Retinoic Acid Receptors and Binding Proteins in Human Skin

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Nuclear retinoic acid receptors (RAR) are likely to mediate many of the pleiotropic cutaneous actions of retinoids by acting as ligand-dependent enhancer factors. The presence of nuclear RAR in skin was confirmed by identification of a 45-kDa nuclear RA binding activity by fast protein liquid chromatography (FPLC). Analysis of RNA extracted from skin specimens demonstrated expression of RAR-α and RAR-γ transcripts, as well as expression of the homologous low-affinity receptor, RXR-α. Both isoforms of RAR-γ RAR-γ1 and RAR-γ2 were detectable, with RAR-γ1 being the more strongly expressed. FPLC analysis also demonstrated a 15-kDa peak of specific RA binding activity, consistent with the presence of cellular retinoic acid binding protein (CRABP). Of the two known forms of CRABP, CRABP-II was much more strongly expressed than CRABP-I at the level of steady-state mRNA. CRABP-II was also expressed in keratinocytes and fibroblasts in vitro. CRABP-II was up-regulated by agents that induce keratinocyte differentiation, and inhibited by prolonged exposure to high concentrations of RA. In contrast, CRABP-II was consistently induced by RA in dermal, but not in lung fibroblasts. CRABP-I was expressed at low to undetectable levels under all these conditions. The presence of tissue-specific and differentiation-related regulation of CRABP-II suggests that it may be an important regulator of RA action in human skin. J Invest Dermatol 98:36S–41S, 1992

Retinoic acid (RA) has marked effects upon keratinocyte growth and differentiation, including inhibition of the terminal differentiation program [1,2] and acceleration of proliferation in human [3] and murine [4] epidermis. Whereas alternative mechanisms of action remain possible, it is likely that many of these effects are mediated by nuclear retinoic acid receptors (RAR) acting through retinoic acid response elements located in the regulatory regions of multiple target genes [5–8]. Three forms of RAR (RAR-α, -β, and -γ) display high affinity for RA [7,8], and all of these are expressed as isoforms generated by the use of multiple transcriptional start sites and/or alternative splicing events [7,9,10,11]. An additional, evolutionarily related member of the steroid receptor superfamily, termed retinoid X receptor (RXR), displays a much lower affinity for RA but is capable of ligand-dependent transactivation [12].

We and others have previously analyzed the pattern of expression of RAR transcripts in human skin, demonstrating very low levels of RAR-α and -β and high, constitutive levels of RAR-γ mRNA in this tissue [14,15]. We found no evidence for induction of any form of RAR after RA treatment, nor after stimulation of various keratinocyte signal transduction pathways in vitro. RAR transcripts were not increased in psoriatic epidermis relative to the internal control, cyclophilin. However, keratinocytes were shown to respond to RA by expression of several growth factors and cytokines known to be abnormally expressed in psoriatic lesions (e.g., IL-1β, IL-8, and TGF-α), suggesting a role for RA-mediated events in the pathogenesis of this disease [13].

Although much attention has recently been focused on RAR, another potential candidate for regulation of RA action is cellular retinoic binding protein (CRABP). Gradients of CRABP expression have been detected in the chick limb bud [15] and the CNS of the mouse embryo [16], suggesting a role for this protein in the regulation of RA action during embryonic development. Two forms of CRABP have been identified in murine [17,18], rat [19], bovine [20], and avian [21] cells and termed CRABP-I and CRABP-II. Recently, we have identified and cloned the human analogues to both molecules, which are highly homologous to the murine, avian, and rat forms [22]. We have demonstrated that CRABP-II is selectively expressed in human skin, as well as in cultured keratinocytes and dermal fibroblasts [22]. These results strongly suggest that altered expression of CRABP-II, but not of CRABP-I, is responsible for the increased levels of CRABP found after topical RA application [23] and systemic retinoid treatment [24], and in response to agents that stimulate keratinocyte differentiation in vitro [25].

In this report we present an overview of previously reported data regarding expression of RAR [13] and CRABP [22] in human skin, and demonstrate expression of RXR and the RAR isoforms in this tissue. In addition, we present examples of selective regulation of CRABP-II by RA in dermal fibroblasts and cultured keratinocytes. From these data, it is apparent that alterations in the expression of

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Abbreviations:

CRABP: cellular retinoic acid-binding protein
FPLC: fast protein liquid chromatography
IL-1: interleukin 1
IL-8: interleukin 8
KGM: keratinocyte growth medium
RA: retinoic acid
RAR: retinoic acid receptors
RAR-α, -β, -γ: retinoic acid receptor-α, -β, -γ
RXR: retinoid X receptor
SDS: sodium dodecyl sulfate
TGF-α: transforming growth factor-α
CRABP-II may play an important role in the regulation of retinoic acid action in human skin.

