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# Terminal Differentiation in Keratinocytes Involves Positive as Well as Negative Regulation by Retinoic Acid Receptors and Retinoid X Receptors at Retinoid Response Elements

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**Terminal differentiation of epidermal keratinocytes is inhibited by 1 μM retinoic acid, a concentration which induces differentiation in a number of cell types, including F9 teratocarcinoma cells. The molecular basis for these opposing retinoid responses is unknown, although retinoic acid receptors (RARs) and retinoid X receptors (RXRs) have been detected in both cell types. When F9 cells are stably transfected with a truncated RARα lacking the E/F domain necessary for ligand binding and RAR/RXR dimerization, action at retinoid response elements is suppressed and cells produce a retinoic acid-resistant phenotype; i.e., they are blocked in differentiation (A. S. Espeseth, S. P. Murphy, and E. Linney, Genes Dev. 3:1647–1656, 1989). If retinoid receptors influence epidermal differentiation only in a negative fashion, then suppression of transactivation at retinoid response elements would be expected to enhance, rather than block, keratinocyte differentiation. In this study, we show that surprisingly, even though constitutive expression of an analogous truncated RARγ in keratinocytes specifically suppressed transactivation at retinoid response elements, keratinocytes were blocked, rather than enhanced, in their ability to undergo morphological and biochemical features of differentiation. These findings demonstrate a direct and hitherto unrecognized role for RARs and RXRs in positively as well as negatively regulating epidermal differentiation. Additionally, our studies extend those of Espeseth et al. (Genes Dev. 3:1647–1656, 1989), indicating a novel RAR function independent of the E/F domain.**

Since their discovery, retinoids have been known to profoundly influence epithelial differentiation. Retinoid deficiency can convert a secretory epithelium to a squamous epithelium (squamous metaplasia [59]), and retinoid excess can convert a stratified squamous epithelium to a secretory epithelium (mucous metaplasia [6, 15]). The effects of retinoids in suppressing terminal differentiation in the epidermis of skin are particularly striking and in many ways are paradoxical, given the more typical role of 1 μM retinoic acid (RA) in accentuating differentiation (8, 11, 29, 49, 52, 58). Nevertheless, differentiation of cultured epidermal keratinocytes is inhibited by 1 μM RA (18), which suppresses expression of differentiation-specific markers, including keratins 1 and 10 (K1 and K10) (18), filaggrin (4), K6 and K16 (32), and cornified envelopes (23, 62). In addition, 1 μM RA has other effects on epidermal cells, such as altering cell surface glycosylation patterns (10), increasing the detachment rate of squames (13, 18), promoting cell motility (18), and enhancing the levels of K13 and K19 (18). K13 and K19 are among the few suprabasal markers whose expression is upregulated in response to retinoids (33).

The molecular mechanisms by which retinoids exert their effects on the epidermis have not yet been elucidated. Early studies showed that 1 μM RA negatively influences the levels of epidermal mRNAs (18), and more recent studies have shown that it mediates inhibitory action at the transcriptional level (50, 54). RA-mediated transcriptional repression of keratin expression appears to be exerted at the level of 5′ promoter sequences (50, 54) and involve RA receptors (RARs) (54). However, direct binding of RARs to keratin or other differentiation-specific epidermal genes has not yet been demonstrated.

In recent years, it was found that skin expresses a novel RAR, RARγ, which is a member of the steroid receptor family (63). RARγ appears to be far more restricted than other RARs in tissue distribution. This is particularly evident during later stages of embryonic development, when RARγ becomes restricted to cartilage and stratified squamous epithelia (45). In epidermal cells in culture, RARγ is the major RAR expressed, with RARα a minor component and little if any RARβ (12, 26, 50). RAR mRNAs seem to be located primarily in the differentiating layers of the skin (12, 42), and coupled with the marked inhibition of suprabasal gene expression by 1 μM RA, it has seemed likely that RARs play an important role in negatively regulating epidermal differentiation. The possible role, if any, of RARs in activating epidermal differentiation has remained obscure, although (i) epidermal cells can differentiate when cultured on collagen lattices floating on normal serum-containing medium ($4 \times 10^{-8}$ M retinoids [18]) (2, 3) and (ii) RARs can be activated by as little as $10^{-9}$ M RA (34).

The control of gene transcription by RARs is extraordinarily complex, involving a multitude of both indirect and direct mechanisms. RARs can heterodimerize with each other (64), with thyroid hormone receptors (TRs) (17, 21), and with newly discovered and related retinoid X receptors (RXRs) (30, 39, 61, 65). At least some of these interactions appear to change the DNA affinity and activity of RARs (27, 64). While RARs can bind to both TR response elements (TREs [7, 55]) and retinoid response elements (RAREs) (10a, 53, 57), TRs appear to be restricted to TREs (20, 31, 56). The repertoire of complex DNA interactions exhibited by RARs is further expanded by their capacity to interact with AP1

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proteins, thereby conferring indirect transcriptional control of genes (9, 41, 46).

While an impressive and fascinating body of knowledge has been accumulated regarding the intricacies of RAR-DNA interactions, little is known about how these interactions play themselves out in the context of cells that are influenced by retinoids. In this regard, by far the most extensively studied system is the teratocarcinoma cell line F9, which differentiates when exposed to 1 μM RA in a fashion seemingly opposite that of epidermal cells (8, 29, 52, 58). Recently, it was shown that F9 cells expressing a truncated RARα (tRARα) do not differentiate when exposed to 1 μM RA (14; see also reference 43). Since differentiation of F9 cells is positively regulated and that of epidermal cells is negatively regulated by this concentration of RA, we wondered what effects an analogously truncated RARγ might have on epidermal differentiation. To investigate this issue, we engineered keratinocyte lines expressing tRARγ that is missing the hormone-binding domain of the receptor. Analyses of these lines and of the behavior of the truncated receptor have revealed a surprising new role for RARs in positively as well as negatively regulating the process of terminal differentiation. Coupled with the knowledge that in vitro, epidermal differentiation can proceed at low but not high concentrations of retinoids, our findings indicate that positive and negative control by RARs and RXRs must occur at different concentrations of RA.

