Negative Feedback at the Level of Nuclear Receptor Coregulation

SELF-LIMITATION OF RETINOID SIGNALING BY RIP140

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Nuclear receptor-mediated gene expression is proposed to be regulated by the ordered recruitment of large protein complexes in which activity depends on mutual interactions and posttranslational modifications. In contrast, relatively little attention has been given to mechanisms regulating the expression of the coregulator proteins themselves. Previously we have shown that the ligand-dependent corepressor, RIP140, is a direct transcriptional target of all-trans retinoic acid (RA). Here we demonstrate that RA induction of RIP140 constitutes a rate-limiting step in the regulation of retinoic acid receptor signaling. Silencing of the RA induction of RIP140 dramatically enhances and accelerates retinoid receptor transactivation, endogenous expression of other RA target genes, and RA-induced neuronal differentiation and cell cycle arrest in human embryonal carcinoma cells. The data suggest that RA induction of RIP140 constitutes a functional negative feedback loop that limits activation of retinoid receptors in the continued presence of RA and that acutely regulated expression of coregulators may be a general regulatory mechanism in hormonal signaling.

Retinoids are ligands for retinoid receptors, which are members of the steroid receptor superfamily (1, 2). All-trans retinoic acid (RA) regulates gene expression primarily through activation of retinoid receptors bound to consensus retinoic acid response elements (RAREs) (1, 2). Detailed molecular mechanisms have been elucidated whereby acetylation, methylation, and phosphorylation of specific histone tail residues by coregulatory complexes regulate the transcriptional activity of nuclear receptors (3–5). Coregulators associated with histone acetylation/deacetylation (coactivators and corepressors) associate with nuclear receptors in a ligand-dependent fashion (3–5). Corepressors N-CoR and SMRT, through direct interaction with the AF-2 domain of nuclear receptors, actively repress transcription in the absence of ligand (6, 7). In the presence of hormone, corepressor complexes disassociate allowing interaction of coactivator complexes such as SRC-1/NCoA-1, TIF2/GRIp1/NCoA-2, and ACTRI/pCIP/AIB1/RAC3 with the AF-2 domain of receptors (3–5). LXXLL motifs present in coactivators SRC-1, TIF2, and AIB1 are essential for hormone-dependent interaction with nuclear receptors (8).

Receptor-interacting protein 140 (RIP140) is one of the most enigmatic nuclear receptor coregulators (9). RIP140 contains 14 LXXLL motifs and interacts preferentially with nuclear receptors in the presence of ligand (10). However, RIP140 inhibits the transactivation function of ligand-bound receptors, including retinoid receptors (11–17). This property sets RIP140 apart from classic corepressors such as N-CoR and SMRT, which repress apo-receptors, as well as classic coactivators, such as SRC-1, which bind and activate holo-receptors (3–5). RIP140 has intrinsic repression activity when fused to the Gal4-DNA binding domain and directly recruits histone deacetylases and the transcriptional repressor, CtBP (C-terminal-binding protein), to nuclear receptors (16, 17). Thus RIP140 is predicted to suppress target gene expression of agonist-bound hormone receptors.

Utilizing expression array profiling we discovered that RIP140 is itself an immediate RA target gene (18, 19). Induction of RIP140 mRNA occurs within 3 h and induction of its protein within 6 h (19). The RA induction of RIP140 mRNA does not require de novo protein synthesis, consistent with RIP140 being a direct transcriptional target of retinoid receptors (19). A promoter fragment of RIP140 contains a near consensus DR5 RARE and is activated by RA (19). The current study utilizes the embryonal carcinoma cell line NT2/D1 as a model of RA-induced differentiation (20) and proposes that RA induction of RIP140 serves as a negative feedback signal limiting RA activity at the level of nuclear receptor coregulation.

EXPERIMENTAL PROCEDURES

Transient Transfection Reporter Assays—NT2/D1 is a clonal line derived from a xenograft of Tera-2 cells (21). The reporters, RARE-TK-Luc and RAR62-Luc were described previously (22). 1.75 × 10⁵ cells/well of a 6-well plate were transfected with 2.75 μg of DNA including 0.75 μg of reporter plasmid, 1.5 μg of expression plasmid, and 0.5 μg of a β-actin-β-galactosidase expression plasmid using a modified calcium phosphate precipitation technique (22). Cells were exposed to precipitates for 14–16 h and then washed and cultured with or without 1 μM RA for an additional 48 h before harvesting. For siRNA, 0.75 μg of RARE-TK-Luc, 0.5 μg of β-actin-β-galactosidase expression plasmid, and indicated amounts of siRNA were co-transfected using Polyfect (Qiagen). Luciferase activities were measured and normalized to β-galactosidase activity as described (22).

