Characterization of Retinoic Acid Receptor-deficient Keratinocytes*

Philipp Goyette†, Chang Feng Chen‡§, Wei Wang†, Francois Seguin‡, and David Lohnes¶†¶

From the Departments of Molecular Biology, Université de Montréal, §Division of Experimental Medicine, McGill University, and the ¶Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins, Ouest, Montréal, Québec H2W 1R7, Canada

Retinoids are essential for normal epidermal growth and differentiation and show potential for the prevention or treatment of various epithelial neoplasms. The retinoic acid receptors (RARα, β, and γ) are transducers of the retinoid signal. The epidermis expresses RARγ and RARα, both of which are potential mediators of the effects of retinoids in the epidermis. To further investigate the role(s) of these receptors, we derived transformed keratinocyte lines from wild-type, RARα, RARγ, and RARγ null mice and investigated their response to retinoids, including growth inhibition, markers of growth and differentiation, and AP-1 activator protein; RARE, retinoic acid response element; P-Jun, phosphorylated P-Jun; CBP, CREB-binding protein; JNK, c-Jun a stress-activated kinase; RXR, retinoid X receptor; RAR, retinoic acid receptor; AP-1, activator protein 1; MOPS, 4-morpholinepropanesulfonic acid; K, keratin; AP, activator protein; RARE, retinoic acid response element; P-Jun, phosphorylated P-Jun.

Vitamin A derivatives (retinoids) play central roles in embryonic development and maintenance of various tissues in the adult (1–3). Retinoids also exhibit potent antitumorigenic properties in diverse model systems and show potential for the treatment of a number of human malignancies, including diverse epithelial cancers or pre-cancerous lesions (4–9).

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid (RA)3 receptors (RARα, β, and γ and their isoforms) and the retinoid X receptors (RXRα, β, and γ) (10–13). RARs function as ligand-inducible transcriptional regulatory binders by binding, together with an RXR partner, to specific cis-acting response elements (RAREs). RARs can be activated by both RA and its stereoisomer, 9-cis RA, whereas RXRs are activated only by 9-cis RA (14). RXRs are also essential heterodimeric partners for a number of other nuclear receptor signaling pathways, including thyroid hormone, vitamin D, and certain orphan receptors (15, 16). Although 9-cis RA is not obligatory for transcriptional regulation via these pathways, some results suggest that RXR-specific ligands can elicit transcriptional activation in certain settings (e.g. Refs. 17–20).

RARs, like several other nuclear receptors, can function in a ligand-dependent manner to inhibit AP-1 activity, and it has been suggested that the effect of retinoids on the growth of transformed cells may occur through this trans-repression mechanism (21–23). This inhibition is believed to be due, at least in part, to competition for limiting amounts of transcriptional co-factors, such as CBP and/or its homologue p300, common to both pathways (24, 25). Other mechanisms, such as inhibition of the expression of AP-1 family members or c-Jun N-terminal kinase (JNK), may also contribute to this cross-talk (26–29).

Gene targeting of the various RARs has revealed essential and diverse roles for these receptors (2, 30, 31). However, because of perinatal or embryonic lethality inherent to many of these RAR null backgrounds, there is a void in our knowledge of RAR function in a number of contexts, such as tumorigenesis.

Exogenous retinoids can attenuate the effects of tumor promoters in the two stage skin carcinogenesis protocol (9, 32). Among the retinoid receptors, normal epidermis expresses RARγ and RARγ as well as RXRα and RXRβ, with RARγ and RXRα as the predominant heterodimer (33, 34). This pattern of expression prompted us to investigate the roles of RARα and RARγ in mediating the antitumorigenic effects of retinoids in epithelial keratinocytes. To this end, we established RARα, RARγ, and RARγ null keratinocyte lines by transformation with a dominant-negative p53 expression vector and compared the properties of these various lines. Our results demonstrate that RARα and RARγ affect different aspects of retinoid response in these transformed cells, with RARγ being the primary mediator of RA-induced growth inhibition. However, other synthetic ligands affected proliferation independent of the RARs. RAR-dependent, but not -independent, growth inhibitory effects generally correlated with the attenuation of AP-1 transcriptional activity. Finally, the effects of RARα and RARγ on expression of certain keratinocyte markers suggests that each RAR may perform a subset of specific functions, which cannot be entirely fulfilled by other RARs in this cell type.

