

Human Neonatal Keratinocytes Have Very High Levels of Cellular Vitamin A-Binding Proteins*

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Since cellular retinol- and retinoic acid-binding proteins (CRBP and CRABP) mediate the effects of vitamin A on epidermal differentiation, the levels of these binding proteins were measured in the epidermal and dermal layers of newborn, human foreskin as well as in primary cultures of keratinocytes and fibroblasts from these layers. Ligand binding assays with saturating concentrations of all *trans*- ^3H retinol or of all *trans*- ^{11-3}H retinoic acid were used to quantitate amounts of binding proteins in cytosols prepared from these skin layers or cultured cells. The epidermal levels of CRABP and CRBP (60.9 ± 14.4 and 7.3 ± 1.7 pmol per mg cytosol protein, respectively) were markedly

higher than that reported for adult epidermis but were comparable to levels in keratinocytes cultured from neonatal foreskin epidermis (61.8 ± 7.8 and 10.7 ± 2.5 , respectively). The levels of CRABP were much lower in the foreskin dermis than in the epidermis and the levels measured in the fibroblasts cultured from this dermis were consistent with the dermal levels. However, CRBP levels in cultured dermal fibroblasts were very low and could not account for the dermal CRBP levels, suggesting that another dermal cell type has high levels of CRBP. *J Invest Dermatol* 88:37-41, 1987

Vitamin A is required for normal epithelial differentiation and growth [1,2]. This vitamin acts directly on keratinocytes since it prevents these cells in pure culture from terminally differentiating and forming the cornified envelope [3]. At the molecular level, vitamin A promotes the expression of the lower-molecular-weight keratins while inhibiting the expression of the higher-molecular-weight keratins [3]. Although this direct effect on keratin expression agrees with the hypothesis [4] that vitamin A controls genomic expression in a steroid hormone-like manner, the exact mechanism of vitamin A action is not known. It is known, however, that 2 separate vitamin A-binding proteins are found in the cytoplasm of many cells [4-6], one for retinol (CRBP) and one for retinoic acid (CRABP). Two major lines of evidence suggest that the biologic effects of vitamin A are mediated by these 2 binding proteins. First, those vitamin A analogs that have high biologic activity bind tightly to one or other of these binding proteins [7,8]. Second, tissues in which vitamin A transport is

essential (liver, kidney) or whose function is dependent on vitamin A (testes, epididymis) have high levels of these binding proteins [9,10].

Because vitamin A is necessary for normal keratinocyte differentiation, high levels of its cellular binding proteins would be expected in skin in general and epidermis in particular, but high levels have not been consistently found [9-14]. While differences in binding protein assays may account for some of these discrepancies [10], the difficulty in completely homogenizing mammalian skin is more likely to be a major source of the discrepancies. Because cultured cells are readily homogenized, CRBP and CRABP levels in primary cultures of keratinocytes or fibroblasts may give an accurate indication of epidermal or dermal levels if these cells are the sole source of these binding proteins in the tissues. The levels of cytoplasmic vitamin A-binding proteins in epidermis and in keratinocytes cultured from that epidermis have not been directly compared, nor have dermal and fibroblast levels been directly compared. We report, here, just such a comparison of the levels of CRABP and CRBP in the epidermal and dermal layers of human neonatal foreskin with the levels in cultured keratinocytes and fibroblasts obtained from these layers.

MATERIALS AND METHODS

Eagle's minimal essential media (MEM) with Hanks' balanced salt solution and 25 mM HEPES buffer, pH 7.2, was obtained from Grand Island Biological Company, and MCDB 153 media came from K C Biologicals and fetal bovine serum (FBS) from Hyclone Tissue Culture Products. Worthington Type III collagenase was purchased from Cooper Biomedical, Inc. Boehringer Mannheim Biochemicals provided dispase II. Unlabeled all-*trans*-retinol, unlabeled all-*trans*-retinoic acid, bovine pancreas ribonuclease A, chicken egg albumin, $2 \times$ crystallized bovine pancreas trypsin, disodium ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Company. Spectrophotometric grade dimethyl sulfoxide was supplied by Aldrich

Manuscript received March 19, 1986; accepted for publication July 8, 1986.

