0026-895X/97/030377-06$3.00/0

MOLECULAR PHARMACOLOGY, **51:**377–382 (1997).

Functional Consequences of Reduced Retinoic Acid Receptor γ Expression in a Human Squamous Cell Carcinoma Line

RICARDO I. MONZON, CATHERINE FILLMORE, and LAURIE G. HUDSON

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, Illinois 60611

Received July 29, 1996; Accepted December 6, 1996

SUMMARY

Nuclear receptors for retinoic acid are important modulators of epidermal cell proliferation and terminal differentiation. Aberrant expression of retinoic acid receptors (RARs) and retinoid X receptors in the epidermis has been associated with altered differentiation capacity and tumor progression. In this study, we describe a human squamous cell carcinoma line, SCC 12F, which displays reduced RARγ expression and diminished responsiveness to retinoic acid. When compared with normal keratinocytes or other squamous cell carcinoma lines that display normal levels of RARγ, several measures of cellular response to retinoic acid are altered in SCC 12F cells, including inhibition of cornified envelope formation, reduction of involucrin mRNA expression, and transcriptional regulation of the involucrin gene. Normal patterns of ligand-dependent transcriptional response were restored upon co-transfection of an expression vector containing either RARα or RARγ. Our findings demonstrate that reduced expression of RAR may have direct functional consequences with regard to keratinocyte differentiation and that the defect may be alleviated by reintroduction of functional receptor.

Retinoids (vitamin A and metabolic derivatives) play a crucial role in cell proliferation, differentiation, and vertebrate development (1–3). Both excesses and deficiencies in these compounds exert profound effects throughout the organism. In epithelial tissues, a characteristic pattern of widespread squamous metaplasia is observed as a consequence of vitamin A deficiency. In contrast, extended exposure to high concentrations of retinoic acid suppresses terminal differentiation of the epidermis and inhibits expression of numerous keratinocyte-specific genes, which are associated with differentiation (4, 5).

The biological actions of retinoids are mediated by multiple retinoid responsive nuclear proteins (1–3). There are three subtypes of each of the RARs and RXRs (α, β, and γ) as well as isoforms generated by alternate promoter usage and splice variants (3). Interestingly, the predominant RAR and RXR subtypes expressed in keratinocytes differ from those generally seen in other tissues, with RAR-γ and RXRα being the predominant type in the epidermis (3–6). Targeted expression of dominant negative RARs in the skin reveals important functions for these receptors in epidermal differentiation. Expression of mutant RARs that efficiently inhibit the activities of endogenous RARs dramatically suppresses epidermal maturation and barrier function *in vivo* (7, 8) and keratinocyte differentiation *in vitro* (9).

Changes in RAR expression in tumor cells suggest additional roles for these proteins. The *RARβ* gene seems to be deleted at high frequency in lung tumors (10), and its mRNA is expressed in only 35% of head and neck SCC tumors compared with 70% in adjacent normal tissue (11). In addition, transfection of RARβ2 seems to have a tumor-suppressive effect when placed into epidermoid derived cells that do not express the gene (12). In SCCs, there seems to be an inverse relationship between RARβ expression and differentiation. RARβ is frequently absent in oral SCCs, and this correlates with aberrant keratin expression (13, 14). Although RARγ expression is not generally lost in SCCs, it is frequently reduced (5, 15, 16). In contrast, potential differences in RARα expression in tumor cells is less clear; it has been reported that RARα levels do not seem to differ substantially between tumor cells and their normal counterparts (15) and that RARα1 transcripts are reduced in malignant tumor cells within mouse epidermis (16). Taken together, these findings suggest that differences in the expression of particular RARs may play a role in the development or progression of SCC and contribute to the altered cellular responsiveness to retinoic acid that is frequently observed in SCC (17–20).

This work was supported by American Cancer Society Grants BE-135 and JFRA-380 and in part by grant 0439 from the Smokeless Tobacco Research Council, Inc. R.I.M. is supported by National Institutes of Health training grant T32-ES07124–11.

ABBREVIATIONS: RAR, retinoic acid receptor; RXR, retinoid X receptor; DME, Dulbecco's modified Eagle's medium; F12, Ham's F12 nutrient mixture; SCC, squamous cell carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; bp, base pair(s); AP1, activator protein 1.

In this study, we identify a human SCC line (SCC 12F) with substantially reduced RARγ expression compared with normal keratinocytes and several other established SCC lines. This cell line displays reduced responsiveness to the differentiation-suppressive effects of retinoic acid, but ligand-dependent transcriptional activity was restored upon introduction of RARα or RARγ. These findings suggest that reduction in RAR abundance may have direct functional consequences for modulation of keratinocyte differentiation by retinoic acid.