EXPERIMENTAL PROCEDURES

Primary Keratinocyte Culture and Immortalization—The RAR null mice used in these studies have been described previously (35, 36). RARα, RARγ, and RARγ mutants were generated from the appropriate matings, whereas wild-type offspring were obtained from RARγ-/- intercrosses. Fetuses were procured by caesarean section at 18.5 days post coitus, and genotype was determined by polymerase chain reaction as described (37). Primary keratinocyte cultures were established from...
the epidermis by standard means (38) and cultured in 8-minimal essential medium with 10% chexil-treated fetal calf serum (calcium concentration of 0.5 mM), insulin (5 μg/ml), hydrocortisone (0.5 μM), MgCl₂ (1.5 mM), cholela toxin (1.2 × 10⁻⁸ μM), adenine (24 μg/ml), and genti- mycin (10 μg/ml). The next day, the cells were fed with medium further supplemented with epidermal growth factor (10 ng/ml) and expanded for several days. Cultures were treated at 3–5 days post-plating with versene (0.5 mM EDTA in phosphate-buffered saline) to remove contaminating fibroblasts. The cells were subcultured at a 1:3 ratio at most 2 times prior to transformation. A single 10-cm plate of cells (∼2 × 10⁶) of each genotype was harvested, and cells were resuspended in 800 μl of medium. The cells were then electroporated (250 mV, 960 microfarads in a 0.4-cm gap cuvette) with 25 μg of a linearized expression vector harboring a mutated p53 from the Friend erythroleukemia cell line CB7 (39). Cells were plated and routinely subcultured until past crisis. All experiments were performed using cultures between passage 16 and 26. Growth Assays—Transformed keratinocytes were seeded into 96-well plates at a cell density of 500 cells/well and were treated the following day with vehicle (Me₂SO) or the appropriate retinoid (RA, 9-cis RA, 4-HPR, or TTNPB). Medium was replenished every second day. Growth was assessed either in response to varying concentrations of retinoid at eight days post-plating or over time in response to 10⁻⁶ M ligand. DNA content was assessed as a measure of cell growth using crystal violet staining as described previously (40). Relative dye binding was assessed by OD at 590 nm using a microplate reader. Results were expressed either as A₅₉₀ values or as growth relative to untreated controls and were derived from the mean (± S.D.) of four replicate wells. Transient Transfection and AP-1 Activity Assay—Transfections were performed using Lipofect ACE reagent (Life Technologies, Inc.). Briefly, cells were plated in 6-well cluster plates at 4 × 10⁴ cells/well. Transfections consisted of 0.5 μg of AP-1 reporter or appropriate control (41), either alone or with expression vectors encoding c-Fos, c-Jun, CBP, p300, or RARs. Total DNA (5 μg; normalized with KS⁺) was mixed with 10 μl of lipid and added to 100 μl of serum-free 8-minimal essential medium. The lipid/DNA mixture was then added to the cells in 1 ml of complete medium and incubated at 37°C overnight. Medium was changed daily, and luciferase activity was assessed 48 h post-transfection. Results were corrected for protein concentration and are expressed as the mean (± S.D.) from three independent transfections. All experiments were repeated at least three times with comparable results. Electrophoretic Mobility Shift Assays and Western Blot Analysis—Cells were cultured in 10-cm plates in the presence of RA (10⁻⁶ M) or vehicle for 48 h prior to harvest. Nuclear proteins were isolated from each cell line, and protein concentration was determined using the DC protein assay kit (Bio-Rad). Electrophoretic mobility shift assays were performed essentially as before (42). Briefly, binding reactions containing ~2 ng of probe (50,000 cpm) and 5 μg of nuclear protein were separated by electrophoresis through a 6% polyacrylamide gel containing 0.25 × Tris borate and EDTA. Specificity of binding was assessed by competition with a 10-fold excess of unlabeled RARE (5'-GGGTAGGC- TTACCGAAAAAGTTTCACTGCA) or AP-1 (5'-GATCCGATGAGTCAGCCA) double-stranded oligonucleotides. For Western blot analysis, 40 μg of nuclear protein from the various cell lines were size fractionated on a 10% SDS-polyacrylamide gel electrophoresis and electroblotted to Immobilon-P polyvinylidene diflu- rode membrane as recommended by the supplier (Millipore). Proteins

