Retinoic Acid Receptor Isotype Specificity in F9 Teratocarcinoma Stem Cells Results from the Differential Recruitment of Coregulators to Retinoic Acid Response Elements

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The retinoic acid receptor (RAR) α, β2, and γ isotypes each regulate specific subsets of target genes in F9 teratocarcinoma stem cells. We used chromatin immunoprecipitation assays to monitor the association of RARγ, retinoic X receptor (RXR) α, and coregulators with the RARB2, Hoxa1, and Cyp26A1 retinoic acid response elements (RAREs) in F9 wild type and RARα, β2, and γ null cells. Additionally we quantitatively monitored expression of the corresponding mRNAs. We demonstrated that the association of RARγ and/or RXRα with a RARE was not sufficient for retinoic acid (RA)-mediated transcription of the corresponding target gene. However, the ability of RARγ and/or RXRα to recruit pCIP (AIB1/ACTR/RAC-3/TRAM-1/SRC-3) and p300 to a RARE did correlate with RA-associated transcription of target mRNAs. Therefore, the specific functions of the RAR isotypes do not manifest at the level of their DNA binding but rather from a differential ability to recruit specific components of the transcriptional machinery. We also demonstrated that RA-mediated displacement of the polycomb group protein SUZ12 from a RARE was inhibited in the absence of RARγ. Thus, transcriptional components of the RAR signaling pathway are specifically required for displacement of SUZ12 from RAREs during RA-mediated differentiation of F9 cells.

Retinoic acid (RA) is an important regulator of vertebrate development and homeostasis because of its role in essential processes such as apoptosis, cell differentiation, and proliferation (1, 2). The effects of RA are mediated through binding to the retinoic acid receptors (RARs) (3) and retinoic X receptors (RXRs) (4), which are members of the nuclear receptor superfamily (5). The RARs and RXRs each have three isotypes (α, β, and γ), which are encoded by distinct genes. In addition, for each RAR isotype, there are several isoforms generated by differential promoter usage and splicing (3, 4). The multiple RAR and RXR isotypes and isoforms are conserved in vertebrate evolution and display distinct spatiotemporal expression patterns in developing embryos and adult tissues, suggesting that each receptor performs some unique functions (1).

RXR-RAR heterodimers bind much more efficiently to retinoic acid response elements (RAREs) than their respective homodimers in vitro (6), and several lines of evidence support the idea that these heterodimers represent the functional units transducing the retinoid signal in vivo (3). RAR-RXR heterodimers are thought to be constitutively associated with RAREs and to actively repress transcription in the absence of ligand through association with the corepressors nuclear receptor corepressor or SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) (7–9). Nuclear receptor corepressor and SMRT are found in repressor complexes containing the histone deacetylase HDAC3 (10, 11).

The F9 murine embryonal carcinoma cell line has been extensively used as a cell autonomous model system to study RA signaling. F9 cells resemble the pluripotent stem cells of the inner cell mass of the early embryo and differentiate into three distinct extraembryonic (primitive, parietal, and visceral) endodermal cell types upon treatment with RA, depending on the cell culture conditions (12). F9 cells express all known RA receptors, but RARB2 mRNA is only present in high amounts after RA addition (13).

Specific functions for each of the RARs have been demonstrated through the use of F9 cells in which each of the individual RARs has been knocked out by homologous recombination as well as through the use of synthetic isotype-selective ligands. F9 RA null cells exhibit marked modulation of a variety of genes when compared with the F9 wild type (WT) cells. For example, the RA-mediated transcription of the homeobox gene Hoxa1 is specifically regulated by RARγ because the RA-induced expression of Hoxa1 mRNA is abrogated in F9 RARγ−/− cells (14). Additionally a synthetic retinoid selective for RARγ can induce expression of Hoxa1 mRNA in F9 WT cells but not in F9 RARγ−/− cells (15). Furthermore RARγ−/− cells fail to exhibit complete morphological differentiation in culture when...
treated with RA (14). Another example of isotype-specific function comes from studies utilizing a F9 RARβ2−/− cell line. In contrast to F9 Wt, RARα−/−, and RARγ−/− cell lines, the F9 RARβ2−/− cell line exhibits no growth arrest in response to RA (16). Additionally a number of genes specifically regulated by the RARβ2 isoform in F9 cells have been identified through use of subtractive hybridization and DNA array analysis (17). RARα also specifically regulates RA target genes in F9 cells as the expression of both Hoxa1 and CRABP-II is reduced in F9 RARα−/− cells compared with F9 Wt and F9 RARγ−/− cells (18). It should be noted that in the F9 RAR null cell lines the expression levels of the undisrupted RARs were similar to those found in F9 Wt cells (19). These results demonstrate that each of the RAR isotypes regulates a specific subset of target genes in F9 cells when the RAR isotypes are expressed at endogenous levels.

A degree of functional redundancy among the three RAR isotypes has also been demonstrated through use of the F9 RAR null cells and isotype-selective ligands. For example, ~10-fold overexpression of RARα in F9 RARγ−/− cells could restore target gene activation of RARγ target genes such as Hoxa1 as well as the differentiation potential of F9 RARγ−/− cells (20). In the same set of experiments, however, overexpression of RARβ2 could not restore Hoxa1 mRNA expression in F9 RARγ−/− cells. Additionally the expression of Hoxa1 mRNA in F9 RARγ−/− cells could be restored by exposure to an RARα-selective ligand (15). However, this same RARα-selective ligand inefficiently induced expression of Hoxa1 mRNA in F9 Wt cells (15), indicating that the presence of RARγ can hinder the ability of RARα, bound to an RARα-selective agonist, to induce RA target genes. Furthermore an RARβ-specific agonist could induce expression of another RA target gene, RARβ2, itself, in F9 RARγ−/− cells but not in F9 Wt or RARα−/− cells (15). These results demonstrate that some of the functional redundancies observed among individual RARs in F9 RAR null cells do not exist in the context of wild type cells.

In this study we used ChIP assays to monitor how the association of RARγ, RXRα, and coregulators with the RAREs regulating expression of the Hoxa1 (21), RARβ2 (22), and Cyp26A1 (23, 24) genes is affected in the various F9 RAR null cell lines as compared with F9 Wt cells. Additionally we quantitatively monitored the expression of these target genes in the above-mentioned F9 cell lines by real time PCR. We demonstrated that RARγ was associated with the RAREs that regulate the transcription of the Hoxa1 and Cyp26A1 mRNAs. We also showed that RARγ was associated with the RARβ2 RARE even though RARγ was not required for the RA-induced expression of RARβ2 mRNA. Furthermore we demonstrated that the presence of RARγ and RXRα at a RARE was not sufficient for the recruitment of factors required for transcription, such as p300 and pCIP, to the corresponding target genes. We also showed that the levels of the polycomb repressive protein SUZ12 associated with the RAREs monitored in this study were significantly higher in RARγ−/− cells as compared with F9 Wt and F9 RARβ2−/− cells. Thus, we demonstrated that components of the RAR signaling apparatus were specifically required for displacement of SUZ12 from RAREs during the RA-mediated differentiation of F9 cells.

MATERIALS AND METHODS

Cell Culture—F9 Wt, RARα−/−, RARβ2−/−, and RARγ−/− embryonal carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in gelatin-coated tissue culture plates ~48 h prior to RNA harvesting (5 × 106 cells/60-mm dish) or formaldehyde fixation (2.5 × 106 cells/20-cm dish). Cells were treated with 1 μM retinoic acid for 24 h.

