Differentiation of human epidermal keratinocytes is accompanied by increased expression of CRABP-II and increased cellular concentration of retinoic acids: retention of newly synthesized retinoic acids by CRABP-II

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Abstract **Keratinocytes differentiating in vitro exhibit greater cytosolic capacity for retinoic acid synthesis from retinol or retinaldehyde as compared to nondifferentiated cells (Siegenthaler et al. 1990. *Biochem. J.* 268: 371–378), and increased expression of CRABP-II (Siegenthaler et al. 1988. *Exp. Cell Res.* 178: 114–126). Based on these observations, the content and disposition of [^{3}H]retinoic acids were determined in intact, nondifferentiated and differentiating keratinocytes incubated with [^{3}H]retinaldehyde or [^{3}H]retinol. Differentiating keratinocytes contained higher levels of [^{3}H] retinoic acids compared to undifferentiated cells when either [^{3}H]retinaldehyde or [^{3}H]retinol was the substrate. The largest increases in [^{3}H]retinoic acids were achieved with [^{3}H]retinaldehyde. Differentiation-associated increases in [^{3}H]retinoic acids correlated with cellular content of retinoid alcohol substrates in incubations with retinaldehyde but not in incubations with retinol. Consistent with previous observations, CRABP-II was significantly increased in differentiating cells. Moreover, newly synthesized [^{3}H]retinoic acids were retained within cells bound to CRABP-II. The results suggest that increasing cellular concentration of retinoic acids in in vitro differentiating keratinocytes is achieved by a combination of increased activity of the retinoic acid synthesis pathway and increased cellular content of CRABP-II.—**Chatellard-Gruaz, D., R. K. Randolph, G. Hagens, J-H. Saurat, and G. Siegenthaler. **Differentiation of human epidermal keratinocytes is accompanied by increased expression of CRABP-II and increased cellular concentration of retinoic acids: retention of newly synthesized retinoic acids by CRABP-II. *J. Lipid Res.* 1998.** 39: **1421–1429.**

Supplementary key words retinoic acid • 3,4-didehydroretinoic acid • retinol • retinaldehyde • retinoid • retinol esters • retinol esterification • cellular retinoic acid-binding protein

It has long been known that the epithelial tissues require an appropriate supply of vitamin A for the maintenance of the normal balance between proliferation and differentiation (1). For most tissues, retinoic acid is the molecular species of vitamin A that exhibits biological activity. This role for retinoic acid follows its function as a ligand for nuclear retinoid receptors, which are potent effectors of gene expression (2, 3).

The epidermis of skin, although a keratinized epithelium, requires an appropriate supply of retinoic acid to sustain normal structure and function (4–7). There are at least two sources of retinoic acid that possess potential to fulfill the epidermis' requirement for retinoic acid. First, retinoic acid is present constitutively in the plasma, at a concentration of 4–14 nm (8, 9), and may be available to the epidermis from the vasculature of underlying dermis. Second, in addition to retinoic acid, retinol is also constitutively present in the plasma at a concentration of approximately 2 μm (10). After its permeation into the epidermis, retinol may serve as a substrate for retinoic acid synthesis in situ. The contribution of each of these sources of retinoic acid to epidermal homeostasis is not known.

Retinoids have been used extensively for many years in the treatment of hyperproliferative, hyperkeratotic, neoplastic, and degenerative skin diseases (7). Among the natural retinoids that have been used as therapeutic agents, retinoic acid has proven to be the most potent. The pharmacological use of retinoic acid and analogues, however, often produces undesirable side effects (7). Therefore, the use of the natural precursors of retinoic acid, e.g., retinyl esters (11), retinol (12), and the more recently described retinaldehyde (13), are interesting clinical alternatives that although ex-

Abbreviations: BSA, bovine serum albumin; CRABP, cellular retinoic acid-binding protein; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, isotonic phosphate-buffered saline.

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hibiting less potency than retinoic acid, exert clinically significant retinoid activity with fewer side effects. It is thought that these retinoids act indirectly to effect the production of an active vitamin A hormone message by increasing the concentration of substrate retinol, which presumably drives retinoic acid synthesis at higher levels. Although this hypothesis makes sense in light of what is known about retinoic acid synthesis in general, the metabolism of retinaldehyde to retinoic acid and other retinoid metabolites in intact keratinocytes has not been described.

In previously published work, cytosolic extracts from differentiating and undifferentiated cultured human keratinocytes were evaluated for their potential to catalyze the synthesis of retinoic acid from retinol and retinaldehyde (14). These investigations demonstrated that cytosol from differentiating keratinocytes possesses a higher capacity to synthesize retinoic acid from retinol or retinaldehyde than does cytosol from nondifferentiated cells. This suggests that the cytoplasmic potential for retinoic acid synthesis increases in differentiating keratinocytes.

This conclusion raises compelling questions with regard to both keratinocyte differentiation and retinoic acid synthesis. Does the increased potential of cytosol from differentiating keratinocytes to synthesize retinoic acid predict increased retinoic acid synthesis in intact differentiating keratinocytes? If so, is increased retinoic acid synthesis explained by increased enzyme activities or substrate levels? As a first step toward answering these questions, the metabolism of [^{3}H]retinaldehyde and [^{3}H]retinol in intact, differentiating, and nondifferentiated cultured keratinocytes has been examined. In addition, the cellular content of CRABP-II and its disposition with regard to newly synthesized [^{3}H]retinoic acid ligands has been measured.