Materials and Methods

Cell culture and cornified envelope formation. Human SCC lines isolated by Rheinwald and Beckett (21) have been characterized with respect to tumorigenicity and defects in terminal differentiation. SCC-9 was obtained from the American Type Culture Collection (Rockville, MD), and SCC line 12F was provided by W.A. Toscano, Jr. (Tulane University, New Orleans, LA). SCC cells were grown in a 1:1 mixture of DME/F12 containing 5% iron-supplemented defined calf serum (Hyclone, Logan, UT). For experiments involving ligand treatment, endogenous ligand concentrations were reduced by charcoal extraction of serum, and calcium-depleted medium was prepared by batchwise extraction with Chelex resin (Bio-Rad Laboratories, Richmond, CA) as previously described (22). For the indicated experiments, cell cultures were then rinsed twice with Ca^{2+}/Mg^{2+}-free phosphate buffered saline and placed in Chelex-treated DME/F12/5% serum supplemented to 1 mM $MgCl_2$ and 50 μM $CaCl_2$ for 48 hr to ensure uniformity of culture conditions for all cell lines. A basal cell morphology was apparent under these conditions. After 48 hr, the cells were placed in medium containing standard calcium concentrations (permissive for differentiation) with or without ligand at the concentrations and times indicated in the figure legends. Stock solutions of dexamethasone (Sigma Chemical, St. Louis, MO) were prepared in ethanol and all-*trans*-retinoic acid (Sigma) and 9-*cis*-retinoic acid (a gift from Ligand Pharmaceuticals, San Diego, CA) were prepared in dimethylsulfoxide. The final concentration of each solvent did not exceed 0.1% in the medium, and appropriate solvent controls were included for each treatment condition. The differentiation capacity of cells treated without or with hormones was determined by evaluating spontaneous and ionophore-induced cross-linked envelope formation after release from basal cell constraint (low calcium medium) as previously described (22).

Isolation and analysis of RNA. Total RNA was isolated from treated cells by extraction in guanidine isothiocyanate followed by centrifugation on a CsCl cushion (23) or using TRI reagent (Molecular Research, Cincinnati, OH) according to the manufacturer's instructions. For Northern analysis of RNA, 10 μg of total RNA was denatured in glyoxal/dimethylsulfoxide (23), fractionated on a 1.0% agarose gel, transferred to nylon membrane (Hybond N; Amersham, Arlington Heights, IL). A complimentary riboprobe was generated to an 850-bp *Pst*I fragment from the involucrin structural gene (24) and 780-bp *Pst*I-*Xba*I fragment of the human *GAPDH* gene was used as a control probe. The blots were hybridized, and the results were visualized by autoradiography and quantitated by phosphorimage analysis using a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji, Tokyo, Japan) as previously described (22). Alternatively, RAR mRNA levels were determined by an RNase protection assay (23). Briefly, 10 μg of total RNA was hybridized with 1×10^5 cpm of antisense ^{32}P-labeled riboprobe, and the reaction was subjected to RNase digestion using an RNase cocktail (Ambion, Austin, TX). Riboprobe for RARα was generated to a receptor linearized by restriction digest with *Pml*I at position 364 bp (25), which generated a 364-bp protected fragment. Riboprobe for RARγ was generated by restriction digest with *Acc*I at position 827 bp (26), which generated a 750-bp protected fragment. The protected fragments were visualized by autoradiography and quantitated by phosphorimage analysis using a Fujix BAS 2000 Bio-Imaging Analyzer. Relative RAR expression levels were generated by normalizing RAR values to those obtained in parallel reactions using complimentary riboprobe generated to GAPDH (Ambion). Reverse-transcription polymerase chain reaction analysis was performed using 25 ng of each primer per reaction and cDNA generated from 625 ng of total RNA. Twenty-five cycles were performed for detection of RARα and 40 cycles were performed for RARβ. The RAR primers have been previously described (27), and parallel reactions using control primers to GAPDH (5′TCCTTGGAGGCCATGTAGGCCAT3′ and 5′TGATGACATCAAGAAGGTGAAG3′) were performed using 30 cycles (28).

Transfection procedure and analysis of reporter gene activity. *Hin*dIII-*Xho*I and −197 bp (single AP1 site) *Apa*I-*Xho*I fragments of the involucrin 5′ untranslated region were each subcloned into the luciferase expression vector pSVOALΔ5′ (22, 29). Subconfluent cells grown on 6-cm plastic tissue culture plates were transfected using a modification of a lipid-mediated protocol, and luciferase activity was measured as previously described (22). In co-transfection experiments, luciferase reporter plasmid was transfected at the ratio indicated in the figure legends with either RSV-human RARα or RARγ expression vectors (a generous gift of Dr. R. Evans, Salk Institute, La Jolla, CA) or control vector containing the neomycin resistance gene (30) and incubated for the times described in the legends of Figs. 3–6. Luciferase activity in each sample was normalized to internalized reporter plasmid DNA or β-galactosidase activity (29).

Results

RARγ levels are reduced in SCC 12F cells. Altered cellular response to retinoic acid is a common characteristic of SCC cells (17–20), and changes in RAR levels or signaling capacity are potential mechanisms that account for this observation. We compared expression of RARα, RARβ, and RARγ mRNA among normal human keratinocytes and several established SCC lines. As shown in Table 1, the expression of RARγ was reduced in several of the SCC lines examined, as has been previously reported (14). The loss of RARγ expression was most pronounced in SCC 12F cells (4- to 5-fold) when compared with normal keratinocytes, and this observation was confirmed by a comparable decrease in RARγ protein as detected by Western blot analysis (data not shown). Some reduction in RARα expression was also noted among normal keratinocytes and certain SCC lines (Table 1), but they were of a lesser magnitude (~2-fold) than those

TABLE 1

Relative RAR levels in SCC lines compared to normal keratinocytes.