Antibodies and Chemicals—All-trans-RA was obtained from Sigma-Aldrich and dissolved in ethanol. Anti-RARγ serum was generated by immunization of rabbits with a peptide corresponding to the F region of RARγ (NH2-PGPHPKASER-DEAPGGQGKRQGS-COOH). Polyclonal anti-RARγ IgG was purified from the crude serum through use of a DEAE Affi-Gel blue gel column (Bio-Rad). Anti-RXRs (D-20, sc-553), anti-pCIP (M-397, sc-9119), anti-p300 (N-15, sc-584), and anti-actin (I-19, sc-1616) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser-5 carboxyl-terminal domain (CTD) of RNA polymerase II (pCTDser5) was purchased from Covance Research Products (Richmond, CA).

RNA Preparation and RT-PCR—RNA was prepared and subjected to semiquantitative or real time RT-PCR analysis as described previously (25).

Characterization of F9 RARα, β2, and γ Null Cell Lines—RNA was prepared and subjected to semiquantitative RT-PCR analysis. The primers used to detect RARα spanned the exon in which the disruption cassette was inserted and were as follows: 5′-ATCGAGACCCACAGACAGCACG-3′ and 5′-CCTGGTGCGCCTTGGCAGAC-3′ (18). The primers used to detect RARγ also spanned the exon in which the disruption cassette was inserted (14) and were as follows: 5′-CAATAAGAGAGACACTCTTGGCC-3′ and 5′-TGCTGACCTTGTGATGAGTT-3′. The primers used to detect RARβ2 (16) were as follows: 5′-GATCCTGGATTCTACACCAGG-3′ and 5′-CACTGACGCCCATGTTGA-3′.

ChIP Assays—ChIP assays were performed as described previously (25).

Semiquantitative and Real Time PCR—Semi-quantitative PCRs and real time PCRs were performed as described previously (25).

Western Blot Analysis—Whole cell extracts were prepared from COS cells that were either mock-transfected or transfected with a plasmid expressing RARα, RARβ, or RARγ. Five micrograms of each of the COS whole cell extracts were resolved by 12% SDS-PAGE followed by transfer to a nitrocellulose membrane (0.45-μm pore size; catalog number 162-0090, Bio-Rad). Primary antibody incubation was done overnight at 4°C. The anti-RARγ blue eluate, as described above, was used at a 1:200 dilution to detect RARγ. After a 1-h incubation with an immunoglobulin G horseradish peroxidase-conjugated secondary antibody at room temperature (anti-rabbit, 1:40,000 dilution; sc-2030, Santa Cruz Biotechnology), the membranes were developed with SuperSignal Substrate (Pierce) for 5 min and exposed to BioMax film (Eastman Kodak...
Co.). Primary and secondary antibodies were diluted in phosphate-buffered saline containing 5% Blotto (Santa Cruz Biotechnology) and 0.1% Tween 20. Blots were stripped with Restore Plus Western Blot Stripping Buffer (Pierce, 46430) and then reprobed with an anti-actin antibody (1:400 dilution) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-goat, 1:2000 dilution; sc-2056, Santa Cruz Biotechnology).

RESULTS

We have previously studied the association of RARγ, RXRα, and other proteins involved in transcriptional regulation with the Hoxa1, RARβ2, and Cyp26A1 RAREs (R1 and R2) during the course of RA treatment in F9 Wt cells (25). The main purpose of this study was to determine how the association of RARγ, RXRα, and coregulators with the aforementioned RAREs is affected by the absence of each of the three RAR isotypes. Therefore, we utilized F9 cells lines in which both alleles of RARα (18), RARβ2 (the predominant isoform of RARβ) (16), and RARγ (14) were individually knocked out by homologous recombination.

To confirm that the RAR isotypes were individually knocked out in our cell lines, we monitored expression of the RAR isotypes in the various F9 RA null cell lines by semiquantitative RT-PCR. F9 Wt and F9 RARα−/−, -β2−/−, and -γ−/− cell lines were either untreated or treated with 1 μM RA for 24 h, and RNA was harvested. RARα mRNA was detected in RARγ−/− and RARβ2−/− cells at levels similar to those in F9 Wt cells irrespective of the presence of RA (Fig. 1A). As expected, no RARα mRNA could be detected in F9 RARα−/− cells (Fig. 1A). The expression of RARγ mRNA could not be detected in RARγ−/− cells, although RARγ mRNA was expressed in RARα−/− and RARβ2−/− cells at levels similar to those seen in F9 Wt cells irrespective of the presence of RA (Fig. 1A). In contrast to RARα and RARγ, the expression of RARβ2 was strongly induced by the presence of RA in F9 Wt cells (Fig. 1A). RA-induced expression of RARβ2 mRNA was observed in RARα−/− and RARγ−/− cells, and as expected, RARβ2 mRNA could not be detected in RA-treated RARβ2−/− cells (Fig. 1A).

To ensure that equivalent amounts of RNA were used in the RT-PCR assays, expression of the ribosomal phosphoprotein 36B4 “housekeeping gene” (26) was monitored. All samples expressed 36B4 mRNA to similar levels (Fig. 1A). Thus, we confirmed that the three different RAR null cell lines each specifically lacked expression of one RAR isotype.

RA Induced Expression of Hoxa1, RARβ2, and Cyp26A1 mRNAs in F9 Wt and RAR Null Cell Lines—We next determined whether Hoxa1, RARβ2, and Cyp26A1 mRNAs could be induced by RA in the F9 RARα, -β2, and -γ null cell lines as these three genes are all strongly induced by RA in F9 Wt cells (27–29). F9 Wt and the RAR null cell lines were either untreated or treated with 1 μM RA for 24 h, and RNA was harvested for use in RT-PCR. Real time PCR was used to quantitate RT-PCR products corresponding to the three RA target genes. As expected, all three RA target genes were strongly induced (>22-fold) by RA treatment in F9 Wt cells (Fig. 1B). Again we also monitored the levels of 36B4 mRNA in the F9 Wt, RARα−/−, RARβ2−/−, and RARγ−/− cell lines. Similar levels of 36B4 mRNA were observed in all of the cell lines both in the presence and absence of RA (Fig. 1B).

Hoxa1 mRNA levels in RARα−/− cells were similar to those seen in F9 Wt cells cultured in the presence or absence of RA (Fig. 1B) in agreement with previous reports (18). This result demonstrates that RARα is not required for expression of the Hoxa1 gene. Additionally in the presence of RA, RARβ2, mRNA levels were similar in F9 Wt and RARα−/− cells, demonstrating that RARα is not required for induction of RARβ2 by RA. However, in untreated RARα−/− cells, as compared with F9 Wt cells, ~2.9-fold higher levels of RARβ2 transcripts (p value <0.0001) were expressed (Fig. 1B). This latter result indicates that in the absence of RA RARα may have a role in the repression of RARβ2 (30). Levels of Cyp26A1 mRNA after RA treatment of F9 RARα−/− cells were ~57% of the levels observed in F9 Wt cells (Fig. 1C), indicating that RARα has a role in the RA-induced expression of this gene.

Previous reports have demonstrated that RARγ is required for the expression of both the Hoxa1 (18) and Cyp26A1 (27) genes in F9 Wt cells. Our results are in agreement with these reports as we did not observe expression of either Hoxa1 or Cyp26A1 mRNA in RA-treated F9 RARγ−/− cells (Fig. 1, B and C), whereas a >22-fold induction of these mRNAs was observed in F9 Wt cells. RA-induced expression of RARβ2 also was reduced in RARγ−/− cells (~43% of F9 Wt) relative to F9 Wt cells (Fig. 1, B and C). Thus, we concluded that RARγ has a prominent role in the regulation of Hoxa1 and Cyp26A1 and a lesser role in the induction of RARβ2 transcripts in response to RA.