Supported in part by Research Funds from the Veterans Administration (REG) and by United States Public Health Service grant CEC 5R25CA19429 (CM) from the National Cancer Institute, Department of Health and Human Services.

*This work was presented in part at the Joint International Meeting of The Society for Investigative Dermatology, Inc. and The Japanese Society for Investigative Dermatology, Washington, D.C., May 1-5, 1985.

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Abbreviations:

- CRABP: cellular retinoic acid-binding protein
- CRBP: cellular retinol-binding protein
- FBS: fetal bovine serum
- MEM: minimal essential media
- PBS: Dulbecco's phosphate-buffered saline (calcium- and magnesium-free)

Chemical Company, Dextran T-70 by Pharmacia Fine Chemicals, and Norit A by Fisher Scientific Company. Methanol, acetonitrile, and hexane were all chromatography grade from Burdick and Jackson Laboratories, Inc. [^3H]Retinol at 15 Ci/mmol prepared as previously described [15] was kindly provided by Dr. David Ong (Vanderbilt University). Batches of all-*trans*-[11- ^3H]retinoic acid with specific activities of 1.54 Ci/mmol and 2.15 Ci/mmol were obtained from the Biological and Chemical Prevention Program, Division of Cancer Cause and Prevention of the National Cancer Institute. Bio-Gel P-60 (100–200 mesh) and Econo-Column (0.7 \times 10 cm) glass columns were purchased from Bio-Rad Laboratories. The Econo-Columns were shortened to 9 cm by cutting off their bed support-efflux tips. These were replaced with the fast-flow cellulose bed support-efflux tips cut from columns supplied by Whale Scientific, Inc., Commerce City, Colorado (catalog no. CC-09).

Separation of Epidermis and Dermis Following circumcision, normal human neonatal foreskins were stored at 4°C overnight in MEM supplemented as described previously [16] and made 5% in FBS. The foreskins were then rinsed 2 \times in fresh sterile MEM, treated for 20 h at 4°C with 1 unit (2 mg) of dispase II per ml of MEM according to Kitano and Okada [17]. Following 3 rinses in calcium- and magnesium-free phosphate-buffered saline (PBS) at 0°C that was 1 mM in phenylmethylsulfonyl fluoride and 0.12 mM in EDTA, the separated epidermis and dermis were centrifuged, weighed after removing excess liquid, and stored frozen at -70°C.

Keratinocyte and Fibroblast Cultures Keratinocytes and fibroblasts were obtained for cell culture by treating the foreskins with 0.25% trypsin in MEM for 20 h at 4°C. Keratinocytes were prepared for culture by incubating trypsin-separated epidermis in MCDB 153 medium at 37°C for 20 min followed by trituration with a pipet. Keratinocytes were cultured using a method similar to that of Yuspa et al [18]. After 10–14 days, when the keratinocytes had become confluent, they were removed with 0.1% trypsin, 0.12 mM EDTA in PBS, then diluted with MEM plus 10% FBS, and collected by centrifugation. The cells were washed once by resuspension in MEM plus 10% FBS and 3 \times by resuspension in PBS that was 1 mM in phenylmethylsulfonyl fluoride and 0.12 mM in EDTA. The final cell pellet was weighed and stored at -70°C.

Isolated fibroblasts were prepared for culture by mincing the trypsin-separated dermis and incubating it in MEM plus 10% FBS and 0.2% collagenase at 37°C for 16 h. The fibroblasts were grown and harvested in a manner similar to the keratinocytes.

Preparation of Cytosols Three milliliters of 0.05 M Tris-HCl (pH 7.5 at 22°C) and 6 ml of H₂O were added for each gram of cell or tissue sample, followed by homogenization at 0°C using an Ultra-Turrax homogenizer (Tekmar Company). The homogenates were filtered through gauze and 1-ml aliquots removed for DNA determination according to Burton [19]. The homogenates were processed to obtain cytosols as described by Ong and Chytil [20]. The cytosols were frozen, lyophilized to dryness, and reconstituted with 2.16 ml of H₂O per gram of initial sample weight. Following the removal of aliquots (10 μl) for protein determination according to Bradford [21], 0.27 ml of 20 mg/ml ovalbumin and 0.27 ml of 20 mg/ml ribonuclease were added. The reconstituted cytosol was stored frozen at -70°C until assayed for CRABP and CRBP.