Data points represent the average of triplicate transfections with similar results obtained in at least three independent experiments.
SiRNA—The sequence of the custom siRNA duplex for RIP140 corresponds to the human RIP140 cDNA starting 331 bp downstream of the ATG start codon (Dharmacon). The siRNA duplex control used was the scramble II sequence from Dharmacon. Transfection of siRNA was performed with OligofectAMINE Reagent (Invitrogen) according to the manufacturer’s instructions. The final concentration of siRNA used in each experiment was ~150 nM. In independent experiments, transfection efficiency was assessed to be greater than 90% using fluorescein-labeled siRNA (Dharmacon).

**Immunophenotypic and Cell Cycle Analysis—**Indirect fluorescence-activated cell sorter (FACS) analysis to evaluate RA-induced neuronal differentiation of NT2/D1 cells was performed using established techniques (23). Briefly, NT2/D1 cells were incubated with a monoclonal antibody to the cell surface antigen A2B5 or an isotype-matched monoclonal control antibody. Cells were assayed indirectly with fluorescein isothiocyanate-conjugated goat-anti-mouse antibody, and fluorescence was measured as described (23). Mean peak fluorescent values and the percentage of positive cells were measured. For cell cycle phase analysis, cells were fixed with 70% ethanol and stained with propidium iodide for 16 h. The percentages of cells in G1, S, and G2/M were then determined on a BD Biosciences FACscan flow cytometer using Modfit LT software and established techniques (23).

**Northern, RT-PCR, and Heteronuclear-PCR (hn-PCR) Analyses—**Total RNA was isolated using TriReagent (Invitrogen). Northern hybridizations were performed as described previously (19). Expression levels of RA targets genes were also measured by semiquantitative RT-PCR. The cDNA was synthetized from 5 μg total RNA using SuperScript II reverse transcriptase (Invitrogen), as described previously (18, 22). Amplification of genes and β-actin was performed using Taq polymerase as described (18, 22). Experiments were performed to determine the optimal number of cycles that would yield linear phase of amplification, and reverse transcriptase was omitted to control for genomic DNA contamination. For certain products, detection in the linear range required transfer to nitrocellulose and probing with a radiolabeled internal primer. The sequence of PCR primers is available upon request. To detect primary, pre-spliced transcripts, hn-PCR was radiolabeled internal primer. The sequence of PCR primers is available upon request. The final concentration of siRNA used in each experiment was ~150 nM. In independent experiments, transfection of siRNA was performed with OligofectAMINE Reagent (Invitrogen) according to the manufacturer’s instructions. The final concentration of siRNA used in each experiment was ~150 nM. In independent experiments, transfection efficiency was assessed to be greater than 90% using fluorescein-labeled siRNA (Dharmacon).

**RESULTS**

**RIP140 Is a Rate-limiting Negative Regulator of Retinoid Receptor Transactivation—**RA induces neuronal differentiation and G1, S, and G2/M cell cycle arrest in NT2/D1 cells (21, 23). Our prior work identified RIP140 as acutely up-regulated upon RA treatment of NT2/D1 cells (18, 19). RIP140 is a direct transcriptional target of retinoid receptors based on rapid, cycloheximide-insensitive induction of RIP140 with RA treatment (18, 19). Because it has been reported that RIP140 can repress ligand-bound retinoid receptors (11–17), the extent to which RA induction of RIP140 represses RA signaling was investigated. Overexpression of RIP140 greatly inhibited a RARE-containing promoter/enhancer (up to 7-fold) in a dose-dependent manner (Fig. 1A). RIP140 transfection had no effect on SV40- and CMV-driven control reporters. Further, the inhibitory effect of RIP140 occurred only in the presence of ligand, a property not shared with classic corepressors such as SMRT or N-CoR which preferentially bind and repress apo-receptors (3–5). Because overexpression studies are susceptible to nonphysiologic effects such as squelching, siRNA was designed to repress RIP140 expression. RIP140 siRNA but not a control siRNA effectively decreased both basal and RA-induced expression of RIP140 mRNA—5-fold (Fig. 1C). RIP140 siRNA dramatically increased (up to 15-fold) RA-dependent RARE-TK-Luc activity in a dose-dependent manner (Fig. 1B). RIP140 siRNA had no effect on basal RARE activity and control siRNA failed to stimulate RARE activity (Fig. 1B). RIP140 siRNA also failed to alter TK-, SV40-, CMV-, and β-actin-driven control reporters. Overexpressed RIP140 and RIP140 siRNA similarly affected RARE reporter activity in MCF-7 cells (data not shown). Together these data strongly indicate that RIP140, in a rate-limiting fashion, represses RARE-mediated transactivation.

