Identification and Characterization of Retinoic Acid Receptor $\beta_2$ Target Genes in F9 Teratocarcinoma Cells

Yong Zhuang,1 Teresa N. Faria,1,* Pierre Chambon,2 and Lorraine J. Gudas1

1Pharmacology Department, Weill Medical College of Cornell University, New York, NY, and 2Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, CU de Strasbourg, France

Abstract
Retinoids, a group of natural and synthetic analogues of vitamin A (retinol), modulate the differentiation of many cell types. Retinoids are also used for the prevention and treatment of cancer. The actions of retinoids are generally mediated by the retinoic acid receptors (RARs $\alpha$, $\beta$, and $\gamma$) and the retinoid X receptors (RXRs $\alpha$, $\beta$, and $\gamma$). One of the RARs, RAR$\beta_3$, is expressed at reduced levels in many human carcinomas, and F9 RAR$\beta_2$ cells do not growth arrest in response to RA. To determine if RAR$\beta_2$ regulates the expression of a unique set of genes, through the use of subtractive hybridization and DNA array analysis we have identified and characterized genes that are differentially expressed in F9 RAR$\beta_2$-teratocarcinoma cells. These genes, which encode transcription factors, cell surface signal transduction molecules, and metabolic enzymes, include c-myc, FOG1, GATA6, glutamate dehydrogenase, glutathione S-transferase homologue (p28), Foxq1, Hic5, Meis1a, Dab2, midkine, and the PDGF-\(\alpha\)-receptor. These genes are regulated specifically by RAR$\beta_2$ in F9 wild-type (Wt) cells as indicated by their expression profiles in F9 RAR$\beta_2$ cells as compared to F9 Wt, RAR$\alpha$-trans, or RAR$\gamma$-trans cells, and their responsiveness to specific retinoid receptor agonists. The basal expression levels of some of these genes, such as c-myc, are higher in the F9 RAR$\beta_2$ cells than in F9 Wt in the absence of exogenous retinoids, suggesting that RAR$\beta_2$ can inhibit gene expression in the absence of a ligand. The RAR$\beta_2$ target genes are transcriptionally activated by retinol, as well as RA, in F9 Wt cells. Because the lack of RAR$\beta_2$ alters both the control of proliferation and differentiation in F9 cells, the genes that we have characterized may mediate key effects of RA, via RAR$\beta_2$, on these processes.

Received 2/18/03; revised 5/9/03; accepted 5/9/03.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Grant support: NIH grant R01 CA43796 to L.J.G.; a research grant from the American Institute for Cancer Research; and in part by a Cancer Pharmacology Training Grant (CA62948-08) (Y.Z.).

Requests for reprints: Lorraine J. Gudas, Room E409, Pharmacology Department, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. Phone: (212) 746-6250; Fax: (212) 746-8858. E-mail: liguadas@med.cornell.edu

*Current address: Bristol-Myers Squibb Company, New Brunswick, NJ 08903.
Copyright © 2003 American Association for Cancer Research.

Introduction
Retinoids, a group of natural and synthetic analogues of vitamin A, exert profound effects on many biological processes, such as vertebrate embryonic development (1) and cell growth and differentiation (2). Vitamin A insufficiency during pregnancy results in the death of the fetus, as well as congenital malformations affecting the eyes and ocular tissues, myocardium, respiratory, urogenital, and circulatory systems (1). Vitamin A deficiency in experimental animals is also associated with a higher incidence of some types of cancer, and with increased susceptibility to chemical carcinogens (3). Clinical models of carcinogenesis have demonstrated the efficacy of pharmacological administration of retinoids in preventing the development of cancers of the skin, oral cavity, lung, mammary gland, prostate, bladder, liver, and pancreas in animals exposed to carcinogenic agents (3). Clinical trials have indicated that retinoids may be useful for prevention of cancers of the upper aerodigestive tract, skin, breast, and ovaries (4). In addition, retinoids have been successfully used in the treatment of acute promyelocytic leukemia (APL) (5, 6), and other cancers (for review, see Refs. 7 and 8).

The biological effects of retinoids are primarily mediated by two classes of nuclear retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (9, 10). RARs and RXRs are members of the nuclear receptor superfamily that also include estrogen, androgen, thyroid hormone, vitamin D, PPAR, and orphan receptors. The retinoid receptors are encoded by six distinct genes: RAR$\alpha$, RAR$\beta$, RAR$\gamma$, RXR$\alpha$, RXR$\beta$, and RXR$\gamma$. Each of these receptors includes several isoforms formed by different splicing and usage of alternative promoters (9, 10). All-trans retinoic acid (RA) binds and activates RARs, and 9-cis-retinoic acid (9-cis RA) binds and activates both RARs and RXRs (11). Retinoid receptors activate transcription in a ligand-dependent manner by binding to retinoic acid response elements (RAREs) located in the promoter regions or enhancers of target genes. RAREs generally consist of two directly repeated half-sites of the consensus sequence AGGTCA spaced by 2 or 5 bp (DR2 and DR5 elements) (12). Recently, various coactivators for the nuclear receptors have been identified, including CBP/p300, SRC-1, TIF-2/Grip1, and ACTR/RAC3/pCIP. They are recruited by ligand-activated receptors and enhance transcription (13, 14). Many of these coactivators possess acetyltransferase activity that can modify histones and may increase promoter access to DNA-binding proteins and the transcriptional machinery (15–18).