## MATERIALS AND METHODS

**CAT reporter constructs.** TRE-CAT, RARE-CAT, and RXRE-CAT were generated as derivatives of pBLCAT2, containing a multiple cloning segment 5′ upstream from the herpes simplex virus thymidine kinase (HSV TK) promoter and the chloramphenicol acetyltransferase (CAT) gene (37). The TREs and RAREs were generated as double-stranded oligomers, with the appropriate restriction endonuclease site extended ends. The TRE is 5′-AGCTTAGGTCAGGGACGT GACCTT-3′ (63), and three copies were inserted at the *Bam*HI site of the vector (32a). The RARE, present in the promoter of the RARβ gene, is 5′-GGGTAGGGTTCACCG AAAGTTCACTCG-3′ (53), and three copies were inserted at the *Sal*I site. The RXR element (RXRE), present in the promoter of the cellular retinol-binding protein type II gene (40), is 5′-GCTGTCACAGGTCACAGGTCACAGGTCACA GTTCA-3′ and was inserted as a single copy at the *Sal*I-*Bam*HI sites. Number and sense orientation of inserts were confirmed by sequence analysis. AP1-CAT, containing five synthetic copies of the collagenase promoter's AP1 sequence inserted 5′ from the HSV TK promoter, was a gift from Michael Karin (University of California, San Diego).

**Preparation of tRARγ lines and detection of transgene and raft cultures.** Human SCC-13 squamous cell carcinoma cells, obtained as a gift from James G. Rheinwald, were cultured as described previously (60). For clonal selection experiments, SCC-13 keratinocytes were cultured in the presence of mitomycin C-treated fibroblasts that had been transfected with pSV2neo to confer G418 resistance (G418$^r$). Keratinocytes were transfected with pβACTtRARγneo (for tRARγ clones) or pβACTneo-CB (for G418$^r$ control clones), using the calcium phosphate precipitation method of Graham and van der Eb (22). Sixty-five hours after transfection, cells were exposed to G418 (GIBCO) at 800 μg/ml for 10 days. Surviving keratinocyte colonies were cloned and replated in G418-containing medium. After identification by Southern blot analysis, positive clones were subcloned and retested for the transgene, after which G418 was withdrawn. Keratinocyte raft cultures were prepared essentially according to Asselineau et al. (3), with media modifications described by Kopan et al. (33). Rafts were cultured at the air-liquid interface for 2 weeks prior to harvesting.

**Transient transfections and CAT assays.** Soluble proteins from transfected SCC-13 or clone cultures were extracted, quantitated, and assayed for CAT protein via a colorimetric sandwich enzyme-linked immunosorbent assay method (5 Prime→3 Prime, Inc., Boulder, Colo.; Boehringer Mannheim) (35). β-Galactosidase expression from cotransfected pCH110 (Pharmacia) was used to monitor transfection efficiency and normalize CAT values (35). For both CAT and β-galactosidase, all extracts were assayed in duplicate, within the linear range of appropriate standard curves to determine picograms of CAT protein per microgram of cell protein. Where indicated, cultures were treated with all-*trans*-RA (Sigma, St. Louis, Mo.) in dimethyl sulfoxide (DMSO). All RA preparation and culture manipulations were performed under reduced lighting conditions. Control cultures received DMSO without RA.

**Immunohistochemistry.** Bouin's fixative-treated, paraffin-embedded sections (5 μm) were hydrated prior to immunohistochemical staining. Antisera and dilutions used were as follows: rabbit polyclonal anti-human K14 antisera, 1:100 (51); rabbit polyclonal antisera against human involucrin (Biomedical Technologies, Inc., Stoughton, Mass.), 1:10; and mouse monoclonal antibody against human filaggrin (Biomedical Technologies), 1:100. Following incubation with primary antisera, slides were subjected to immunogold enhancement (Amersham, Arlington Heights, Ill.) as described previously (32).

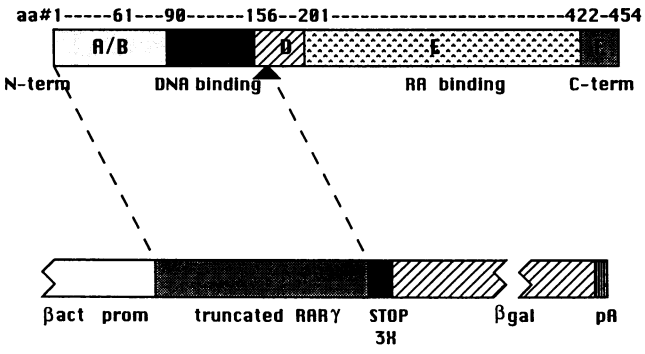


FIG. 1. Expression vector pβACTtRARγneo. The *Eco*RI-*Sac*I fragment of plasmid pSG5RARγ (hRAR-γ0 [34]) contains sequences encoding the first 188 amino acid (aa) residues of human RARγ. This fragment was inserted into the polylinker cloning region of Bluescript KS$^+$ along with an oligomer containing three stop codons in staggered reading frames. Sequence analysis confirmed that the first stop codon was in the same reading frame and immediately 3′ to the truncated RARγ coding sequences. This hybrid, tRARγ-3X-STOP, was subcloned as a *Hin*dIII-*Bam*HI fragment into the corresponding sites of a β-actin (βact) expression vector, pβACTneo-CB (24), to yield pβACTtRARγneo (see reference 14 for a similar strategy). The β-galactosidase (βgal) sequences were 3′ from the stop codons, providing 3′ untranslated nucleotides and a polyadenylation signal (pA). The vector contains a simian virus 40-*neo* gene for G418 selection. N-term and C-term, N terminus and C terminus; prom, promoter.