Inhibition of RA-induced Expression of RIP140 Enhances Expression of Endogenous RA Target Genes—As depicted in Fig. 2A, RIP140 siRNA but not control siRNA increased the endogenous expression of the known RA target genes RARβ and LEFTYA in NT2/D1 cells (18). RIP140 siRNA failed to inhibit the expression of the nuclear receptor coregulators SRC-1 and N-CoR, which served as specificity controls (data not shown). Increased expression of RARβ and LEFTYA in response to RIP140 RNAi was apparent at 3–6 h of RA treatment, which is within the time frame of RIP140 protein induction with RA (Fig. 2A) (19). An increase in target gene expression of ~6-fold was seen at 24 h of RA treatment in RIP140
siRNA cells as compared with cells treated with control siRNA. A similar pattern of expression in response to RIP140 siRNA was seen for the RA target gene HAS2 (data not shown). The effects of knocking down RIP140 expression on RA target genes was largely ligand-dependent, which supports a role for RIP140 as a ligand-dependent corepressor based on prior studies of in vitro interactions. This result supports a negative feedback model by which RA induction of RIP140 inhibits retinoid receptor activity in the continued presence of ligand.

Others have shown that estrogen receptor target gene expression is phasic in the constant presence of ligand (24, 25). For ER target genes, peak transcription rate occurs within 1–3 h in the constant presence of estrogen. Thereafter the rate gradually declines over 24 h despite continued estrogen treatment (24). To address whether RA target genes demonstrate a similar pattern of expression and the generality of RIP140 effects on RA target gene regulation, the expression of the RA target genes CYP26A1 and CRABP2 was measured in the continuous presence of RA in MCF-7 cells. As depicted in the top portion of Fig. 2B, total steady state levels of CRABP2 and CYP26A1 mRNA steadily increased in the continued presence of RA, and this was enhanced by RIP140 siRNA. The rate of CRAB2 and CYP26A1 expression was then measured by hN-PCR. In control cells the rate of transcription of CRABP2 and CYP26A1 peaked at 1 and 6 h of RA treatment, respectively, and then declined (Fig. 2B bottom). Although the timing of the peak transcription rate was slightly altered by RIP140 siRNA, the major effect was an abrogation of the decline in transcription rate, which occurred in control cells at the later time points. In RIP140 siRNA cells, the rate for CRABP2 held steady at 8 and 24 h of RA treatment, although the decline at 6 h was still present suggesting that other mechanisms of attenuation independent of RIP140 occur at this earlier time point for CRABP2. In RIP140 siRNA cells the transcription rate for CYP26A1 continued to increase through 6, 8, and 24 h of RA treatment (Fig. 2B bottom). These data indicate that the greater accumulation of total RA target gene mRNA by RIP140 siRNA is not due primarily to an increase in transcription rate per se but rather is due to a prolongation of peak transcription rate. These data indicate that like the estrogen receptor, retinoid receptors also mediate phasic transactivational activity in the constant presence of cognate ligand, and supports a role for RIP140 in this process.