Values given for RARα, RARβ and RARγ represent expression levels relative to normal human keratinocytes (NHK) defined as 1.0. For all receptor analysis, expression was normalized with respect to GAPDH in parallel or concurrent reactions. RARα expression was obtained using RNase protection analysis and independently verified using reverse transcriptase-polymerase chain reaction analysis. RARβ expression was obtained using reverse transcriptase-polymerase chain reaction analysis. Values for RARγ were obtained by RNase protection analysis and independently verified by Northern blot analysis.

Cell line	Receptor		
	RARα	RARβ	RARγ
NHK	1.0	1.0	1.0
SCC-9	0.64	0.88	0.93
SCC 12F	0.47	0.94	0.26
SCC 13	1.08	ND	0.46
SCC 25	0.50	ND	0.62

ND, not determined.

observed for RARγ. RARβ expression was detectable only by polymerase chain reaction analysis and did not differ in the cells examined. We selected SCC 12F and SCC-9 cells for further analysis because RARγ represents >85% of all functional RAR protein in the epidermis (6); therefore, the difference in RARγ expression detected in these two cell lines might be expected to result in altered ligand-dependent cellular responses.

Comparison of cornified envelope repression by retinoic acid in SCC-9 and SCC 12F cells. Cornified envelopes are a marker of keratinocyte terminal differentiation, and differences in cornified envelope formation were observed among SCC lines that differed in RARγ expression (Fig. 1). Retinoic acid decreased cornified envelope formation to 15% of control in SCC-9 cells but to only approximately 55% of control in SCC 12F cells. Similarly, SCC lines 13 and 25 retained retinoic acid modulation of cornified envelope formation with a ligand-dependent inhibition to 22% and 16% of control, respectively. Another mechanism by which retinoids regulate keratinocyte differentiation is through interference with the differentiation-promoting activities of glucocorticoids (17, 22). The ability of retinoic acid to inhibit glucocorticoid-mediated induction of cornified envelope formation was also impaired in SCC 12F cells when compared with SCC-9 cultures (Fig. 1). Thus, in a functional measure of retinoic acid repression of keratinocyte differentiation, SCC 12F cells were markedly less responsive than SCC-9 cells.

Modulation of tissue specific gene expression by retinoic acid is reduced in SCC 12F cells. Because involucrin is the major cornified envelope structural protein expressed in the early stages of keratinocyte differentiation (31), we wanted to determine whether the reduced retinoic acid responsiveness of SCC 12F cells was also reflected in modulation of endogenous involucrin mRNA expression. Under the same culture conditions used for determination of induced cornified envelope formation, retinoic acid markedly inhibited involucrin expression in SCC-9 cells (Fig. 2). In contrast, all-*trans*- and 9-*cis*-retinoic acid (100 nM) decreased involucrin mRNA levels in SCC 12F cells to only 78 ± 5.4% and 63 ± 5.5% of control levels, respectively (3 independent experiments, mean ± standard deviation). The differences in retinoic acid-dependent response among cell lines detected for involucrin mRNA expression (Fig. 2) are consistent with those observed for regulation of cornified envelope formation (Fig. 1).

Reduced retinoic acid responsiveness in the transcriptional activity of tissue-specific genes. Our recent studies demonstrate that retinoic acid transcriptionally regulates involucrin gene expression (22); therefore, we wanted to compare the ligand-dependent responses of SCC-9 and SCC 12F cells in transient transfection analysis using the involucrin promoter. Notably, the magnitude of retinoic acid-dependent repression of luciferase activity differed between the cell lines (Fig. 3). In SCC-9 cells, exposure to retinoic acid for 4 days typically reduced luciferase activity to 12% of the untreated control, which is comparable with the response observed in normal keratinocytes (data not shown). In contrast, activity was inhibited to only ~60% of control in SCC 12F cells. Increasing the concentration of retinoic acid to 1 μM did not result in further repression of luciferase activity (data not shown). In both cell lines, the apparent EC_{50} for retinoic acid was ~0.3 nM (Fig. 3), which suggests that the observed difference in retinoic acid receptor abundance (Table 1), rather than altered receptor affinity, accounts for the differential response in SCC 12F cells.