**FIG. 1.** Electrophoretic mobility shift assay of an RARE sequence by nuclear extracts from transformed keratinocyte lines. Nuclear extracts (5 μg) were incubated with a labeled double-stranded RARE oligonucleotide probe (50,000 cpm) and bound and free probe separated by polyacrylamide gel electrophoresis. Binding was competed with 10-fold excess RARE probe (C) but not by a nonspecific probe comprised of an AP-1 recognition sequence (NSC). Note the presence of a nonspecific complex (N/S) migrating slightly faster than the RAR-containing complex (RAR). WT, wild type.

**FIG. 2. Growth of transformed keratinocyte lines.** The plots indicate growth of transformed keratinocyte lines in the absence (closed circles) or presence of 10⁻⁶ M RA (open circles) over 11 days. Microphotographs show representative cultures after 6 days of growth in the presence of vehicle (DMSO, Me₂SO) or 10⁻⁶ M RA as denoted on the top of the columns. Genotypes of the cultures are indicated to the left. WT, wild type.
of interest were detected by incubation with the desired antibodies and detection with an ECL kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Antibodies were purchased from Santa Cruz Biotechnology.

Northern Blot Analysis—Fifteen micrograms of total RNA, isolated by Trizol reagent (Life Technologies, Inc.), were size fractionated on a 1% agarose-formaldehyde gel in MOPS buffer and transferred to a MAGNA nylon membrane (MSI). Fragments were isolated by restriction digestion of cDNAs followed by purification by Geneclean and used to generate probes by labeling with $^{32}\text{P}$CTP by random priming with an oligo labeling kit (Amersham Pharmacia Biotech). Membranes were hybridized according to the manufacturer's directions.

RESULTS

Generation of RAR Null Cell Lines—Primary cultures of wild-type and RAR null keratinocytes showed no major differences in morphology, growth, or immortalization with dominant-negative p53. All lines grew well for at least 40 passages, suggesting that they were immortalized. None of the lines formed colonies in soft agar or were tumorigenic in nude mice.$^2$

Electrophoretic mobility shift assay revealed that, relative to wild-type extracts, disruption of RAR$\alpha$, and to a greater extent RAR$\gamma$, decreased specific binding to an RARE, and association was completely abolished in extracts from RAR$\alpha\gamma$ double null cultures (Fig. 1). Northern blot analysis confirmed the disruption of RAR$\alpha$ and/or RAR$\gamma$ message in the appropriate cell line (data not shown). RAR$\beta$ transcripts were undetectable in all lines by Northern blot or polymerase chain reaction approaches, consistent with previous studies indicating that this receptor type is not expressed in epidermal keratinocytes (33, 43). These data suggest that there is no compensatory up-regulation of the remaining receptors in response to disruption of a given RAR.

Contribution of Specific RARs to Retinoid-mediated Growth Inhibition—All transformed cell lines exhibited similar morphology and growth characteristics in the absence of retinoid treatment (Fig. 2). However, wild-type and RAR$\alpha^{-/-}$ cultures were growth inhibited by $10^{-6}$ M RA (Fig. 2). In marked contrast, RAR$\gamma^{-/-}$ cells were highly resistant and RAR$\alpha\gamma^{-/-}$ cultures were completely resistant to these effects. The growth arrest observed in wild-type and RAR$\alpha$ null cultures was likely because of the inhibition of proliferation as opposed to apoptosis, as judged by thymidine incorporation and programmed cell death assays (data not shown).

Dose-response experiments were performed to determine the relative sensitivity of the various cell lines to growth arrest by RA, 9-cis RA, TTNPB, or 4-HPR. As shown in Fig. 3, wild-type keratinocytes exhibited a significant reduction in proliferation at $10^{-9}$ M RA, with the maximal affect at $10^{-7}$-$10^{-6}$ M RA. RAR$\alpha^{-/-}$ keratinocytes exhibited a similar profile, although their response to RA was slightly more pronounced than wild-type cultures. Consistent with time-course analysis, RAR$\gamma^{-/-}$ keratinocytes were only marginally

**FIG. 3. Analysis of keratinocyte proliferation in response to RA, 9-cis RA, TTNPB, and 4-HPR.** Cells were grown in the presence of vehicle or retinoids (from $10^{-13}$ M to $10^{-6}$ M) for 8 days. Growth was assayed by DNA content as described under “Experimental Procedures.” Results are the mean ± S.D. of quadruplicate samples and are expressed relative to untreated cultures for each line. WT, wild type.