As expected, expression of RARβ2 mRNA was abrogated in RARβ2−/− cells as RARβ2 transcripts detected in RARβ2−/− cells were less than 1% of the levels observed in F9 Wt cells (Fig. 1, B and C). The low levels of RARβ2 transcripts detected in RARβ2−/− cells may represent fusion transcripts generated through the RARβ2/RARE that remains intact in these cells. Additionally the RA-induced expression of Hoxa1 (~28%) and Cyp26A1 (~34%) transcripts was lower in F9 RARβ2−/− cells relative to levels observed in F9 Wt cells (Fig. 1C), consistent with data from our laboratory (16).

The Association Patterns of RARγ and RXRα with the RAREs Regulating Expression of the Hoxa1 Gene in F9 Wt and F9 RARα, -β2, and -γ Null Cell Lines—We previously demonstrated that RARγ and RXRα, both in the presence and absence of RA, are associated with the RAREs that regulate expression of the Cyp26A1 (R1 and R2), Hoxa1, and RARβ2 genes in F9 Wt cells (25). These results are consistent with previous studies that have also demonstrated that RAR-RXR heterodimers are constitutively associated with RAREs (31, 32). In this study we wanted to determine whether the absence of each of the RAR isotypes in F9 cells would affect the association of RARγ and RXRα with these RAREs. Therefore, we monitored the association of RARγ and RXRα with the aforementioned RAREs through use of a two-step ChIP assay (33). F9 Wt and F9 RARα, -β2, and -γ null cell lines were cultured in the presence or absence of RA for 24 h and then subjected to the protein–protein cross-linking reagent disuccinimidyl glutarate. Cells were then formaldehyde-fixed as in conventional ChIP assays, and soluble chromatin was prepared as described under "Materials.
and Methods.” Antibodies to RARγ and RXRα were used to immunoprecipitate protein-DNA complexes from soluble chromatin. Nonspecific rabbit IgG antibodies were also used as a negative control in the two-step ChIP assays.

The anti-RARγ IgG used in the two-step ChIP assays was generated in our laboratory (see “Materials and Methods”). To determine the specificity of the anti-RARγ IgG, we prepared individual COS cell extracts from cells overexpressing each of the RAR isotypes. COS cell extract was also prepared from cells that were mock-transfected. The COS cell extracts were resolved by SDS-PAGE and subsequently subjected to immunoblot analysis against the anti-RARγ IgG. The anti-RARγ IgG specifically recognized antigen from COS cells overexpressing RARγ, and the positive signal was at the expected molecular mass for RARγ (Fig. 2B). These results demonstrate that the anti-RARγ IgG specifically recognizes the RARγ isotype.

The Hoxa1 RARE is located ~2 kb downstream of the Hoxa1 gene, whereas the RARβ2 RARE is located ~55 bp upstream of the transcription start site (Fig. 2A). Cyp26A1 contains a RARE ~70 bp upstream of the transcription start site denoted as R1 (23) as well as a more recently described RARE denoted as R2 (24) found ~1950 bp upstream of R1 (Fig. 2A). As a control for the nonspecific IP of DNA in ChIP assays, we also measured a gene-free region located ~18 kb downstream of the Hoxb1 gene (Hoxb1 ~18 kb). Levels of these five DNA regions recovered in ChIP assays were quantitated by real time PCR assays. We define -fold enrichment as the percentage of input of a specific locus in an IP divided by the percentage of input of the
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FIGURE 2. A, diagram of the loci monitored in ChIP assays in relation to respective transcription start sites. DNA is represented with a thin black line. Gray boxes denote introns; black boxes refer to exons. The location of RAREs are represented by arrows pointing downward, and the actual RARE sequences are shown directly below the arrows with bold letters denoting binding sites. Bent arrows indicate transcription start sites; hatch marks signify exon/intron gene architecture that is not detailed. Thick black lines indicate regions of DNA amplified during ChiP assays. Schematics were drawn approximately to scale. B, demonstration of the specificity of the anti-RARγ antibody. Whole cell extracts were prepared from COS cells that were either mock-transfected (m) or transfected with a plasmid expressing RARα (α), RARβ (β), or RARγ (γ). Five micrograms of each of the COS whole cell extracts were resolved by 12% SDS-PAGE followed by Western blot analysis. Anti-RARγ blue eluate was used at a 1:200 dilution to detect RARγ. The experiment was performed three times. PP, promotor proximal region.

Hoxb1 18 kb 3' negative control region in the same IP (Fig. 3). The data are presented as -fold enrichment to normalize for the higher levels of nonspecific DNA (Hoxb1 18 kb 3' locus) found in RXRα IPs as compared with RARγ IPs in the two-step ChIP assays.

The levels of RARγ associated with the Hoxa1 RARE in the presence and absence of RA in F9 Wt cells were similar (Fig. 3A, middle panel; -fold enrichment). However, the levels of RARγ associated with the Hoxa1 RARE increased in F9 RXRα−/− (−3.5-fold, p value < 0.05) and RARβ2−/− (−2.7-fold, p value < 0.01) cells as a result of RA treatment (Fig. 3A, middle panel). Additionally higher levels of RARγ were associated with the Hoxa1 RARE in RXRα−/− cells (−2.7-fold, p value < 0.05) and in RARβ2−/− cells (−1.7-fold, p value < 0.06) treated with RA as compared with RA-treated F9 Wt cells (Fig. 3A, middle panel). The levels of Hoxa1 RARE DNA immunoprecipitated with RARγ when using soluble chromatin derived from RARγ−/− cells (Fig. 3B, middle and right panels) were comparable to the background levels of Hoxa1 RARE DNA immunoprecipitated with nonspecific rabbit IgG (Fig. 3A, right panel). This result further confirms the isotype specificity of the anti-RARγ IgG used in this study.

We also examined the association of RXRα with the Hoxa1 RARE in F9 Wt and the F9 RXRα−/−, -β2, and -γ null cell lines (Fig. 3A, left panel). Previous studies have demonstrated that RXRα is required for the RA-induced expression of Hoxa1 in F9 cells because Hoxa1 is not expressed in F9 RXRα−/− cells treated with RA (34). Although Hoxa1 mRNA is not expressed in RARγ−/− cells (Fig. 1B), RXRα was associated with the Hoxa1 RARE in these F9 RARγ−/− cells presumably as a heterodimer with either RARβ2 or RARα. However, the levels of RXRα associated with the Hoxa1 RARE in untreated (~39% of Wt) and RA-treated (~75% of Wt) F9 RARγ null cells were lower (p value < 0.05 for both comparisons) than those in F9 Wt cells (Fig. 3A, left panel), indicating that RAR-RXRα heterodimer association with the Hoxa1 RARE is reduced, although not eliminated, in F9 RARγ−/− cells as compared with F9 Wt cells.

The Association Patterns of RARγ and RXRα with the RAREs Regulating Transcription of Cyp26A1 and RARβ2 mRNAs in F9 Wt and F9 RARα−/β2−/− Cells—Consistent with our previous report (25) high levels of RARγ were associated with the Cyp26A1 R2 RARE in the absence and presence of RA in F9 Wt cells (Fig. 3B, middle panel). The levels of RARγ associated with the R2 RARE in the F9 RXRα null and F9 RXRβ2 null cells were similar to the levels observed in F9 Wt cells (Fig. 3B, middle panel). Again the Cyp26A1 R2 RARE was not detected in RARγ IPs utilizing soluble chromatin derived from F9 RXRα−/− cells; only backgrounds levels were seen (Fig. 3B, middle panel).