MATERIALS AND METHODS

Retinoids

All-*trans*-[11, 12-^{3}H]retinol (42.7 Ci/mmol) and all-*trans*-[11, 12-^{3}H]retinoic acid (48.5 Ci/mmol) were purchased from DuPont-NEN (Bad Homburg, Germany). All-*trans*-retinol, all-*trans*-retinoic acid, all-*trans*-retinyl palmitate, all-*trans*-retinaldehyde, and citral were obtained from Sigma (St. Louis, MO). All-*trans*-3,4-didehydroretinoic acid and all-*trans*-3,4-didehydroretinol were gifts from Dr. Klaus, Hoffman-La Roche (Basel, Switzerland).

All-*trans*-[^{3}H]retinaldehyde was synthesized from all-*trans*-[^{3}H]retinol by MnO_2 oxidation in hexane according to Ball, Goodwin, and Morton (15) and was purified by high performance liquid chormatography (HPLC) as previously described (16). Unlabeled retinoid stock solutions, diluted in absolute ethanol, were prepared on the day of experiments. The authentic retinoid standards used for HPLC analysis (all-*trans*-3,4-didehydroretinoic acid, all-*trans*-retinoic acid, all-*trans*-3,4-didehydroretinol, all-*trans*-retinol, all-*trans*-retinaldehyde, all-*trans*-retinol palmitate) were dissolved at 1 mm in ethanol containing 50 μg/ml of butylated hydroxytoluene. All experiments involving retinoids were performed in subdued light.

Cell culture

Newborn foreskin keratinocytes were cultured in medium containing a calcium concentration of 1.3 mm according to the method of Rheinwald and Green (17). Keratinocytes were grown in a mixture of Dulbecco-Vogt and Ham's F12 (3:1) medium containing 10% fetal calf serum (FCS) (Seromed, Berlin, Germany), insulin (5 μg/ml), adenine (24 μg/ml), transferrin (6 μg/ml), triiodothyronine (2 ng/ml), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 ng/ml), and epidermal growth factor (10 ng/ml).

For some experiments, differentiating and nondifferentiated keratinocytes were compared. Separation of these two populations of keratinocytes was accomplished in the following manner. Ten-day-old cultures, which were confluent and stratified, received medium containing delipidized fetal bovine serum and low calcium (0.05 mm). Incubations were then continued for an additional 48 h. During this 48-h period, differentiating (suprabasal) keratinocytes became detached from the cultures, while nondifferentiated cells remained attached to tissue culture plates. Experiments with radiolabeled retinoids were conducted in the last 24 h of this 48-h period by adding radiolabeled retinaldehyde or retinol to the cultures containing the low calcium medium. Experimental incubations were then continued for the indicated periods of time. After experimental incubations, detached differentiating keratinocytes were harvested by aspiration, and adherent nondifferentiated keratinocytes were harvested by scraping (see below). This method for producing and separating differentiating and nondifferentiated keratinocytes has been previously characterized (18).

Detached keratinocytes were confirmed to be differentiating by the presence of cornified envelopes (19), and CRABP-II expression (18, 20). Attached nondifferentiated keratinocytes did not express or only weakly expressed these markers of differentiation.

Incubation of cell cultures with radiolabeled retinoids and cell harvesting

On the day of experiment, tritiated retinol or retinaldehyde (8.5 Ci/mmol) were dissolved in ethanol and were added to low calcium medium also containing 1 mg/ml of fatty acid-free BSA. This solution was added to the culture medium yielding a final radiolabeled retinoid concentration of 50 nm. The concentration of ethanol in the culture medium never exceeded 0.01%. This medium was added to cultures and incubation was continued at 37°C. Detached differentiating cells were harvested by aspiration, were washed three times in ice-cold phosphate-buffered saline (PBS) containing 1 mg/ml BSA and 100 μm citral. Cells were subsequently lysed in Tris-HCl buffer (50 mm Tris-HCl, 25 mm NaCl, 2 mm EDTA, 1 mm DTT, pH 7.5) containing 100 μm citral. Adherent, nondifferentiated keratinocytes were washed three times with PBS and were scraped with a rubber policeman into the Tris-HCl buffer containing citral as described above. Citral was included in buffers to inhibit retinoic acid synthesis during cell harvesting (21; see below). Control cultures were incubated in the absence of radiolabeled retinoid, and 50 nm [^{3}H]retinaldehyde was added at the end of the experimental incubation period immediately after harvesting. Cells from control cultures were analyzed identically to experimental cultures to control for the possibility that retinoid metabolites were formed during cell harvesting and analysis. In no instance were any metabolites formed during analysis (data not shown). After harvesting, keratinocytes were stored at −20°C before being analyzed by polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC; see below).