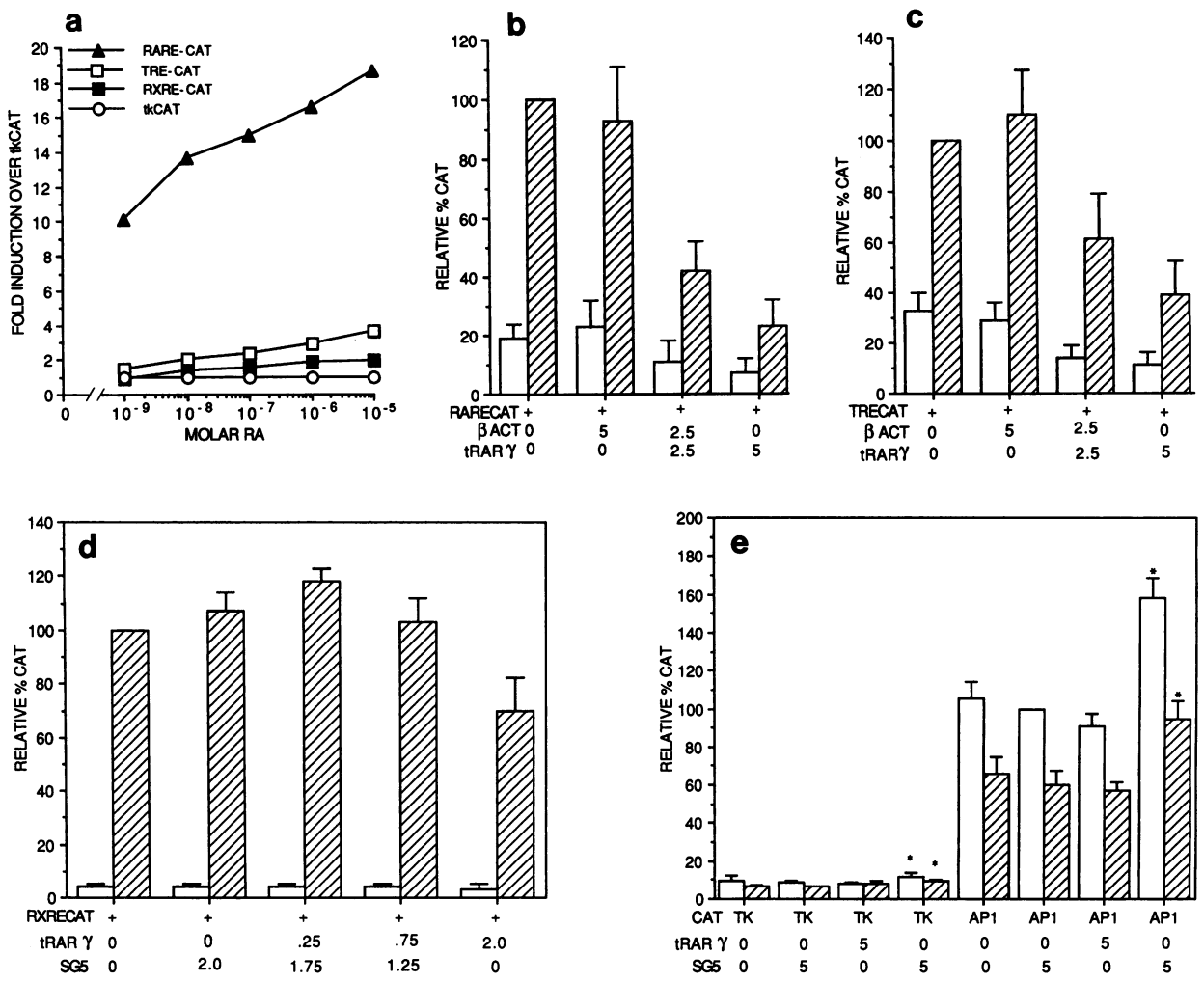


FIG. 2. Evidence that tRARγ inhibits ligand-dependent transactivation at TREs and RAREs but does not strongly affect RXREs or AP1 elements. Human epidermal keratinocytes (SCC-13) (a to c and e) (60) or monkey kidney epithelial (CV-1) cells (d) were transfected with 5 μg of RARE-CAT, TRE-CAT, RXRE-CAT, TK-CAT, or AP1-CAT in the presence or absence of increasing amounts of tRARγ plasmid. Where indicated, vector plasmid (β-actin or pSG5) was added to maintain the same DNA levels. For panel d, cells were cotransfected with a mammalian RXRα expression vector (RXRα cDNA in pSG5 [1a]). After transfection, cells were treated with DMSO (0 in panel a; open bars in panels b to e) or RA in DMSO (molarity as in panel a; cross-hatched bars represent 1 μM in panels b to e) for 48 h prior to CAT assays. + in panels b to d denotes 5 μg; * in panel e denotes TPA treatment at 100 ng/ml for 24 h prior to harvest. Plasmid amounts are in micrograms. For panels b to d, 100% CAT expression is the level obtained with reporter gene alone in the presence of RA. For panel e, 100% CAT expression is the level obtained with AP1-CAT in the absence of RA. Means ± standard deviations of three transfection experiments are shown. Because of the extremely low endogenous activity of RXRE-CAT in SCC-13 cells, effects of tRARγ on RXRE-CAT expression were measured in CV-1 cells cotransfected with the RXRα expression vector and treated with 10 μM RA.