The strong conservation of each RAR across vertebrates suggests that each receptor performs unique functions (19).
our laboratory, we generated F9 teratocarcinoma cells with mutations in both alleles of \( RAR_a, RAR_{b2}, \) or \( RAR_y \) by homologous recombination and showed that different RARs have different functions (20–23). Mice homozygous for \( RAR_a, RAR_b, \) or \( RAR_y \) have also been generated to understand the function of each receptor. Mutant mice lacking \( RAR_a, RAR_{b2}, RAR_b, \) or \( RAR_y \) are viable but exhibit abnormalities in several tissues (24–27). For example, \( RAR_{b2} \) mice exhibit greatly reduced hippocampal CA1 long-term potentiation (LTP) and long-term depression (LTD) (28), activities which are likely to play a role in learning and memory.

The \( RAR_b \) gene has four isoforms: \( \beta_1, \beta_2, \beta_3, \) and \( \beta_4. \) The \( \beta_2 \) isoform is the most abundant \( RAR_b \) isoform and the major RA inducible form (29). An RARE that mediates RA-induced \( RAR_{b2} \) gene expression in many different cell types was identified in the promoter region (30, 31). The \( RAR_{b2} \) RARE consists of two direct repeats of the core motif sequence AGGTCA separated by five nucleotides (31, 32). Activation of the \( RAR_{b2} \) promoter is mediated by RAR/RXR heterodimers (33). A thyroid hormone receptor/RXR heterodimer can also bind strongly to the \( \beta \)RARE and activates the \( RAR_{b2} \) promoter in response to RXR ligands (34). \( RAR_b \) exhibits a pattern of expression during development and in the mature organism (35) which is different from those of the other RARs. This suggests that \( RAR_b \) performs specific functions.

\( RAR_b \) is not expressed, or is expressed at low levels, in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, breast cancer cell lines, and esophageal carcinoma (36–40). \( RAR_b \) is encoded by a gene located on the short arm of chromosome 5 (p24) (41). In breast cancer, loss of heterozygosity (LOH) has frequently been detected at chromosome 3p22–25 (42). All of these findings support the concept that the specific loss of \( RAR_b \) expression may be an important event in tumorigenesis.

Recent studies have indicated that a decrease in \( RAR_b \) expression results in resistance to the growth inhibitory actions of retinoids. Indeed, transfection of \( RAR_b \) into \( RAR_b \)-negative cervical, breast, and lung cancer cells increased responsiveness to growth inhibition and induction of apoptosis by retinoids (43–47), and the restoration of \( RAR_b \) expression in \( RAR_b \)-negative lung cancer cell lines inhibited tumorigenicity in nude mice (48, 49). Administration of 13-cis RA to patients with premalignant oral lesions can restore the expression of \( RAR_b \), as well as reverse the lesions (50). Conversely, the loss of both alleles of the \( RAR_b \) in an F9 teratocarcinoma line, generated in this laboratory by homologous recombination, resulted in the loss of growth inhibition by RA (21).

We hypothesized that each RAR regulates a specific subset of target genes. Thus, to understand the functions of the transcription factor \( RAR_{b2} \), the target genes specifically regulated by \( RAR_{b2} \) must be identified and analyzed. Very few \( RAR_b \) target genes have been identified to date. By comparing RA-treated F9 wild-type (Wt) teratocarcinoma cells and F9 \( RAR_{b2} \) cells, we identified some of the \( RAR_{b2} \) target genes through the use of subtractive hybridization and DNA expression microarray techniques. The target genes identified include transcription factors, protein tyrosine kinases, homeobox proteins, and oncoproteins. We have initiated the further characterization of eleven target genes in this study.

**Results**

**Identification of \( RAR_{b2} \) Target Genes in F9 Cells**

We hypothesized that \( RAR_{b2} \), which is induced by RA in many cell types, mediated the growth inhibitory actions of RA in F9 cells. To test this hypothesis, an F9 teratocarcinoma cell line that contained targeted disruptions in both alleles of the \( RAR_{b2} \) gene was generated by homologous recombination. We showed that the F9 Wt and \( RAR_{b2} \)-heterozygous lines could undergo RA-induced growth arrest, but that RA did not cause growth arrest in the F9 \( RAR_{b2} \) cells (21). While these data confirmed our hypothesis, more information about the target genes regulated by \( RAR_{b2} \) was then required to understand the downstream effects of \( RAR_{b2} \). We anticipated that \( RAR_{b2} \) positively regulated the transcription of some of its target genes, and negatively regulated other target genes.

To isolate \( RAR_{b2} \) target genes, F9 Wt cells and F9 \( RAR_{b2} \) cells were treated with 1 \( \mu \)M all-trans RA for 24 h. Subtractive hybridization and microarray analysis were then employed. By comparing the gene expression patterns between F9 Wt cells and the F9 \( RAR_{b2} \) cells, we identified approximately 80 genes from subtractive hybridization and 300 target genes from the microarray analyses (see “Materials and Methods”). Approximately one-half of the putative target genes were expressed at higher levels in RA-treated F9 Wt cells as compared to F9 \( RAR_{b2} \) cells, while the remaining genes were expressed at higher levels in RA-treated \( RAR_{b2} \) cells than in F9 Wt.