The levels of RXRα associated with the Cyp26A1 R2 RARE in F9 RXRβ2 null and RXRα null cells were similar to those seen in F9 Wt cells (Fig. 3B, left panel). Therefore, although RARγ is required for expression of Cyp26A1 (Fig. 1B), RXRα can still associate with the R2 RARE in the absence of RARγ presumably as a heterodimer with RARβ2 or RARα. This result suggests that RARβ2-RXRα or RARα-RXRα heterodimers bound at the R2 RARE are not able to transduce a RA signal, culminating in transcription of the Cyp26A1 gene, in response to RA. Similar patterns of association of RXRα and RARγ with the Cyp26A1 R1 RARE compared with the R2 RARE were observed, although -fold enrichment levels were lower at the R1 RARE compared with the R2 RARE (Fig. 3, B and C).

We wanted to address whether RARγ associates with the RARβ2 RARE in F9 RXRβ2−/− cells even though these cells do not express RARβ2 mRNA in response to RA (Fig. 1, B and C). In contrast to the Hoxa1 and Cyp26A1 genes, RARγ was not required for transcription of RARβ2, although RA-induced
FIGURE 3. RARγ and RXRα associate with the target RAREs in F9 Wt and F9 RARγ, -βγ, and -γ null cells. F9 Wt and F9 RARα, -βα, and -γ null cell lines were either untreated or treated with 1 μM RA for 24 h. Cells were then fixed with disuccinimidyl glutarate and formaldehyde and processed into soluble chromatin. Chromatin samples were immunoprecipitated with antibodies to RARγ, RXRα, or IgG, and bound DNA was quantitated by real time PCR. The data are presented as -fold enrichment (mean ± S.E.). -Fold enrichment is defined as the percentage of input of a specific locus in an IP divided by the percentage of input of the Hoxa1 18 kb 3′ negative control region in the same IP. Error bars indicate standard error of three biological replicates with each quantitative PCR performed in triplicate. KO, knock-out.

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A. RXRα

B. Cyp26A1 R2 RARE

C. Cyp26A1 R1 RARE

D. RARβ2 RARE

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expression levels of RARβ2 were reduced in RARγ−/− cells as compared with F9 Wt cells (Fig. 1, B and C). RARγ was associated with the RARβ2, RARE in both the presence and absence of RA (Fig. 3D, middle panel) in F9 Wt cells. Additionally the levels of RARγ associated with the RARβ2, RARE in the F9 RARβ2 null and F9 RARα null cell lines were comparable to the levels seen in the F9 Wt cells (Fig. 3D, middle panel). Therefore, we have demonstrated that RARγ associated with the RARβ2, RARE is incapable of transducing the RA signal required for expression of RARβ2 transcripts in the absence of RARβ2 protein.

We also determined whether the association level of RXRα with the RARβ2, RARE is perturbed in F9 RARβ2−/− cells as compared with F9 Wt, F9 RARγ−/−, and F9 RARα−/− cells. In contrast to RARγ, lower levels of RXRα were associated with the RARβ2, RARE, both in the presence (∼43% of Wt) and absence (∼25% of Wt) of RA, in F9 RARβ2−/− cells as compared with F9 Wt cells (Fig. 3D, left panel; p value < 0.05 for both comparisons). Additionally the levels of RXRα associated with the RARβ2, RARE were lower in the F9 RARβ2−/− cells as compared with the F9 RXRα and γ null cell lines (Fig. 3D, left panel). Therefore, the absence of RARβ2 impedes the association of RXRα with the RARβ2, RARE, consistent with previous reports that demonstrated an autoregulatory role for the RARβ2 protein (15, 20, 35).

RA-mediated Recruitment of pCIP and p300 to the RAREs That Regulate Expression of the Hoxa1 and Cyp26A1 Genes Is Compromised in F9 RARγ−/− Cells as Compared with F9 Wt and RARβ2−/− Cells—We demonstrated that RARγ was required for the RA-induced expression of Hoxa1 and Cyp26A1 mRNAs (Fig. 1, B and C) in F9 cells. Additionally we showed that RARβ2 transcripts were not expressed in F9 RARβ2−/− cells. Therefore, we wanted to examine whether the recruitment of coregulators to the RAREs regulating the aforementioned genes is perturbed in F9 RARβ2−/− and F9 RARγ−/− cell lines. We were able to utilize conventional one-step ChIP assays to monitor the association of the p160 coactivator pCIP (also referred to as AIB1/ACTR/RAC-3/TRAM-1/SRC-3) (36) as well as the histone acetyltransferase coactivator p300 (37) to the RAREs in the F9 RARβ2−/− and F9 RARγ−/− cell lines as well as in the F9 Wt cells.

Consistent with our previous results (25), the levels of pCIP associated with the Hoxa1 RARE rose ∼4.3-fold in F9 Wt cells as a result of RA treatment (Fig. 4A, left panel). The levels of pCIP associated with the Hoxa1 RARE in untreated RA-treated RARβ2−/− cells were similar to those seen in F9 Wt cells (Fig. 4A, left panel), although the RA-induced expression of Hoxa1 mRNA was reduced in F9 RARβ2−/− cells to ∼25% of the level observed in F9 Wt cells (Fig. 1, B and C). However, in F9 RARγ−/− cells, which do not express Hoxa1, the levels of pCIP associated with the Hoxa1 RARE were reduced both in the absence (∼3-fold) and presence (∼4-fold) of RA relative to F9 Wt cells (Fig. 4A, left panel). Additionally the levels of pCIP associated with the Hoxa1 RARE in RA-treated F9 RARγ−/− cells were similar to the basal levels of pCIP associated with the Hoxa1 RARE in F9 Wt and F9 RARβ2−/− cells (Fig. 4A, left panel). Therefore, we concluded that coactivators are not recruited by RAR-RXR heterodimers bound at the Hoxa1 RARE in F9 RARγ−/− cells treated with RA.

We also monitored pCIP association with the Cyp26A1 R1 RARE located immediately upstream of the Cyp26A1 transcription start site (Fig. 2A). The recruitment patterns of pCIP to the Cyp26A1 R1 RARE in the three F9 cell lines were similar to those seen for the Hoxa1 RARE (Fig. 4A, compare left panel and middle panel). The levels of pCIP associated with the Cyp26A1 R1 RARE rose in the F9 Wt by ∼3.9-fold and in F9 RARβ2−/− cells by ∼5.4-fold as a result of RA treatment, and this RA-associated increase was not observed in the F9 RARγ−/− cell line (Fig. 4A, middle panel). Therefore, we concluded that recruitment of pCIP to the RAREs that regulate the transcription of Hoxa1 and Cyp26A1 requires RARγ. Furthermore the lack of RA-induced recruitment of pCIP to the Hoxa1 and Cyp26A1 RAREs likely explains why these genes are not expressed in RARγ−/− cells.