Kinetics of tritium-labeled retinaldehyde uptake by cultured keratinocytes

The uptake of [^{3}H]retinaldehyde by cells of confluent stratified cultures was studied at different time points, encompassing a 32-h incubation period. For these experiments, nondifferenti-

ated and differentiating keratinocytes were not separated. At the end of the incubation period, keratinocytes were washed three times with ice-cold PBS containing 1 mg BSA/ml, detached from the dishes by incubation with 0.05% trypsin for 5 min at 37°C, and were collected by centrifugation. The resulting cell pellets were lysed by adding 500 μl of Soluene-350 (Packard) and incubating at 37°C for 1 h. After this, 4 ml of Ultima Gold (Packard) was added to the lysed cell pellet, and radioactivity was determined by liquid scintillation counting.

Retinoid-binding protein analysis

The [^{3}H]retinoid acid–CRABP-II complexes generated during incubations with [^{3}H]retinaldehyde or [^{3}H]retinol were analyzed by a previously described nondenaturing PAGE-radiobinding assay (18, 20). Nondifferentiated and differentiating cultured keratinocytes were disrupted by three cycles of freezing and thawing, and cell extracts were obtained by centrifugation at 10,000 *g* for 30 min at 4°C. Protein concentration was determined by the method of Lowry et al., using BSA as standard (22). Protein extract (250 μg) was subjected to PAGE on a 7.5% acrylamide slab gel. After electrophoresis, the gel was divided into lanes and cut into 2-mm bands that were incubated overnight with 300 μl each of Solvable (DuPont NEN) to hydrolyze proteins. The resulting fractions were mixed with 4 ml of Ultima Gold scintillation fluid and were assayed for radioactivity by liquid scintillation counting. Total CRABP-II was quantitated by incubating cytosolic protein extracts (250 μg) of keratinocytes from control cultures with a saturating concentration, 600 nm, of [11,12-^{3}H] all-*trans*-retinoic acid, for 1 h at 37°C, before analysis by PAGE.

To control for the possibility that ^{3}H-labeled ligands originated during analysis, [^{3}H]retinoid-binding experiments were performed in the presence of 100 μm citral. In these studies we found that citral did not compete with [^{3}H]retinoic acid for binding to CRABP-II.

Analysis of [^{3}H]retinoids by HPLC

Approximately one nmole of each unlabeled retinoid standard, dissolved in 0.5 ml of ethanol containing butylated hydroxytoluene, was added to 0.5 ml of broken cell homogenate (obtained by freezing and thawing). The mixture was adjusted to pH 3.0 by adding 10 μl of 1 m HCl. Two milliliters of hexane was added and the mixture was vortexed for 30 s, centrifuged 5 min at 700 *g*, and the upper organic phase was collected. This extraction procedure was performed 3 times for each sample. The combined organic phases were evaporated under N_2 and the dry residue was dissolved in acetonitrile (50 μl). Aliquots of 40 μl were subsequently analyzed by HPLC. The recovery of the radiolabeled material and of the internal standards was greater than 85%. The remaining aqueous layer containing cell debris was used for DNA measurement (23).

Analyses by HPLC were carried out on an Ultrasyl-ODS reversed-phase column (4.6 mm × 25 cm; Beckman, Geneva, Switzerland). Separations of 3,4-didehydroretinoic acid, all-*trans*-retinoic acid, 3,4-didehydroretinol, all-*trans*-retinol, all-*trans*-retinaldehyde were performed with an isocratic elution using a solvent system composed of 62% acetonitrile, 30% water (0.05 m ammonium acetate, pH 7) and 8% tetrahydrofuran, at a flow rate of 2 ml/min. Retinyl esters were eluted as a group by increasing acetonitrile to 70% and tetrahydrofuran to 30%. Internal retinoid standards were detected by measuring u.v. absorbance at 350 nm. Fractions of 1 ml were collected and each fraction was mixed with 4 ml of Ultima Gold scintillation fluid before determination of radioactivity. The concentration of retinoids was determined by summing the radioactivity in identified metabolite peaks and is expressed as pmol/mg DNA.

RESULTS

Kinetics of [^{3}H]retinaldehyde uptake by cultured keratinocytes

Confluent, stratified cultures of keratinocytes were incubated with 50 nm [^{3}H]retinaldehyde for various periods of time. Cells were harvested and the total radioactivity associated with the cells was measured as a function of time (**Fig. 1**). The uptake of retinaldehyde increased steadily over a 8-h period, reaching a maximum of about 30% of the total radioactivity in the culture. Cellular radioactivity remained at this level throughout the next 24 h. All subsequent experiments were 24 h in duration.

