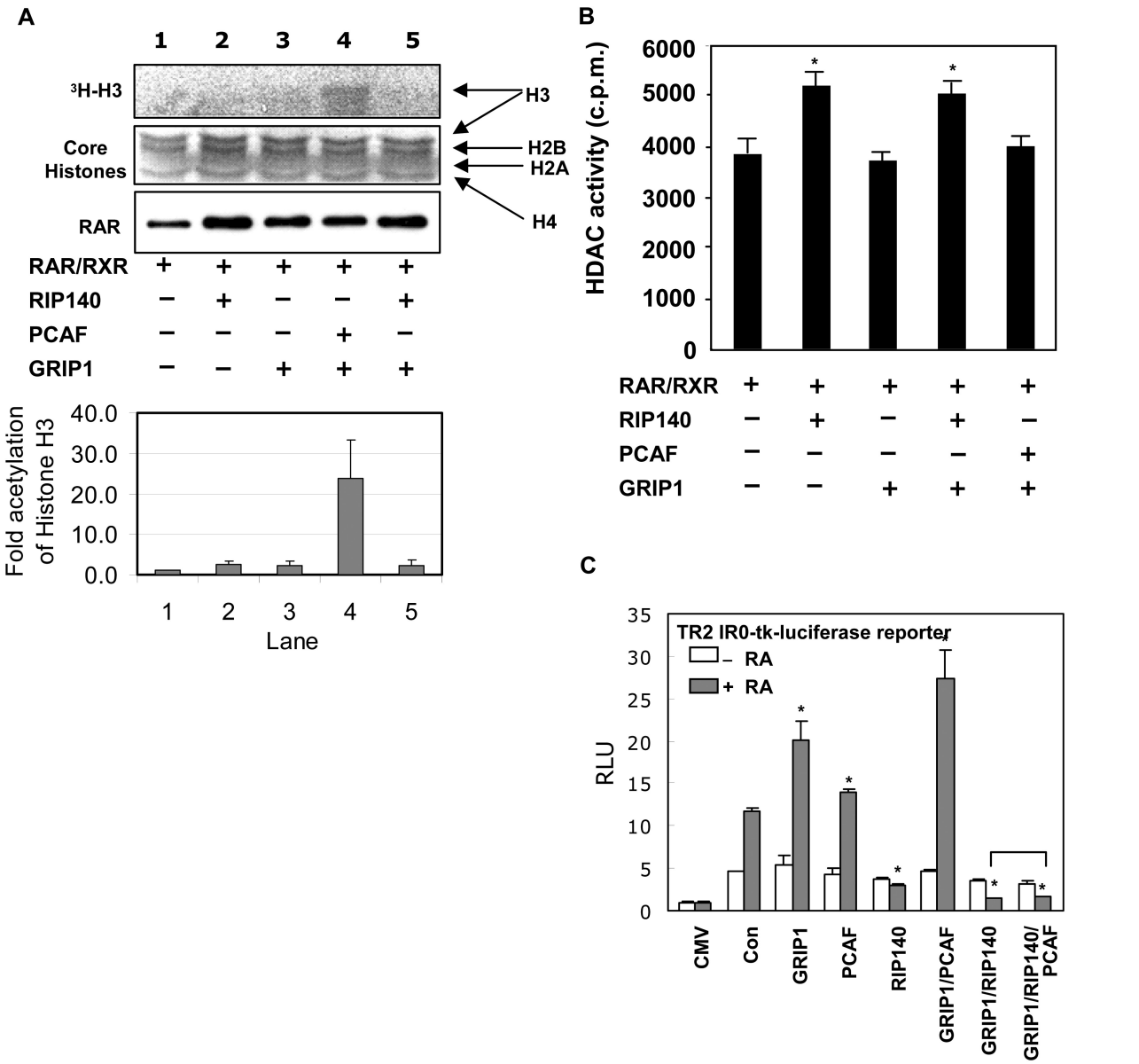

Figure 5. Activating (HAT) and repressive (HDAC) enzymatic activities of receptor–coregulatory complexes. Receptor complexes were precipitated using anti-RAR from COS1 cells expressing various combinations of RAR, RXR, GRIP1, PCAF, RIP140 in the presence of RA. The RAR-associated complex was monitored for (**A**) HAT activity (**B**) HDAC activity. $^{*}P<0.05$ for difference from the RAR–RXR control. (**C**) TR2 reporter gene (IRO-tk-luciferase) activity regulated by different receptor–coregulator complexes in the presence of RA or vehicle was monitored in COS1. The fold relative luciferase activity (RLU) was obtained by normalizing to the internal control lacZ activity. $^{*}P<0.04$ for difference from the control. Bracket indicates no significant difference.