Glucocorticoids not only promote cornified envelope formation but also induce the expression of genes such as involucrin and type I (keratinocyte) transglutaminase, which are involved in envelope assembly (17, 22). As an additional measure of retinoic acid responsiveness in the two cell lines, we evaluated interactions between retinoic acid and dexamethasone in the regulation of involucrin promoter activity. We observed that retinoic acid modulation of the glucocorticoid response was diminished in SCC 12F cells compared with SCC-9 cells (Fig. 4). These findings are in good agreement with those obtained for ligand regulation of cornified

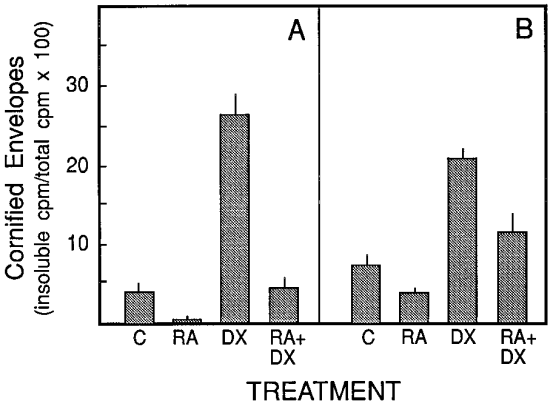

Fig. 1. Ligand regulation of cornified envelope formation. An enriched basal cell population of (A) SCC-9 or (B) SCC 12F cells was selected by incubation in reduced-calcium DME/F12 for 2 days before hormone treatment. Upon addition of hormones (100 nM) to standard calcium medium containing 5% delipidized serum, cells were labeled with [^{35}S]methionine at a specific activity of 2 μCi/ml. After 4 days, the cells were collected, and envelope formation was determined by measuring incorporation of labeled methionine into detergent-insoluble cross-linked protein, as described in Materials and Methods. Envelope competence was calculated as the percentage of cross-linked protein [detergent insoluble (cpm)]) divided by total protein [detergent insoluble (cpm) + trichloroacetic acid precipitable (cpm)]. *C,* Solvent control; *RA,* all-*trans*-retinoic acid; *Dx,* dexamethasone. Values shown are the results (mean ± standard deviation) obtained from triplicate cultures and are representative of multiple independent experiments.

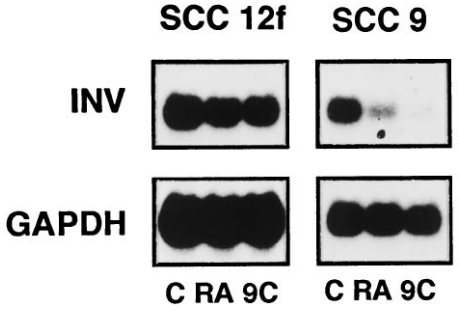

Fig. 2. Hormone regulation of involucrin mRNA expression. Enriched basal cell cultures of SCC 12F and SCC-9 cells were returned to medium containing standard calcium concentrations in the absence or presence of the indicated ligand (100 nM). Total RNA was collected after 4 days. Ten micrograms of total RNA was fractionated on a 1% agarose gel and transferred to Hybond N (Amersham). Blots were probed with an involucrin riboprobe, stripped, and reprobed with GAPDH as an internal control, and values quantitated by phosphorimage analysis as described in Methods. *C,* control; *RA,* all-*trans*-retinoic acid; *9C,* 9-*cis*-retinoic acid.

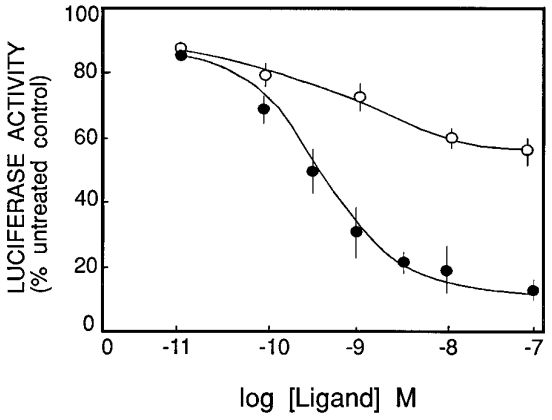

Fig. 3. Concentration dependence for inhibition of involucrin gene expression by retinoic acid. SCC-9 (●) or SCC 12F cells (○) were treated with the indicated concentration of all-*trans*-retinoic acid for 2 days before transfection with a *Hin*dIII-*Xho*I fragment of the involucrin 5′-untranslated region in a luciferase expression vector (22) and incubation for an additional 2 days. Transfections and analysis of luciferase activity were performed as described in Materials Methods. The values shown represent the (mean ± standard deviation) of triplicate determinations and are representative of two independent experiments.

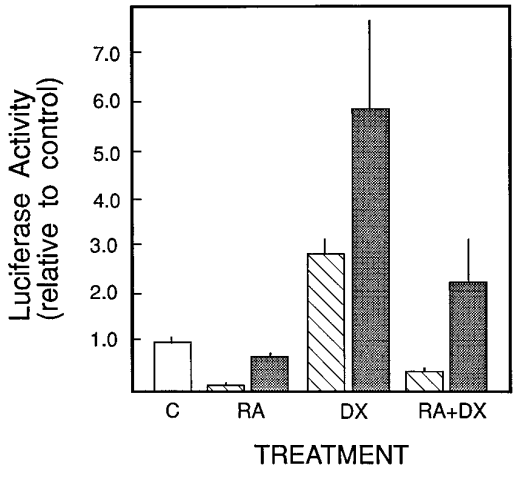

Fig. 4. Comparisons of responses to ligand treatment in SCC-9 and SCC 12F cells. Results obtained from five (SCC-9) or three (SCC 12F) independent experiments were normalized with respect to the untreated control in each experiment (□). The values shown represent the mean ± standard deviation. ▧, SCC-9 cells; ▩, SCC 12F cells.

envelope formation after 4 days of hormone treatment (Fig. 1).