The levels of p300 increased at both the Hoxa1 (∼6.9-fold) and Cyp26A1 R1 (∼4-fold) RAREs in F9 Wt cells as a result of RA treatment (Fig. 4B, left and middle panels). Additionally comparable levels of p300 were associated with the Hoxa1 and Cyp26A1 R1 RAREs in F9 RARβ2−/− cells as compared with F9 Wt cells both in the absence and presence of RA (Fig. 4B, left and middle panels). In contrast, RA-associated p300 recruitment to the Hoxa1 RARE was reduced by ∼4.5-fold in F9 RARγ−/− cells relative to F9 Wt cells. Moreover p300 levels did not increase at the Cyp26A1 R1 RARE as a result of RA treatment in F9 RARγ−/− cells. These results further demonstrate that RA-mediated coregulator recruitment to the RAREs that control transcription of Hoxa1 and Cyp26A1 is compromised in F9 RARγ−/− cells.

The Levels of pCIP and p300 Associated with the RARβ2, RARE Do Not Increase in Response to RA in F9 RARβ2−/− Cells—RA-induced transcription of RARβ2 is abrogated in F9 RARβ2−/− cells and reduced ∼2-fold in F9 RARγ−/− cells relative to F9 Wt cells (Fig. 1, B and C). Thus, we monitored the association of pCIP and p300 with the RARβ2, RARE in F9 Wt, RARβ2−/−, and RARγ−/− cell lines. The levels of p300 associated with the RARβ2, RARE increased ∼2.3-fold (p value < 0.08) as a result of RA treatment in F9 Wt cells (Fig. 4B, right panel). Levels of p300 also increased at the RARβ2, RARE by ∼1.8-fold as a result of RA treatment (p value < 0.05) in the F9 RARγ−/− cell line. The high basal levels of pCIP and p300 observed at the RARβ2, RARE are consistent with a previous study that demonstrated that much of the transcriptional machinery is associated with the RARβ2, RARE in P19 embryonal carcinoma cells prior to RA treatment (32). In contrast, we did not observe an RA-induced increase of p300 at the RARβ2, RARE in the F9 RARβ2−/− cell line (Fig. 4B, right panel), although the basal level of p300 associated with the RARβ2, RARE was higher in RARβ2−/− than in the F9 Wt (p value < 0.01) and the F9 RARγ−/− cell lines (p value < 0.01). Recruitment patterns of pCIP to the RARβ2, RARE mirrored those seen for p300 in the three cell lines (compare Fig. 4B, right panel, with Fig. 4A, right panel). The fact that p300 and pCIP levels did not increase as a result of RA treatment at the RARβ2, RARE in the F9 RARβ2−/− cell line indicates that RARβ2 protein itself is required to
increase the levels of the coregulators necessary for \( \text{RAR}\beta_2 \) transcription.

Levels of RNA Polymerase II Associated with Transcription Initiation Were Reduced at the RAREs Regulating Expression of the Hoxa1 and Cyp26A1 Genes in F9 \( \text{RAR}^{-/-} \) Cells as Compared with F9 Wt and \( \text{RAR}\alpha, \beta, \gamma \) null cells—We also determined how the association of the initiating form of RNA polymerase II (initiating pol II) with the RAREs monitored in this study is perturbed in the F9 \( \text{RAR}\beta_2^{-/-} \) and the F9 \( \text{RAR}\gamma^{-/-} \) cell lines as compared with F9 Wt cells. We monitored the association of pol II by using an antibody that recognizes phosphorylated serine 5 of the CTD of initiating pol II (38). In other systems, serine 5 phosphorylation of the CTD has been associated with transcriptional initiation (39). Similar basal levels of initiating pol II were associated with the Hoxa1 RARE in F9 Wt, F9 \( \text{RAR}\beta_2^{-/-} \), and F9 \( \text{RAR}\gamma^{-/-} \) cells (Fig. 5A, left panel, 0 h). Treatment of F9
Wt cells with RA resulted in a ∼9.3-fold ($p$ value <0.02) increase in the levels of initiating pol II associated with the Hoxa1 RARE. In the F9 RARβ2−/− cells, RA treatment resulted in a similar increase (∼8.7-fold) in the levels of initiating pol II associated with the Hoxa1 RARE as compared with F9 Wt cells (Fig. 5A, left panel). However, in the F9 RARγ−/− cell line, initiating pol II levels rose only ∼1.7-fold at the Hoxa1 RARE after RA treatment. Additionally the levels of initiating pol II associated with the Hoxa1 RARE in the F9 RARγ−/− cells never rose above the basal levels observed at this RARE in F9 Wt cells (Fig. 5A, left panel). These results suggest that in the absence of RARγ, RAR (α + β)-RXRα heterodimers (Fig. 3A) bound at the Hoxa1 RARE are unable to associate and/or increase the levels of initiating pol II in response to RA treatment.

The recruitment patterns of initiating pol II to the Cyp26A1 R1 RARE in the F9 RARβ2−/− and F9 RARγ−/− cell lines, as
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compared with F9 Wt cells, were as follows. Initiating pol II levels rose much higher at the Cyp26A1 R1 RARE (~9.0-fold in F9 Wt cells) as compared with the Hoxa1 RARE (~9.3-fold in F9 Wt cells) in response to RA (Fig. 5A, compare middle and left panels). The increase of initiating pol II levels at the Cyp26A1 R1 RARE (~12.3-fold) was much lower (p value <0.02) in the F9 RARγ−/− cells as compared with the increase observed in F9 Wt cells (~90-fold) (Fig. 5A, middle panel). Therefore, in the absence of RARγ, the RAR (α + β)-RXR heterodimers bound at the Cyp26A1 RAREs (Fig. 3, B and C) were also unable to recruit and/or increase the levels of initiating pol II efficiently in response to RA treatment.

Differences in the Association of Initiating pol II with the RARB2 RARE in F9 RARβ2−/− Cells as Compared with F9 Wt and F9 RARγ−/− Cells—The recruitment patterns of initiating pol II to the RARB2 RARE in the F9 Wt, RARB2−/−, and RARγ−/− cells were different from those seen for the Hoxa1 and Cyp26A1 RAREs. In contrast to the levels of initiating pol II recruitment observed for the Hoxa1 and Cyp26A1 RAREs, the increase in initiating pol II recruited to the RARB2 RARE in RA-treated F9 RARγ−/− cells was ~5.4-fold (p value <0.02), and this increase was similar to the ~4-fold increase (p value <0.02) in levels of initiating pol II recruited to the RARB2 RARE in F9 Wt cells (Fig. 5A, right panel). No RA-mediated increase in initiating pol II associated with the RARB2 RARE was observed in the F9 RARB2−/− cells, and these data are consistent with the observation that RARB2 transcripts were not expressed in F9 RARB2−/− cells (Fig. 5A, right panel). The high basal levels of initiating pol II associated with the RARB2 RARE in the three basal cell lines studied are consistent with previous reports (32, 40). We concluded that although RARγ was associated with the RARB2 RARE in F9 RARB2−/− cells (Fig. 5D) the presence of RARγ is not sufficient for RA-mediated recruitment and/or increase in the levels of initiating pol II (Fig. 5A) or the RA-induced transcription of the RARB2 gene (Fig. 1, B and C).

The Polycomb Group Protein SUZ12 Is Not Efficiently Displaced from the Hoxa1 and Cyp26A1 R1 RAREs in F9 RARγ−/− Cells in Response to RA—We have previously shown that the polycomb group protein SUZ12 is associated with the Hoxa1, Cyp26A1 R1, and RARB2 RAREs in F9 Wt cells and that dissociation of SUZ12 occurs upon exposure to RA (25). Polycomb proteins have been shown to negatively regulate Hox genes (41–43), and the association of SUZ12 with numerous RAREs suggests that polycomb proteins may have a more global role in the repression of RA target genes. We therefore addressed the question of whether there were differences in the association patterns of SUZ12 with the Hoxa1, Cyp26A1, and RARB2 RAREs in the F9 RARB2−/− and F9 RARγ−/− cell lines as compared with F9 Wt cells.