followed by PCAF recruitment (at the second time point, 12 h). This coincided with histone H3 hyperacetylation and RNA polymerase II recruitment. RIP140 was never detected on the *TR2* promoter in these undifferentiated cells during the entire course of RA treatment. In contrast, in differentiated adipocytes before RA treatment, while GRIP1 was constantly associated with this promoter, PCAF association with the *TR2* promoter was substantially reduced as compared to the undifferentiated condition. RIP140 was, initially, weakly recruited to the *TR2* promoter without RA treatment. After RA addition, the weakly associated PCAF then left the *TR2* promoter (6 h after RA treatment), coinciding with the significantly enhanced recruitment of RIP140 and HDAC3 to this promoter. This also coincided with the reduced histone H3 acetylation and weaker association of RNA pol II with this promoter after RA treatment.

To further examine if GRIP1 was physically associated with different types of coregulatory complexes binding to the *TR2* promoter, repeated ChIP was carried out for both undifferentiated and differentiated cells. The first ChIP was conducted using anti-GRIP1, followed by the subsequent ChIP using anti-PCAF or anti-RIP140 (Figure 6B). PCAF was recruited increasingly to the

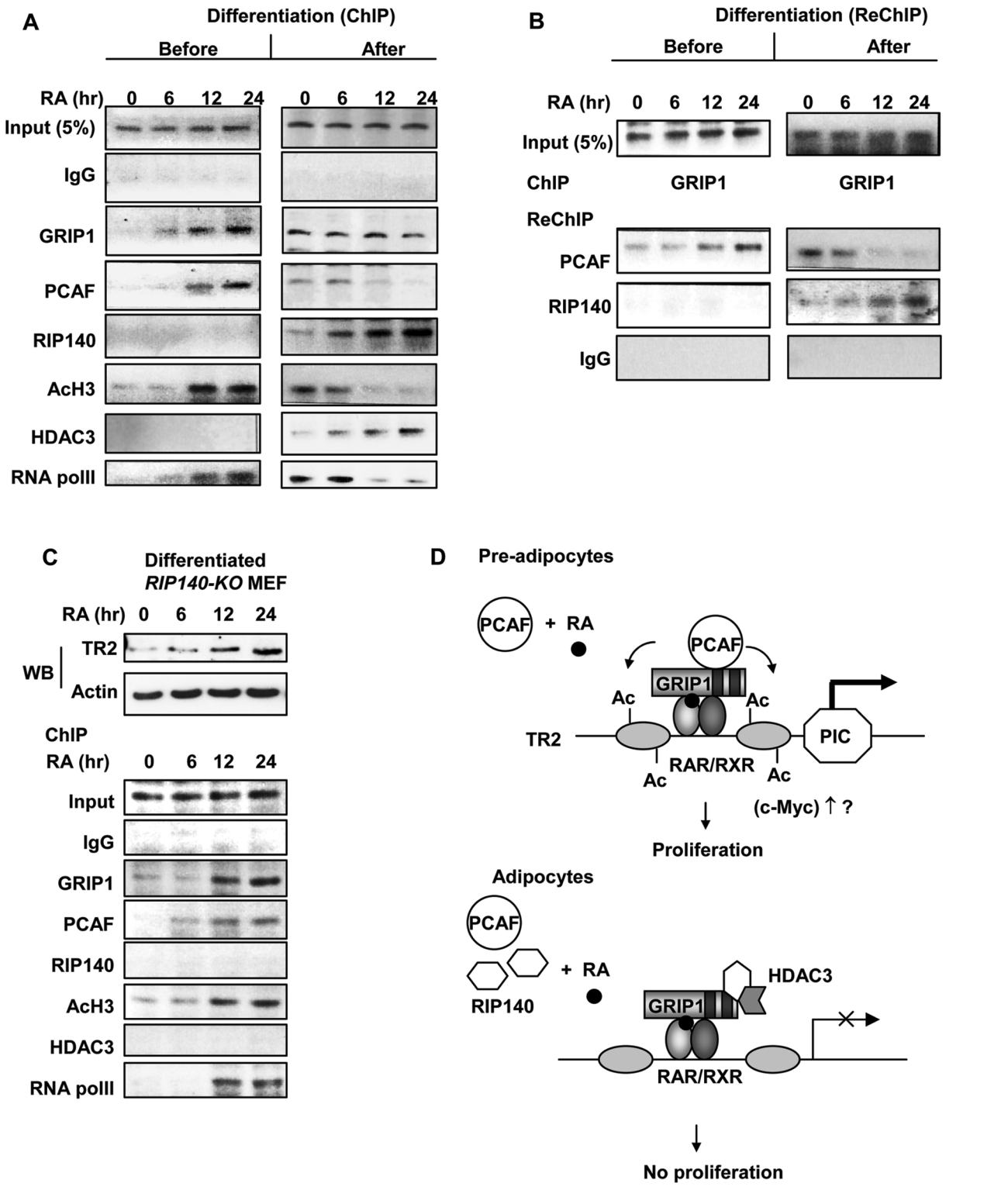

Figure 6. The dynamics of complex formed on the endogenous TR2 gene promoter in 3T3 L1. (**A**) ChIP assay for RA-induced complexes formation on TR2 in preadipocyte (left, before) and differentiated (right, after) 3T3-L1 cells. Specific antibodies used in immunoprecipiation are shown on the left-hand side of panels. (**B**) Repeated ChIP assays first with anti-GRIP1, followed by a specific second antibody indicated on the left. (**C**) TR2 expression (upper panel) and ChIP analysis (lower panel) of TR2 promoter in differentiated, RIP-knockdown MEFs. (**D**) A schematic representation of RA-induced receptor–coregulator complex formation on TR2 promoter before and after cell differentiation.

GRIP1 complex binding to the *TR2* promoter in undifferentiated preadipocytes during the course of RA induction. However, RA triggered its gradual dissociation from this promoter in differentiated cells. In contrast, RIP140 was never detected on the GRIP1 complex binding to the *TR2* promoter in undifferentiated cells during the entire course of RA induction, but it was increasingly recruited to this promoter in differentiated cells following RA treatment.