We have shown that the actions of retinoic acid localize to an AP1-containing proximal fragment of the involucrin promoter (22, 32). In addition, treatment of keratinocytes with retinoic acid antagonizes phorbol ester-induced transcriptional activity mediated through this proximal enhancer region (22). Using TPA induction as a tool to induce AP1 activity, we evaluated interaction between the retinoic acid and AP1 signaling pathways. Under these conditions, retinoic acid abolishes TPA-induced involucrin activity in SCC-9 cells (Fig. 5) or normal keratinocytes (data not shown); however, only slight inhibition by retinoic acid was detected in SCC 12F cells (Fig. 5). This same pattern of response was also observed with the keratinocyte transglutaminase promoter (data not shown). Thus, by multiple measures of retinoic acid-mediated activity, which include modulation of cornified envelope formation, repression of endogenous involucrin mRNA expression, and inhibition of the involucrin and keratinocyte transglutaminase promoters, SCC 12F cells are markedly less responsive compared with SCC-9 cells or normal keratinocytes.

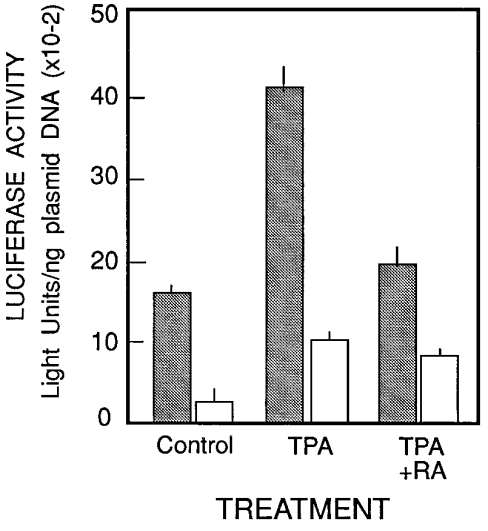

Fig. 5. Comparisons of ligand-dependent suppression of TPA-induced promoter activity in SCC-9 and SCC 12F cells. SCC-9 (▨) and SCC 12F (□) cells were transfected with the *Hin*dIII-*Xho*I luciferase construct and treated with the indicated ligand (retinoic acid, 100 nM; TPA, 20 nM) immediately after transfection. Cells were collected after 24 hr, and luciferase activity was measured. Values shown represent the mean ± standard deviation of triplicate determinations.

Functional consequences of reintroduced RAR. Loss of cellular response to retinoic acid could be the result of decreased receptor expression or of a signaling defect unrelated to RAR abundance. To determine whether the impaired retinoic acid responsiveness of SCC 12F cells could be alleviated by reintroduction of receptor, we co-transfected RARs into SCC 12F (Fig. 6A) cells, then evaluated retinoic acid modulation of AP1-mediated transcriptional activity. In the presence of a control vector, retinoic acid inhibited TPA-stimulated activity in SCC-9 cells (Fig. 6B). Only a slight response (~20% decrease) was detected in SCC 12F cells (Fig. 6A, *white bars*). Co-transfection of RARα restored ligand-dependent inhibition of TPA-induced luciferase activity in SCC 12F cells (Fig. 6A, *gray bars*) and enhanced the ligand-dependent response in SCC-9 cells (data not shown). Similarly, co-transfection of RARγ (Fig. 6A, *black bars*) restored ligand-dependent repression of AP1-mediated transcriptional activity in SCC 12F cells to a level equivalent to that observed for RARα (Fig. 6A, *gray bars*) or stimulation of endogenous receptors in SCC-9 cells (Fig. 6B). These findings indicate that expression of either RARα or RARγ is sufficient to restore a ligand-dependent function in the RARγ-deficient SCC 12F line.

Discussion

The retinoic acid signaling pathway is vital for maintenance of normal epithelial cell function. Loss of receptor abundance or function, differences in vitamin A metabolism, or a functional defect downstream of the RARs have all been suggested as possible contributors to the aberrant responses to retinoic acid observed in tumor cells (5, 10–16, 33). In

