Differential Regulation of Retinoic Acid Receptors and Binding Proteins in Human Skin

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Many of the pleiotropic effects of retinoids are likely to be mediated by nuclear retinoic acid receptors (RAR) acting as ligand-dependent enhancer factors. However, in previous studies we have been unable to document altered RAR expression at the RNA level in response to retinoic acid (RA) treatment or in psoriatic lesions, conditions characterized by marked alterations in keratinocyte proliferation and differentiation, which are either caused by or responsive to RA. In an attempt to identify other potential regulators of RA responsiveness, we have used RNA blot hybridization to study the expression of the cellular retinoic acid binding proteins (CRABP) CRABP-I and CRABP-II, the RAR-γ isoforms RAR-γ1 and RAR-γ2, and the low-affinity RAR homologue RXR in normal, RA-treated, and psoriatic human epidermis. CRABP-II is selectively and markedly induced by RA in adult human skin (J Biol Chem 266:17662–17666. 1991). However, in submerged, serum-free keratinocyte cultures, CRABP-II mRNA could not be induced by RA. Comparisons of intact human skin, submerged keratinocyte cultures, and human skin equivalent cultures indicated that induction of CRABP-II by RA requires epidermal stratification, dermal-epidermal interactions, or both. CRABP-II transcripts were also expressed in heat-separated human dermis at levels similar to those found in epidermal keratocyte biopsies, whereas CRABP-I transcripts were undetectable in dermal RNA. CRABP-II transcripts were markedly elevated in psoriatic lesions, as they were in RA-treated skin. In contrast, CRABP-I mRNA was undetectable and not increased in psoriatic lesions. Expression of RAR-γ isoforms and RXR was not detectably altered in either psoriatic lesions or in RA-treated skin. Thus, altered expression of CRABP-II appears more likely to regulate the cutaneous actions of RA than does altered expression of CRABP-I, RXR, or RAR-γ isoforms. From these and other results, a model for regulation of RA action involving sequestration of RA by CRABP-II is proposed. J Invest Dermatol 98:673–679, 1992

Retinoic acid (RA) is a pleiotropic effector of keratinocyte growth and differentiation in vitro and in vivo. In particular, RA inhibits expression of the terminal differentiation program in vitro [1,2] and accelerates proliferation after topical application in vivo in human [3] and murine [4] epidermis. The discovery of nuclear retinoic acid receptors (RAR) capable of acting as trans-activating enhancer-binding factors [5,6] has provided a plausible mechanism by which RA might exert its pleiotropic effects. Three high-affinity forms of RAR (RAR-α, -β, and -γ) have been described, and of these, RAR-γ is the prominent receptor mRNA expressed in human [7,8] and murine [9] skin. In addition, homologous receptors displaying low affinity for RA have been cloned, and termed RXR [10]. Nuclear RA binding activity of the size expected for RAR (45 kDa) has been demonstrated in human skin [8]; however, the molecular identity and relative abundance of the receptor proteins expressed in the skin remains to be established. Despite this gap in our current knowledge, it is remarkable that no alteration in the expression of RAR-γ or detectable induction of RAR-α or RAR-β transcripts [8] is observed in psoriasis, a condition known to be characterized by marked alterations in both keratinocyte proliferation [11] and differentiation [12]. Moreover, no alteration in expression of any of the receptor transcripts was observed in skin treated topically with RA [8]. Therefore, we have searched for additional mechanisms that might regulate the pleiotropic effects of RA via its nuclear receptors.

One protein that might display such regulatory properties is cellular retinoic acid binding protein (CRABP). Human epidermal CRABP exhibits a K_d of 13.7 nM [13], well within the range of RA concentrations required for trans-activation by RAR (0.5–50 nM, [14]). Therefore, it is plausible to hypothesize that CRABP may interact with RAR in some fashion to effect the cellular actions of RA. Gradients of CRABP expression have been detected in the developing chick limb buds [15,16] and the murine central nervous system [17], suggesting a role for this protein in the regulation of RA action during embryonic development. Two forms of CRABP have been characterized in murine [18,19], rat [20], bovine [21], and avian [22] cells and termed CRABP-I and CRABP-II. Recently, we
have isolated human CRABP-I and CRABP-II cDNA, which are highly homologous to the murine, avian, and rat forms [23]. CRABP-II was markedly and selectively induced in human skin by topical retinoid treatment in vivo and by agents that induce keratinocyte differentiation in vitro [23], suggesting that it might function to regulate the cutaneous actions of RA in the context of epidermal differentiation.