Inhibition of RA-induced Expression of RIP140 Accelerates Differentiation and G1 Cell Cycle Arrest—The data thus far indicate that inhibiting RA induction of RIP140 increases retinoid receptor transactivation and RA target gene expression. The effects of perturbing RIP140 expression on RA-induced differentiation and G1 arrest were studied. Remarkably, RIP140 siRNA but not a control siRNA accelerated the time...
course of RA-induced neuronal differentiation as assayed by the neuroectodermal marker A2B5 (Fig. 3, A and B). We have previously demonstrated that 1 µM RA treatment maximally induces expression of A2B5 within 5 days (23). At 2 and 3 days of RA treatment, control siRNA cells were essentially A2B5-negative. In contrast, substantial RA-induced A2B5 expression was seen in RIP140 siRNA cells within 2 days. The maximal differential effect of RIP140 siRNA was at 3 days of RA treatment, where values for mean fluorescence and percent positive cells were 460 and 57%, respectively, for RIP140 siRNA-treated cells versus 88 and 17.6%, respectively, for control siRNA (Fig. 3B). Likewise, accelerated loss of expression of the stem cell marker SSEA3 was seen upon RA treatment of RIP140 siRNA-transfected cells as compared with controls (data not shown). G_{0} cell cycle arrest is closely associated with RA-induced differentiation of NT2/D1 cells (23). RIP140 siRNA also accelerated RA-induced G_{0} arrest in NT2/D1 cells at 2 and 3 days of RA treatment as compared with controls (Fig. 3C). Together, these data indicate that RIP140 is rate-limiting for repressing ligand-dependent receptor transactivation, endogenous RA target gene expression, and RA-induced differentiation in NT2/D1 cells.

**DISCUSSION**

Models have been proposed to explain the self-limitation of transcriptional activation by nuclear receptors. Chen et al. (24) reported that the rate of estrogen receptor target gene expression peaks early in response to estrogen and gradually declines over 24 h in the constant presence of ligand. This correlated with transient formation of co-activator complexes on estrogen target gene promoters (24, 25). A mechanism proposed for this attenuation involved p300 acetylation of the p160 activator, ACTR (activator of thyroid and retinoid acid receptors), which led to disassembly of the coactivator complex (24). Thus post-translational modification is one mechanism for attenuation of nuclear receptor signaling. In contrast, relatively little attention has been given to transcriptional regulation of coregulators. Based on the current study, we propose that an additional attenuation mechanism for nuclear receptors is classical negative feedback inhibition. In this model, activated retinoid receptors induce the production of a nuclear receptor corepressor, RIP140. That RIP140 inhibits retinoid receptors in a ligand-dependent fashion is a key feature of this model. RIP140 overexpression inhibited ligand-dependent RARE promoter activity. Notably, prevention of RA induction of RIP140 via siRNA dramatically induced RARE activity and increased levels of RA target gene expression. Remarkably, RIP140 silencing also accelerated RA-induced terminal differentiation and cell cycle arrest. This indicates the novel finding that RIP140 can be a rate-limiting factor in regulating nuclear receptor activity.

RIP140 is widely expressed and can inhibit distinct agonist-bound nuclear receptors (9, 26). Further RIP140 has been reported to be modestly induced by estrogen (27). It will be important to further define the extracellular cues regulating the expression of RIP140 and other coregulators. Because RIP140, as supported here and by prior in vitro interaction and transactivation studies, may inhibit a number of agonist-bound nuclear receptors, the possibility exists that RIP140 may participate in cross-talk among distinct nuclear receptor classes and contribute to the integration of diverse hormonal signals. Based on our findings, careful analysis of the relative potency of RIP140 toward distinct nuclear receptors is warranted. Although the precise interplay between RIP140 and other coregulators in receptor regulation is unclear, RIP140 and p160 coactivators have been reported to be at least partially competitive in binding nuclear receptors (12, 15, 28). Recently, the RNA helicase, DP97, and the C-terminal-binding protein CBLP, CLoR, were shown to repress nuclear receptors in a ligand-dependent fashion, suggesting that this form of corepression could be broadly engaged in diverse cells and tissues (29, 30).

In summary, these data support a straightforward model of regulatory control involving negative feedback inhibition mediated by RA induction of a ligand-dependent corepressor. We have shown that part of the coregulator machinery, RIP140, is itself a direct target of RA. This is of particular importance considering that coregulators are proposed, as demonstrated here for RIP140, to be a rate-limiting component of nuclear receptor activity. Further, we have demonstrated that RIP140 has potent effects both in modulating retinoid receptor activity and on the efficacy of retinoids to signal growth suppression and differentiation. Further studies will be required to uncover the full biological and therapeutic impact of this form of regulation.