**Target Genes Identified by Subtractive Hybridization**

We examined 20 of the genes isolated by subtractive hybridization by Northern analysis of F9 Wt versus F9 \( RAR_{b2} \) cells and found that 7 out of 20 target genes were reproducibly altered in the Northern blot analysis. We sequenced these seven target genes and identified them using NCBI Blast Programs. Among them, six are known genes (Table 1) and one is an uncharacterized gene. The six known genes are \( Dab2 \) (p96 form), glutamate dehydrogenase, \( Foxq1, Meis1a, midkine, \) and \( glutathione \) \( S \)-transferase homologue (p28). To determine whether or not these genes were specific \( RAR_b \) targets, the regulation of these genes by RA was also examined by Northern blot analysis in F9 \( RAR_o \) and \( RAR_y \) lines, generated by homologous recombination in our laboratory (22, 23). We also examined \( RAR_{b2} \) mRNA expression. As expected, \( RAR_{b2} \) mRNA was not expressed in F9 \( RAR_{b2} \) cells and its expression was stimulated by RA in F9 Wt, \( RAR_o \), and \( RAR_y \) cell lines (Fig. 1).

\( Dab2 \) (disabled-2), one of the two mammalian orthologues of the \( Drosophila \) Disabled gene, is thought to be a tumor suppressor in ovarian cancer since its expression is lost or greatly reduced in 85% of breast and ovarian cancers (51). We show here that \( Dab2 \) mRNA expression was up-regulated by RA in F9 Wt cells, while \( Dab2 \) mRNA levels were not affected by RA in F9 \( RAR_{b2} \) cells (Table 1 and Fig. 1, C and D).
Table 1. Summary of Genes Differentially Expressed in RA-Treated F9 Wt and F9 RARβ2−/− Cells

<table>
<thead>
<tr>
<th>GenBank Identity</th>
<th>GenBank Accession No.</th>
<th>Method of Identification</th>
<th>Regulation by RA in Wild-Type F9 Cells</th>
<th>RARβ2 Specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelocytomatosis oncogene (c-myc)</td>
<td>L00039</td>
<td>Microarray</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Friend of GATA-1 (FOG)</td>
<td>AF006492</td>
<td>Microarray</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>GATA-binding protein 6</td>
<td>U51335</td>
<td>Microarray</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (GLUD)</td>
<td>X57024</td>
<td>Subtractive hybridization</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Glutathione S-transferase homologue (p28)</td>
<td>MMU380819</td>
<td>Subtractive hybridization</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Foxq1 (Hfh-1L)</td>
<td>AF010405</td>
<td>Microarray and subtractive hybridization</td>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>Hic-3</td>
<td>L22485</td>
<td>Microarray</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Meis1a</td>
<td>U33629</td>
<td>Subtractive hybridization</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Mitogen-responsive phosphoprotein p96 (Dab2)</td>
<td>U18869</td>
<td>Microarray and subtractive hybridization</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>midkine</td>
<td>M35833</td>
<td>Subtractive hybridization</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>PDGFRα receptor</td>
<td>M57683</td>
<td>Microarray</td>
<td></td>
<td>N</td>
</tr>
</tbody>
</table>

Note: NC, no change; Y, yes; N, no.

Furthermore, Dab2 basal mRNA levels were about 10-fold lower in the F9 RARβ2−/− cells as compared to Wt. This indicates that in the absence of exogenous RA, RARβ2 can positively regulate the Dab2 gene. Dab2 mRNA expression in F9 RARY−/− cells was also low, both before and after RA treatment, as compared to Wt cells (Fig. 1, A and C), indicating that RARY may also modulate its expression.

Foxq1 (formerly Hfh-1L) belongs to the family of the FOX (Forkhead box) transcription factors (previously called HNF-3/forkhead transcription factors) (52). RA strongly induced Foxq1 expression in F9 Wt cells, but not in F9 RARβ2−/− cells. For instance, Foxq1 mRNA was induced by 5.6- and 7.8-fold after 48 and 72 h RA treatment in F9 Wt cells. By contrast, in F9 RARβ2−/− cells, after 48 and 72 h, Foxq1 mRNA was only slightly induced (Fig. 1, A and B; Table 1). However, Foxq1 appeared not to be a specific RARβ2 target. Foxq1 mRNA expression in F9 RARY−/− cells was also low, both before and after RA treatment, as compared to Wt cells (Fig. 1, A and B), indicating that RARY may also modulate its expression.

Meis1a, a member of a family of genes encoding the TALE (three-amino-acid loop extension) subset of homeodomain-containing proteins (53), acts as a cofactor of certain Hox proteins and may play a major role in normal hematopoiesis (54). Meis1a mRNA was expressed in F9 Wt, RARα−/−, and RARY−/− cell lines in the absence of RA. In contrast, Meis1a mRNA is barely detectable in the untreated F9 RARβ2−/− cells, indicating that the basal expression level of Meis1a mRNA in F9 Wt, RARα−/−, and RARY−/− cells may be regulated by RARβ2 in the absence of exogenously added ligand. After 24 h of RA treatment, the Meis1a mRNA level was increased by 3.3-, 3.3-, and 2.5-fold in the F9 Wt, RARα−/−, and RARY−/− cell lines, respectively, but it was not changed in the F9 RARβ2−/− cells (Fig. 1, A and B). Meis1a mRNA was only slightly induced in the F9 RARβ2−/− cells, even after 48 or 72 h RA treatment, indicating that Meis1a is a specific RARβ2 target gene in these cells.