MATERIALS AND METHODS

Skin Biopsies  Keratome biopsies were obtained with informed consent from normal volunteers as previously described [26]. Retin-A cream, 0.1%, or its vehicle (Ortho Pharmaceuticals) was applied for 4 d under plastic wrap occlusion as described [13]. Biopsies were immediately frozen in liquid nitrogen and stored at -70°C.

Cell Culture  Cultures of normal human keratinocytes and fibroblasts were prepared and treated as previously described [13].

Preparation of Nuclear Extracts and RA Binding Assay  Fresh keratome biopsies were homogenized, subjected to low-speed centrifugation, and extracted with 0.6 M KCl as previously described [13]. After overnight incubation of nuclear extracts with 100 nM [3H] RA at 4°C in the presence or absence of 10 μM unlabeled RA, size fractionation of epidermal RA binding proteins was performed on a GF 250 column (DuPont) as previously described [13].

RNA and DNA Isolation and Blot Hybridization  RNA was extracted from keratome biopsies by guanidinium isothiocyanate lysis and ultracentrifugation [13,27]. Keratinocytes and fibroblasts were harvested for preparation of RNA by lysis in RNAzol (Cinna/Biotech, Houston, TX), as previously described [13,27]. Methods used for RNA blot hybridization, quantitative densitometry, preparation of CRABP-I, CRABP-II, RAR-γ, and cyclophilin probes, and isolation of polyadenylated RNA have all been described [13,22,27]. Isoform-specific RAR-γ1 and RAR-γ2 probes were prepared by PCR amplification and subcloning of residues 25-586 of the RAR-γ1 sequence and residues 47-219 of the RAR-γ2 sequence [11]. RXR-α probes were prepared by PCR amplification of human skin cDNA as described [22] using primers derived from the published RXR-α sequence [12,28].

RESULTS

RA Binding Proteins  As shown in Fig 1, FPLC analysis of [3H] RA binding to 0.6 M KCl nuclear extracts prepared from human epidermis revealed peaks of bound radioactivity at eluent volumes consistent with RA binding to proteins of M, approximately 45 kDa and 15 kDa, as determined by comparison with the elution volumes of known protein standards. A third peak eluted at a M, of less than 10 kDa. The elution positions of the 45- and 15-kDa peaks in human epidermis correspond to the predicted elution volumes of RAR and CRABP, respectively [13]. Each peak was reduced by at least 95% by a 100-times molar excess of unlabeled RA, indicating the specificity of binding. The presence of a peak similar in size to CRABP in the extracts is expected due to cytosolic contamination of the nuclear preparations. Chromatography of in vitro translated RAR-γ1 under conditions identical to those employed for nuclear epidermal extracts, confirmed the identity of the 45-kDa peak as RAR [13].

Expression of RAR-γ Isomers and RXR in RA-Treated Skin  RAR-γ is expressed as two principal isoforms, called RAR-γ1 and γ2 in mouse [11], and RAR-γA and RAR-γE in human [7]. These isoforms differ in their N-terminal coding regions and could differ in terms of transactivation function [7,11]. We have compared the expression of RAR-γ1 and γ2 transcripts in vehicle-treated and RA-treated human skin by means of isoform-specific probes (Fig 2). The specificity and sensitivity of these probes has been verified by hybridization against genomic DNA blots (Elder et al, submitted). However, the RNA bands detected by each probe are very similar in size (3.3 kb for RAR-γ1 versus 3.1 kb for RAR-γ2 [7,11]). As shown in Fig 2, the RAR-γ1 isoform-specific probe detects bands very similar in size to those detected by the 3′-terminal 400 Nrl-EcoRI fragment of the RAR-γ cDNA, a probe that detects both RAR-γ1 and RAR-γ2 isoforms [7]. Re-hybridization of the same blot against the RAR-γ2 probe (not shown) failed to reveal bands; however, low levels of RAR-γ2 transcripts have been observed in other experiments [28].

To test for expression of the low-affinity RXR receptor in human skin, we generated an RXR-specific probe by PCR amplification of cDNA generated from human skin RNA and used it as a probe for blot hybridization. Figure 2 (right panel) demonstrates expression of the 5.0-kb RXR-α mRNA in polyadenylated RNA extracted from keratome biopsies of normal epidermis. We have not observed modulation of RXR mRNA levels in response to RA treatment [28].