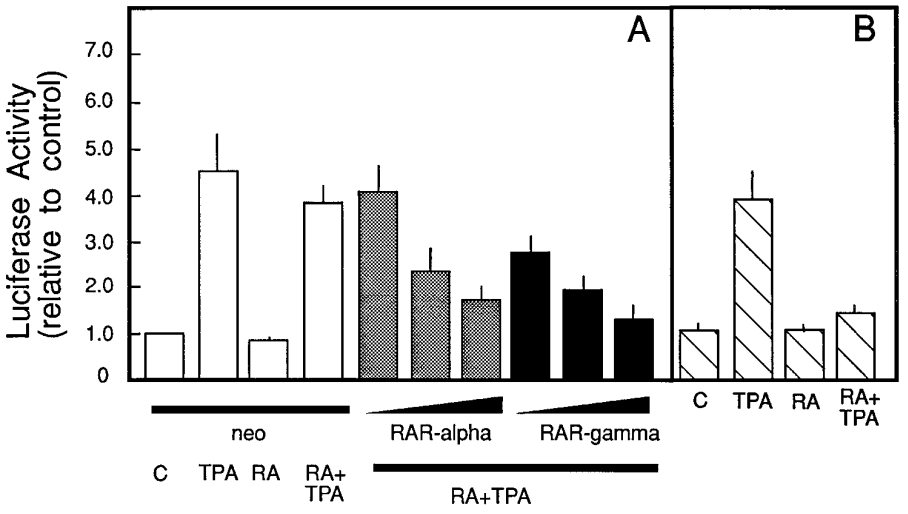

Fig. 6. Consequences of RARα or RARγ co-transfection on ligand-dependent suppression of TPA-stimulated activity. SCC 12F (A) or SCC-9 (B) cells were transfected with 5 μg of the −197 bp (one AP1 site) involucrin deletion construct and 5 μg of a neomycin (*neo*) control expression vector (□), 0.05–5.0 μg of RARα (▨), or 0.05–5.0 μg of RARγ (■) expression vector. Five micrograms of a β-galactosidase expression vector was included as an internal control for transfection efficiency. Cells were treated for a total of 24 hr with retinoic acid (*RA;* 100 nM) with TPA (20 nM) added for the final 12 hr of incubation, then cultures were collected for measurement of enzymatic activities. Values shown represent the mean ± standard deviation of triplicate determinations compared with the untreated cells transfected with the neomycin control vector in three independent experiments.

these studies, we observed diminished ligand-dependent responses with regard to keratinocyte differentiation and tissue-specific gene expression (Figs. 1–5) in a cell line displaying a substantial reduction in RARγ expression (Table 1). Ligand-dependent activity was restored upon reintroduction of RAR in a transient transfection assay (Fig. 6). This finding suggests that the diminished responsiveness to retinoic acid observed in this cell line is a direct consequence of reduced RAR abundance rather than of a signaling defect downstream of the receptor or altered retinoid metabolism. RARα or RARγ were equally able to restore a ligand-dependent response in SCC 12F cells (Fig. 6); thus, there seems to be functional overlap between these receptor subtypes with regard to repression of involucrin gene transcription.

Given the multiplicity of nuclear retinoic acid receptors (RARs and RXRs), redundancy in their functional roles has been proposed (1–3). Targeted disruption of specific RAR subtypes *in vivo* supports this conclusion; loss of individual RAR subtypes does not lead to apparent disruption in the development or function of certain retinoic acid-responsive tissues, including the epidermis (1–3). However, there is also evidence in support of specific roles for RAR subtypes. Direct evidence for unique roles of RAR subtypes in differentiation has been provided through targeted disruption of specific RARs in the F9 embryonal carcinoma cell line (34, 35). Loss of either RARα or RARγ results in impaired induction of a different subset of RAR-inducible genes and opposite changes in the metabolism of retinoic acid (34, 35). These studies clearly show that RARα and RARγ exhibit specificity in the regulation of differentiation-specific genes and suggest that each RAR may possess unique functions. In addition, at least partial functional redundancy among receptors is observed within the F9 model system (36). Re-expression of RARγ or over-expression of RARα1 in RARγ-null F9 cells restores retinoic acid regulation of target gene expression and cell differentiation. However, RARβ2 over-expression restored only target gene regulation, not differentiation potential (36).

The abundance of RARγ and RXRα in the epidermis compared with other tissues suggests that these receptor subtypes may mediate distinct responses in keratinocytes. However, to date, there are relatively little direct data in support of different functional roles for RAR subtypes in the epidermis. Some evidence is provided through the use of RARγ-selective retinoids, which implicate RARγ in the mediation of topical retinoic acid efficacy and irritation *in vivo* (37). Yet it remains unclear whether this observation represents an RARγ-specific function or is primarily the result of the preponderance of RARγ in the epidermis.

RARγ expression levels do seem to play a role in the ligand-dependent inhibition of keratinocyte growth. Manipulation of RARγ levels in SqCC/Y1 cells through introduction of sense and antisense expression vectors results in an increase of the growth-inhibitory effects of retinoic acid in the sense-transfected clones and a decrease in the antisense transfectants (38). Because only the functions of RARγ were reported, it is uncertain whether this represents an RARγ-specific response in SqCC/Y1 cells or whether modulation of RARα expression would similarly regulate the growth-inhibitory response to retinoic acid.

Our studies suggest that RARγ expression is an important determinant of the ability of retinoic acid to inhibit keratinocyte differentiation and tissue-specific gene expression. In contrast, decreased RARγ levels in SCC/Y1 antisense transfectants does not seem to interfere with ligand-dependent repression of keratin 1, involucrin, or keratinocyte transglutaminase expression (38). The difference in observations may reflect the extended time course (7 days) and higher retinoic acid concentration (1 μM) used in the SqCC/Y1 studies. Under these conditions, changes in secondary responses modulated by retinoic acid might contribute to the ligand-dependent repression detected in the RARγ antisense cells, or, alternatively, endogenous RARα might mediate the response in these stable cell lines.