The levels of SUZ12 associated with the Hoxa1 RARE in F9 Wt cells decreased ~33-fold in response to RA treatment (Fig. 5B, left panel). An ~8.3-fold decrease in the level of SUZ12 associated with the Hoxa1 RARE was seen in F9 RARB2−/− cells after RA treatment. Although the basal levels of SUZ12 associated with the Hoxa1 RARE were similar in F9 Wt and F9 RARB2−/− cells (Fig. 5B, left panel, 0 h), RA treatment caused a ~3.4-fold greater decrease in the levels of SUZ12 associated with the Hoxa1 RARE in F9 Wt cells as compared with F9 RARB2−/− cells (Fig. 5B, left panel, 24 h; p value <0.01).

The basal levels of SUZ12 associated with the Hoxa1 RARE in F9 RARγ−/− cells were also statistically similar to the basal levels of SUZ12 associated with this RARE in F9 Wt cells (Fig. 5B, left panel, 0 h; p value >0.10). However, ~26-fold lower levels of SUZ12 remain associated with the Hoxa1 RARE in F9 Wt cells as compared with F9 RARγ−/− cells after RA treatment (Fig. 5B, left panel, 24 h). In fact, the level of SUZ12 associated with the Hoxa1 RARE in RA-treated F9 RARγ−/− cells was similar to (~2-fold difference) the level seen in untreated F9 RARγ−/− cells (Fig. 5A, left panel; p value <0.05).

The association patterns of SUZ12 with the Cyp26A1 R1 RARE before and after RA treatment in the F9 Wt, RARB2−/−, and RARγ−/− cell lines were as follows. The basal levels of SUZ12 associated with the Cyp26A1 R1 were similar in the three F9 cell lines (Fig. 5B, middle panel, 0 h). However, after RA treatment the levels of SUZ12 associated with the Cyp26A1 R1 were ~18.3-fold lower in F9 Wt cells than in F9 RARγ−/− cells (p value <0.0001) and ~2.7-fold lower in F9 Wt cells as compared with F9 RARB2−/− cells (Fig. 5B, middle panel, 24 h; p value <0.01).

These results demonstrate that the addition of RA does not result in the efficient displacement of SUZ12 from the Hoxa1 and Cyp26A1 RAREs in F9 RARγ−/− cells. Additionally, the levels of SUZ12 displaced from the Hoxa1 RARE after RA treatment in the three cell lines were inversely related to the expression levels of Hoxa1 in these same lines. Whereas transcription of Hoxa1 was strongly induced by RA in F9 Wt cells, transcription of Hoxa1 was reduced by ~50% in F9 RARB2−/− cells, and it was almost completely abrogated in the F9 RARγ−/− cell line (Fig. 1A). Conversely after RA treatment the highest levels of SUZ12 were associated with the Hoxa1 RARE in the RARγ−/− cells with lower levels in F9 RARB2−/− cells and the lowest level in the F9 Wt cells (Fig. 5A, left panel). An inverse relationship between the expression of Cyp26A1 mRNA in the three cell lines and the level of SUZ12 associated with the Cyp26A1 R1 RARE in these three same cell lines was also observed (Fig. 1, B and C, and Fig. 5A, middle panel).

The Levels of SUZ12 Associated with the RARB2 RARE Correlate with Transcription and the Presence of RARγ—The levels of SUZ12 associated with the RARB2 RARE decreased ~3.4-fold (p value <0.01) in F9 Wt cells and ~7.9-fold (p value <0.06) in the F9 RARγ−/− cell line after exposure to RA (Fig. 5B, right panel). However, in the F9 RARB2−/− cell line, which does not express RARB2 transcripts, an RA-dependent decrease in association of SUZ12 with the RARB2 RARE was not observed (Fig. 5A, right panel). Therefore, these results indicate that RA-associated transcription of the RARB2 gene is correlated with the displacement of SUZ12 from the RARB2 RARE.

Interestingly higher levels of SUZ12 were associated with the RARB2 RARE in untreated F9 RARγ−/− cells as compared with untreated F9 RARB2−/− cells (p value <0.06) and untreated F9 Wt cells (p value <0.06). Additionally the levels of SUZ12 that were associated with the RARB2 RARE were similar in RA-treated RARγ−/− cells and RA-treated F9 RARB2−/− cells (Fig. 5B, right panel, 24 h), although transcription of RARB2 mRNA was higher in RA-treated RARγ−/− cells as compared...
with RA-treated F9 RARβ2−/− cells (Fig. 1, B and C). Furthermore the levels of SUZ12 associated with the RARβ2 RARE were ~3.5-fold higher in RA-treated F9 RARγ−/− cells as compared with RA-treated F9 Wt cells (Fig. 5B, 24 h; p value <0.01). Therefore, irrespective of the transcriptional status of a gene, the levels of SUZ12 associated with a RARE may be influenced by the presence of RARγ. The binding of RARγ to a RARE may block the association of SUZ12 with the aforementioned RARE. In the absence of RARγ, higher levels of SUZ12 may be able to associate with a RARE.

**DISCUSSION**

Previous studies have demonstrated that the RARα, -β2, and -γ isoforms each regulate a specific subset of target genes (44). In this study, we used chromatin immunoprecipitation assays to monitor the association of RARγ, RXRα, and other proteins involved in transcription with the RARβ2, Hoxa1, and Cyp26A1 RAREs in F9 Wt cells. We then monitored the association patterns of these factors to the aforementioned RAREs in F9 RARα, -β2, and -γ null cell lines and compared these results to what was observed in the F9 Wt cells. Additionally we determined the expression of the corresponding target genes in the F9 Wt and the F9 RARα, -β2, and -γ null cell lines via quantitative real time RT-PCR. By using this approach we were able to demonstrate that the association of RARγ and/or RXRα with a RARE does not suffice for the RA-mediated transcription of the corresponding target gene. However, the ability of RARγ and/or RXRα to recruit the coactivators pCIP and p300 to an RARE did correlate with the RA-mediated transcription of the corresponding target mRNAs. Additionally the ability of RARγ and/or RXRα to recruit and/or increase the levels of initiating RNA polymerase II to a RARE in response to RA mirrored the ability to recruit pCIP and p300. From these results we conclude that the specific functions of the RAR isoforms do not manifest at the level of DNA binding but rather from a differential ability to recruit specific components of the transcriptional machinery. We also demonstrated that the RA-mediated displacement of SUZ12 from a RARE was greatly inhibited in the absence of RARγ. Thus we demonstrated that components of the RAR signaling machinery are specifically required for the displacement of SUZ12 from RAREs during the RA-mediated differentiation of F9 cells.