Expression of CRABP mRNA in Human Skin  FPLC analysis (Fig 1) revealed a specific RA-binding peak of M, approximately 15 kDa, consistent with the presence of CRABP in human skin extracts. To determine which form(s) of CRABP might be expressed in human skin, volunteers were treated with 0.1% RA cream, 0.2% SDS, or RA vehicle under occlusion for 16 h. Keratome biopsies were obtained from each treated site and a nearby untreated site, total RNA was extracted, and 40 μg total RNA was analyzed by blot hybridization. Some of these data have been published previously [22]. CRABP-I transcripts were undetectable or

![Figure 1](image1.png)  Figure 1. FPLC size-exclusion chromatography of epidermal nuclear extracts. Solid dots, extracts incubated in the presence of 100 nM [3H] RA. Open dots, parallel extracts incubated in the presence of 10 μM excess unlabeled RA. Arrows, elution volumes of standards: yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocyte carbonic dehydrrogenase (29 kDa), and horse heart cytochrome c (12.4 kDa). M, values estimated for the RA-binding peaks by interpolation from the standards are indicated above the peaks.

![Figure 2](image2.png)  Figure 2. Expression of RAR, RAR-γ isoforms, and RXR in normal human skin. RNA blots containing 5 μg polyadenylated RNA from four normal individuals were sequentially rehybridized against the probes shown above the autoradiograms. Mobilities of 18S and 28S ribosomal RNA are shown to the left, and mobilities of RAR-γ and RXR transcripts are shown to the right.
detectable at only extremely low levels under all treatment conditions (not shown). In contrast, CRABP-II transcripts were detectable in all of the samples, and were markedly and selectively induced by RA (Fig 3). Similar inductions were observed after 12 and 48 h, but not after 4 h of RA treatment [22].

Expression of CRABP in Fibroblasts. Treatment of human dermal fibroblasts with RA resulted in marked, dose-dependent induction of CRABP-II mRNA, as shown in Fig 4. Marked (two- or three-fold or greater) inductions of CRABP-II mRNA were observed at RA concentrations as low as 3 × 10^{-10} M (Fig 4A) and reached a plateau between 3 × 10^{-7} and 3 × 10^{-6} M (Fig 4B). This figure also demonstrates that the kinetics of this response are delayed, as CRABP-II transcripts remain at basal levels after 4 h but undergo a marked and sustained increase over 24-48 h of RA treatment. As previously reported [22], this response is not observed in lung fibroblasts propagated under identical conditions (Fig 4B). The failure to observe induction CRABP-II mRNA was not due to a general lack of RA responsiveness, as lung fibroblasts displayed marked induction of RAR-β by RA (Fig 4C, center panel). RAR-γ transcripts displayed no consistent pattern of RA responsiveness in this cell type (Fig 4C, right panel). CRABP-I transcripts were undetectable in dermal and lung fibroblasts and were not induced by any concentration of RA (data not shown).

Expression of CRABP in Keratinocytes. As shown in Fig 5A, CRABP-II mRNA was markedly induced in cultured keratinocytes upon reaching confluence, and persisted at elevated levels in the postconfluent state. The figure also demonstrates that CRABP-II mRNA was also markedly induced by raising the medium calcium concentration from 0.15 mM to 2 mM, even in subconfluent keratinocytes. In contrast to its effects on dermal fibroblasts, RA treatment of keratinocytes caused a marked reduction in CRABP-II levels (Fig 5B). As was the case in fibroblasts and in skin specimens, CRABP-I mRNA was undetectable by these methods in cultured keratinocytes (data not shown).

DISCUSSION

Retinoic Acid Receptors in Human Skin. Nuclear retinoic acid receptors act as ligand-dependent transcriptional enhancer factors [5-12] and may explain many, if not all, of the pleiotropic cutaneous effects of retinoids [13]. However, the first two forms of RAR to be discovered, RAR-α and RAR-β, were found to be expressed only at very low levels in the skin [5,6,13]. A third member of this family, RAR-γ, is expressed at high levels in skin [8,10,13,14], suggesting that this form of the receptor is likely to mediate many of the cutaneous effects of RA action. As presented here (Fig 1) and described in more detail in a recent report [13], we identified specific RA-binding activities of approximately 45 and 15 kDa in normal adult human epidermis. RNA blot hybridization revealed very low levels of RAR-α and high levels of RAR-γ transcripts in this tissue, whereas RAR-β transcripts were undetectable [13] (Fig 2). These results suggest that the bulk of the 45-kDa RA binding activity is RAR-γ, although this conclusion may need to be revised if translational control and protein stability prove to be important determinants of the content of RAR proteins in the skin.