Binding Protein Assays The ligand binding assay for quantitating the amount of CRBP and CRABP in the tissue cytosols was similar to the method previously described [22]. Both [^3H]retinol and [^3H]retinoic acid were purified by high-performance liquid chromatography within 2 days of use in the assay exactly as described previously [23]. After purification the specific activity of [^3H]retinol was always reduced to 3.0 Ci/mmol by adding unlabeled retinol. When nonspecific binding was determined a 50-fold excess of appropriate unlabeled ligand was

added. The binding mixture was analyzed on small (3.4 ml) molecular-sieving columns as previously described [22]. This column procedure was modified by using a longer glass column and by packing and eluting the column at unit gravity rather than by centrifugation. The columns were eluted and fractionated by repetitively (12 times) adding 250- μl aliquots of 0.05 M Tris-HCl, pH 7.4 at room temperature, at 4-min intervals. Fractions were collected every 4 min coincident with the addition of eluent. The amount of tritiated ligand in each of the last 10 column fractions was calculated from the amount of tritium determined by liquid scintillation counting. Specific binding was determined and used to calculate the amount of CRBP or CRABP per amount of tissue as described previously [23]. The significance of the difference found between 2 different types of samples was evaluated using a Student's *t*-test. A paired *t*-test was used where samples could be paired and equal variances were always assumed unless the number of values in the 2 groups being compared was not identical.

RESULTS

Dispase II treatment has been reported [17] to cleanly separate dermal and epidermal layers while preserving the architectural details of the epidermis. By histologic examination, we did not detect cross-contamination of the separated epidermal and dermal layers (data not shown). Furthermore we found that keratinocytes cultured after trypsinizing the dispase-separated epidermis only occasionally had small areas of fibroblast contamination.

To minimize the potential degradation of CRBP and CRABP during the preparation of cytosols by residual dispase, skin samples were washed several times with a buffer containing EDTA, a known [17] inhibitor of dispase. To evaluate the loss of binding protein due to dispase proteolysis, foreskins were cut into halves longitudinally along the midline and one half was treated with dispase while the other half was treated identically without dispase. Both halves were washed and processed to obtain cytosol

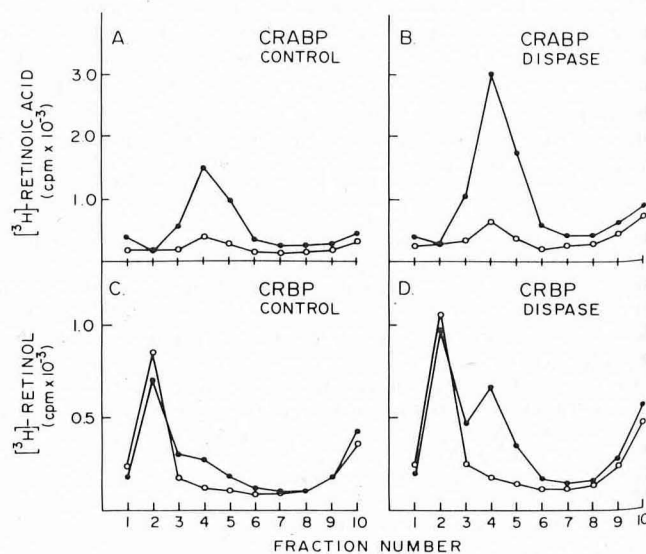


Figure 1. Ligand binding assays for CRABP and for CRBP in control and dispase-treated human neonatal foreskins. Cytosols (150 μl) from control (A,C) and from dispase-treated (B,D) foreskins were incubated with all-*trans*-[11- ^3H]retinoic acid (A,B) or with all-*trans*-[^3H]retinol in a total volume of 200 μl and fractionated on a small molecular-sieving column as described in *Materials and Methods*. Cytosols were reconstituted to give 3 ml per g of pretreatment weight of identical foreskin halves. Control samples were treated identically to the dispase-treated samples except dispase was omitted. For each of the skin samples, specific binding was computed as the difference between total ($\bullet\text{---}\bullet$) radioactivity bound minus radioactivity nonspecifically ($\text{---}\circ\text{---}$) bound in fractions 3 through 6 or 7.