Based upon these ChIP and Re-ChIP data, it was concluded that, in undifferentiated cells RA first enhanced GRIP1 recruitment to receptors occupying the *TR2* promoter, followed by the recruitment of HAT-encoding PCAF to form a receptor–GRIP1–PCAF complex on this promoter. This resulted in the initial phase of RA activation of the *TR2* gene in the undifferentiated cells and was required for its proliferation. On the *TR2* promoter in the differentiated cells where RIP140 levels are elevated and PCAF levels are maintained, the receptor-associated PCAF was replaced with RIP140 in the presence of RA, forming the repressive complex that encodes HDAC activity. This resulted in the second phase of RA action, i.e. the repression of *TR2* probably because TR2 was no longer needed in differentiated cells. The dynamics of differential recruitment of various RA-dependent coregulators to the target *TR2* promoter underlined the biphasic effects of RA on the expression of TR2 in this culture system.

Other studies on MEFs showed an elevation of RIP140 in differentiated cells (38). To determine if RIP140 plays a role in the transition from hormone-activated to hormone-repressed phases in MEFs, and if it is important for formation of different complexes in the MEF cells, RIP140-knockdown MEFs were generated, which were monitored for adipogenesis and by ChIP assays. TR2 expression, both before and after differentiation, was similarly upregulated by RA in the RIP140-knockdown cells, supporting a central role for RIP140 in the biphasic regulation of this gene by RA in the wild-type MEF system (Figure 6C, top panel). Consistently, ChIP assays performed on differentiated RIP140-knockdown MEFs revealed that GRIP1 was recruited to the *TR2* promoter in the differentiated cells treated with RA (Figure 6C, lower panel). Consistently, PCAF recruitment and histone hyperacetylation, as well as RNA polymerase II recruitment, all occurred in these RIP140-knockdown and differentiated MEFs, mimicking the events occurred in the proliferating preadipocytes. This validated a functional role for RIP140 in the biphasic regulation of the RA target gene, *TR2*, in this system. The model for the biphasic regulation of the RA target gene in MEFs is illustrated in Figure 6D. This mechanism could potentially equip cells with the capacity to regulate the expression of certain important genes, such as *TR2*, at a level that was required for efficient proliferation in preadipocytes.