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$^2$ P. Goyette, C. F. Chen, W. Wang, F. Seguin, and D. Lohnes, unpublished observation.
inhibited by the highest dose of RA examined (10^{-6} M), and RARγγ−/− keratinocytes were not significantly affected by RA at any dose tested.

9-cis RA is a ligand for both RARs and RXRs, and RXR agonists have been shown to induce effects on growth or differentiation in several model systems. Proliferation of both wild-type and RARα−/− cultures was inhibited by 9-cis RA, although higher concentrations were required compared with RA (Fig. 3). Interestingly, 9-cis RA had no significant outcome on the growth of either RARγ−/− or RARγγ−/− cultures. This finding suggests that RXR activation does not lead to growth arrest in this model system, at least in the absence of RARs. Whether RXR-specific signaling has other biological consequences remains to be investigated.

The RAR agonist TTNPB was a very potent inhibitor of growth in wild-type or RARα−/− cultures with an effect evident at 10^{-11}-10^{-10} M (Fig. 3). However, TTNPB affected RARγ−/− and RARγγ−/− cultures only at the highest dose tested (10^{-6} M). Whether this is indicative of effects on other pathways or is because of nonspecific cytotoxicity is unknown.

The synthetic retinoid 4-HPR has been shown to be a potent inducer of growth arrest and/or apoptosis in several model systems (44–46). This compound was the least efficient of all those tested in inhibiting proliferation of wild-type and RARα−/− cultures (Fig. 3). However, in marked contrast to the other retinoids, 4-HPR affected the growth of RARγ and RARγγ cultures at high doses, consistent with receptor-dependent and -independent mechanisms of action for this compound (47–50).

RAR Regulation of AP-1 Transcriptional Activity—RARs can repress AP-1 transcriptional activity, and this mechanism of action has been proposed to underlie at least some of the antitumorogenic effects of retinoids (8, 9, 43, 51). In transient transfection assays, we found that RA (10^{-6} M) inhibited AP-1 activity 8–10-fold in wild-type and RARα−/− cultures (Fig. 4A). AP-1 activity in RARγ−/− cultures was more modestly affected, typically exhibiting 10–30% reduction, whereas activity in RARγγ−/− cultures was not affected. The latter line was capable of response following re-introduction of either RARα or RARγ by transient transfection (Fig. 4B). Thus, attenuation of AP-1 activity requires the presence of at least one functional RAR, although there does not appear to be discrimination between receptor types for this outcome.

Dose-response studies revealed a close parallel between AP-1 activity and growth inhibition mediated by all four compounds in wild-type cultures (Fig. 5). However, growth arrest induced by 4-HPR in RARγ and RARγγ mutant lines never correlated with a reduction of AP-1 activity (data not shown). This finding underscores a unique and unknown mechanism of action for this retinoid in affecting proliferation.

We next determined the effect of RA on the expression of AP-1 members in wild-type and RAR null lines. Both the basal mRNA levels and RA response of several of the AP-1 members varied across the different RAR null lines. In untreated cells, c-fos expression was comparable across all four lines, although it was slightly reduced in RARγ cells (Fig. 6). RA strongly inhibited c-fos in both wild-type and RARα−/− lines but had no effect in RARγ or RARγγ cultures. This pattern was also observed at the protein level (Fig. 7). In contrast, treatment affected c-jun expression only in RARα null cultures, although basal mRNA levels varied across the lines. However, c-Jun protein did not reflect its cognate mRNA levels and was reduced by RA treatment in wild-type, RARα−/−, and RARγ−/− cultures. Phosphorylated c-Jun (P-Jun) levels paralleled those of c-Jun, suggesting that variations in phosphorylation were because of alterations in total c-Jun levels, rather than affects on JNK activity.