Table I. CRABP and CRBP Levels in Untreated and Dispase-Treated Human Neonatal Foreskin

	CRABP (pmol/g wet wt)	CRBP (pmol/g wet wt)	Protein (mg/g wet wt)	DNA (mg/g wet wt)
Untreated	81.6 ± 19.7	9.0 ± 2.2	7.2 ± 1.6	0.42 ± 0.12
Dispase-treated	168.3 ± 72.9	17.0 ± 9.4	10.7 ± 1.3	0.56 ± 0.14

without attempting to separate the dermis and epidermis and the cytosols were analyzed for CRBP and CRABP. The results of one such experiment are shown in Fig 1. The peak of specific binding, which is the difference between total and nonspecific binding, is clearly seen in fraction 4, which corresponds to where CRBP and CRABP elute. The dispase-treated samples (Fig 1B, D) have nearly twice as much specific binding, and therefore CRABP or CRBP, as the control samples (Fig 1A,C). Since these results are based on the wet weight of identical samples before treating with dispase, this treatment does not destroy CRBP or CRABP but instead increases their measured levels.

To understand why dispase treatment increased the level of cytoplasmic vitamin A-binding proteins, homogenate DNA and cytosolic protein values were also determined on untreated and dispase-treated contralateral foreskin halves. Table I shows the mean and standard deviation of these determinations and of CRABP and CRBP levels in 5 separate sample pairs. CRBP and CRABP levels increased an average of 2-fold following dispase treatment, while the cytosolic protein and homogenate DNA levels increased by 3/2 and 4/3, respectively. Considered along with the observation that dispase treatment caused the skin to swell, these increases strongly suggest that proteolysis by dispase degrades the intracellular matrix making the skin easier to homogenize and resulting in better recovery of cytosolic components.

The mean and standard deviation of the CRBP and CRABP levels determined for 5 batches of dispase-separated epidermis and dermis are shown in Table II. CRABP levels are about 9-fold higher than CRBP levels in the epidermis while both levels are much lower in the dermis and are nearly identical to each other. As seen in Table I CRABP levels are also 9- to 10-fold higher than CRBP levels in either untreated or dispase-treated skin. Because the ratio of binding protein levels is identical in whole skin and epidermis but not in dermis, the majority of the binding proteins must be in the epidermis. Based on the wet weight of separated epidermis and dermis (weight increase due to swelling = 31.0 ± 6.5%), only 10.4% of the skin was epidermis and yet 88% of CRABP and 56% of CRBP were in the epidermis.

To determine whether the amount of CRABP and CRBP in epidermis and dermis could be attributed to the main cell type of each skin layer, cultured keratinocytes and fibroblasts were assayed for the vitamin A-binding proteins. Only keratinocyte cultures without detectable fibroblast contamination were used for these assays. Table III shows the mean and standard deviation for separate batches of keratinocytes (n = 4) and fibroblasts (n = 5). Cultured keratinocytes like dispase-separated epidermis have high levels of CRABP and also have CRBP. While cultured fibroblasts have appreciable levels of CRABP, CRBP levels are very low (Table III). A comparison of the binding protein levels in isolated

Table II. CRABP and CRBP Levels in Epidermis and Dermis of Human Neonatal Foreskin

	CRABP (pmol/mg protein)	CRBP (pmol/mg protein)	Protein/DNA (mg/mg)
Epidermis	60.9 ± 14.4	7.3 ± 1.7	11.7 ± 1.2
Dermis	2.4 ± 1.9	1.7 ± 0.6	35.6 ± 10.8

