Expression of CRABP-I and -II in human epidermal cells

Alteration of relative protein amounts is linked to the state of differentiation

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The physiological role of cellular retinoic acid-binding proteins (CRABPs) may be to influence the intracellular level of free retinoic acid in the cell. In the present study two isoforms of CRABP, CRABP-I and CRABP-II were partially characterized in various human Malpighian epithelia and in human cultured keratinocytes expressing various patterns of differentiation. We have developed a new sensitive radiobinding assay using a PAGE/autoradioblotting technique which effectively separates CRABP-I and CRABP-II. This method allows the simultaneous quantification of these proteins. We show that CRABP-I and -II have similar M_r values (15000), but differ in their dissociation constant towards retinoic acid (K_d of 16.6 nM and 50 nM respectively), in pI (4.86 and 5.13) and in their relative mobilities (R_f) on PAGE under non-denaturing conditions (R_f values 0.65 and 0.44). In addition, we show that CRABP-II is the major isoform expressed in human keratinocytes, *in vivo* as *in vitro*. Furthermore, we demonstrate that CRABP-II is actually the CRABP previously studied in epidermal cells by a PAGE assay (Siegenthaler & Saurat (1987) Eur. J Biochem. 166, 209–214) and whose levels are dramatically increased by retinoic acid and its analogues in human epidermis. Keratinocytes, in the absence of full terminal differentiation, as well as hyperplasia, such as cultured human differentiating keratinocytes, psoriatic plaques, and non-keratinized oral mucosa, contained high levels of CRABP-II. CRABP-I was not detected in cultured keratinocytes, whereas normal skin (at full terminal differentiation) expressed CRABP-I and CRABP-II at a ratio of approx. 1:1.4. This value was approx. 1:17 in lesional psoriatic skin and 1:8 in oral mucosa. These observations suggest that CRABP-I and -II are regulated differently in human keratinocytes. The sharp increases in CRABP-II levels are associated with an alteration in the differentiation programme, as well as with cell response to retinoic acid overload, whereas CRABP-I might be a marker for terminal differentiation.

INTRODUCTION

Retinoids have profound effects on epithelial differentiation. They inhibit terminal differentiation in epidermis and stratified squamous epithelia [1,2]. Retinoic acid is thus the most potent physiological metabolite of retinol [3–5]. The mechanisms by which retinoic acid elicits these effects are largely unknown. However, the discovery of nuclear retinoic acid receptors (RARs and RXRs) has been a major step towards understanding the action of retinoic acid at the genomic level. Three major isoforms of RARs have been identified in mammalian skin: RAR α , β , and γ [6–9]. Retinoic acid and its analogues have specific binding affinities to RARs [10] and activate differently the RARs [11,12]. Thus synthesis of retinoic acid may be an important step in the metabolism of retinol, with the purpose of maintaining an optimal intracellular concentration of retinoic acid for the targeting of a specific RAR. In epidermal cells, retinoic acid is formed from retinol by a cytosolic enzymic process that appears to be distinct from alcohol dehydrogenase activities [13–15].

In the cytoplasm, retinol and retinoic acid are bound to specific proteins, cellular retinol-binding proteins (CRBPs) [16,17] and retinoic acid-binding proteins (CRABPs) [16,17], all belonging to the superfamily of fatty acid-binding proteins (FABPs) found in many tissues [18]. The precise function of CRABPs and CRBPs in the cell remains to be elucidated. It has been proposed that CRABPs participate in the mechanism of action of retinoic acid by regulating the level of retinoic acid reaching the nucleus

and RARs [19,20]. Very recently it has been suggested that holo-CRABP might also be a substrate in retinoic acid metabolism [21].

A novel species of CRABP, termed CRABP-II, distinct from the previously characterized CRABP [22], has been isolated from neonatal rat [23]. A cDNA encoding CRABP-II was identified by molecular cloning [24]. CRABP-II mRNA is expressed at high levels during mouse embryogenesis and also predominantly in the adult mouse [25] and human skin [25]. Moreover, CRABP-II mRNA levels are induced upon treatment of F9 teratocarcinoma cells [24] and human skin [25] with retinoic acid.