TR2 mediates the proliferating effect of RA in 3T3-L1 preadipocytes

The target genes of TR2 that could mediate cell proliferation were searched. Myelocytomatosis oncogene (*Myc*), which is known to play a role in 3T3-L1 preadipocyte proliferation and differentiation (39,40), was first examined. c-Myc expression directly correlated with TR2 expression in the initial screening. To validate this finding, the expression pattern of *Myc* in RA-treated, or TR2-overexpressing, preadipocytes was examined (Figure 7A). The expression of *Myc* was elevated following RA treatment (bottom panel). Interestingly, the kinetics of *Myc* expression also closely paralleled the kinetics of *TR2* elevation induced by tetracycline (top panel). A direct causal relationship of TR2 and c-Myc expression was further confirmed in *TR2*-knockdown cells (Figure 7B), where c-Myc expression could no longer be effectively activated by RA. As a control, scramble RNA was included and normal RA-stimulated c-Myc expression was observed.

RA is known as a natural proliferating agent for preadipocytes (21) but not for differentiated cells. Our data (Figure 2A) revealed that thymidine incorporation was gradually increased in the preadipocyte culture following RA treatment, coinciding with the parallel activation of TR2 by RA in these cells. Knockdown of TR2 in these cells reduced their basal proliferative rates (Figure 1A). The functional role of TR2 in RA-stimulated proliferation was then examined in *TR2* knockdown and control cells that were then treated with RA. As shown in Figure 7C, the scramble RNA-treated cells incorporated normal levels of labeled thymidine, which were increased approximately 5–6-fold by RA treatment. In contrast, TR2-knockdown cells incorporated labeled thymidine at approximately 50% of the control level without additional RA. The addition of RA to these *TR2*-knockdown cells could no longer significantly enhance thymidine incorporation. Therefore, these *TR2*-knockdown cells were defective in their normal proliferation. The effect was especially severe in the case of RA-stimulated cellular proliferation. These data strongly supported our hypothesis that TR2 mediates the proliferative effect of RA in the undifferentiated 3T3-L1 preadipocytes. Its action involved the activation of a potential downstream effector, c-Myc.

As a result of reduced cellular proliferation, *TR2* knockdown in these cells significantly improved their capacity for fat accumulation, as monitored qualitatively by oil-red-O staining (Figure 7D) and quantitatively by measuring the accumulation of tri-glyceride/mg protein (Figure 7E). This coincided with the physiological effects of RA in reduced adipogenesis (22,41,42). It is likely that as these preadipocytes became deficient in proliferation, they might differentiate better. This requires further studies to monitor marker genes for differentiation in cells with complete knockdown of *TR2* in the future.

DISCUSSION

A functional role for TR2 in preadipocyte proliferation

In studies using tetracycline to induce TR2 expression, it was found that TR2 elevation could stimulate preadipocyte proliferation. In contrast, *TR2*-knockdown