Metabolites formed in keratinocytes incubated with [^{3}H]retinaldehyde or [^{3}H]retinol

The cell-associated [^{3}H]metabolites synthesized during incubation of keratinocyte cultures with [^{3}H]retinaldehyde were identified and quantitated by reverse phase HPLC. A representative radiochromatogram for differentiating keratinocytes is shown in **Fig. 2**. Reflecting robust retinaldehyde reduction and retinol esterification reactions in keratinocytes, the most abundant retinoids arising from [^{3}H]retinaldehyde in differentiating keratinocytes were [^{3}H]retinoid esters (indicated in Fig. 2 as peak 6). In this mobile phase system, because individual retinoid esters exhibited overlapping retention times, it was not possible to identify the individual acyl species of ester. Also present but in considerably smaller amounts, were [^{3}H]retinol (peak 4), [^{3}H]3,4-didehydroretinol (peak 3), and the carboxylic acids of both of these vitamin A alcohols, [^{3}H]retinoic and [^{3}H]3,4-didehydroretinoic acids (peak 2 and peak 1, respectively). In the control cultures (open

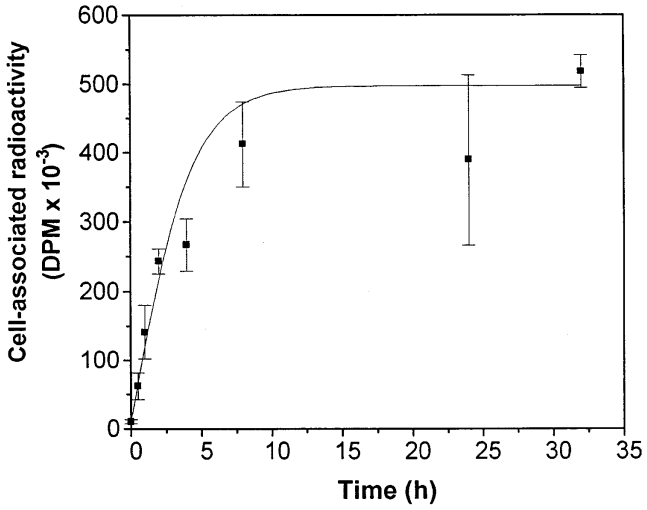

Fig. 1. Time course for the uptake of **[^{3}H]**retinaldehyde by cultured keratinocytes. Confluent, stratified cultured keratinocytes were incubated with 50 nm [^{3}H]retinaldehyde at 37°C for the indicated times. Cells were washed, harvested, hydrolyzed, and total cell-associated radioactivity was determined by scintillation counting. Cellular content of radioactivity at time points beyond 8 h represents 30% of the total amount of radioactivity added to the culture medium. Values are mean ± SD from 3 experiments.

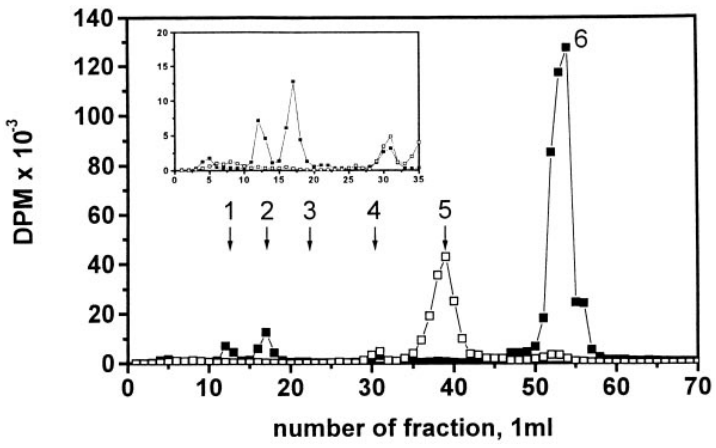

Fig. 2. HPLC analysis of [^{3}H]retinoid metabolites arising from [^{3}H]retinaldehyde in differentiating keratinocytes. HPLC elution profile of ^{3}H-labeled metabolites obtained from cultured keratinocytes incubated with 50 nm [^{3}H]retinaldehyde for 24 h (closed symbols). Also shown is a radiochromatogram from a control culture, in which 50 nm [^{3}H]retinaldehyde was added just after cell harvesting and immediately before analysis (open symbols). One milliliter fractions were collected and assayed for radioactivity by liquid scintillation counting. Elution of added authentic retinoid standards was monitored at 340 nm. The labeled peaks correspond to the retention times of these authentic standards and are as follows. 1) 3,4-didehydroretinoic acid, 2) all-*trans*-retinoic acid, 3) all-*trans*-3,4-didehydroretinol, 4) all-*trans*-retinol, 5) all-*trans*-retinaldehyde, 6) all-*trans*-retinyl palmitate.

symbols), in which [^{3}H]retinaldehyde was added after cell harvest and at the beginning of the analysis, the only radioactive peak recovered was that of the parent retinoid (peak 5).

The mass and relative quantities of these retinoid metabolites for differentiating and nondifferentiated keratinocytes (radiochromatogram not shown) are summarized in **Table 1**. Differentiating keratinocytes contained about 3-fold more total retinoid than did nondifferentiated cells, the bulk of which was accounted for as retinoid ester. Retinoic and 3,4-didehydroretinoic acid each increased greater than 3-fold in differentiating keratinocytes incubated with retinaldehyde. This increase in retinoic acids was not accompanied by approximately parallel increases in the content of the proximate substrate, retinaldehyde, which was the same in both cell populations. The 3-fold increase in total retinoic acids in differentiating cells compared to a 2-fold increase in retinol and 3,4-didehydroretinol, considered together, as their distal, respective substrates. The pmol retinoic acid/mg DNA values shown in Table 1 translate into total retinoic acid concentrations of 278 nm and 80 nm for differentiating and nondifferentiated keratinocytes, respectively. Overall, despite the differences between the two cell populations' absolute content of the individual retinoid metabolites, the relative abundance of each metabolite was similar.