We observed pronounced differences between SCC-9 and SCC 12 F cells with regard to retinoic acid-dependent inhibition of phorbol ester-induced involucrin and keratinocyte transglutaminase expression. Other examples of interactions between the AP1 transcription complex and RAR are well documented (3, 39) and seem to be the result of direct interactions between nuclear receptors and AP1 proteins (3, 39) or competition for the cAMP response element-binding protein/p300 integrator complex (40). Because SCC 12F responsiveness to retinoic acid is restored by co-transfection of functional RAR (Fig. 6), it seems that reduction in total RAR content significantly impairs ligand-dependent transcriptional regulation of at least two keratinocyte genes. Because

RARα and RARγ are equally effective in restoring function, we propose that reduction in total RAR content, rather than loss of RARγ *per se*, is the critical determinant in loss of ligand-dependent suppression of cornified envelope formation and keratinocyte gene expression in SCC 12F cells.

References

1. Lohnes, D., M. Mark, C. Mendelsohn, P. Dolle, D. Decimo, M. LeMeur, A. Dierich, P. Gorry, and P. Chambon. Developmental roles of the retinoic acid receptors. *J. Steroid Biochem. Mol. Biol.* **53:**475–486 (1995).
2. Gudas, L. J. Retinoids and vertebrate development. *J. Biol. Chem.* **269:** 15399–15402 (1994).
3. Giguere, V. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocr. Rev.* **15:**61–79 (1994).
4. Fuchs, E., and C. Byrne. The epidermis: rising to the surface. *Curr. Opin. Genet. Dev.* **4:**725–736 (1994).
5. Lotan, R. M. Squamous differentiation and retinoids, in *Head and Neck Cancer* (W. K. Hong and R. S. Weber, eds.). Kluwer Academic Publishers, Norwell, MA, 43–72 (1995).
6. Fisher, G. J., H. S. Talwar, J. H. Xiao, S. C. Datta, A. P. Reddy, M. P. Gaub, C. Rochette-Egly, P. Chambon, and J. J. Voorhees. Immunological identification and functional quantitation of retinoic acid and retinoid X receptor proteins in human skin. *J. Biol. Chem.* **269:**20629–20635 (1994).
7. Saitou, M., S. Sugai, T. Tanaka, K. Shimouchi, E. Fuchs, S. Narumiya, and A. Kakizuka. Inhibition of skin development by targeted expression of a dominant negative retinoic acid receptor. *Nature (Lond.)* **374:**159–162 (1995).
8. Imakado, S., J. R. Bickenbach, D. S. Bundman, J. A. Rothagel, P. S. Attar, X. J. Wang, V. R. Walczak, S. Wisniewski, J. Pote, J. S. Gordon, R. A. Heyman, R. M. Evans, and D. R. Roop. Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev.* **9:**317–329 (1995).
9. Aneskievich, B. J., and E. Fuchs. The A/B domain of truncated retinoic acid receptors can block differentiation and promote features of malignancy. *J. Cell Sci.* **108:**195–205 (1995).
10. Houle, B., M. Pelletier, J. Wu, C. Goodyer, and W. E. C. Bradley. Fetal isoform of human retinoid acid receptor beta expressed in small cell lung cancer lines. *Cancer Res.* **54:**365–369 (1994).
11. Xu, X., J. Ro, J. Lee, D. Shin, W. Hong, and R. Lotan. Differential expression of nuclear retinoid receptors in normal, premalignant and malignant head and neck tissues. *Cancer Res.* **54:**3580–3587 (1994).
12. Houle, B., C. Rochette-Egly, and W. E. C. Bradley. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* **90:**985–989 (1993).
13. Crowe, D. L., L. Hu, L. J. Gudas, and J. G. Rheinwald. Variable expression of retinoic acid receptor *beta* mRNA in human oral and epidermal keratinocytes: relation to keratin 19 expression and keratinization potential. *Differentiation* **48:**199–208 (1991).
14. Hu, L., D. Crowe, J. Rheinwald, P. Chambon, and L. Gudas. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* **51:**3972–3981 (1991).
15. Lotan, R. Suppression of squamous cell carcinoma growth and differentiation by retinoids. *Cancer Res.* **54:**1987S–1990S (1994).
16. Darwiche, N., G. Celli, T. Tennenbaum, A. B. Glick, S. H. Yuspa, and L. M. De Luca Mouse skin tumor progression results in differential expression of retinoic acid and retinoid X receptors. *Cancer Res.* **55:**2774–2782 (1995).
17. Ponec, M., A. Weerheim, J. Kempenaar, and J. Boonstra. Proliferation and differentiation of human squamous carcinoma cell lines and normal keratinocytes: effects of epidermal growth factor, retinoids and hydrocortisone. *In Vitro Cell. Dev. Biol.* **24:**764–770 (1988).
18. Rubin, A., and R. Rice. Differential regulation by retinoic acid and calcium of transglutaminases in cultured neoplastic and normal human keratinocytes. *Cancer Res.* **46:**2356–2361 (1986).