Using a PAGE radiobinding assay [26,27] we have previously reported our findings concerning the level of CRABP and CRBP in human epithelial cells *in vitro* and *in vivo* [26–31]. We have demonstrated that CRABP is expressed at high levels in diseased epithelial tissues with a defect of keratinization, as well as in cultured human differentiating keratinocytes, as compared with cultured non-differentiated keratinocytes [26]. In addition, CRABP levels were increased 5–6-fold in the skin of patients given retinoic acid or its synthetic analogues by systemic or topical routes [30]. At that time, no indication was available about the type of CRABP present in the skin. After comparison of our results with the subsequent findings of Giguère *et al.* [24], it is probable that the CRABP of human epidermal cells may correspond to CRABP-II.

The two CRABPs share many similar properties, and, owing

Abbreviations used: RARs and RXRs, nuclear retinoic acid receptors; CRABP-I and -II, cellular retinoic acid-binding protein type I and type II; CRBP-I and -II, cellular retinol-binding protein type I and type II; serum RBP, serum retinol-binding protein; FABP, fatty acid-binding protein; PAGE, polyacrylamide gel electrophoresis; PBS, iso-osmotic phosphate-buffered saline (5 mM-Na₂HPO₄/1.54 mM-KH₂PO₄ phosphate/130 mM-NaCl, pH 7.2); i.e.f., isoelectric focusing.

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to the low amounts expressed, their analysis is very difficult in crude human tissue extracts. The use of an anion-exchange h.p.l.c. technique has been suggested for the separation of CRABP-I and -II [32]. This technique requires a large amount of sample, has a low resolution, and is relatively time-consuming. We have therefore developed an assay which can separate these two proteins and excludes other proteins, not specific for retinoic acid, such as the FABP [31].

We report here the partial characterization and measurement of CRABP-I and CRABP-II by a PAGE/autoradioblotting assay in normal human skin, oral mucosa, psoriatic skin and in cultured human epidermal keratinocytes. We show that CRABP-II is the major isoform expressed in human keratinocytes *in vivo* and *in vitro*, and that it corresponds to the CRABP species that we have previously found in epidermal cells and which is induced after retinoic acid treatment. In contrast, CRABP-I was not detected in cultured keratinocytes, suggesting that CRABP-I and -II are regulated separately.

MATERIALS AND METHODS

Materials

Retinol, retinoic acid and oleic acid were purchased from Sigma. [11,12-³H]all-*trans*-retinol (49.3 Ci/mmol), [11,12-³H]all-*trans*-retinoic acid (50.6 Ci/mmol), and [9,10-³H]oleic acid (10 mCi/mmol) were obtained from du Pont/New England Nuclear Corp., Bad Homburg, Germany). The analyses of the retinoids were performed under yellow light. The labelled and unlabelled retinoids were checked by h.p.l.c. analysis for purity, which consistently exceeded 95%. All ethanolic solutions of retinoids contained butylated hydroxytoluene [27]. The other chemicals were of the best grade commercially available.

Human keratinocyte culture

Keratinocytes, isolated from human foreskin, were cultured in the presence of irradiated 3T3 fibroblasts using the Rheinwald-Green feeder technique [33]. The culture medium was a mixture of Dulbecco-Vogt and Ham's F12 (DVH) medium (3:1, v/v) supplemented with 5% (v/v) fetal-calf serum (FCS), cortisol (0.4 µg/ml), 1 µM isoprenaline and epidermal growth factor (10 ng/ml). The separation of differentiating (detached) from non-differentiated (attached) cells was performed when the cells, grown to confluency at normal calcium concentrations (1.2 mM), were switched to low-calcium medium (0.06 mM) {calcium-free DVH medium (3:1, v/v) supplemented with 5% Chelex-treated FCS for 2-3 days [34]}. The cells were washed three times with ice-cold iso-osmotic phosphate-buffered saline (PBS) before being stored at -70 °C. Cytosolic fractions were obtained as described for tissue preparation (see below). The term 'non-differentiated' is used for attached cells, whereas 'differentiating' is used for shed keratinocytes, as previously reported [29].