As earlier work (14) had established that the cytosol of differentiating keratinocytes catalyzed retinoic acid synthesis from retinol, in addition to retinaldehyde, to a greater extent than the cytosol from nondifferentiated cells, it was of interest to determine whether increased retinoic acid content could be observed in intact differentiating cells incubated with retinol. The results of these experiments are summarized in **Table 2**. Similar to the results of incubations with retinaldehyde, differentiating keratinocytes contained about 4-fold more total retinoid than did nondifferentiated cells, the majority of which was retinoid ester. Different than the result obtained in cells incubated with retinaldehyde, the content of retinoic acid in both differentiating and nondifferentiated keratinocytes was similar, approximately 8 pmol/mg DNA (approximately 30 nm). There was, however, more 3,4-didehydroretinoic acid present in the differentiating cells than their nondifferentiated counterparts. When retinoic and 3,4-didehydroretinoic acids were considered together, total retinoic acids were increased about 50% in differentiating keratinocytes even though their respective alcohol precursor substrates, when considered together, decreased by 50%.

Significantly, for both cell populations, the concentration of retinoic acid was only one-half to one-fourth that in cells incubated with retinol (Table 2) as compared to cells incubated with retinaldehyde (Table 1).

TABLE 1. [^{3}H]metabolites in keratinocytes incubated with [^{3}H]retinaldehyde

	Cultured Keratinocytes	
[^{3}H]metabolites	Nondifferentiated	Differentiating
	pmol/mg DNA ± SD (% of total retinoid)	
[^{3}H]retinoic acid	16.9 ± 9.9 (5.3)	59.5 ± 30.7 (5.9)
[^{3}H]retinol	5.3 ± 2.5 (1.6)	11.5 ± 6.3 (1.1)
[^{3}H]retinaldehyde	2.9 ± 2.1 (0.9)	2.8 ± 1.7 (0.3)
[^{3}H]3,4-7didehydroretinoic acid	8.3 ± 4.3 (2.6)	27.6 ± 15.4 (2.7)
[^{3}H]3,4-didehydroretinol	0.2 ± 0.1 (0.1)	0.8 ± 1.3 (0.1)
[^{3}H]retinyl esters	288.1 ± 150.1 (89.5)	914.1 ± 572.9 (89.9)

Values represent the mean pmole [^{3}H]retinoid/mg DNA ± SD for experiments with four strains of keratinocytes. The values in parentheses reflect the percentage of total cell retinoid as that metabolite.

TABLE 2. [^{3}H]metabolites in keratinocytes incubated with [^{3}H]retinol

[^{3}H]metabolites	Cultured Keratinocytes	
	Nondifferentiated	Differentiating
	pmol/mg DNA ± SD (% of total retinoid)	
[^{3}H]retinoic acid	7.6 ± 8.5 (1.8)	8.1 ± 6.5 (0.5)
[^{3}H]retinol	24.4 ± 15.1 (5.9)	17.2 ± 7.5 (1.0)
[^{3}H]3,4-didehydroretinoic acid	1.9 ± 1.0 (0.5)	6.1 ± 4.3 (0.4)
[^{3}H]3,4-didehydroretinol	0.8 ± 0.2 (0.2)	0.7 ± 0.4 (0.1)
[^{3}H]retinyl esters	380.3 ± 264.8 (91.6)	1616.5 ± 1389.8 (98.1)

Values represent the mean pmole [^{3}H]retinoid/mg DNA ± SD for experiments with four strains of keratinocytes. The values in parentheses reflect the percentage of total cell retinoid as that metabolite. Cell-associated [^{3}H]retinaldehyde was not detected in any cell preparations.

Effect of retinaldehyde concentration on the synthesis of retinoid metabolites

As the difference in retinoic acid synthesis potential between differentiating versus nondifferentiated keratinocytes was most evident when cells were incubated with retinaldehyde, the two groups of cells were next compared for their capacity to metabolize retinaldehyde as a function of its concentration in the medium (**Fig. 3**). The quantity of retinoic acid increased linearly in a concentration-dependent manner in both differentiating and nondifferentiated cells across the entire retinaldehyde concentration range. There were significantly higher levels of retinoic acid in differentiating cells at all retinaldehyde concentrations tested. Moreover, there was no evidence of saturation at the highest retinaldehyde concentration, 200 nm. This trend for cellular retinoic acid content compared similarly to the cellular content of retinol, indicating that increased retinoic acid levels were the result, at least in part,