Midkine is a member of a family of heparin-binding growth factors and was originally isolated as a retinoic acid-responsive gene (55). midkine mRNA was increased by RA in F9 Wt, RARα−/−, and RARY−/− cells, and after 48 h, RA increased midkine mRNA levels by 5.5-, 4.6-, and 5.5-fold, respectively. In contrast, RA led to a slight reduction in the midkine mRNA level in F9 RARβ2−/− cells (Fig. 1, C and D). Thus, midkine displays the features of a RARβ2 specific target gene.

Glutamate dehydrogenase (GDH) is a mitochondrial enzyme that catalyzes the oxidative deamination of glutamate to α-ketoglutarate using NAD⁺ or NADP⁺ as a cofactor (56). In the brain, besides its function in metabolism, GDH influences glutamate neurotransmitter availability (57). The basal GDH mRNA level was lower in F9 RARβ2−/− cells than in F9 Wt cells (data not shown). RA treatment caused an induction of glutamate dehydrogenase mRNA expression in F9 Wt cells, but not in F9 RARβ2−/− cells (Table 1).

Finally, glutathione S-transferase homologue (p28) is a small stress response protein which belongs to a family of GST-like (glutathione S-transferase) proteins (58). p28 expression was not affected by RA at either 24 or 48 h in Wt, RARα−/−, or RARY−/− cells, and its expression was 4-fold lower in F9 RARβ2−/− cells compared to the other three cell lines (Fig. 1, C and D). Thus, p28 exhibits the properties of an RARβ2 specific target gene.

Target Genes Identified by Microarray Analyses

We used gene microarray analyses to identify RARβ2 target genes, and obtained EST clones for the genes showing the largest fold changes between F9 Wt cells and F9 RARβ2−/− cells. Seven out of the seven target genes from microarray analysis were confirmed by semi-quantitative RT-PCR or Northern analysis, indicating that the microarray analysis was generally reliable. These genes are listed in Table 1. Dab2 and Foxq1 were also identified by subtractive hybridization and were discussed above.

c-myc is a proto-oncogene that is activated in various animal and human tumors. It is a transcription factor that plays a pivotal role in many biological functions including cell growth and differentiation (59, 60). c-myc mRNA in untreated RARβ2−/− cells was expressed at a 3.4-fold higher level than in untreated F9 Wt, RARα−/−, or RARY−/− cells. Furthermore, c-myc mRNA expression in F9 RARβ2−/− cells is 4.5-fold higher than in F9 Wt cells after 6 h of RA treatment (Fig. 1, A and B). After 24 h of RA treatment, the expression of c-myc mRNA is reduced by 3.7-, 4-, and 4.9-fold in F9 Wt cells, RARα−/−, and RARY−/− cells, respectively, while in the RARβ2−/− cells, the c-myc mRNA level is still higher than in Wt cells. These data suggest that RARβ2 suppresses c-myc mRNA expression in F9 Wt cells even in the absence of exogenously added RA.
RARβ2
FOG-1
Foxq1
GATA6
Meis1
c-myc
β-actin

Dab2
p28
midkine
β-actin

Foxq1
Meis1
c-myc

Dab2
midkine
p28
Fog1 is a member of the FOG (for Friend of GATA) family of multitype zinc finger proteins that interact with GATA and play essential roles in development (61). Fog1 is expressed in erythroid cells and megakaryocytes, liver, and testis (61). GATA6 is a member of the GATA family of transcription factors which regulate gene expression during development (62). GATA6, together with GATA4 and GATA5, is involved in the formation of the extra-embryonic and embryonic endoderm, as well as the cardiogenic mesoderm (63). Fog1 and GATA6 mRNAs were increased by RA in the F9 Wt, RARα−/−, and RARY−/− cell lines, but were not increased by RA or increased to a lesser extent in the F9 RARβ2−/− cells (Fig. 1A). Thus, Fog1 and GATA6 are RARβ2 specific target genes.

Hic5 is a focal adhesion protein that is very similar to paxillin (64). Studies have suggested that Hic5 is involved in the negative regulation of cell growth, since overexpression of Hic5 results in cell growth inhibition (65). Like p28, the expression of Hic5 is not affected by RA treatment, but basal Hic5 mRNA expression is much higher in F9 Wt cells than in F9 RARβ2−/− cells (Table 1).

PDGF-α receptor (PDGFrα) is one of the two receptors for platelet-derived growth factor (PDGF); it has been suggested that the PDGFrα plays a role in embryonic development (66). It was previously shown that PDGFrα expression is up-regulated during RA-induced F9 cell differentiation (67). Our results show that the PDGFrα mRNA level is increased by RA in F9 Wt cells, but not in F9 RARβ2−/− cells (Table 1). PDGFrα mRNA levels are also lower in F9 RARα−/− and F9 RARY−/− cells, indicating that multiple RARs can regulate the expression of this gene.