Fra-1 expression was barely detectable in wild-type, RARα−/−, and RARγγ−/− lines but was elevated in RARγ−/− cultures; Western blot analysis was inconclusive, as the signal was too weak to be distinguished (data not shown). junB and junD expression did not vary significantly with the exception that junB levels were slightly reduced in the RARγγ−/− line (Fig. 6 and data not shown).

A number of mechanisms have been suggested to underlie retinoid repression of AP-1 activity. These include inhibition of JNK activity, which is unlikely given the observation that P-Jun levels appear to change as a function of c-Jun levels. Alternatively, the observed down-regulation of c-Fos and/or c-Jun proteins might play a role, especially if either of them are limiting. A third mechanism involves competition for limiting ancillary factors common to both RAR and AP-1 transcriptional complexes, such as p300/CBP (24). We addressed the latter two possibilities by assessing the ability of exogenous CBP, p300, c-Fos, or c-Jun to negate the effects of RA treatment on AP-1 activity.

CBP or p300 transfection in wild-type cells resulted in a dose-dependent increase in AP-1 activity in the absence of RA (Fig. 8A). Interestingly, p300 appeared to be more potent in affecting AP-1 activity, suggesting that it may be preferred...
over CBP in this context. Despite this increase in activity, expressing the data as fold inhibition indicated that both factors resulted in only a modest reversal of inhibition (Fig. 8B).

Overexpression of either c-Fos or c-Jun also resulted in an increase in basal AP-1 activity, again with only a marginal reduction in fold repression mediated by RA (Fig. 8). Although this rescue effect was more pronounced when both c-Jun and c-Fos were co-transfected, repression was not completely abolished (Fig. 8B). These observations suggest that several mechanisms, including titration of limiting co-factors and inhibition of expression of AP-1 family members, act in concert in an RAR-dependent manner to attenuate AP-1 activity in these transformants.

**Effect of RAR Ablation on Gene Expression—**Northern blot analysis was performed to study the effect of receptor disruption on the expression levels of several genes implicated in keratinocyte growth and differentiation. The major integrin isoforms found in epidermis are integrin α2, α3, α6, β1, and β4. These are expressed in basal keratinocytes, and a decrease in integrin expression is generally correlated with differentiation and loss of proliferative potential (52, 53). Northern blot analysis revealed that, with the exception of the α3 isoform, all integrins were down-regulated by RA treatment in wild-type, RARα−/−, and RARγ−/− cultures in a manner that correlated with the effects of treatment on proliferation (Fig. 9). Moreover, although integrin expression was not affected by RA treatment in RARγ cultures, basal expression of integrins α2, α3, and β4 was substantially reduced. The seemingly contradictory observation that both RA excess and RAR loss can reduce expression of several integrins is perhaps indicative of an altered differentiation state in the double mutant line. Interestingly, integrin β1 expression decreased in untreated RARα−/− cells and was up-regulated in untreated RARγ mutant cultures.

Keratin expression patterns reflect the differentiation states of the various epithelial strata (52, 53). K5/K14 are expressed in basal epidermal cells, whereas K1/K10 are associated with early differentiation steps and predominate in suprabasal cells. K6 and K19 are not expressed in normal epidermal keratinocytes but are often observed in situations of aberrant proliferation, such as psoriasis, wound healing, and propagation in tissue culture. With the exception of RARγ null cultures, RA treatment repressed expression of K10 (Fig. 9). This observation may be related to the fact that RA excess can inhibit keratinocyte differentiation (54, 55). However, RAR disruption did not result in up-regulation of this differentiation marker, suggesting that K10 is not normally regulated by the RARs but responds to pharmacological levels of RA.
RA suppressed K6 expression in wild-type, RARα⁻/⁻, and (to a lesser extent) RARγ⁻/⁻ cultures, consistent with the effect of treatment on both AP-1 activity and proliferation. K19 expression was induced in wild-type and RARα²/² cultures. Interestingly, this gene was also up-regulated in RARγ²/² cells in the absence of treatment. These data indicate that, as for the integrins, the roles of the various RARs on keratin expression vary depending on both the receptor and the gene of interest. Moreover, most of the integrin and keratin markers were affected in both RARα and RARγ null cultures. This demonstrates that both receptor types transduce effects on expression of many responsive genes.