19. Breitkreutz, D., H. J. Startk, P. Plein, M. Baur, and N. E. Fusenig. Differential modulation of epidermal keratinization in immortalized (HaCaT) and tumorigenic human skin keratinocytes (HaCaT-ras) by retinoic acid and extracellular calcium. *Differentiation* **54:**201–217 (1993).
20. Lotan, R. Squamous cell differentiation markers in normal, premalignant and malignant epithelium: effects of retinoids. *J. Cell. Biochem.* **17F:**167–174 (1993).
21. Rheinwald, J. G., and M. A. Beckett. Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. *Cell* **22:**629–632 (1980).
22. Monzon, R. I., J. J. LaPres, and L. G. Hudson. Regulation of involucrin gene expression by retinoic acid and glucocorticoids. *Cell Growth Differ.* **7:**1751–1759 (1996).
23. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York (1989).
24. Eckert R. L., and H. Green. Structure and evolution of the human involucrin gene. *Cell* **46:**583–589 (1986).
25. Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature (Lond.)* **330:**444–450 (1987).
26. Krust, A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon. A third human retinoic acid receptor, RAR gamma. *Proc. Natl. Acad. Sci. USA* **86:**5310–5314 (1989).
27. Ferrari, N., U. Pfeffer, F. Tosetti, C. Brigati, and G. Vidali. An improved RT-PCR protocol for the quantitation of human retinoic acid receptor RNA. *Exp. Cell Res.* **211:**121–126 (1994).
28. Ercolani, L., B. Florence, M. Denaro, and M. Alexander. Isolation and complete sequence of a functional glyceraldehyde-3-phosphate dehydrogenase gene. *J. Biol. Chem.* **263:**15335–15341 (1988).
29. LaPres, J. J., and L. G. Hudson. Identification of a functional determinant of differentiation dependent expression in the involucrin gene. *J. Biol. Chem.* **271:**23154–23160 (1996).
30. Hudson, L. G., J. B. Santon, C. K. Glass, and G. N. Gill. Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promoter expression. *Cell* **62:**1165–1175 (1990).
31. Eckert, R. L., M. B. Yaffe, J. F. Crish, S. Murthy, E. A. Rorke, and J. F. Welter. Involucrin—structure and role in envelope assembly. *J. Invest. Dermatol.* **100:**613–617 (1993).
32. Welter, J. F., J. F. Crish, C. Agarwal, and R. L. Eckert. Fos-related antigen (Fra-1), junB and junD activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects and promoter activity. *J. Biol. Chem.* **270:**12614–12622 (1995).
33. Gudas, L. J. Retinoids, retinoid-responsive genes, cell differentiation and cancer. *Cell Growth Differ.* **3:**655–662 (1992).
34. Boylan, J. F., D. Lohnes, R. Taneja, P. Chambon, and L. J. Gudas. Loss of RARγ function by gene disruption results in aberrant hoxa-1 expression and differentiation upon retinoic acid treatment. *Proc. Natl. Acad. Sci. USA* **90:**9601–9605 (1993).
35. Boylan, J. F., T. Lufkin, C. C. Achkar, R. Taneja, P. Chambon, and L. J. Gudas. Targeted disruption of retinoic acid receptor α (RARα) and RARγ results in receptor specific alterations in retinoic acid mediated differentiation and retinoic acid metabolism. *Mol. Cell. Biol.* **15:**843–851 (1995).
36. Taneja, R., P. Bouillet, J. F. Boylan, M.-P. Gaub, B. Roy, L. J. Gudas, and P. Chambon. Reexpression of retinoic acid receptor (RAR)γ or overexpression of RARα or RARβ in RARγ-null F9 cells reveals a partial functional redundancy between the three RAR types. *Proc. Natl. Acad. Sci. USA* **92:**7854–7858 (1995).
37. Chen, S., J. Ostrowski, G. Whiting, T. Roalsvig, L. Hammer, S. J. Currier, J. Honeyman, B. Kwasniewski, K.-L. Yu, R. Sterzycki, C. U. Kim, J. Starret, M. Mansuri, and P. R. Reczek. Retinoic acid receptor gamma mediates topical retinoid efficacy and irritation in animal models. *J. Invest. Dermatol.* **104:**779–783 (1995).
38. Oridate, N., N. Esumi, D. Lotan, W. K. Hong, C. Rochette-Egly, P. Chambon, and R. Lotan. Implication of retinoic acid receptor γ in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cell. *Oncogene* **12:**2019–2028 (1996).
39. Glass, C. K. Differential recognition of target genes by nuclear receptor monomers, dimers and heteromers. *Endocr. Rev.* **15:**391–407 (1994).
40. Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. A CBP integrator complex mediates transcriptional activation and AP1 inhibition by nuclear receptors. *Cell* **85:**403–414 (1996).

Send reprint requests to: Dr. Laurie Hudson, Dept. of Molecular Pharmacology and Biological Chemistry, Searle 8–565, Northwestern University, 303 East Chicago Ave., Chicago, IL 60611.