Target Genes Are Transcriptionally Regulated by RARβ2

To determine if the target genes were transcriptionally regulated by RARβ2, we inhibited transcription by using actinomycin D, an inhibitor of RNA polymerase II (68). F9 Wt cells and F9 RARβ2−/− cells were first treated for 12 h with or without 1 μM RA. At 12 h, RNA was isolated from some dishes of cells, while actinomycin D (2 μg/ml) (69) was added to other dishes of cells to inhibit RNA transcription. RNA was isolated after an additional 6 h. The RNA samples were analyzed by Northern blot analysis (Fig. 2). Consistent with results shown in Fig. 1, RA induced target gene expression in F9 Wt cells, but not in the F9 RARβ2−/− cells. It is also worth noting that the stimulatory effect of RA can be seen as early as 12 h. Treatment of Wt cells with actinomycin D alone resulted in a decrease in target gene mRNA. Furthermore, RA induction of target gene mRNA was completely blocked by treatment with actinomycin D, indicating that ongoing transcription is required for RA-induced expression of target genes. The c-myc mRNA level increased by RA in the F9 Wt, but not in the F9 RARβ2−/− cells (Table 1).

RARβ Agonists, in the Presence of a RXR Agonist, Stimulate the Expression of Target Genes in F9 Wt but not in F9 RARβ2−/− Cells

To study further the function of RARβ2 in the regulation of its target genes, F9 Wt and RARβ2−/− cells were treated for 48 h with 1 μM RA or RARβ selective agonists (100 nM BMS185411 or 1 μM CD2314), and/or a pan-RXR agonist (1 μM BMS188649 [BMS649]) (Fig. 3). Neither BMS185411 nor CD2314, the RARβ selective agonists, alone could induce the expression of any target genes in F9 Wt cells. Similarly, BMS649 (pan-RXR) alone had no effect on the expression of target genes. However, BMS649 (pan-RXR) and the RARβ selective agonists (BMS185411 or CD2314) synergistically induced the expression of all RARβ2 target genes in F9 Wt cells, but not in RARβ2−/− F9 cells. Thus, RXR-RARβ2 heterodimers appear to play a major role in

**FIGURE 1.** Northern blot analysis of the clones obtained from microarray analysis and subtractive hybridization analysis. A, F9 Wt cells, RARβ2−/−, α−/−, and γ−/− F9 cells were treated with 1 μM RA for different times and total RNA was extracted. After size fractionation on the gel, the RNA was transferred to nylon and hybridized to different cDNAs for the target genes. β-Actin was used as an RNA loading control. This experiment was repeated three to five times with different RNA preparations and very similar results were obtained. A representative experiment is shown. B, Quantitative analysis of the effect of 1 μM RA on the mRNA levels of some target genes. C, Northern blot analysis of three clones obtained from subtractive hybridization analysis. This experiment was repeated three to five times with different RNA preparations and very similar results were obtained. One representative experiment is shown. D, Quantitative analysis of the effect of 1 μM RA on the mRNA levels of the target genes. Quantitative analyses in panels B and D were performed using NIH Image 1.62 or Image Quant 1.2. The mRNA levels are normalized to β-actin mRNA and are expressed relative to the value derived from Wt cells not treated with RA. Columns, means from three to five experiments; bars, SE. Y-axes, arbitrary density units.

**FIGURE 2.** Northern blot analysis of the effects of actinomycin D on RARβ2 target gene expression. F9 Wt cells and F9 RARβ2−/− cells were grown for 12 h in the absence (−) or presence (+) of 1 μM RA. At 12 h, RNA was isolated or 2 μg/ml transcriptional inhibitor actinomycin D (ACT D) was added to the medium and RNA was isolated after 6 h. After size fractionation on the gel, the RNA was transferred to nylon and hybridized to different cDNAs for the target genes. This experiment was performed twice with similar results; one experiment is shown.

seen after 18 h of RA treatment in F9 RARβ2−/− cells was greatly reduced after 6 h of actinomycin D treatment, which is consistent with the previous finding that the half-life of c-myc transcripts in F9 cells is about 40 min (70).
mediating the induction of the expression of RARβ2 target genes. This is consistent with the conclusion that RXR-RAR heterodimers are the functional units transducing the retinoid signal (9).

Expression of RARβ2 Target Genes Is Induced in F9 Wt Cells Treated With RACT, but not in F9 RARβ2/C0 Cells

RACT (retinoic acid, dibutyryl cAMP, and theophylline) treatment causes F9 Wt cells to differentiate into parietal endoderm, while RA alone results in primitive endoderm (71). We examined the regulation of the expression of some of the RARβ2 target genes in F9 cells treated with RACT. F9 Wt or F9 RARβ2/C0 cells were treated with RACT, or different combinations of RARβ agonist and/or RXR agonist in the presence of CT (dibutyryl cAMP and theophylline) for 48 h. The RNA was transferred onto nylon and hybridized to different cDNAs for the target genes. The experiments were performed two times with different RNA preparations. β-Actin is used as an RNA loading control. Quantitation of some of the mRNAs is shown below the figure. As expected, CT alone did not have an effect on expression of target genes (72). Like RA, RACT induced the expression of target genes in F9 Wt cells. Addition of the pan-RXR agonist BMS649 decreased the Foxq1, FOG1, and Meis1a expression induced by RACT in F9 Wt cells, but increased the GATA6 expression induced by RACT. In the presence of CT, BMS649 (pan-RXR) and the RARβ selective agonist BMS185411 or CD2314 induced the expression of

FIGURE 3. Expression of RARβ2 target genes is increased by a RARβ-specific agonist and pan-RXR agonist. A. F9 Wt cells and RARβ2/C0 cells were treated for 48 h with 1 μM RA, 1 μM or 100 nm RARβ-specific agonist (BMS185411 or CD2314), 1 μM pan-RXR agonist (BMS188649), or different combinations of drugs. Total RNA was then extracted and size fractionated on the gel. The RNA was transferred onto nylon and hybridized to different cDNAs for the target genes. The experiments were repeated with different RNA preparations. β-Actin is used as an RNA loading control. One representative experiment is shown here. B. Quantitation of the effects of different agonists on the mRNA levels of the target genes in F9 Wt cells. The mRNA levels are normalized to β-actin mRNA and are expressed relative to the value derived from Wt cells not treated with RA. Columns, means from three experiments; bars, SE. Y-axes, arbitrary units.