**DISCUSSION**

We present, for the first time, the effects of RAR disruption on the characteristics and RA response of transformed epidermal keratinocytes. These data indicate that each RAR type plays both specific as well as overlapping roles in events related to keratinocyte growth and gene expression.

**RARs Are Not Necessary for Survival and Growth of Transformed Keratinocytes**—Previous work using a dominant-negative RARα under the control of a basal-keratinocyte-specific promoter suggested that RA signaling is essential for normal keratinocyte differentiation (56). We found that wild-type and RARαγ null keratinocytes are comparable in regards to growth and morphology, although expression of some markers, such as integrin α2, did differ. It is unlikely that RARβ plays any compensatory role in these cells, as we have never observed expression of this receptor in these cultures irrespective of RAR status. Moreover, the RARαγ line was completely resistant to excess RA with respect to all outcomes examined. Thus, these cells are likely completely devoid of functional RARs. The difference between the relatively mild effects observed in the present study, compared with a dominant-negative RAR (56), suggests that transgene expression affects other pathways, perhaps by sequestration of RXRs. Alternatively, we cannot exclude an unrecognized compensation mechanism in the RAR null animals and derivative cells that may mask certain roles for these receptors in skin.

**RARγ Is the Principle Mediator of Growth Arrest in Transformed Keratinocytes**—Analysis of the effects of the various RARs on growth inhibition suggests that the major player in transducing this effect is RARγ with only a negligible contribution by RARα. This may simply be because of the prevalence of the former receptor type in keratinocytes (57) rather than indicative of receptor-specific function. Nevertheless, irrespective of the basis for this finding, these data suggest that targeting RARγ is a logical strategy to affect disorders of keratinocyte proliferation.

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**FIG. 8. Rescue of AP-1 repression.** Wild-type keratinocytes were transfected with an AP-1 reporter in the absence or presence of various amounts of expression vectors encoding CBP, p300, c-Fos (0.1, 0.5 or 1.0 µg) or c-Jun (0.5 µg), or c-Fos plus c-Jun (0.5 µg each). A, cells were treated with vehicle (closed bars) or 10⁻⁶ M RA (open bars), and luciferase activity was assessed 48 h post-transfection. Results are expressed as percentage activity relative to untreated wild-type control. B, the results from A were expressed as fold AP-1 activity relative to untreated transfected cultures. Results are the mean ± S.D. of three independent transfections for both A and B.
In the absence of the RARs, the RXR ligand 9-cis RA had no effect on proliferation, suggesting that liganded RXRs do not impact on keratinocyte growth, at least in the absence of RARs. This is in contrast to previous reports, which suggests that growth inhibitory effects can be mediated by RXR-selective agonists in several other transformed cell types (17, 19, 58–61). This suggests either that RXRs ligands inhibit growth in a cell type-specific manner or that these synthetic agonists have effects that cannot be mimicked by 9-cis RA. It will be of interest to determine if such ligands exert an effect in the RARα null line.

Although 9-cis RA inhibited the growth of wild-type and RARα null lines, it was consistently less potent than RA or TTNPB. Because both RA and 9-cis RA have comparable affinities for the RARs (62), this observation suggests either that 9-cis is more labile than RA or is titered away from RAR signaling. In contrast to 9-cis RA, the synthetic RAR agonist TTNPB was a strong inhibitor of growth. As displacement assays suggest that TTNPB binds to the RARs with a lower affinity than RA, the greater relative potency of this analog likely lies in its enhanced stability and/or weaker association with cellular RA-binding proteins relative to RA (63). Moreover, although full manifestation of growth inhibition by TTNPB required the RARs, a slight inhibition at the highest dose used was seen in RARγ and RARαγ null cultures, suggesting either nonspecific cytotoxicity or effects via nonreceptor-mediated pathways.