**RAR Isotype-specific Function Does Not Manifest at the Level of DNA Binding—RAR-RXR heterodimers bind to RAREs, which are composed typically of two direct repeats of a core hexameric motif, PuG(G/T)TCA where Pu is a purine, spaced by 5 bp (referred to as DR5) or less commonly 2 bp (DR2) (6). The DNA binding domain is the most highly conserved region among the RAR isoforms (6). In vitro studies have demonstrated that RAR isoforms can bind to the same sequences as each RAR isotype could bind to the Hoxa1/RARβ2 RARE in gel shift mobility assays (14, 20). Additionally although the RA-induced expression of Hoxa1 and RARβ2 mRNAs is regulated by the same DR5 element (Fig. 2A), expression of Hoxa1 in F9 cells requires RARγ, whereas RARβ2 is required for its own expression (Fig. 1, B and C). These results suggest that each of the RAR isoforms has the ability to associate with a given RARE and that the specific functions of a RAR do not manifest at the level of DNA binding. Consistent with this conclusion, we demonstrated that RXRα, presumably bound as a heterodimer with RARα or RARβ, was associated with the Hoxa1 and Cyp26A1 RAREs in F9 RARγ−/− cells (Fig. 3, A, B, and C, left panels) even though these two genes are not induced by RA in this cell line (Fig. 1, B and C). Additionally we showed that RARγ was associated with the RARβ2 RARE in F9 Wt, F9 RARα−/−, and F9 RARβ2−/− cells (Fig. 3D, middle panel) even though RARγ was not required for the expression of RARβ2 mRNA (Fig. 1, B and C).

Future ChIP studies using antibodies specific for the RARα and RARβ isoforms could be used to test our conclusion further that each of the RAR isoforms can bind to a given RARE. We unsuccessfully tried numerous commercially available RARα and RARβ antibodies in our ChIP studies. Unfortunately these antibodies generated a signal in the corresponding F9 RAR null cell line comparable to what was seen in the F9 Wt cell line, or the antibodies generated a signal that was not sufficiently above background levels. Thus, we were unable to show definitively that RARα and/or RARβ were associated with the Hoxa1 and Cyp26 RAREs in F9 RARγ−/− cells.

Previous studies have shown that RARβ is required for the RA-associated transcription of Cyp26 mRNA in P19 embryonal carcinoma cells. We showed that neither RARα nor RARβ was required for expression of Cyp26A1 in F9 cells, whereas RARγ was required for the expression of Cyp26A1 mRNA (Fig. 1, B and C). However, previous studies indicate that the functional roles of RAR isoforms in different cell types vary (20, 35). The molecular mechanisms governing the differential requirement of the RAR isoforms in the regulation of Cyp26A1 in P19 and F9 cells remain to be determined.

**RA-mediated Coactivator Recruitment to RAREs Is an RAR Isotype-specific Event—**We also monitored the association of the coactivators pCIP and p300 with the Hoxa1, Cyp26A1 (R1), and RARβ2, RAREs. The levels of pCIP and p300 associated with these RAREs increased as a result of RA treatment in F9 Wt cells (Fig. 4, A and B). However, RA-mediated increases in the levels of pCIP and p300 associated with a particular RARE were not observed if the corresponding target genes were not transcribed in the F9 RAR null cells. Additionally we demonstrated that RXRα was associated with the Hoxa1 and Cyp26A1 R1 RAREs, presumably bound as heterodimers with RAR (α + β) in RARγ−/− cells, even though Hoxa1 and Cyp26A1 mRNAs are not transcribed RARγ−/− cells. Therefore, we have shown that the association of RAR-RXR heterodimers with an RARE is not sufficient for the recruitment of factors required for subsequent transcription. We conclude that specific functional roles for RAR isoforms arise from a differential ability to recruit specific components of the transcriptional machinery. Future studies could address whether the recruitment of other coactivators to RAREs by RAR-RXR heterodimers are also compromised in the RAR null cell lines.

There is precedent for the proposal that RARs interact with corepressors in an isotype-specific manner. RARα has been shown to strongly interact with the nuclear corepressor SMRT in the absence of ligand, whereas unliganded RARβ and RARγ only weakly interact with SMRT (30). The inability of RARβ and RARγ to interact with SMRT results from intramolecular...
interactions between helix 3 and helix 12 of the hormone binding domains of these isotypes, which occlude corepressor binding (45). RARα differs from RARβ and RARγ at three residues in helix 3, which prevents the interaction of helix 3 with helix 12 in RARα (45). As a result, the corepressor docking site is exposed in RARα. Transient reporter assays were used to demonstrate that in the absence of RA RARs is the only RAR isotype that can repress transcription (46). RARβ and RARγ actually mediated substantial levels of transcriptional activation in the absence of ligand in this same report (30). These results illustrate how coregulator recruitment by RAR can differ between isotypes.

A differential ability to recruit coactivators has also been demonstrated for the estrogen receptors (ERs), which consist of two isotypes, ERα and ERβ (47). Glutathione S-transferase interaction assays were used to demonstrate that ligand-dependent differences exist between ERα and ERβ in their ability to interact with the coactivators TIF2 and SRC1a despite the similarity in binding affinity of the various ligands used in the study for both ER subtypes (48). However, other groups have demonstrated that certain compounds show ER receptor selectivity with regard to ligand binding and/or efficacy (47), similar to the development of synthetic retinoids selective for each of the RAR isotypes.

The peroxisome proliferator-activated receptor class of nuclear receptors is similar to RARs in that there are three isotypes, PPARα, PPARγ, and PPARδ, all of which can bind to similar DNA sequences as heterodimers with RXRs (49). Each of the PPAR isotypes regulates the expression of distinct but overlapping sets of target genes (50). Additionally analysis of chimeric PPAR receptors points to the amino terminus of each receptor as the key determinant of isotype-selective gene expression. For example, the amino terminus of PPARγ confers the ability to promote adipocyte differentiation when fused to the PPARδ DNA binding domain and ligand binding domain. Therefore, RAR isotype specificity may also, in part, arise from differences in the amino termini among RAR isotypes.

RAR Isotype-dependent Association of the Initiating Form of RNA Polymerase II to RAREs Mirrors the Recruitment Patterns of p300 and pCIP—We showed that the RA-mediated increase in the level of initiating pol II associated with an RARE was correlated with the transcription levels of the corresponding target gene. Initiating pol II levels increased in response to RA at the Hoxa1 RARE and Cyp26A1 R1 RARE in F9 Wt and F9 RARβ2−/− cells but not in F9 RARγ−/− cells (Fig. 5A). Conversely initiating pol II levels rose at the RARβ2, RARE in F9 Wt and F9 RARγ−/− cells in response to RA but not in F9 RARβ2−/− cells (Fig. 5A, right panel). The RA-mediated recruitment of initiating pol II to the target RAREs also correlated with the RA-mediated recruitment of p300 and pCIP to the same RAREs. We note that in F9 Wt cells the association patterns of pol II with target RAREs in ChIP assays using an antibody that recognizes the largest subunit of pol II were similar to the results obtained with the antibody that recognizes the initiating form of pol II. Previous studies implementing in vitro transcription assays have demonstrated that RAR-RXR heterodimers recruit p300 and pCIP prior to pol II and that p300 and p160 recruitment is essential for RA-mediated transcription (31, 51). Additionally p300 is essential for the RA-mediated differentiation of F9 cells (52). Our results are consistent with the observation that p300 and pCIP are required for the association and/or increase in the levels of initiating pol II during RA-mediated transcription.