FIGURE 4. Expression of RARβ2 target genes in F9 Wt cells and RARβ2/C0 cells treated with RACT. F9 Wt cells and F9 RARβ2/C0 cells were treated for 48 h with 1 μM RA, 100 nm (CD2314) or 1 μM (BMS185411) RARβ-specific agonist, 1 μM pan-RXR agonist, or different combinations in the presence of CT (dibutyryl cAMP and theophylline) for 48 h. The RNA samples were analyzed by Northern blot analysis (Fig. 4). As expected, CT alone did not have an effect on expression of target genes (72). Like RA, RACT induced the expression of target genes in F9 Wt cells. Addition of the pan-RXR agonist BMS649 decreased the Foxq1, FOG1, and Meis1a expression induced by RACT in F9 Wt cells, but increased the GATA6 expression induced by RACT. In the presence of CT, BMS649 (pan-RXR) and the RARβ selective agonists (BMS185411 or CD2314) induced the expression of
all RAR\textsubscript{\(\gamma\)} target genes in F9 Wt cells. In addition, retinol (Rol; vitamin A) induced the expression of all of the RAR\textsubscript{\(\gamma\)} target genes in F9 Wt cells. In contrast, in F9 RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} cells, expression of \textit{FOG1}, \textit{Foxq1}, \textit{GATA6}, and \textit{Meis1a} mRNA was very low with various treatments (Fig. 4).

We also studied the regulation of expression of the \textit{Hoxa1} gene, a gene we have studied previously (23, 69, 73, 74). Unlike the RAR\textsubscript{\(\gamma\)} target genes, a similar pattern of \textit{Hoxa1} mRNA expression was observed in F9 Wt and F9 RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} cells (Fig. 4). \textit{Hoxa1} mRNA expression was induced by RA in both F9 Wt and the RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} cells. Furthermore, \textit{Hoxa1} mRNA expression was not significantly increased by the RXR agonist (BMS664) and RAR\textsubscript{\(\gamma\)} selective agonists (BMS185411 or CD2314) in the presence of CT (Fig. 4). Rol only slightly induced \textit{Hoxa1} mRNA expression in F9 Wt cells; thus, Rol treatment was more effective in inducing several of the RAR\textsubscript{\(\gamma\)} target genes, especially \textit{GATA6} and \textit{Foxq1}, than in inducing \textit{Hoxa1} mRNA, consistent with our prior genetic data that the \textit{Hoxa1} gene is primarily transcriptionally regulated by RAR\textsubscript{\(\gamma\)} and not RAR\textsubscript{\(\gamma\)} in F9 cells (23).

**Discussion**

In this study, using subtractive hybridization and microarray analyses, we identified some of the RAR\textsubscript{\(\gamma\)} targets in F9 cells. To our knowledge, this is the first time that specific RAR\textsubscript{\(\gamma\)} target genes have been identified and confirmed by a comparison of Wt, RAR\textsubscript{\(\gamma\)}\textsubscript{+/−}, RAR\textsubscript{\(\alpha\)}\textsubscript{+/−}, and RAR\textsubscript{\(\beta\)}\textsubscript{+/−} cell lines. Most of the target genes are regulated by RA, though our data suggest that some genes, such as \textit{Dab2}, \textit{Hic5}, and \textit{p28}, are also regulated by RAR\textsubscript{\(\gamma\)} in the absence of exogenously added RA. The RAR\textsubscript{\(\gamma\)} target genes are also induced by Rol (Fig. 4) to a greater extent than a primary RA target gene such as \textit{Hoxa1}, which is not an RAR\textsubscript{\(\gamma\)} target (23). As we have shown previously that Rol is not metabolized to RA in F9 cells (75), a Rol metabolite, 4-oxoRol, is likely to be involved in the regulation of these target genes in F9 cells. We have not yet determined the number of primary versus secondary RAR\textsubscript{\(\gamma\)} target genes, as this requires much more extensive characterization of the promoters of the genes. The target genes identified in this study encode a wide range of proteins that regulate a variety of biological processes. This suggests that, in addition to the regulation of cell proliferation, the transcription factor RAR\textsubscript{\(\gamma\)} participates in the regulation of many biological processes via the transcription of multiple target genes.

We found that some target genes are RAR\textsubscript{\(\gamma\)} specific (Table 1) in that the F9 RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} cells exhibited reduced or no expression of these target genes, but the F9 RAR\textsubscript{\(\alpha\)}\textsubscript{+/−} and F9 RAR\textsubscript{\(\beta\)}\textsubscript{+/−} cell lines exhibited expression levels similar to those in F9 Wt cells. These data provide evidence that each RAR isofrom has distinct functions.