Tissue preparation

Human skin samples were obtained with a keratome set at 180 µm from patients undergoing plastic surgery and from psoriatic patients after informed consent (as previously reported [35]). Normal human oral mucosa was obtained from patients undergoing impacted-wisdom-teeth extraction as previously described [36]. The tissue samples were washed with PBS, to remove traces of blood, before being frozen. Human embryo (12-week-old) debris was obtained by suction abortion after ethical committee approval. Frozen rat testes and human embryo debris were homogenized in the same manner as epithelial tissues, but without prior freeze-drying.

Approx. 15 mg of freeze-dried tissue were homogenized in 800 µl of ice-cold buffer (100 mM-Tris/HCl/50 mM-NaCl/5 mM-EDTA/2 mM-dithiothreitol, pH 7.4) using a Polytron-PT7 (Kinematica, Luzern, Switzerland) tissue homogenizer. Supernatants were obtained by ultracentrifugation at 100 000 *g* at 4 °C for 60 min. Protein content was determined by the method of Lowry *et al.* [37], with BSA as standard.

Incubation with labelled ligands

Binding proteins were analysed after incubation of 100 µl of supernatant (300 µg of protein) with all-*trans*-[11,12-³H]retinoic acid, all-*trans*-[11,12-³H]retinol and [9,10-³H]oleic acid at 600 nM for 16 h at 4 °C in the presence or absence of a 200-fold excess of unlabelled ligand as described previously [28].

PAGE/autoradioblotting

The supernatants incubated with tritiated ligand were subjected to vertical-slab non-denaturing PAGE with 7.5% acrylamide, as previously described [27,28]. At the end of electrophoresis (determined as the front of migration reaching the bottom of the gel), the gel was either divided into lanes and cut into 2 mm bands, which were then assayed for the determination of the radioactivity profiles, or transferred on to a nitrocellulose sheet [28]. The transfer was performed at 13 °C in 30 mM-sodium phosphate solution, pH 6.5, for 2.5 h under the electrical conditions recommended by the manufacturer (Bio-Rad). After the transfer, the nitrocellulose sheet was dried, and a direct autoradiography analysis with Hyperfilm-³H (Amersham International) was performed. The film was developed after 12 days of exposure. The relative amounts of CRABP-I and -II was estimated by densitometric evaluation of the intensity of the bands on the film using a computing-densitometer apparatus (Molecular Dynamics, Kemsing, Sevenoaks, Kent, U.K.) We obtained a linear relationship between retinoid-binding activities and protein concentration of supernatants over the range of 100-900 µg of protein.

Immunoblotting of CRABP-I

CRABP-I was detected by immunoblotting with an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to a sequence from bovine CRABP-I [38-40]. After the transfer and without drying, the nitrocellulose sheet was blocked in PBS containing 3% defatted powdered milk at pH 7.2 for 1 h at 37 °C, before a 2 h 30 min incubation at room temperature in 50 mM-Tris/HCl buffer, pH 8.0, containing 150 mM-NaCl, 0.5% BSA, 0.2% Tween 20 and 10 µg of anti-(CRABP peptide) IgG. The nitrocellulose was then washed and further incubated for 1 h at room temperature with phosphatase-labelled affinity-purified antibody to rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) in the same buffer as the first antibody. The immunoreactive bands were revealed by incubating the nitrocellulose sheet for 30 min at 37 °C in 0.9 M-amino-2-methyl-2-propan-1-ol buffer, pH 9.5, containing 1 mM-MgSO₄ and 1.9 mM-5-bromo-4-chloroindol-3-yl phosphate.