**REFERENCES**

1. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) The Retinoids: Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 319–349, Raven Press, Ltd., New York
2. Chamberlain, P. (1996) FASEB J. 10, 940–954
3. Gamble M. J., and Freedman L. P. (2002) Trends Biochem. Sci. 27, 165–167
4. McKenna, N. J., and O’Malley, B. W. (2002) Cell 108, 465–474
5. Hassmann, O., Glass, C. K., and Rosenfeld, M. G. (2002) Trends Endocrinol. Metab. 13, 55–60
6. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Glass, B., Kurokawa, R., Ryan, A., Kaimer, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Nature 377, 297–304
7. Chen, J. D., and Evans R. M. (1995) Nature 377, 454–457
8. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1995) Nature 377, 723–726
9. Parker, M. G., Leonardsson, G., White, R., Steel, J., and Miligan, S. (2003) FEBS Lett. 546, 149–153
10. Cavailles, V., Dauvois, S., L’Hesbor, P., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) EMBO J. 14, 3741–3751
11. Lee, C. H., and Wei, L. N. (1999) J. Biol. Chem. 274, 31320–31326
12. Treuter, E., Albrechtsen, T., Johansson, L., Leers, J., and Gustafsson, J. A. (1998) Mol. Endocrinol. 12, 864–881
13. Miyata, K. S., McCaw, S. E., Meertens, L. M., Patel, H. V., Rachubinski, R. A., and Capone, J. P. (1998) Mol. Cell. Endocrinol. 146, 69–76
14. Eng, F. C., Barsalou, A., Akutsu, N., Mercer, I., Zechel, C., Mader, S., and White, J. H. (1998) J. Biol. Chem. 273, 28371–28377
15. Tessyssier, C., Guilouise, K., Galtier, F., Cavailles, V., and Chalbos, D. (2003) Mol. Endocrinol. 17, 297–309
16. Wei, L. N., Xu, H., Chandra, D., Seto, E., and Farooqui, M. (2000) J. Biol. Chem. 275, 40782–40787
17. Vo, N., Fjeld, C., and Goodman, B. H. (2001) Mol. Cell. Biol. 21, 6181–6188
18. Freemantle, S. J., Kerley, J. S., Olsen, S. L., Gross, R. H., and Spinella, M. J. (2002) Oncogene 21, 2880–2889
19. Kerley, J. S., Olsen, S. L., Freemantle, S. J., and Spinella, M. J. (2001) Biochem. Biophys. Res. Commun. 285, 969–975
20. Spinella, M. J., Kerley, J. S., White, A. J., and Curtin, J. C. (2003) J. Nutr. 133, 2738–2743
21. Andrews, P. W. (1998) Acta Pathol. Microbiol. Immunol. Scand. 106, 158–168
22. Curtin, J. C., Dragnev, K. H., Sekula, D., Christie, A. J., Dmitrovsky E., and Spinella, M. J. (2001) Oncogene 20, 2559–2569
23. Spinella, M. J., Freemantle, S. J., Sekula, D., Chang, J. H., Christie, A. J., and Dmitrovsky, E. (1999) J. Biol. Chem. 274, 22013–22018
24. Chen, H., Lin, R. J., Xie, W., Wiltz, D., and Evans, R. M. (1999) Cell 98, 675–686
25. Shang, Y., Hu, X., DiBenzo, J., Lazar, M. A., and Brown, M. (2000) Cell 103, 843–852
26. Lee, C. H., Chinpaisal, C., and Wei, L. N. (1998) Mol. Cell. Biol. 18, 6745–6755
27. Thenot, S., Charpin, M. Bonnet, S., and Cavailles, V. (1999) Mol. Cell. Endocrinol. 156, 85–93
28. Subramanian, N., Treuter, E., and Okret, S. (1999) J. Biol. Chem. 274, 18121–18127
29. Rajendran, R. R., Nye, A. C., Frazer, J., Balsara, R. D., Balsara, R. P., Martini, P. G., and Katzenellenbogen, B. S. (2003) J. Biol. Chem. 278, 4628–4638
30. Fernandez, I., Bastien, Y., Wai, T., Nygard, K., Lin, H., Curnier, O., Lee, H. S., Eng, F., Bertos, N. R., Pelletier, N., Mader, S., Han, V. K., Yang, X., and White, J. H. (2000) Mol. Cell 11, 139–150