## RESULTS

**A C-terminal truncated RAR has a major effect on RARE- and TRE-containing, but not RXRE- or AP1-containing, promoters in keratinocytes.** The construct used in these studies is illustrated in Fig. 1. The vector has a simian virus 40-*neo* cassette for G418 selection and a β-actin promoter for expression of inserted cDNAs. The tRARγ cDNA was an exact parallel to the tRARα cDNA described by Espeseth et al. (14). The mutant encompassed amino acids 1 to 188 of the 454 amino acids in RARγ and contained the amino terminus (A/B), putative DNA-binding domain (C), and nuclear localization signal in the hinge region (D) but not the ligand-binding domain (E/F). Recent mutational analyses have shown that a heptad repeat segment in the E domain (17, 21, 47) is essential for homodimerization and for heterodimerization with T3 receptor, other RARs, and RXRs, features thought to be essential for high-affinity DNA-receptor interactions (19, 30, 61, 65). Furthermore, the E/F domain also seems to be required for interactions with AP1 proteins (9, 41, 46). While no studies have yet revealed at a molecular level how a C-terminally truncated RAR can act to suppress RA-mediated action, Espeseth et al. (14) showed that F9 teratocarcinoma lines expressing a tRARα were unable to activate a TRE.

To first demonstrate the presence of functional endogenous RAR and RXR, we transfected the human epidermal line SCC-13 with CAT reporter genes driven by promoters containing multiple TREs (activated by TRs, RARs, and RXRs [21, 39, 65]), RAREs (activated by RARs and enhanced by RXRs [30, 40, 65]), or RXREs (activated by RXRs

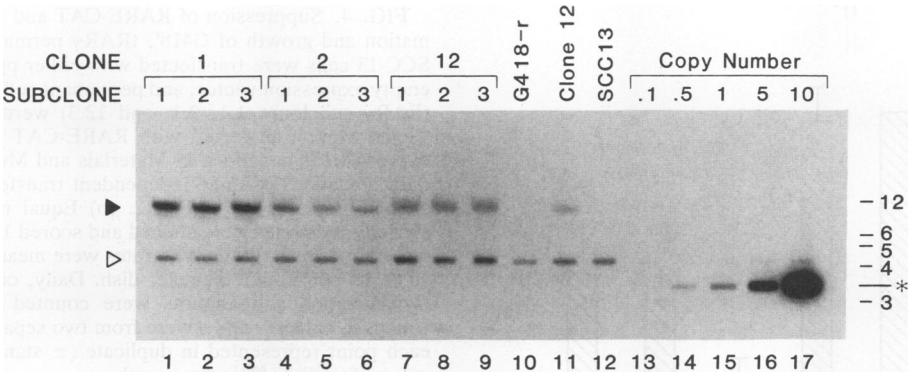


FIG. 3. Detection of the transgene in G418$^r$ SCC-13 clones. Southern blot analysis was used to measure transgene copy number. Total cellular DNAs (20 μg) and various amounts of control plasmid (tRARγ-3X-STOP) were digested with *Bam*HI and subjected to agarose gel electrophoresis. After transfer to nitrocellulose, the RARγ-related bands were detected with a [α-$^{32}$P]dCTP-labeled random-primed probe made from the entire RARγ cDNA. Sources of DNA samples: lanes 1 to 3, subclones of clone 1; lanes 4 to 6, subclones of clone 2; lanes 7 to 9, subclones of clone 12; lane 10, a representative G418$^r$ clone from SCC-13 cells transfected with pSV2neo; lane 11, the master tRARγ clone 12; lane 12, the parental SCC-13 line; lanes 13 to 17, ptRARγ-3X-STOP diluted to 0.1, 0.5, 1.0, 5.0, and 10 copies per human genome. The solid triangle denotes migration of the integrated transgene, and the open triangle marks the ~4.5-kb *Bam*HI fragment from the endogenous RARγ gene (see also reference 12). Numbers (in kilobases) denote migration of DNA markers, and the asterisk denotes migration of the control plasmid. On the basis of copy number standards and signal intensity, subclones of clones 1, 2, and 12 contained approximately five, one, and three copies, respectively, of the transgene. Note the uniformity in intensity for the individual subclones of each master clone.

and suppressed by RARs [30, 40, 65]) (Fig. 2). In keratinocytes, the RARE had substantially more activity than did the TRE or RXRE in response to RA (Fig. 2a). Given the differential responses of RARs and RXRs to RA, it was not surprising that RARE activity was high even at low (1 nM) RA, while RXRE activity was appreciable only at >1 μM RA. Cotransfection with tRARγ decreased RARE-CAT and TRE-CAT expression in a dose-dependent fashion (Fig. 2b and c). The effects of tRARγ on RXRE-CAT expression appeared to be considerably less (Fig. 2d), a finding consistent with those of a previous study (30).

To investigate whether tRARγ was able to influence AP1 activity in keratinocytes, the mutant was coexpressed with a CAT gene containing a minimal HSV TK promoter with or without multimer AP1 sites (Fig. 2e). AP1 activity in keratinocytes was appreciable, as judged by (i) comparison between TK-CAT and AP1-CAT expression levels and (ii) tetradecanoyl phorbol acetate inducibility of AP1-CAT expression. However, addition of tRARγ had no appreciable effect on AP1-mediated CAT expression, consistent with the notions that (i) the E/F domain is required for receptor-mediated interference of AP1 action (41, 46) and (ii) action by tRARγ at RAREs is specific and not a general squelching of basal transcription factors. In agreement with previous findings for tRARα (14), these data demonstrated that the amino-terminal segment of RARγ could negatively and specifically affect RAR-dependent transcription.