**Target Genes and Cell Growth and Cancer**

Consistent with our data showing that the induction of RAR\textsubscript{\(\gamma\)} is necessary for the growth arrest of F9 cells, two of the target genes we identified, c-\textit{myc} and \textit{Dab2}, from this study have been shown to play major roles in cell growth and proliferation. The c-\textit{myc} proto-oncogene, the cellular homologue of the retroviral \textit{v-myc} oncogene, is activated in various animal and human tumors (76). c-Myc is a transcription factor which regulates many biological processes, including the cell cycle and cell differentiation (77). The c-Myc protein can regulate some cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors, which are cell cycle regulators (78). Deregulated c-\textit{myc} expression often results in increased cyclin E and cyclin A expression (79, 80). In addition, expression of c-\textit{myc} can decrease p27 levels and interfere with its function (81).

Our data indicate that expression of c-\textit{myc} mRNA is repressed by RAR\textsubscript{\(\gamma\)} in F9 Wt cells, even in the absence of exogenous RA, and that c-\textit{myc} mRNA is expressed at a much higher level in untreated and 24 h RA treated F9 RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} than F9 Wt cells (Fig. 1). We conclude that in F9 Wt cells, a low level of RAR\textsubscript{\(\gamma\)} expression (without exogenous RA addition but in the presence of low levels of Rol from serum) inhibits c-\textit{myc} mRNA expression. The reduction of c-\textit{myc} mRNA by RA in F9 Wt cells has been observed in several studies, and down-regulation of c-\textit{myc} mRNA by RA is associated with RA-induced growth arrest of F9 cells (82). The increased level of c-\textit{myc} mRNA in RAR\textsubscript{\(\gamma\)}\textsubscript{+/−} cells may play a role in preventing these cells from undergoing growth arrest on RA treatment. Since changes in the c-\textit{myc} gene or its expression are associated with approximately one-seventh of U.S. cancer deaths (76), experiments will be carried out to determine if the loss of RAR\textsubscript{\(\gamma\)} expression in tumors causes the overexpression of c-\textit{myc} transcripts and concomitant abnormal regulation of tumor cell growth.

RA has been shown to regulate \textit{Dab2} expression during embryonic development (83). Moreover, \textit{Dab2} has been suggested to be a major mediator of RA cell growth inhibition. Transient transfection of Dab2 was sufficient to up-regulate MAPK activation and c-fos expression, and was associated with growth inhibition in F9 cells (84). Since we found \textit{Dab2} to be a RAR\textsubscript{\(\gamma\)} target in this study, RAR\textsubscript{\(\gamma\)} could mediate the growth inhibitory effects of RA by up-regulating \textit{Dab2} expression. Studies of GATA6-deficient mouse embryos suggest that \textit{Dab2} expression is regulated by GATA6 (85). Since we have shown that GATA6 is also a RAR\textsubscript{\(\gamma\)} specific target, \textit{Dab2} may be an indirect target of RAR\textsubscript{\(\gamma\)} and may be regulated by RAR\textsubscript{\(\beta\)} through GATA6. The lack of a known RARE in the \textit{Dab2} promoter (86) supports this suggestion. The observation that the expression of \textit{Dab2} mRNA is eliminated in 85–95% of breast and ovarian tumors suggests that it is a tumor suppressor gene (51). Indeed, overexpression of Dab2 reduced the tumorigenicity of carcinoma cells (87). Conversely, the reduction of RAR\textsubscript{\(\beta\)} levels in human breast tumors (88) could result in the down-regulation of \textit{Dab2}, which in turn could alter the control of cell proliferation.

**Target Genes and Development**

Several targets identified in this study provide insight into the roles of RAR\textsubscript{\(\gamma\)} in development. Meis1a is a member of the homeobox gene family of transcription factors and has been suggested to play a role in peripheral neural system development. Correct expression of Meis1a is essential for correct patterning in the embryonic peripheral nervous system (89). Our finding that \textit{Meis1a} is a target of RAR\textsubscript{\(\gamma\)} is consistent with the finding that RAR\textsubscript{\(\beta\)} is expressed and plays a role in the developing nervous system (90).
FOG1 is the founding member of the FOG family of proteins that interact with GATA transcription factors and modulate their activity (61). FOG1 knockout mice die at embryonic day 11.5 of gestation because of arrested erythroid cell maturation and complete failure of megakaryopoiesis (91). As FOG1 was shown to be a target of RARβ2 in these F9 cells, RARβ2 may play a role in megakaryopoiesis.

The GATA6 transcription factor is involved in the formation of the extra-embryonic and embryonic endoderm, as well as in cardiogenic mesoderm (92). GATA6 is essential for lung epithelial differentiation in the mouse embryo (63). Our result that GATA6 is a RARβ2 specific target suggests that RARβ has a role in the lung epithelial cell differentiation. Consistent with this, RARβ is expressed in the epithelia of the lungs (93).