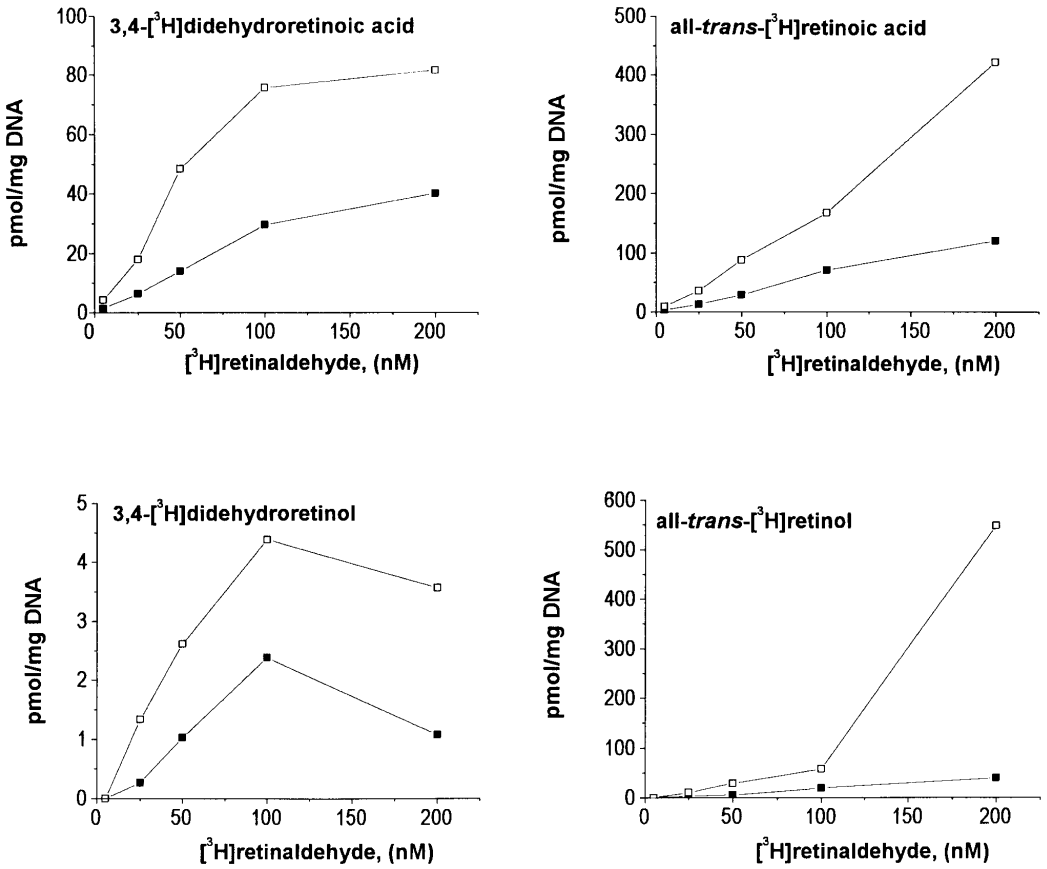

Fig. 3. Effect of [^{3}H]retinaldehyde concentration on [^{3}H]retinoid metabolites in cultured keratinocytes. [^{3}H]retinaldehyde was added to the culture medium of confluent and stratified cultured keratinocytes for 24 h over a concentration range extending between 25 nm–200 nm. Radioactive metabolites associated with keratinocytes were extracted from nondifferentiated (closed symbols) and differentiating (open symbols) cells and analyzed by HPLC as described in Material and Methods. The amounts of the respective retinoid metabolites are indicated as pmol/mg DNA.

of increased synthesis due to higher availability of substrate retinol with increasing retinaldehyde concentration.

The cellular content of 3,4-didehydroretinoic acid was higher in differentiating cells at all retinaldehyde concentrations but, unlike retinoic acid, showed a tendancy to plateau at concentrations above 100 nm. The higher content of 3,4-didehydroretinoic acid in differentiating versus nondifferentiated keratinocytes was reflected in the respective cells' content of 3,4-didehydroretinol, suggesting that substrate might be limiting under these conditions. Consistent with this interpretation, cellular levels of 3,4-didehydroretinol plateaued or decreased slightly at retinaldehyde concentrations higher than 100 nm.

Complexes of CRABP-II with radiolabeled retinoid in intact keratinocytes

Although the technique used for separation of differentiating and nondifferentiated keratinocytes did not accomplish a complete separation of the respective cell populations, both cell populations possessed the capacity to synthesize retinoic and 3,4-didehydroretinoic acids from retinaldehyde and retinol (Tables 1, and 2, respectively). Considering the two retinoic acids together, cellular content of retinoid acids was increased in differentiating cells. As it has been previously established that CRABP-II levels also increase in differentiating keratinocytes (18, 20), it was of interest to quantitate [^{3}H]ligand–CRABP-II complexes in keratinocytes incubated with [^{3}H]retinaldehyde. Using the previously characterized PAGE-radiobinding technique for CRABP-II (18, 20; **Fig. 4**), both nondifferentiated and differentiating keratinocytes expressed a radioactive peak (band 28) with a relative mobility corresponding to CRABP-II (20). In all experiments, [^{3}H]ligand–CRABP-II complexes were higher in differentiating cells than in nondifferentiated cells (Table 1). The identity of the tritium-labeled ligand bound to CRABP-II is not known, but presumably includes both retinoic and 3,4-didehydroretinoic acids.