**Generation of keratinocyte lines expressing tRARγ.** Under appropriate conditions, SCC-13 cells can recapitulate many morphological and biochemical features of differentiation (32, 60). To assay the effects of tRARγ on keratinocyte differentiation, permanent lines (G418$^r$) were generated. Depending on the clone, the tRARγ transgene was present at two to five copies, as determined by Southern blot analysis (Fig. 3). Transgene mRNA was detected by reverse transcription-polymerase chain reaction analysis, although neither endogenous RARγ nor tRARγ could be detected by immunoblot analysis (RARs are generally present at very low levels in cells [44]). That our cDNA could generate a tRARγ protein of the expected size (21 kDa) was verified by immunoblot analysis of tRARγ transiently overexpressed in COS cells (not shown).

**Expression of tRARγ promotes growth and inhibits terminal differentiation in SCC-13 keratinocytes.** Levels of tRARγ in three clonal cell lines were assessed by the ability of the cells to suppress RARE transactivation relative to a G418$^r$ control line and parental SCC-13 lines. Whereas the G418$^r$ control line exhibited RARE-CAT expression comparable to that of parental cells, tRARγ lines had a reduced response (Fig. 4a), similar to that observed in transiently transfected keratinocytes. Surprisingly, colony-forming efficiencies and growth plateau levels were higher in tRARγ-expressing cells than in G418$^r$ clones or parental SCC-13 cells (Fig. 4b and c). Thus, the growth behavior of these clones was opposite that expected from a keratinocyte population in a retinoid-deficient environment, even though RARE suppression verified their reduction in RA responsiveness.

In addition to changes in growth characteristics of our tRARγ-expressing lines, colony morphology was markedly altered (Fig. 5). The parental SCC-13 and G418$^r$ lines displayed irregular borders and generally larger squame-like cells, particularly at colony centers (Fig. 5a and b, respectively). In contrast, tRARγ-expressing clones exhibited a morphology which deviated from that of the wild type (Fig. 5c to e). Clones with the poorest RARE transactivation produced colonies that were very tightly packed with small cells (Fig. 5c [clone 1.1] and d [clone 2.1]). Colonies had smooth and refractile edges, reflective of the uniform appearance of their cells. Confluent cultures exhibited focus-like mounds, atypical of control cultures (Fig. 5f to j).

The growth characteristics and colony morphologies of the tRARγ clones suggested that these clones were blocked, rather than enhanced, in their ability to terminally differentiate. To test this possibility, we subjected cultures to a trichrome stain that produces red keratinized cells and gray-brown basal-like cells (5). Parental SCC-13 and G418$^r$ keratinocytes formed colonies that exhibited bright red centers, indicative of keratinization (Fig. 5k and l, respectively). In contrast, tRARγ clones stained only gray-brown, reflective of a basal-like state (Fig. 5m to o).

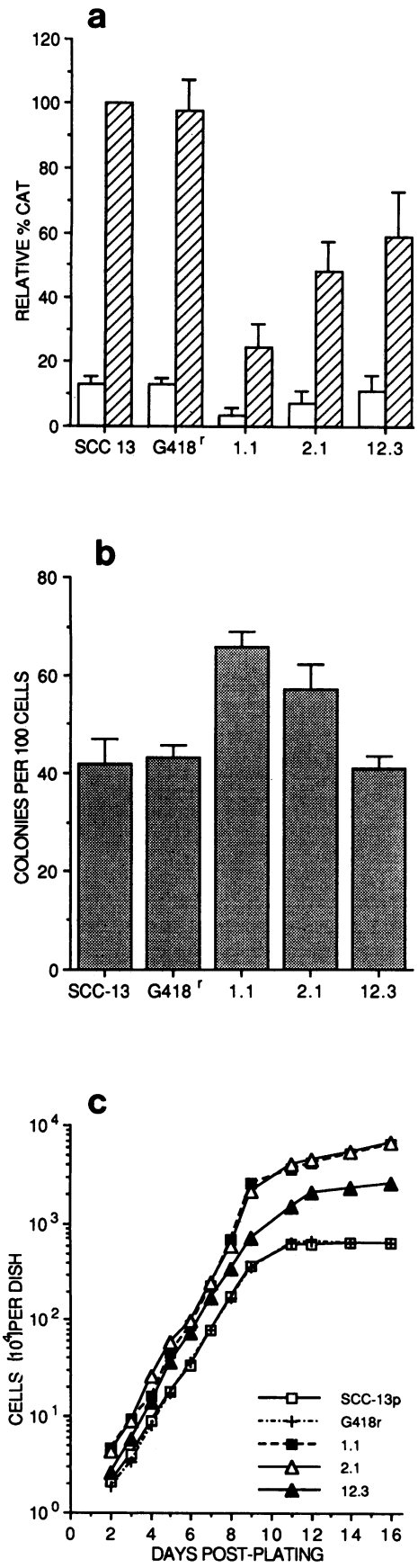


FIG. 4. Suppression of RARE-CAT and increase in colony formation and growth of G418$^r$, tRARγ permanent SCC-13 lines. (a) SCC-13 cells were transfected with either pβACTtRARγneo or an empty expression vector, and permanent cell lines (G418$^r$ control or tRARγ subclones 1.1, 2.1, and 12.3) were selected and cloned. Clones were transfected with RARE-CAT and assayed for CAT expression as described in Materials and Methods. Means ± standard deviations of three independent transfection experiments are shown. Bars are as in Fig. 2. (b) Equal numbers of cells were seeded, and plates were stained and scored 14 days later for colony number per dish. (c) Growth rates were measured by seeding 2.5 × $10^4$ cells per 60-mm-diameter dish. Daily, cells were harvested by trypsinization and aliquots were counted in a hemocytometer. Values in panels b and c were from two separate experiments, with each point represented in duplicate (± standard deviation for the colony-forming efficiency assay).