Table III. CRABP and CRBP Levels in Keratinocytes and Fibroblasts Cultured from Human Neonatal Foreskin

	CRABP (pmol/mg protein)	CRBP (pmol/mg protein)	Protein/DNA (mg/mg)
Keratinocytes	61.8 ± 7.8	10.7 ± 2.5	21.3 ± 2.7
Fibroblasts	9.2 ± 2.0	0.6 ± 0.2	16.4 ± 3.1

keratinocytes and fibroblasts with epidermal or dermal levels is shown in Table IV. In keratinocytes each binding protein level is the same as or higher than its corresponding binding protein level in epidermis whether expressed per mg protein or per mg DNA. Apparently keratinocytes can account for virtually all of the vitamin A-binding proteins found in epidermis. While fibroblasts have the same or higher levels of CRABP than the dermis does, CRBP levels are significantly lower in fibroblasts than in dermis whether based on protein or DNA. Therefore, fibroblasts can account for the CRABP levels in dermis, but they cannot account for the dermal CRBP levels.

A unique vitamin A-binding protein that binds both retinol and retinoic acid has been reported [24] and has been detected in human skin [25]. Because epidermis contains the majority of the vitamin A-binding proteins, we looked for this unique binding protein in human foreskin epidermis. Since this unique binding protein binds both retinol and retinoic acid, unlabeled retinoic acid competes with the binding of labeled retinol to this protein [24]. Figure 2 shows the results of this kind of competition experiment on cytosol from isolated epidermis. The solid circles in the upper panels show the binding of [³H]retinoic acid in the absence (A) and presence (B) of excess unlabeled retinol. Similarly the solid circles in the lower panels show the binding of [³H]retinol in the absence (C) and presence (D) of excess unlabeled retinoic acid. Clearly, no significant competition can be detected. Labeled ligand is readily displaced by an excess of the same unlabeled ligand as shown by the open circles. Therefore, the epidermis of human neonatal foreskin does not appear to have a unique vitamin A-binding protein that binds both retinol and retinoic acid. A similar conclusion applies to the dermis, since the majority of the specific binding of [³H]retinol in dermal cytosol could not be displaced by excess cold retinoic acid (data not shown).

DISCUSSION

In this study very high CRABP levels and much lower CRBP levels were found both in human epidermis and in cultured keratinocytes. However, both the levels of CRABP and CRBP are always lower in epidermis than in cultured keratinocytes when levels are expressed per mg DNA. This probably reflects the difficulty in completely homogenizing the epidermis and recovering the cytosolic binding proteins as compared to the nearly total recovery of binding proteins from the easily homogenized cultured keratinocytes. This comparison of retinoid-binding protein levels in cytosols from epidermis and cultured keratinocytes is also complicated by the potential differences between in situ

Table IV. Comparison of Vitamin A-Binding Protein Levels in Keratinocytes to Epidermal Levels and in Fibroblasts to Dermal Levels

	CRABP per mg of:		CRBP per mg of:	
	Cytosol Protein	DNA	Cytosol Protein	DNA
Ratio of keratinocyte to epidermal levels	1.0 (NS) ^a	1.9 (p < 0.02)	1.5 (NS)	2.6 (p < 0.01)
Ratio of fibroblast to dermal levels	3.8 (p < 0.001)	1.5 (NS)	0.4 (p < 0.01)	0.2 (p < 0.01)

^aNot significant since p > 0.05.