Gel-filtration analysis

Human normal skin supernatant (500 µl, containing 4.5 mg of protein) was subjected to gel-filtration chromatography on a Sephadex G-150 column (2.5 cm × 37 cm) equilibrated with 50 mM-sodium phosphate / 0.2 M-NaCl / 2 mM-dithiothreitol / 1 mM-phenylmethanesulphonyl fluoride, pH 7.4, at 4 °C. The column was calibrated by using Blue dextran, albumin, ovalbumin, chymotrypsin, myoglobin, human epidermal CRABP-[³H]retinoic acid complex and cytochrome *c*. The column was eluted at a flow rate of 0.1 ml/min, and fractions (3 ml each)

were collected. The elution profile of proteins was monitored by a u.v. absorbance detector set at 280 nm. The elution position of the radiolabelled ligand-binding proteins was determined in a liquid-scintillation counter with a 100 μ l aliquot of each eluted fraction in 4 ml of Ultima Gold scintillant (Packard). Fractions containing the radioactivity, eluted at an M_r of 15 kDa, were pooled and concentrated to a final concentration of 1 mg of protein/ml using Centricon-10 centrifugal microconcentrators, with a 10 kDa cut-off (Amicon). This fraction (hereafter termed the '15 kDa protein fraction'), which is enriched in CRABPs, CRBPs, FABP and others small proteins, was frozen until further analysis.

Saturation experiments

Saturation experiments were carried out to determine the apparent dissociation constant (K_d) for both CRABP-I and CRABP-II in the 15 kDa protein fraction of human skin. In typical assays, 80 μ g of 15 kDa protein fraction in 100 μ l of homogenization buffer were incubated overnight at 4 °C in 8 mm $\times$ 40 mm glass microtubes containing increasing concentrations (2.4–600 nM) of [3 H]retinoic acid. The samples were subsequently analysed by PAGE, and binding activities corresponding to CRABP-I and -II were measured. Scatchard-plot analyses were calculated as described previously [27].

Isoelectrofocusing

To determine the pI of CRABP-I and -II, i.e.f. studies were performed using vertical-slab polyacrylamide gels. The gels contained 5.8% acrylamide, 13.3% sucrose and 6.3% of ampholyte (Pharmacia) for the pH interval 4–6.5. Electrode solutions were; anode: 0.04 M-glutamic acid; cathode: 0.05 M-NaOH.

The 15 kDa protein fractions were incubated with 600 nM- [3 H]retinoic acid in the presence or absence of a 200-fold excess of unlabelled ligand before being subjected to i.e.f. To identify the position of CRABPs, the radioactive bands corresponding to CRABP-I and CRABP-II obtained after PAGE analysis were cut out and introduced separately into the wells. For the control experiment, [3 H]retinoic acid without protein extract was added. After electrophoresis, the gels were separated into lanes, cut into 2 mm bands, and the radioactivity counted as for the PAGE technique.

RESULTS

PAGE electrophoresis

When normal skin supernatant was incubated with 600 nM- [3 H]retinoic acid and subsequently analysed by the PAGE technique, the migration profile shown in Fig. 1 was obtained. Free [3 H]retinoic acid moved with the front of the gel (bands 48–49). Two additional radioactive peaks were detected. One peak migrated at band 23 and corresponds to the CRABP from keratinocytes previously described [26–30,36]. The second peak (at band 31), which had the same mobility as albumin, was only partially abolished by an excess of unlabelled retinoic acid. The shoulder at band 33 was almost completely abolished, suggesting that this second radioactive peak might contain specific binding for retinoic acid.

In order to remove the albumin contamination, the protein extracts were first partially purified by gel filtration on Sephadex G-150. The fraction eluted at a molecular mass of 15 kDa was subsequently analysed by a PAGE radiobinding assay after incubation with 600 nM- [3 H]retinoic acid. Fig. 2 shows one large radioactive peak (at band 23) and a smaller one (at band 33). These two peaks were completely abolished by a 200-fold excess

of retinoic acid, demonstrating the specificity of the binding. A 200-fold excess retinol or oleic acid did not abolish the radioactive peaks (results not shown). Moreover, neither [3 H]retinol nor [3 H]oleic acid at 600 nM showed any binding to these two radioactive peaks, as shown in Fig. 3, but did bind to CRBP-I and FABP, respectively [31]. These results demonstrate that the two retinoic acid-binding proteins are specific for retinoic acid, have a molecular mass close to 15 kDa and should thus correspond to CRABP-I and -II.