To test more rigorously the inability of tRARγ keratinocytes to differentiate, cells were cultured at the air-liquid interface (3, 33). The G418$^r$ control exhibited a morphology indistinguishable from that of the parental SCC-13 line, with mitotically active, basal-like cells in the first three to five layers and differentiating, spinous-like, and granular cells in upper layers (Fig. 6a; for parental SCC-13 raft cultures, see reference 32). In contrast, while the tRARγ clones stratified, cells were relatively small and basal-like, with a low cytoplasm nucleus ratio and numerous mitotic figures in all layers (Fig. 6b [clone 2.1]). The striking presence of mitotically active cells in the upper layers of tRARγ cultures was also visualized by immunohistochemical staining with antibodies against proliferating cell nuclear antigen (not shown). Despite the increase in mitotic figures and the extension of dividing cells to the upper layers of the tissue, tRARγ-expressing cultures did not invade the artificial dermis, in contrast to some highly transformed keratinocyte cell lines (see, for example, reference 51). The trichrome stain was again used to identify the more terminally differentiated keratinocytes within the stratified raft. Granular layer-like keratinocytes were seen as blue-gray cells with condensed nuclei in the G418$^r$ raft (Fig. 6c) but were absent from the tRARγ raft (Fig. 6d). Again, more frequent and suprabasally located mitotic figures were seen.

The absence of differentiation in tRARγ cultures was confirmed by immunohistochemistry. Whereas only basal layers of G418$^r$ rafts stained strongly with an antiserum specific for the basal K14 (Fig. 6e), tRARγ rafts showed intense anti-K14 staining throughout all layers (Fig. 6f). Stainings for the suprabasal K6 (not shown) and the cornified envelope protein involucrin (Fig. 6g and h) were notably reduced in tRARγ cultures, with no preference of positive cells for basal or suprabasal location. Moreover, staining with antiserum against a late differentiation marker, filaggrin, was present in the uppermost layers of G418$^r$ cultures (Fig. 6i) but was completely suppressed in tRARγ cultures (Fig. 6j). While some variation in the behavior of raft cultures was observed among tRARγ clones, they all exhibited a marked reduction in differentiation relative to control G418$^r$ clones.

The failure of tRARγ clones to express appreciable levels of differentiation markers was verified by isolating their keratins and resolving them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 7). Parental and G418$^r$ clones expressed both the differentiation-specific keratins (K1/K10 and K6/K16) and the basal keratins (K5 and K14) (lanes 1 and 2, respectively). In