SLUZ2 Is Not Efficiently Displaced from RAREs after RA Treatment in the F9 RARγ−/− Cell Line as Compared with F9 Wt and F9 RARβ2−/− Cell Lines—We have previously demonstrated that SUZ12 associates with the RARBα, Hoxa1, and Cyp26A1 RAREs in F9 Wt cells and that this association is disrupted upon RA treatment (25). In this study we demonstrated that SUZ12 levels remained high at the Hoxa1 and Cyp26A1 R1 RAREs in RA F9 RARγ−/− cells after RA treatment as compared with RA-treated F9 Wt and F9 RARβ2−/− cells (Fig. 5B, 24 h). Additionally Hoxa1 and Cyp26A1 mRNAs were not transcribed in RA-treated F9 RARγ−/− cells (Fig. 1, B and C). Therefore the question arises as to whether transcription is a prerequisite for the displacement of SUZ12 from RAREs after RA treatment. This could be tested by abolishing transcription of Hoxa1 mRNA in F9 Wt cells through mutation of the Hoxa1 TATA box and monitoring the association of SUZ12 with the Hoxa1 RARE and Hoxa1 promoter-proximal region before and after RA treatment. If RARs recruit the polycomb group complexes directly to RAREs then we would expect SUZ12 to be displaced from the Hoxa1 RARE in the absence of transcription. Alternatively if transcription of Hoxa1 mRNA is required for the displacement of SUZ12, then SUZ12 levels should remain high at the Hoxa1 RARE after RA treatment in a cell line that has a mutated Hoxa1 TATA box.

We observed comparable levels of SUZ12 associated with the RARBα, RARE after RA treatment in RARγ−/− cells and F9 RARβ2−/− cells. However, significantly higher levels of RARB2 transcripts were expressed in the F9 RARγ−/− cell line (Fig. 1, B and C) as compared with the F9 RARβ2−/− cell line. Therefore, the levels of SUZ12 associated with a RARE do not necessarily correlate to the transcription levels of the corresponding target gene. These results are consistent with a report demonstrating that SUZ12 associated with actively transcribed genes during differentiation of mouse embryonic stem cells (53). Additionally SUZ12 has been shown to associate with transcribed genes prior to and during their down-regulation in human embryonic fibroblasts induced to differentiate by RA (54).

The fact that RARB2 mRNA was transcribed in RA-treated F9 RARγ−/− cells even though comparable levels of SUZ12 were associated with the RARB2 RARE in F9 RARγ−/− cells and F9 RARβ2−/− cells (in which RARB2 mRNA is not expressed) suggests that SUZ12 does not have a prominent role in the repression of RARB2 transcription. Consistent with this hypothesis, higher levels of SUZ12 were associated with the Hoxa1 and Cyp26A1 R1 RAREs as compared with the RARB2 RARE. Approximately 1.0% of the Hoxa1 RARE input and ~2.7% of the Cyp26A1 R1 RARE input were immunoprecipitated in SUZ12 ChIP assays with soluble chromatin derived from untreated F9 Wt cells as compared with 0.1% of the RARB2 RARE input. Therefore the association parameters of SUZ12 with the RARBα, RARE may be different from those seen for SUZ12 with the Hoxa1 and Cyp26A1 R1 RAREs.
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**A model for the mechanism by which RAR isotype specificity is manifested through RA-associated transcriptional regulation of the Hoxa1 gene.** In the absence of ligand, each of the RARs (α, β, and γ) can associate with the 3′ Hoxa1 RARE as a heterodimer with RXR (α, β, and γ) (see Fig. 2A for gene structure). SUZ12 covers the entire Hoxa1 locus in the absence of RA (58). However, when unliganded RARγ is associated with the Hoxa1 RARE, lower levels of SUZ12 associate with this RARE as compared with when RARα and RARβ are associated with the Hoxa1 RARE. Upon RA treatment, RARγ simultaneously recruits pCIP and p300, and SUZ12 is displaced from the Hoxa1 RARE. The RARγ-mediated recruitment of pCIP and p300 allows for the subsequent recruitment of initiating pol II to the Hoxa1 RARE. Initiating pol II recruited to the 3′ Hoxa1 RARE can then communicate with the Hoxa1 promoter-proximal region (PP) located ~4.5 kb upstream, which already has initiating pol II prebound (25), culminating in the RA-associated transcription of Hoxa1 mRNA. Neither RARα nor RARβ can recruit pCIP or p300 to the Hoxa1 RARE in the presence of RA. Therefore, initiating pol II is not recruited to the 3′ Hoxa1 RARE, and SUZ12 is not displaced from the Hoxa1 locus after RA treatment.

It remains to be determined how SUZ12 is targeted to RARβ, Hoxa1, and Cyp26A1 RAREs in F9 cells and how mechanistically SUZ12 is displaced from these RAREs upon RA treatment. As we discussed previously, the association of PcG proteins with RA target genes may arise through interactions with RAR-RXR heterodimers (25). Support for such a model comes from a recent study demonstrating that the human tumor antigen PRAME (preferentially expressed antigen in melanoma) can bind to ligand-bound RAR and repress transcription through recruitment of PcG proteins (55). Additionally, the authors showed that stable expression of PRAME in F9 cells blocked RA-induced differentiation and inhibited RA-induced gene expression. In the context of wild type F9 cells, we posit that an as yet unidentified protein may simultaneously interact with PcG proteins and RAR-RXR heterodimers in the absence of RA, allowing for the recruitment of PcG proteins to RA target genes.

Alternatively SUZ12 may be displaced from RAREs after RA treatment in F9 cells due to an undefined feature of the differentiation process. It has been demonstrated that SUZ12 dissociates from numerous genes during RA-mediated neuronal differentiation of the human embryonal carcinoma cell line NT2/D1 (54). Moreover SUZ12 dissociation from target genes, resulting in the derepression of these genes during differentiation, has also been observed in murine (53, 56) and human (57) embryonic stem cells. However, our results clearly demonstrate that components of the RAR signaling apparatus are involved in the displacement of SUZ12 because SUZ12 was not displaced from the Hoxa1 and Cyp26A1 RAREs after RA treatment in the absence of RARγ (Fig. 5B). Additionally SUZ12 mRNA levels did not decrease during the RA-mediated differentiation of F9 cells (data not shown). Furthermore SUZ12 reassociates with the Hoxa1 and Cyp26A1 RAREs when RA is removed from the media of F9 Wt cells (25), demonstrating that some of the molecular events underlying the differentiation process are reversible.

The results of this study demonstrate that components of the RAR signaling apparatus are required for the displacement of SUZ12 from RAREs during the RA-mediated differentiation of F9 Wt cells. We present a model for how SUZ12 may be displaced from the Hoxa1 locus in response to RA (Fig. 6). Previous studies have demonstrated that SUZ12 can bind throughout the whole Hox A cluster domain in F9 cells (58) as well as in human embryonic stem cells (57). Additionally we have shown that SUZ12 is associated with the 3′ Hoxa1 RARE and Hoxa1 promoter-proximal region (25). Therefore, we depict SUZ12 covering the whole Hoxa1 locus. Based on the results of this study, we propose that the RARγ isotype is specifically required for the RA-mediated displacement of SUZ12 from the Hoxa1 gene. In response to RA, RARγ can recruit pCIP and p300 to the Hoxa1 RARE, whereas the RARα and RARβ isotypes are unable to do so. The RA-dependent recruitment of pCIP and p300 by RARγ-RXR to the Hoxa1 RARE allows for the subsequent recruitment and/or increase in the levels of initiating pol II to the 3′ Hoxa1 RARE. Factors associated with the 3′ Hoxa1 RARE are then able to communicate with initiating pol II prebound to the Hoxa1 promoter region (25), culminating in transcription of Hoxa1 mRNA. RARγ may directly cause the disassociation of SUZ12 from the Hoxa1 RARE and/or may indirectly cause SUZ12 disassociation through the transcription of the Hoxa1 gene.

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RAR Isotype Specificity through Coregulator Recruitment

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