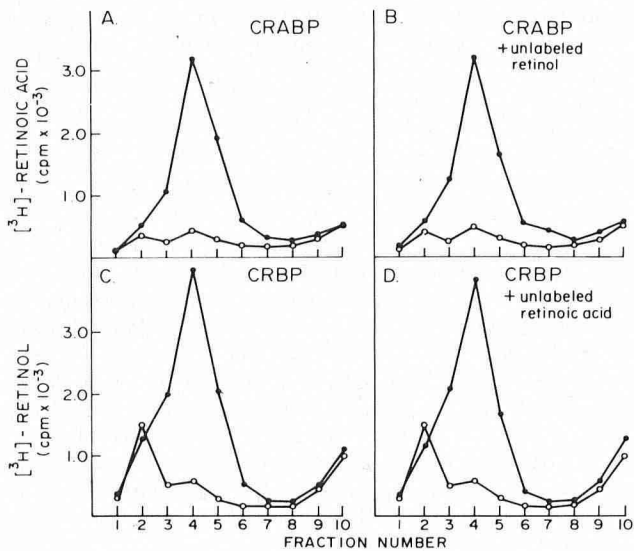


Figure 2. Competition by unlabeled retinol in the CRABP assay and by unlabeled retinoic acid in the CRBP assay. Epidermal cytosol (20 μ l) was incubated with all-*trans*-[11- 3 H]retinoic acid in the presence (B) or absence (A) of 50 μ M unlabeled retinol to determine total binding (—●—) of retinoic acid. Epidermal cytosol (150 μ l) was incubated with all-*trans*-[3 H]retinol in the presence (D) or absence (C) of 50 μ M unlabeled retinoic acid to determine total binding (—●—) of retinol. The incubation, fractionation, and subsequent calculation were done as described in the legend to Fig 1. The nonspecific binding (—○—) in A and B was determined using 50 μ M unlabeled retinoic acid in the absence of unlabeled retinol while the nonspecific binding (—○—) in C and D was determined using 50 μ M unlabeled retinol in the absence of unlabeled retinoic acid.

and cultured keratinocytes. Given these experimental limitations, the major cell type in human epidermis, the keratinocyte, appears to contain almost all of the epidermal cellular vitamin A-binding proteins.

The CRABP level in human neonatal foreskin epidermis of 61 pmol/mg protein detected in this study is considerably higher than previously reported levels for normal adult human epidermis (3.7–26.5 pmol/mg protein) [11–14,26]. While this difference could suggest that the level of epidermal CRABP decreases with age, we found that keratinocytes cultured from newborns or from young adults have comparable levels of both CRABP and CRBP (R. E. Gates and L. E. Allred, unpublished observations). Others [27] found CRABP levels in newborn keratinocytes much lower than we detected in newborn keratinocytes (13.5 vs 62 pmol/mg protein). Therefore, the higher levels of CRABP found here are probably not due to age differences. The higher levels are more likely due to the higher values measured in our improved ligand binding assay [22] and, as noted in the *Results*, to the nearly 2-fold increase in recovery of the binding proteins in homogenates of disperse-treated skin.

When dermis is digested with collagenase to obtain fibroblasts for culturing, fragments of microvessels containing erythrocytes are seen microscopically (data not shown). Since these erythrocytes contain soluble protein but no DNA, they may account for the high protein to DNA ratio in the dermis which results in artificially low levels for CRABP and CRBP in the dermis when expressed per mg protein. The possible presence of serum retinol-binding protein in the microvessels could contribute significantly to the retinol binding measured in the dermis. Although the small amount of retinol binding in the dermis is difficult to quantitate, the majority of the retinol binding in the dermal cytosol was not abolished by excess retinoic acid. Since excess retinoic acid does compete for and abolish the binding of labeled retinol to serum retinol-binding protein [28], the majority of the dermal retinol

binding must be due to CRBP and not to serum retinol-binding protein.

In the fibroblast, levels of CRABP were higher and levels of CRBP were lower than in the dermis whether expressed per mg protein or per mg DNA. Therefore, fibroblasts as the major dermal cell type can account for all of the dermal CRABP, but cannot account for most of the dermal CRBP. One explanation for the higher dermal levels of CRBP would be marked differences in the expression of the binding proteins between in situ and cultured fibroblasts. With this explanation, as seen in Table IV, CRABP levels would increase significantly when fibroblasts are cultured while CRBP levels would decrease significantly. Another, perhaps more probable, explanation is that some cell type in the dermis other than the fibroblast, such as the endothelial cell, contains high levels of CRBP.

We thank Dr. Lloyd King for helpful discussions and suggestions. We acknowledge the expert technical assistance of Ms. Rebecca Gariglietti and of Ms. Judith Swanson.

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