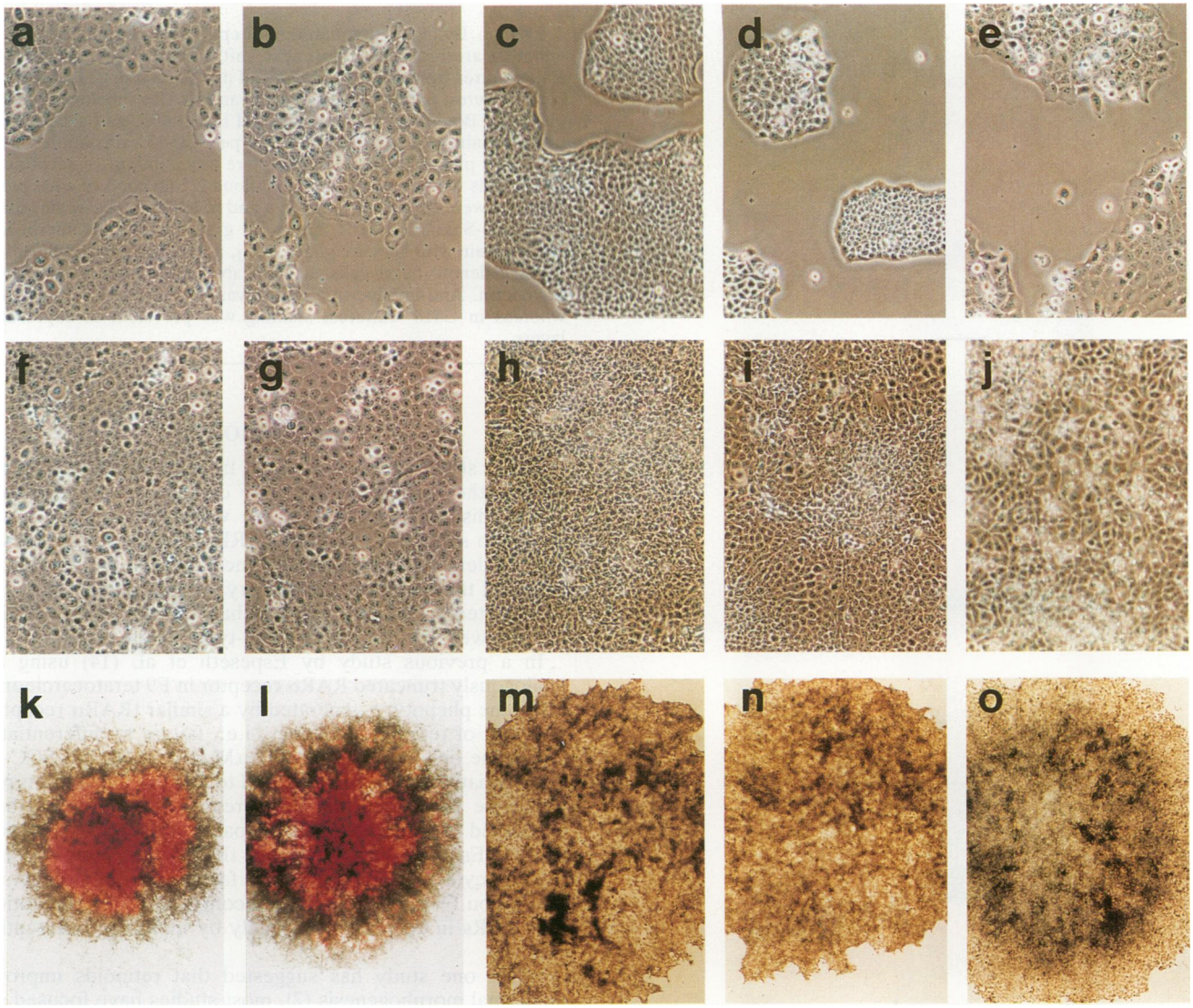


FIG. 5. Histological differentiation and cell morphology in cell clones expressing tRARγ. SCC-13 cells, a representative G418$^r$ control clone, and three tRARγ-expressing subclones (1.1, 2.1, and 12.3) were cultured as described in Materials and Methods. Prior to reaching confluence, cells were examined by phase-contrast microscopy or after staining with Ayoub-Shklar solution. (a to j) Cell morphology of preconfluent (a to e) and postconfluent (f to j) cultures of SCC-13 cells (a and f), G418$^r$ cells (b and g), clone 1.1 (c and h), clone 2.1 (d and i), and clone 12.3 (e and j). (k to o). Same order as panels a to e but with Ayoub-Shklar staining.

contrast, the tRARγ-expressing clone 2.1 expressed predominantly K5/K14, with no K1/K10 and reduced levels of K6/K16 (lane 3). Collectively, tRARγ-expressing raft cultures behaved as a population of undifferentiated, basal keratinocytes. Paradoxically, the behavior was similar to that of SCC-13 raft cultures in 1 μM RA (32).

A priori, since filaggrin, K1/K10, and K6/K16 have all been shown to be negatively regulated by 1 μM RA, one might argue that the effect of the tRARγ was to selectively suppress those genes known to be negatively regulated by RA. To assess the extent to which this notion might be valid, we investigated the patterns of keratins produced by tRARγ-expressing clones cultured on plastic. Under these circumstances, K13 and K19 are known to be expressed by the parental SCC-13 line (60). These keratins are also known to be positively regulated by 1 μM RA (18) and expressed suprabasally in RA-treated raft cultures of primary epidermal keratinocytes (32). Interestingly, while K13 and K19 were expressed by G418$^r$ keratinocytes cultured on plastic (and in raft cultures; data not shown), these keratins were dramatically suppressed in the two clones expressing higher tRARγ levels (Fig. 8). In addition, expression of K6/K16 was also markedly downregulated relative to that of K5/K14, as noted previously for tRARγ raft cultures. These findings provided an important demonstration that (i) the truncated receptor was acting in a fashion clearly distinct from that of the intact receptor in modulating RA effects and (ii) both positively and negatively regulated RA-responsive keratins were suppressed by tRARγ expression. Thus, the effects of tRARγ seemed to be a general suppression of all suprabasal markers of differentiation rather than merely those that are negatively regulated by 1 μM RA. This phenomenon occurred in the presence of normal levels of retinoids in the culture medium (~$4 \times 10^{-8}$ M [18]).

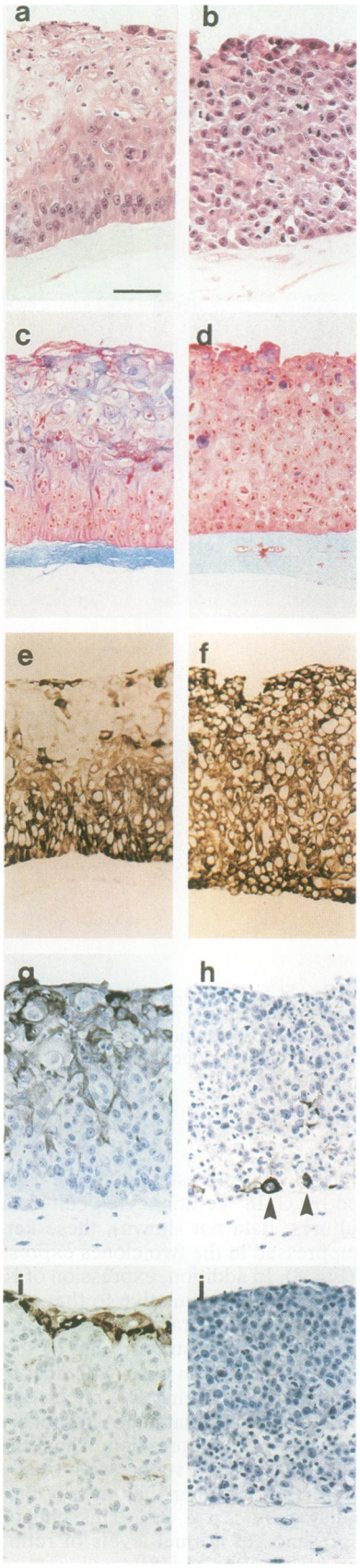


FIG. 6. Evidence that markers of epidermal keratinocyte differentiation are reduced or absent in raft cultures of tRARγ clones. Raft cultures from a G418$^r$ subclone of pSV2neo-transfected SCC-13 keratinocytes, parental SCC-13 cells, and tRARγ subclone 2.1 were placed in Bouin's fixative, embedded in paraffin, and sectioned (5 μm). Stainings of sections of wild-type SCC-13 raft cultures were published previously (32). Shown here are stainings of sections of G418$^r$ cells (first of each pair) and clone 2.1 (second of each pair). Sections were stained as follows: a and b, hematoxylin-eosin; c and d, Ayoub-Shklar; e and f, anti-K14; g and h, anti-involucrin and counterstain with hematoxylin; i and j, antifilaggrin. Arrowheads in panel h denote basal cells that are aberrantly staining with anti-involucrin. Anti-involucrin staining was rare in clone 2.1 cultures, whereas in G418$^r$ cultures, staining was prevalent in suprabasal layers.