Evidence for RAR-independent Mechanisms of Growth Inhibition by 4-HPR—4-HPR can inhibit growth and induce apoptosis in a number of model systems (44, 64, 65). Although this compound can act directly via the RARs, some of its effects may also be mediated by receptor-independent mechanisms (47, 66). Indeed, we found that 4-HPR inhibited proliferation in all lines assessed but that this occurred at lower concentrations in RAR-positive cultures. This is consistent with 4-HPR acting through both RAR-dependent and -independent mechanisms. As 4-HPR is well tolerated at high doses (65), it should mediate effects even in those epithelial tumors that lack retinoid responsiveness to “pure” RAR agonists.

RARs and Inhibition of AP-1 Activity—RA is a potent inhibitor of AP-1, and this mechanism of action has been proposed to underlie some of the antitumorigenic effects of retinoids (2, 67–70, 72, 73). We found that both RARα and RARγ can repress AP-1 activity in p33-transformed keratinocytes in culture. However, attenuation of AP-1 activity in RARγ null cultures (−30%) did not agree well with growth inhibition in this cell line, which was minimal. A lack of correlation was especially notable in the case of 4-HPR, which never repressed AP-1 reporter activity in RARγ or RARαγ null cultures at concentrations that inhibited their growth. This observation supports the existence of retinoid-mediated mechanisms of growth inhibition unrelated to AP-1 activity, at least in this model.

Multiple Pathways for RA in Inhibition of AP-1 Activity—Several mechanism have been proposed to underlie the crosstalk between AP-1 and RA signaling pathways. These include titration of common transcriptional co-regulators, such as CBP/p300 (24), effects on expression of AP-1 family members (27, 74, 75), or inhibition of JNK activity (28). We found evidence that supports the two former possibilities in our model.

RA inhibited c-Fos and c-Jun (and P-Jun) protein levels in a manner that partially correlated with the observed attenuation of AP-1 activity. Although c-Jun levels paralleled the effect of treatment on AP-1 across the various cell lines, c-Fos was not affected by RA in RARγ or RARαγ null cultures. These data underscore a specific role for RARγ in inhibiting c-Fos expression, whereas either RARα or RARγ affected c-Jun. In this regard, it is interesting to note that c-Jun was also elevated in untreated RARα and RARαγ lines, indicating that RARα, presumably in its unliganded state, represses c-Jun expression. Consistent with this possibility, prior studies also suggest that certain RARs may be antagonistic to one another in F9 cells (71, 76).
Transfection of either CBP or p300 induced basal AP-1 expression in wild-type keratinocytes and modestly attenuated the effects of RA on AP-1 activity. The persistent inhibition of AP-1 by RA, even following transfection of higher levels of CBP/p300 expression vectors, suggests that the RARs remain in excess. Alternatively, as c-Fos and/or c-Jun also appear to contribute to this relationship, the combination of several distinct events probably underlies retinoid antagonism of AP-1.

RARs and RARγ Play Both Overlapping and Specific Roles in Keratinocytes—The phenotype of RAR mutant mice clearly indicates that these receptors are highly but not completely redundant. This is supported by work using RAR null F9 embryocarcinoma cells (e.g. Refs. 71 and 76). Our present findings suggest that specificity also exists regarding RAR function in keratinocytes. Although certain aspects of this selectivity may be explained by the relative levels of expression, with RARγ being the predominant receptor type, this is not always the case. For example, repression of integrin α6 and integrin β3 was more affected by the loss of RARα than the loss of RARγ.

In addition to specific functions, it is also interesting to note that RARα may actually attenuate the effects of certain RARs in certain instances. For example, growth inhibition mediated by RA, 9-cis RA, or TTPNB was greater in RAR null cells relative to wild-type cultures, suggesting that RARα is a more efficient inducer of growth arrest in the absence of RARα. Other complex interactions were also observed. RA induced keratin 19 expression in wild-type cells, and this induction was greatly reduced in RARα expression vectors and AP-1 reporters, Jana Krosl for several of the cases. For example, repression of integrin α6 and integrin β3 was more affected by the loss of RARα than the loss of RARγ.

Acknowledgments—We thank P. Chambon for the RAR null mice, M. Nemer, K. McBride, M. Karin, R. Goodman, and S. Benchimol for expression vectors and AP-1 reporters, Jana Krosl for several of the antibodies, and members of the laboratory for support and suggestions.
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J. Biol. Chem. 2000, 275:16497-16505.
doi: 10.1074/jbc.M909382199 originally published online March 19, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909382199

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