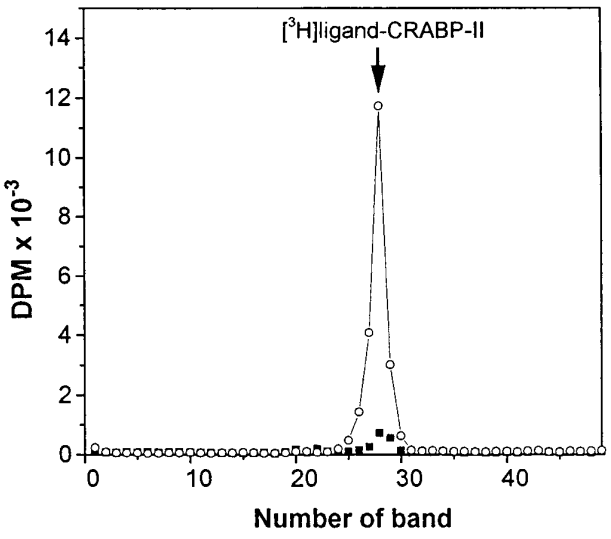

Fig. 4. Formation of [^{3}H]ligand–CRABP-II complexes in differentiating and nondifferentiating keratinocytes incubated with [^{3}H] retinaldehyde. Cultured keratinocytes were incubated for 24 h with 50 nm [^{3}H]retinaldehyde. Differentiating (open symbols) and nondifferentiated (closed symbols) keratinocytes were isolated and cytosolic proteins were prepared. Three hundred micrograms of cytosolic protein was then subjected to PAGE analysis as described in Material and Methods. The electrophoretic mobilities of [^{3}H]retinoic acid–CRABP-II complexes (band n 28) are indicated.

To assess whether the tritium-labeled ligands bound to CRABP-II were produced by on-going retinoic acid synthesis, cytosol preparations from differentiating keratinocytes were incubated simultaneously with citral, an inhibitor of retinoic acid synthesis, and [^{3}H]retinaldehyde prior to characterization by the PAGE-radiobinding technique (20). The presence of tritium-labeled ligand with CRABP-II was clearly dependent upon retinoic acid synthesis as no [^{3}H]ligand–CRABP-II complex was observed in incubations containing citral (data not shown).

To determine the total quantity of CRABP-II in the two cell populations, the cytosolic fraction of keratinocytes was incubated with a saturating concentration of [^{3}H]retinoic acid. The data in **Table 3** show that for both cell populations, the total [^{3}H]retinoic acid-binding capacity ([^{3}H]retinoic acid–CRABP-II complexes) exceeded the quantity of [^{3}H]retinoic acid. As [^{3}H]retinaldehyde does not bind to CRABP-II at these concentrations (data not shown), the [^{3}H]retinaldehyde present in cytosolic extracts would not be expected to compete with [^{3}H]retinoic acid for total CRABP-II binding activity.

Consistent with previous observations (18, 20), differentiating keratinocytes contained significantly more CRABP-II than did nondifferentiated cells (Table 3). In accord with the differences in the content of total [^{3}H]retinoic acids in differentiating versus nondifferentiated cells incubated with [^{3}H]retinaldehyde or [^{3}H]retinol, differentiating cells contained more CRABP-II-bound [^{3}H]ligand than did nondifferentiated cells.

DISCUSSION

The concentration of retinoic acids increases in intact differentiating keratinocytes in vitro. These results are consistent with earlier in vitro results demonstrating that the cellular content of retinoic and 3,4-didehydroretinoic acids increases in keratinocyte cultures as suprabasal, differentiating cells become increasingly abundant in the cultures (24). These confirming results are supportive of a role for active retinoids in the differentiation program of epidermal keratinocytes in vitro (25), and argue against the common misconception that retinoids inhibit differentiation of epidermal keratinocytes. Even though it is not known whether retinoic acid concentrations increase in differentiating keratinocytes in vivo, it is clear that active retinoids play a supportive role as a physiological supply of active retinoid is essential for the maintenance of normal epidermal phenotype (4–7); either excess or deficiency of retinoid results in altered, abnormal differentiation.

Increased retinoic acid synthesis from retinol may play a role in increasing retinoic acids in differentiating kera-

TABLE 3. [^{3}H]ligand–CRABP-II complexes in keratinocytes incubated with [^{3}H]retinaldehyde or [^{3}H]retinol

Incubation	[^{3}H]ligand-CRABP-II Complexes	
	Nondifferentiated Cells	Differentiating Cells
	pmol/mg protein (% saturation of CRABP-II)	
[^{3}H]retinaldehyde	0.9 (1.8)	1.6 (7.0)
[^{3}H]retinol	0.2 (3.5)	0.4 (1.7)
Total CRABP-II binding activity	4.5 (100)	22.8 (100)

Content of [^{3}H]ligand–CRABP-II complexes was determined in cytosol preparations from keratinocytes incubated with either [^{3}H]retinaldehyde or [^{3}H]retinol as described in Material and Methods. Total CRABP-II binding activity was determined as described in Methods. Values are from one experiment, which was typical of three.

tinocytes in vitro. This conclusion follows from the observation that the concentration of total retinoic acids in differentiating keratinocytes increases by about 50% (Tables 2, 3) without a corresponding change in substrate retinoid alcohols (Table 2). Consistent with this, previous observations have demonstrated that cytosol from differentiating keratinocytes catalyzes a higher rate of retinoic acid synthesis from retinol than does cytosol from nondifferentiated cells (14). The lack of retinaldehyde accumulation in either cell population suggests that the initial, rate-limiting reaction catalyzed by retinol dehydrogenase may be increased in differentiating cells. Confirmation of this hypothesis, however, awaits further experimentation.