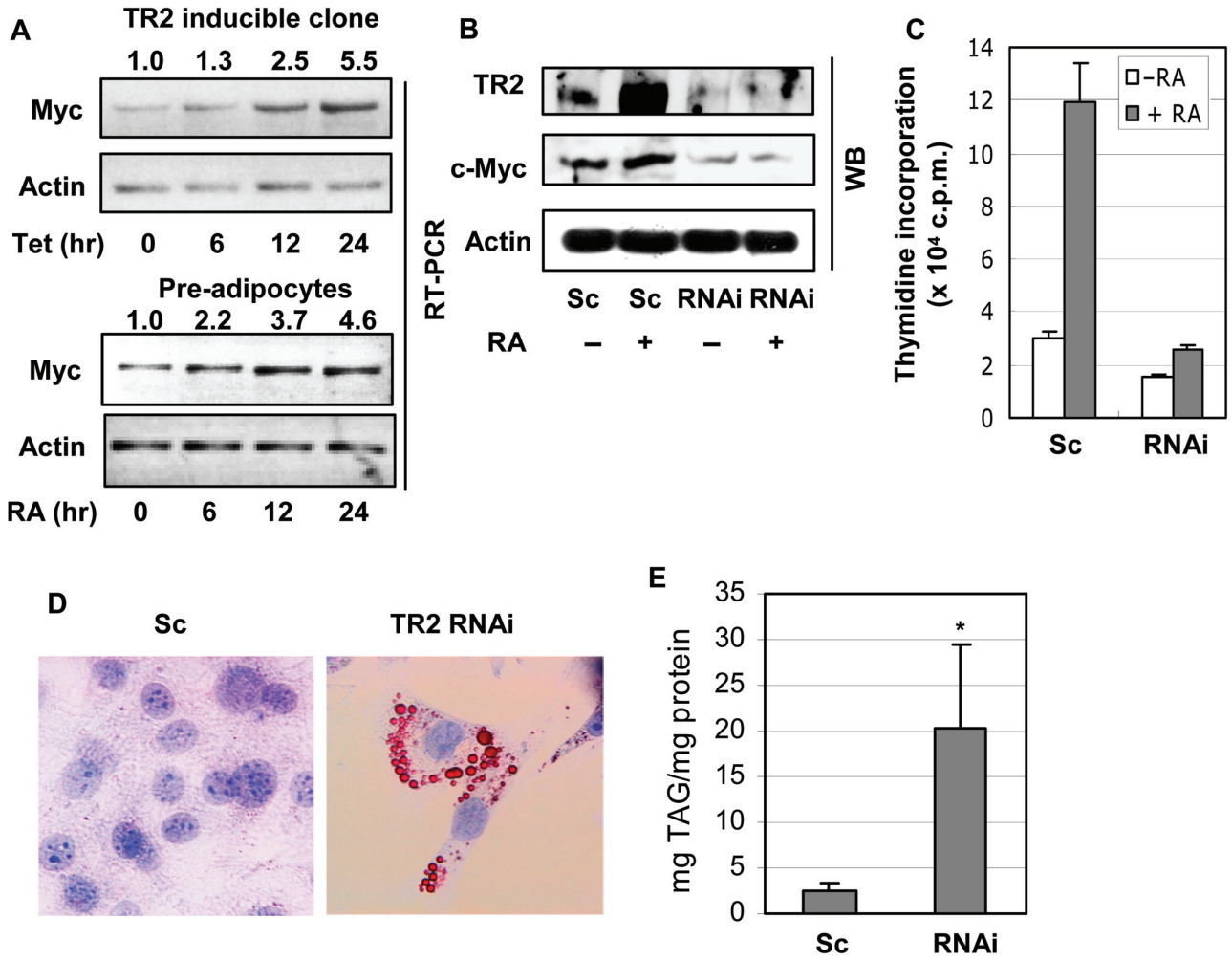

Figure 7. TR2 as a mediator of RA-stimulated proliferation of 3T3-L1 preadipocytes. (**A**) Expression of *Myc* a possible downstream target gene of TR2 was monitored in TR2-inducible clones (top panel) and in RA-stimulated regular preadipocytes (bottom panel). (**B**) Expression of c-Myc protein and (**C**) preadipocyte proliferation in TR2-silenced 3T3-L1 preadipocyte, with or without RA. (**D**) Parameters for fat accumulation was monitored qualitatively by oil-red-O staining and (**E**) quantitatively by tri-glycerides/mg protein as a function of TR2 silencing.

preadipocytes proliferated less well. Studies of downstream effectors of TR2 confirmed a causal relationship of TR2 action in the expression of c-Myc, a known cell growth and differentiation factor of 3T3-L1 (39,40). But the exact mode of TR2 action, in terms of the regulation of *Myc* gene and other downstream effectors, remains to be determined.

The role of GRIP1 in the formation of different receptor–coregulator complexes

The *TR2* gene responds differentially to the RA signal, depending upon the availability of the types of coregulators in the cells. PCAF consistently acted as an RA-dependent coactivator and RIP140 as an RA-dependent corepressor. The role of GRIP1 appeared to be more complicated. GRIP1 belongs to the p160 family of proteins that includes SRC-1, TIF2/GRIP1 and RAC3/ACTR/pCIP/AIB-1, all interacting with nuclear receptors through LXXLL-motifs called NR boxes. They also seemed to be involved in recruiting secondary coactivators [reviewed in (34,43)]. Recently, GRIP1 was suggested to act as a indirect repressor by unknown mechanisms (44–46). Our findings provide a mechanistic basis for a role for GRIP1 in the formation of either ligand-dependent coactivators or ligand-dependent corepressors. Its role might be better described as a platform molecule that functions by directly interacting with various coregulators and facilitating the formation of receptor–coregulator complexes. It would be important to determine the molecular basis underlying the differential interaction of GRIP1 with various coregulators. In terms of its biological function, it remains to be determined whether GRIP1 is essential for all the complexes with which it can associate. As PCAF and RIP140 can directly associate with RAR–RXR (17,18), it is possible that the platform function of GRIP1 might not be required for all complexes. Rather, it could be specific to certain promoters and in particular cell types