In the midgestation period, midkine is expressed in the endoderm-derived lung epithelium and the adjacent mesenchyme, which suggests a key role for midkine in the regulation of lung development (94). Interestingly, RARβ is also expressed in the endoderm-derived lung epithelium and the adjacent mesenchyme at the same stage of embryogenesis. In the embryo, PDGF-A is expressed primarily by epithelial cells, whereas the PDGFα receptor is expressed primarily in mesenchymal cells. This ligand-receptor pair is thought to be important to epithelial-mesenchymal signaling in the lung during development (95). These findings, together with our results that midkine and the PDGFα receptor are RARβ2 target genes, suggest that RARβ plays a role in epithelial-mesenchymal signaling during lung development.

Fosq1 is expressed throughout embryonic development of the mouse (52). Although its function remains unclear, it is likely to play a crucial role during embryogenesis, given its similarity to other family members. Another family member, Foxp2, is involved in the development of speech (96).

Further characterization of the RARβ2 target genes identified in this study will be helpful in increasing our understanding of the functions of RARβ2 in a variety of biological processes. Future studies should also provide important insights into the molecular mechanisms by which the silencing of the RARβ2 gene leads to cancer.

**Materials and Methods**

**Materials**

The RARβ agonist BMS185411 and the pan-RXR agonist BMS188649 are from Bristol-Myers Squibb (Buffalo, NY). The RARβ agonist CD2314 is from CIRD Galderma (Sophia Antipolis, Valbonne, France). Actinomycin D was purchased from Sigma Chemical Co. (St. Louis, MO) and used at a concentration (2 μg/ml) shown to inhibit RNA synthesis (69).

**Cell Culture, RNA Extraction, and Northern Blot Analysis**

F9 Wt, RARβ2/−, RARα−/−, and RARγ−/− cells were maintained in DME supplemented with 10% bovine calf serum and 2 mM glutamine. Total cellular RNA was extracted using RNA-STAT 60 (Tel-Test, Friendswood, TX) following the manufacturer’s protocol. Northern blot analysis was performed as described previously (97). Briefly, total RNA (10 μg) was resolved on 1% agarose gels containing 5% formaldehyde and RNA was transferred to nylon filters. Nylon filters were prehybridized, and hybridized at 42°C in 50% (v/v) formamide, 5× SSC, 50 mM Na2HPO4-NaH2PO4 (pH 7.4), 5 mM EDTA, and 0.1 mg/ml salmon sperm DNA. cDNA probes of target genes were labeled with [32P]dCTP using a random primer labeling kit (Roche, Indianapolis, IN). cDNA inserts for target genes were either from the F9 cDNA library described below or were commercially available EST clones. EST clones for GATA6 (AI894084), PDGFα-receptor (BE630572), and FOG1 (AW763708) were obtained from Incyte (Palo Alto, CA). EST clones for c-myc (BG234302) and Hic5 (BF536993) were from ATCC. Northern blots were quantitated by phosphorimage analysis, and the software NIH Image 1.62 or Image Quant 1.2.

**Subtractive Hybridization**

A PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) was used to carry out subtractive hybridization analysis. F9 Wt teratocarcinoma cells and the F9 RARβ2/− line were treated with 1 μM all-trans RA for 24 h and total RNA was extracted. After reverse transcription, cDNAs from the two cell lines were subjected to subtractive hybridization according to the manufacturer’s protocol. Products from the secondary PCR reactions were radiolabeled with [α-32P]dCTP and hybridized to duplicate filters containing phage clones from a cDNA library in a Lambda ZAP vector; this cDNA library was synthesized from F9 Wt cells treated with RA for 72 h. After autoradiography, the filter patterns of F9 Wt cells and of F9 RARβ2/− cells were compared for each spot. Differentially hybridized clones were picked and the inserts were subjected to Northern blot analysis.

**DNA Microarray Analysis**

Total cellular RNA was extracted from F9 Wt teratocarcinoma cells and the F9 RARβ2/− cells, both of which were treated with 1 μM all-trans RA for 24 h. Microarray analysis was carried out according to the Affymetrix Genechip expression analysis technical manual. Briefly, total RNA from both cell lines was reverse transcribed into cDNA. After second-strand synthesis, cDNAs were then in vitro transcribed into cRNA with biotinylated ribonucleotides (Enzo Diagnostics, Farmingdale, NY). cRNA (20 μg) was fragmented by heating at 94°C for 35 min. A cocktail containing fragmented cRNA, control oligonucleotide B2, control cRNA (Biotin B, Biotin C, Biotin D, and Cre), and herring sperm DNA was hybridized to microarray chips (MG-U74Av2) for 16 h at 45°C. After washing and staining, the distribution of fluorescent material on the array was measured using a laser scanner. The resultant image was processed with MAS (Microarray Suite) software (Affymetrix, Santa Clara, CA). The microarray experiments were repeated three times with different RNA preparations. Only genes which exhibited different expression patterns between the F9 Wt and F9 RARβ2/− cells in three independent array experiments were further analyzed.

**Microarray Data Analysis**

The Microarray Core of the Weill Medical College of Cornell performed data preparation and data analysis.

1Thompson and Gudas, unpublished data.
The expression level of each gene and the fold change between genes from F9 Wt cells and F9 RARγ−/− cells were calculated using a computer program (GeneSpring, Redwood, CA). This computer program was also used for functional gene grouping analyses.

Acknowledgments
We thank members of the Gudas laboratory for scientific discussions.

References


Identification and Characterization of Retinoic Acid Receptor β2 Target Genes in F9 Teratocarcinoma Cells

Yong Zhuang, Teresa N. Faria, Pierre Chambon, et al.