Determination of the isoforms of CRABP

The PAGE technique was standardized by using partially purified CRABPs from rat testes and human embryos, as the

Fig. 1. PAGE analysis of [3 H]retinoic acid-binding proteins in cytosol of normal human skin

Aliquots of supernatant containing 300 μ g of protein in 100 μ l of buffer were incubated with 600 nM- [3 H]retinoic acid in the absence (▲) or presence (○) of a 200-fold excess of unlabelled retinoic acid. Peak 1 corresponds to CRABP; peak 2 has the same mobility as albumin; the shoulder (band 33) is specific for retinoic acid binding; peak 3 corresponds to free [3 H]retinoic acid; 2 mm sections of gel were analysed.

Fig. 2. PAGE analysis of [3 H]retinoic acid-binding proteins from the 15 kDa protein fraction (normal human skin) after Sephadex G-150 column chromatography

Aliquots of 80 μ l of the 15 kDa protein fraction containing 80 μ g of protein were incubated with 600 nM- [3 H]retinoic acid in the absence (▲) or presence (○) of a 200-fold molar excess of unlabelled retinoic acid. The technique was standardized with CRABP-I from rat testes and for CRABP-I and -II with that of human embryo cytosol. Band 23 corresponds to CRABP-II, whereas band 33 corresponds to CRABP-I.

Fig. 3. PAGE/autoradioblotting of human epithelial-cell extracts

Cytosol extracts (300 µg) were incubated without (lane 1) or with [3 H]retinoic acid (lanes 2–8) before being subjected to PAGE. Lane 1, CRABP-I identified by immunoblot of normal human skin extracts with anti-peptide-IgG to CRABP-I. After separation, the proteins were transferred onto a nitrocellulose sheet and the radioactive bands were revealed by direct autoradiography, as described in the Materials and methods section. Lane 2, human embryo; lane 3, human embryo with a 200-fold excess of unlabelled retinoic acid; lane 4, normal skin; lane 5, psoriatic plaque; lane 6, human oral mucosa; lane 7, cultured differentiating keratinocytes; lane 8, cultured non-differentiated keratinocytes. The arrows indicate the mobility of the standards used.

Fig. 4. PAGE/i.e.f. analysis of the 15 kDa protein fraction from human skin

An aliquot (80 µl) of the 15 kDa protein fraction (80 µg) was incubated with 600 nM [3 H]retinoic acid before being subjected to i.e.f. analysis. Three radioactive peaks were detected at pI: 5.13 for CRABP-II, 4.86 for CRABP-I, and 4.55 for the unbound [3 H]retinoic acid. Standardization was performed with partially purified CRABP-I and -II and with [3 H]retinoic acid alone, as described in the Materials and methods section. Abbreviation: RA, retinoic acid.

relative expression of CRABP-I and CRABP-II is distinct in these tissues [16,32,41]. CRABP-I is the major form in rat testes [22,42], whereas CRABP-II is present at approx 5% of the predominant CRABP-I form in rat embryo [32]. Cytosolic extracts from rat testes and human embryo tissues were partially purified by gel filtration on Sephadex G-150, and the 15 kDa protein fraction was analysed by PAGE. The CRABP-I from rat testes co-migrated with the radioactive peak at band 33 as did CRABP-I from human embryo. The second CRABP peak from human embryo migrated at band 23, indicating that it corresponds to CRABP-II. These results were confirmed using the PAGE/autoradioblotting technique, as seen in Fig. 3. Human