## DISCUSSION

In this study, we showed that a truncated RARγ receptor, missing the hormone-binding E/F domain, acts in a fashion clearly distinct from that of the wild-type receptor in its ability to suppress action at RAREs. While we do not yet fully understand the molecular mechanisms underlying the action of this receptor, preliminary studies indicate that it is transported to the nucleus but has DNA-binding activity much lower than that of the wild-type receptor (1a).

In a previous study by Espeseth et al. (14) using an analogously truncated RARα receptor in F9 teratocarcinoma cells, the phenotype generated by a similar tRARα receptor was that of retinoid deficiency, i.e., failure to differentiate. Given the opposing effects of 1 μM RA on F9 and SCC-13 differentiation, we were surprised to find that both cell types would be blocked in an undifferentiated state when they expressed truncated receptors capable of suppressing action at RAREs. The fact that our tRARγ receptor affected keratinocyte differentiation in a fashion similar to that of tRARα on F9 differentiation is incompatible with the notion that RARs in epidermis act solely by inhibiting differentiation.

While one study has suggested that retinoids improve epidermal morphogenesis (2), most studies have focused on the ability of retinoids to suppress differentiation, and no prior studies have directly demonstrated the importance of RARs and RXRs in the differentiative process. Most notably, our studies demonstrate that (i) functional RARs are

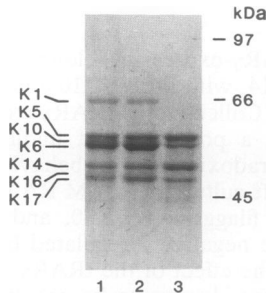


FIG. 7. Suppression of negatively RA-regulated keratins in raft cultures of clones expressing tRARγ. Keratins were isolated from raft cultures as described previously (32), resolved by SDS-PAGE, and stained with Coomassie blue. Samples were from SCC-13 (lane 1), G418$^r$ (lane 2), or clone 2.1 (lane 3). Keratins are identified by number at the left. Molecular masses of standards are shown at the right. Note that relative levels of K1/K10 and K6/K16 were significantly reduced in clone 2.1.

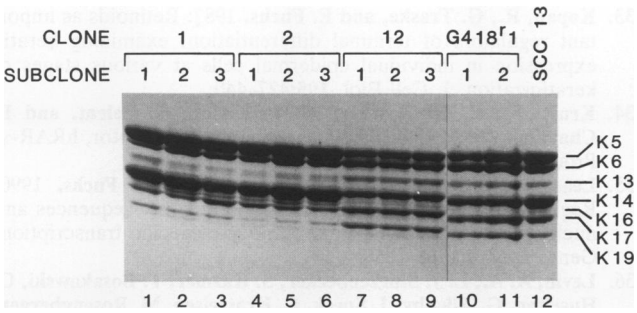


FIG. 8. Suppression of positively RA-regulated keratins in clones expressing tRARγ. Keratins were isolated from keratinocytes cultured on plastic, resolved by SDS-PAGE, and stained with Coomassie blue. Samples were from tRARγ clones (lanes 1 to 9; the subclone is indicated above each lane), two G418$^r$ SCC-13 control clones (lanes 10 and 11), or the SCC-13 parental line (lane 12). Keratins are identified by number at the right. Note that the levels of K13 and K19 were significantly reduced in samples from tRARγ subclones, as were the levels of K6/K16, relative to levels of K5/K14.

essential for triggering differentiation and (ii) this action is mediated through RAREs rather than by receptor-mediated inhibition of AP1 function. This latter point is important, since expression of the AP1 protein, c-Fos, changes as epidermal cells commit to terminally differentiate (16, 48).

An interesting question is how RARs can be necessary for both enhancing and suppressing differentiation. A priori, it is possible that RARs with different activities are expressed differently in basal versus differentiating keratinocytes and that this difference gives rise to a multifaceted response. In this regard, it was recently shown that in transfected CV-1 cells, RARγ1 negatively regulated some RAREs which other RARs positively regulated (28). A model not requiring differential localization of receptors is that action at an RARE may be involved in activating differentiation, while RXRs may be involved in inhibiting terminal differentiation. In this regard, it is noteworthy that RARs act in response to RA concentrations as low as $10^{-9}$ M, while RXRs seem active only at very high RA concentrations ($>10^{-6}$ M), presumably because a small fraction of this RA is then converted to 9-*cis*-RA, the natural ligand for RXRs (25, 30, 36, 38, 65). Also relevant is that epidermal raft cultures differentiate in the presence of medium containing serum ($4 \times 10^{-8}$ M retinoids) but not in medium supplemented with $10^{-6}$ M RA (4, 33).

In closing, if inhibition of terminal differentiation by 1 μM RA is mediated indirectly through RXRs and enhancement of terminal differentiation is mediated directly through RARs at much lower concentrations of RA, then a truncated receptor that interferes with the action of RARs, irrespective of its interaction with RXRs, would block induction of terminal differentiation. While future studies will be necessary to test this hypothesis, our studies present the first in vivo demonstration that (i) RARs and RXRs are involved in positively as well as negatively influencing epidermal differentiation and (ii) positive action must be mediated by a lower concentration of RA than is negative action. This knowledge necessitates a radical change in the prevailing models as to how RARs might influence epidermal differentiation. Furthermore, our finding that RARs are required for inducing terminal differentiation opens the possibility that truncated RARs contribute to epidermal cancers, or other differentiation-suppressing disorders, in a fashion perhaps analogous to that of certain leukemias (reference 1 and references therein). Further studies will be necessary to test the ability of tRARγ to immortalize or malignantly transform normal human keratinocytes.

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