Making use of isoform-specific probes (Fig 2), we found that RAR-γ1 is likely to be the predominant isoform expressed in human skin, as is the case in murine skin [7]. A cDNA probe prepared from human skin RNA by PCR demonstrated expression of RXR transcripts in normal human skin (Fig 2). The evolutionary conservation of RAR-γ isoforms and RXR as well as their presence in skin tissues of human and murine species [7,11,12] suggests that genes...
lar in magnitude to that of CRABP protein reported by Saurat and co-workers [25] after topical retinoid treatment. Moreover, as these workers reported for CRABP protein, we also found that a nonspecific irritant (0.2% SDS) did not affect CRABP-II mRNA levels after short times (16 h) of RA treatment. Finally, the very low levels of CRABP-I expression we observed strongly suggest that the form of CRABP induced by RA in human skin is CRABP-II, and not CRABP-I.

**Regulation of CRABP Expression In Vitro** CRABP-II transcripts were induced by RA in cultured human dermal fibroblasts in a dose-dependent fashion (Fig 4). This dose response correlates well with reported binding affinities of RAR for RA [5–7] as opposed to the higher concentrations required for trans-activation by RXR [12], suggestive of an RAR-mediated response. Involvement of RXR cannot be ruled out, however, as intracellular accumulation of RA might have occurred during the prolonged time periods (24–48 h) required for maximal induction of CRABP-II mRNA (Fig 4B). As previously reported [22], CRABP-II is not induced by RA in lung fibroblasts, indicative of cell lineage specificity (Fig 4B). RAR-β was strongly induced by RA in these cells (Fig 4C), demonstrating that the non-inducibility of CRABP-II is selective and does not reflect a general refractoriness to RA in lung fibroblasts. As is the case in human skin, CRABP-I was not detectably expressed in dermal or in lung fibroblasts [22].

In keratinocytes grown in serum-free medium, CRABP-II transcripts were selectively induced by growth to confluence and by increasing the medium calcium concentrations (Fig 5A). These results indicate that increased expression of CRABP-II, and not CRABP-I, accounts for the reported induction of CRABP protein by agents that promote keratinocyte differentiation in vitro [25]. In contrast to the situation with dermal fibroblasts, CRABP-II transcripts were not induced by RA treatment of cultured keratinocytes using conditions under which other RA responses (e.g., induction of IL-1β mRNA) can be clearly documented [13]. In fact, CRABP-II mRNA was strongly inhibited by prolonged (48 h) treatment with high concentrations (3 × 10^−6 M) of RA [22] (Fig 5B). These results also stand in marked contrast to the strong increase in CRABP-II mRNA after topical RA treatment [22] (Fig 3). These differences may reflect the degree of stratification of the target keratinocytes [28] and imply that CRABP-II is under complex regulation by multiple factors in the cutaneous microenvironment.

**SUMMARY**

In identifying and characterizing the expression of RAR and CRABP in human skin and skin cells, we have begun the task of defining the molecules that mediate the pleiotropic cutaneous retinoid response. The results thus far available suggest that RAR-γ is a major mediator of RA action in the skin, although the roles of RXR, RAR-γ isomers, and other RAR in these processes demand further investigation. Although the identification of genes that are regulated directly by RA in the skin is in its infancy, it is already clear that RA can simultaneously regulate the expression of multiple genes, either positively or negatively. Therefore, it will be very important to understand the factors that regulate the overall concentration of RA available to nuclear receptors, including the enzymes responsible for RA biosynthesis and metabolism and the levels and subcellular distributions of RA binding proteins. The selective expression of CRABP-II in skin and its constituent cells suggests that it may act to regulate cutaneous RA action. The selective stimulation of CRABP-II expression by RA in vivo and by agents that stimulate keratinocyte differentiation in vitro suggests that CRABP-II may exert its effects by controlling RA availability. Future studies will be directed towards understanding the regulation of and identifying the molecular function(s) of CRABP-II in the cutaneous environment.
Figure 5. Effects of confluence and retinoic acid on CRABP-II expression in cultured keratinocytes. A) CRABP-II mRNA is induced at confluence in human keratinocytes. Keratinocytes were trypsinized, seeded at $6 \times 10^3$ cells/cm² in 100-mm dishes and grown in Keratinocyte Growth Medium (KGM, Clonetics, San Diego, CA), with or without the addition of CaCl₂ to a final concentration of 2 mM and/or retinoic acid ($3 \times 10^{-9}$ M) as indicated above the autoradiograms. Medium was changed every other day. After the indicated number of days, cells were lysed and RNA analyzed by blot hybridization against the CRABP-II probe ($20 \mu$g total RNA per lane, upper panel). An ethidium bromide minigel used to confirm equal loadings of total RNA is shown in the lower panel. B) Prolonged RA treatment reduces CRABP-II mRNA levels in adult human keratinocytes. Keratinocytes were seeded as described in (A) above and treated with the indicated concentrations of RA for either 2 or 5 days. Mobilities of 28S and 18S ribosomal RNA are indicated to the left. The results shown have been previously published elsewhere [22] and are reproduced here with permission.

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