Confirming earlier results in keratinocyte cytosol preparations (14), retinaldehyde is a more efficient substrate for retinoic acid synthesis in intact cells than is retinol. Incubation of cells with retinaldehyde (Table 1) raises cellular concentrations of retinoic acids to a greater extent than incubation with the same concentration of retinol (Table 2). The difference between these two retinoids' ability to raise cellular retinoic acid levels reflects the fact that retinoic acid synthesis from retinaldehyde bypasses the rate-limiting step in retinoic acid synthesis, the oxidation of retinol (26), and demonstrates that retinaldehyde dehydrogenase is present in both nondifferentiated and differentiating cells. Both of these conclusions are supported by the demonstrated potential of retinaldehyde (13), but not retinol (12), to increase retinoic acid concentration in intact epidermis.

In contrast to the case for differentiating cells incubated with retinol, increased retinoic acids in differentiating cells incubated with retinaldehyde is accompanied by parallel increases in cellular content of retinoid alcohol substrates (Table 1, Fig. 3). This suggests that differences in retinoic acid concentrations of differentiating versus nondifferentiated cells in retinaldehyde incubations are due to differences in substrate availability for retinoic acid synthesis. Although the reason for this difference is not known, it is interesting in light of the considerations of topical retinoid therapies. Topical retinoid regimens that provide substrate for retinoic acid synthesis contain retinol (12) or retinaldehyde (13), although less potent than those containing retinoic acid, provide good therapeutic results with less risk for the undesirable side-effects normally associated with retinoic acid treatment. This observation clearly reflects the capacity of keratinocytes in vivo to synthesize retinoic acid. This, in fact, has been demonstrated in mouse epidermis treated with topical retinaldehyde (27). It is worth noting that in a side-by-side comparison of topical retinoic acid, retinaldehyde, and retinol in human skin, retinaldehyde exhibited significantly greater potency than retinol as measured by the induction of CRABP-II (13), a result that is consistent with the greater potential for retinaldehyde, as compared to retinol (12), to increase the concentration of retinoic acids in epidermis via synthesis.

Retinoic acid synthesis in differentiating keratinocytes is important, not only in the context of the ability of exogenous retinol or retinaldehyde to produce an active retinoid signal, it is also important in the context of the major pathway of retinoid metabolism in keratinocytes, retinol esterification. The vast majority of exogenous retinaldehyde or retinol taken up by keratinocytes is sequestered as intracellular stores of retinoid ester. The robust esterification reaction in keratinocytes has been shown to function in the maintenance of low cellular concentrations of retinol, both in vitro (28, 29) and in vivo (12). In this role, the esterification reaction is effectively positioned to control the synthesis of retinoic acids by regulating cellular levels of substrate. One might predict, for example, that reducing the activity of the esterification reaction would result in increased cellular content of retinol, which could in turn, drive retinoic acid synthesis and increase cellular concentration of retinoic acids. In this way, a therapeutic dose of active retinoid could conceivably be delivered to the skin utilizing endogenous retinoids.

In addition to exhibiting increased content of retinoic acids, differentiating keratinocytes increase expression of CRABP-II. This result confirms earlier observations (18) and those of others (30). Significantly, however, the present work extends these observations by demonstrating that the increasingly abundant CRABP-II in differentiating cells binds newly synthesized retinoic acids. Although the identity of the retinoid ligands bound to CRABP-II was not identified, they likely include both retinoic and 3,4-didehydroretinoic acids. This conclusion follows from the inhibition of radiolabeled ligand–CRABP-II complexes by citral. In addition, both of these active retinoids exhibit similar affinities for CRABP-II binding (31).

In either case, coordinated increases in CRABP-II and synthesis of retinoic acids may contribute to increasing the cellular concentration of retinoic acids in differentiating keratinocytes. This possibility positions CRABP-II in the role of sequestering retinoic acid in differentiating cells and enabling their concentration to increase. However, whether the binding of retinoic acids by CRABP-II prevents their loss from cells or protects them from inactivating metabolic reactions remains to be determined.

The early differentiation events discussed here, concomitantly increasing retinoic acid synthesis, concentration of retinoic acids and CRABP-II, may be linked to each other in vivo. This prediction is based on the well-known sensitivity of the CRABP-II gene to induction by retinoic acid in vivo (13, 32–38). This scenario would place increased retinoic acid synthesis ahead of increased CRABP-II expression in the differentiation program. Increased retinoic acid synthesis could raise cellular levels of retinoic acid, inducing expression of CRABP-II, retention of retinoic acid and further increasing the retinoic acid concentration. However, as CRABP-II gene expression is high in vitro and is not induced further by retinoic acid (35), the association between CRABP-II expression and retinoic acid concentration in differentiating keratinocytes may be controlled by factors in addition to retinoic acid. This temporal relationship holds much promise for elucidating the regulation and the function of CRABP-II in the epidermis.

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