embryo cytosol extract showed two radioactive bands corresponding to the complexes [3 H]RA-CRABP-I and -II, with identical mobilities to those observed in human normal skin, human psoriatic plaque and oral mucosa. PAGE/immunoblotting using anti-(CRABP-I peptide) IgG showed only one immunoreactive band with the same mobility as CRABP-I detected by PAGE/autoradiobinding assay, thus confirming that these two binding proteins are identical [40]. When cytosol extracts from human skin were incubated with either [3 H]retinol or [3 H]oleic acid and analysed by PAGE/autoradiobinding, two distinct radioactive peaks could be detected, corresponding to CRBP type I ([27]; G. Siegenthaler, D. Chatellard-Gruaz & S. Jaconi, unpublished work) and to FABP [31] respectively, confirming the specificity of CRABP-I and -II for retinoic acid. The second radioactive band (R_f 0.44) should correspond, by deduction, to CRABP-II, since: (i) only two genes have been reported for CRABPs [24,25,43]; (ii) only CRABP-II mRNA levels are increased by exogenous retinoic acid [24,25]; (iii) from the two CRABPs proteins identified in the present study, the CRABP of R_f 0.44 (CRABP-II) was shown to be increased by retinoic acid and analogues [26,30].

Isoelectric point of CRABP-I and -II

Fig. 4 shows the migration profile of the 15 kDa protein fractions obtained from normal human skin incubated with [3 H]retinoic acid before isoelectrofocusing analysis. Standardization of the gel was performed again with partially purified CRABP-I and -II, obtained after PAGE separation of the 15 kDa protein fractions. Since the pK_a of the carboxy group of retinoic acid is near 4.5, [3 H]retinoic acid was added to the gel. After electrophoresis, three radioactive peaks were detected at pI 5.13, 4.86 and 4.55. CRABP-II and CRABP-I corresponded to the radioactive peaks at pI 5.13 and 4.86 respectively, whereas [3 H]retinoic acid corresponded to the broad radioactive peak which appeared at pI 4.55. We have observed that the ligand dissociated from the complexes [3 H]retinoic acid-CRABPs only at pH values close to their pI (results not shown). This explains the large amounts of free retinoic acid observed during i.e.f. The fact that the free retinoic acid peak overlaps with the CRABP-I peak reduced dissociation, and may account for the higher CRABP-I peak as compared with PAGE assay.

Saturation experiments

Saturation curves at equilibrium for CRABP-I and -II were determined using the 15 kDa protein fractions obtained by the PAGE/radiobinding assay, using increasing amounts of [3 H]retinoic acid (Fig. 5, insert A and B respectively). Scatchard-plot analysis of the mean of values obtained in two experiments gave a mean value of a K_d of 16.6 nM for CRABP-I (Fig. 5a) and a K_d of 50 nM for CRABP-II (Fig. 5b). The values obtained in the two experiments did not differ by more than 10%. Remark: the release of the ligand from CRABP during PAGE could render these results questionable, since dissociation could affect the K_d estimates. The arguments for a non-significant release are the following: (i) with the PAGE technique higher amounts of CRABP-II were consistently obtained than when charcoal-dextran technique were used (total CRABPs levels) [26]; (ii) serum retinol-binding protein (RBP), whose affinity for retinol and retinoic acid is lower (approx. 200 nM for both [44]) than that of CRABPs did not show a significant amount of released ligand during PAGE (less than 2%) [45]; (iii) a release of [3 H]ligand from CRABPs should have given a smear from the CRABP bands. No smear released from the CRABPs could be detected on the autoradiograms. One explanation of this could be that ligand dissociation in the highly hydrophilic environment in the gel is hindered by the high hydrophobicity of retinoids.]

Fig. 5. Scatchard plot analysis of the binding of [3H]retinoic acid to CRABP-I and -II of the 15 kDa protein fraction from human skin

A 100 µl portion ([3H]RA) of the 15 kDa protein fraction was incubated with increasing amounts of [3H]retinoic acid, and the levels of CRABP-I and -II were measured by the PAGE technique described in the Materials and methods section (see insets to a and b respectively). Scatchard plots for CRABP-I (a) and for CRABP-II (b) were calculated from the saturation curves.

Expression of CRABP-I and -II in epithelial tissues

In order to measure directly the levels of CRABP-I and CRABP-II without using the gel-filtration step, we developed a PAGE/autoradioblotting technique. Instead of cutting the PAGE gels into 2 mm bands, the separated proteins were transferred on to a nitrocellulose sheet. During the electrophoretic transfer of proteins, the non-specifically bound radiolabelled retinoic acid was almost completely removed. For example, [3H]retinoic acid bound to albumin, which interferes in the CRABP-I determination, was almost completely removed by this procedure. This is illustrated in Fig. 3 by the absence of the radioactive band corresponding to albumin.