that expressed high levels of GRIP1 (47). It is worth noting that TIF2 is highly expressed in preadipocytes and has been documented to exert a physiological role in obesity (48). In terms of the enzymatic activity, GRIP1 is important for PCAF complex formation, but by itself encodes no HAT activity. Further, it does not seem to affect the HDAC enzyme activity recruited by the RIP140 complex. Quite likely, the role of GRIP1 in different receptor–coregulator complexes might vary in different contexts. This is further supported by the observation that GRIP1 interacts with RIP140, CARM1 and PRMT1 through its C-terminal domain (AD2) (49), while it interacts with HAT proteins such as p300/CBP, GCN5 and PCAF through its AD1 (34). Dissociation of PCAF from AD1 domain of GRIP1 in differentiated cells could be attributed to conformational changes in AD1 domain caused by the binding of RIP140 to AD2, steric interference by bound RIP140, or different affinities. Recently, we found that PRMT1 expression was elevated during adipogenesis and that RIP140 could be methylated by directly associating with PRMT1 (50). It would be interesting to examine the potential role for PRMT1 in the adipogenesis model.

Differential recruitment of various ligand-dependent regulatory complexes to the same hormone target gene

The dogma of differential coregulator recruitment to a specific target gene has focused on the recruitment of typical aporeceptor–corepressor complexes in the absence of hormones and the recruitment of typical holoreceptor–coactivator complexes in the presence of ligands (24,25). This current study proposes a modified version of differential recruitment of coregulator complexes to the same target, especially in the presence of hormone. This modified model could address the molecular basis for the very different behaviors of a gene in response to ligands. This model also provides insight into the complexity of hormonal regulation that requires a very precise physiological tuning. While it is unclear whether the behavior of the *TR2* gene in response to hormonal inputs in the 3T3-L1 system can be generalized and applied to many other hormone-regulated genes, it is likely that most hormone target genes require an efficient mechanism to fine-tune their expression in order to effectively respond to the specific need of the cells (51–53). The presence of certain potent tissue- or cell-specific negative coregulators such as RIP140 is likely to be one important factor in fine-tuning the expression of important cellular components.

Our initial model of differential ligand-dependent coregulator recruitment was based largely upon *in vitro* kinetic studies and transfection experiments using reconstituted expression of coregulators (13,15,54). This current study presents the first example where this model could apply in a natural, physiologically relevant condition. However, due to the technical limitation of ChIP and Re-ChIP experiments, it was difficult to evaluate many other components coexisting in the molecular complexes in real time and *in vivo*. Future challenges remain with regard to the identification of the exact components of physiologically relevant receptor–coregulator complexes for a specific target gene and under specific physiological conditions. The kinetic factors deduced from *in vitro* protein interaction tests could provide clues for building models to be tested (17,26). Ultimately, it requires examination of the model on a real gene by taking into account the various cellular environments, where the availability of individual component may fluctuate widely.

A functional role for TR2 in mediating the physiological action of RA in preadipocytes

Adipocytes are a specialized cell type that store energy in the form of lipids. Obesity results from the over-development of white fat and high adiposity is associated with systemic disturbances in cellular differentiation or proliferation such as deregulation of adipocyte differentiation or preadipocyte proliferation (55,56). RA is known to enhance preadipocyte proliferation (21) and inhibit adipocyte differentiation (19,20,57–60). Our study presents the first evidence for the function of TR2 in mediating RA-stimulated preadipocyte proliferation. The possibility of other RA mediators (or effectors) in preadipocyte proliferation has not been excluded.

As a mediator for the RA signal to stimulate preadipocyte proliferation, TR2 would need to be activated by RA in the preadipocytes. This is consistent with its rapid induction by RA in these cells and confirmed in studies using tetracycline to induce TR2 expression without RA addition. Consistently, *TR2*-knockdown preadipocytes were less proliferative and can no longer effectively respond to the mitogenic effect of RA. We also identified c-Myc as a probable downstream effector of TR2. Thus, one of the signaling pathway of RA in preadipocyte proliferation can be mediated by RARs and RXRs (22,41,42) that regulate *TR2*, which in turns regulates *Myc*.

The second phase of RA effect on the expression of TR2 in the differentiated cells suggests that TR2 expression is no longer needed in the differentiated cells because the differentiated cells cease proliferation. Alternatively, it is possible that TR2 needs to be reduced in these differentiated cells when they are exposed to RA. This may be due to a potential anti-differentiation effect of TR2 that remains to be evaluated. Based upon the expression studies, TR2 levels are generally high in early developmental stages and in proliferating cells, but are usually not detected in differentiated cells. Previous studies of ectopic, forced TR2 expression showed that it mostly resulted in apoptosis (4). This would suggest a potential detrimental effect of TR2 in certain cells when they undergo differentiation or are fully differentiated. This also requires further studies.

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