The PAGE/autoradioblotting technique permits a more accurate measurement of the mobility of the radiolabelled binding proteins than when the gel is cut in bands and the radioactivity measured. Table 1 shows the relative mobilities (R_F), as determined by PAGE/autoradiobinding assay, of various binding proteins detected when [3H]retinoic acid, [3H]retinol, and [3H]oleic acid were incubated with epidermal extracts. CRABP-I and -II, serum RBP [27], keratinocyte-FABP (G. Siegenthaler, D. Chatellard-Gruaz & S. Jaconi, unpublished work), CRBP-I and -II (D. Chatellard-Gruaz & G. Siegenthaler, unpublished work) and albumin were well separated by this technique.

Table 1. Relative mobilities (R_F) of various ligand-binding proteins detected in human epidermal cells by the PAGE/autoradiobinding assay

Binding ligands were: (1) [3H]retinoic acid; (2) [3H]retinol; and (3) [3H]oleic acid.

Ligand-binding protein	R_F
FABP ⁽³⁾	0.30
CRABP-II ⁽¹⁾	0.44
Holo-RBP ⁽²⁾	0.47
CRBP-II ⁽²⁾	0.50
Albumin ⁽¹⁾	0.59
CRABP-I ⁽¹⁾	0.65
CRBP-I ⁽²⁾	0.75

Table 2. Percentage of CRABP-I and -II in various human epidermal cells

The data are expressed as a percentage of the total amounts of CRABP-I and -II in the sample. Values ($\pm$ S.D.) were obtained by densitometric analysis after the PAGE/autoradioblotting assay (see the Materials and methods section).

Sample	n	CRABP-I (%)	CRABP-II (%)	CRABP-I/-II ratio
Normal skin	3	41.9 $\pm$ 4.9	58.1 $\pm$ 4.9	1:1.39
Psoriatic plaque	3	5.5 $\pm$ 0.6	94.5 $\pm$ 0.6	1:17.18
Oral mucosa	1	10.5	89.5	1:8.52
Cultured keratinocytes				
Non-differentiated	3	< 0.5	100	
Differentiating	3	< 0.5	100	
Human embryo	3	82.0 $\pm$ 3.7	18.0 $\pm$ 3.7	4.55:1

The percentage, and the ratio of CRABP-I and -II per sample, calculated from densitometric analysis, is shown in Table 2. It can be observed that, in human embryonic tissues, CRABP-I was strongly expressed, whereas CRABP-II was poorly expressed. In contrast, CRABP-II was the major species in human keratinocytes. Also, cultured human differentiating keratinocytes, lesional psoriatic skin and oral mucosa contained high levels of CRABP-II. CRABP-I was detected in normal human skin at approximately the same levels as the CRABP-II, whereas oral mucosa and lesional psoriatic skin contained less CRABP-I. In contrast, cultured keratinocytes expressed only CRABP-II and not CRABP-I.

DISCUSSION

We have developed a PAGE/autoradioblotting technique that allows the simultaneous identification and measurement of the relative amounts of CRABP-I and CRABP-II in crude tissue extracts. This method is easier to perform, and is faster, than anion-exchange h.p.l.c. [32]. It allows the measurement of several samples on one gel, providing a better inter-sample comparison. The sensitivity of this method is in the femtomolar range of [3H]retinoic acid bound per mg of soluble proteins. The present study also shows that binding-protein analysis is superior to transcript analysis for CRABP-I. Indeed, detection of CRABP-I mRNA in normal skin by hybridization *in situ* [46,47] and by RNA-blot [25,47] analysis has not been successful.

Our results strongly suggest that the 'CRABP' previously measured by the PAGE assay [28] in human epidermis and dermis [27], cultured human keratinocytes [29], human oral

mucosa [36], psoriatic plaques [26] and cultured human fibroblasts [26] actually corresponds to CRABP-II, owing to their identical R_f values. Such a conclusion cannot be made when CRABP levels are measured with the gel-filtration technique [48], since CRABP-I and -II have similar molecular masses. Surprisingly, adult rat skin showed no detectable amounts of CRABP-II, when analysed by anion-exchange h.p.l.c. [32]. This is in contrast with the presence of CRABP-II mRNA in mouse [24], the presence of CRABP-II mRNA [25] and, now, the presence of the protein in human skin. The different techniques used may explain this discrepancy.

Binding studies show that human CRABP-I and II bind specifically retinoic acid but not retinol or oleic acid. In a previous report [49] we demonstrated that CRABP, now shown to be CRABP type II, had poor affinity for the 13-*cis*-isomers of retinoic acid and acitretin. Similarly it was recently reported that bovine CRABP-I [21] and rat CRABP-II [23] do not bind 13-*cis*-retinoic acid. A dissociation constant of 50 nM was calculated for human epidermal CRABP-II, whereas CRABP-I showed a higher affinity ($K_d = 16.6$ nM). These values are similar to those reported for rat testis CRABP-I [22] and for neonatal-rat CRABP-II [23].

In contrast with the embryonic human tissues, where CRABP-I predominates, CRABP-II appears to be the major isoform in human keratinocytes both *in vivo* and *in vitro*. We have observed, however, striking differences in the ratio of CRABP-I and CRABP-II under these conditions. In culture, keratinocytes appear to have lost their capability to express CRABP-I, although this isoform is present in human epidermis. CRABP-II is expressed at high levels in differentiating keratinocytes, whereas small amounts were found in non-differentiating cells. This indicates that very little CRABP-II is necessary for the maintenance of the non-differentiated phenotypes. On the other hand, a sharp increase of CRABP-II is associated with the commitment to differentiation. The small amount of CRABP-II in cultured non-differentiated keratinocytes may be due to their contamination by some differentiating keratinocytes, since the detachment of differentiating keratinocytes may be incomplete. CRABP-I was found in higher amounts in keratinizing epithelium (skin), with decreasing amounts in epithelia showing a defect in keratinization (psoriatic plaque) or with an incomplete programme of keratinization (oral mucosa) and was not detected in cultured keratinocytes, suggesting that CRABP-I might be responsible for terminal keratinocytes differentiation (cornification). In contrast, overexpression of CRABP-II and decrease in CRABP-I expression seems to be associated with a decrease in the differentiation of keratinocytes. Higher expression of CRABP-I in human skin might support the hypothesis that this binding protein may enhance the metabolism of retinoic acid to inactive derivatives, thus limiting the retinoic acid specific action to hindered differentiation [21]. This idea is supported by the observation that CRABP-I was found to be expressed in human skin with an increasing concentration gradient from the basal layer (non-differentiated) towards the more differentiating suprabasal layers [40].

The mechanism underlying normal epidermal differentiation involves the regulation of the expression of a number of structural and non-structural genes (reviewed in [50]). Cultured keratinocytes show an increased growth rate in the presence of low concentrations of retinoids, whereas higher concentrations are inhibitory [51,52]. Moreover, the most obvious effect of retinoids in keratinocyte differentiation is the suppression of terminal differentiation [51,52]. If CRABP-II levels reflect the retinoic acid increases in the cells [15], keratinocytes containing high amounts of CRABP-II should require a higher amount of retinoic acid than keratinocytes expressing lower amounts of

CRABP-II. This hypothesis is strengthened by the fact that keratinocytes containing high levels of CRABP-II also express to a higher level the enzymic system that transforms retinol into retinoic acid [13–14], whereas the keratinocytes containing lower levels of CRABP-II, such as epidermal cells *in vivo* and cultured non-differentiated keratinocytes, show only trace amounts of retinoic acid formation from retinol [13]. CRABP-I appears to be related only to the situation *in vivo*, whereas CRABP-II appears to be associated with alterations in the differentiation programme and with the cell response to retinoic acid synthesis or endogenous overload.

The biological significance of these observations remains to be further investigated; for example, the true endogenous ligand for CRABP-II needs to be identified.

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