Another potential mechanism for regulation of RA action in the skin is altered expression of RAR isomers, which are generated largely by alternative splicing events [7,24,25]. It is possible that the nuclear actions of RA might be mediated by differential expression of RAR isomers with different target genes or trans-activating effects. Another mechanism could involve altered expression of RXR [10]. RXR can interact with target DNA sequences but is relatively inefficient in RA binding [10]. Therefore, it could positively or negatively affect RA responsiveness by competing with high-affinityRAR for binding sites on chromatin or by formation of heterodimers with altered responsiveness to RA.

In this report, we make use of stratified human skin equivalent (HSE) cultures to extend our previous evidence [23] for selective up-regulation of CRABP-II in human epidermis by RA and by agents that promote epidermal differentiation. In addition, we examine the expression of CRABP-II mRNA in human dermis and in psoriatic lesions. Extending our previous studies of the nuclearRAR [8], we make use of probes generated by the polymerase chain reaction (PCR) to estimate the relative abundance of RAR-γ1 and RAR-γ2 isomers and to examine the expression of RAR-γ isomers and RXR in normal, psoriatic, and retinoid-treated human skin. Our results indicate that expression of CRABP-II is positively regulated by RA in the context of appropriate dermal/epidermal interactions and epidermal stratification, and suggest that variable expression of CRABP-II may be an important determinant of RA actions via constitutively expressed nuclear receptors in human skin.

MATERIALS AND METHODS

**Skin Biopsies** Keratome biopsies consisting primarily of epidermis were obtained with informed consent from normal volunteers or psoriatic patients as previously described [26]. For in vivo RA treatment, 0.1% Retin-A cream or its vehicle (Ortho Pharmaceuticals) was applied for 4 d as described [8]. Biopsies were frozen in liquid nitrogen immediately after removal and stored at −70°C.

**Heat Separation of Human Dermis** Full-thickness breast or thigh skin (5–20 g wet weight) was obtained from plastic surgery procedures and transported to the laboratory on saline-soaked gauze at 20°C within 1 h of removal. The fat was trimmed, and 2–4 cm squares of tissue were placed for 2 min in calcium and magnesium-free Dulbecco’s phosphate-buffered saline, pH 7.4 (PBS, Gibco) at 60°C. The samples were immediately plunged into ice-cold PBS and the dermis and epidermis were separated on ice using fine curved forceps. The epidermal and dermal tissue was then immediately frozen in liquid nitrogen and stored at −70°C until use.

**Cell Culture** Secondary cultures of normal human keratinocytes were prepared and treated as previously described [8]. Human skin equivalent (HSE) was purchased from Organogenesis (Rogers, MA) and was maintained in living skin equivalent (LSE) assay medium (Organogenesis) without added serum at 37°C in a 10% CO₂ atmosphere overnight prior to RA treatment. RA added to the upper surface of the HSE was diluted in LSE Assay Medium and applied to the upper surface of the culture in a volume of 200 μl.

**RNA and DNA Isolation and Blot Hybridization** RNA was extracted from frozen keratome biopsies and HSE by guanidinium isothiocyanate lysis and ultracentrifugation or from keratinocyte cultures by lysis in RNAsol (Cinna/Biotex, Houston, TX), as previously described [8,27]. Methods used for RNA blot hybridization, alkaline genomic DNA blot hybridization, quantitative densitometry, the preparation of hCRABP-I, hCRABP-II, RAR-γ, and cyclophilin probes and polyadenylated RNA have all been described [8,23,27]. RAR probes were prepared by PCR amplification of human skin cDNA as described [23] using primers derived from the published RAR sequences [10]. The forward and reverse primer sequences (numbered as in [10]) are given below (italic lower case letters indicate XhoI and XbaI linkers, and the remaining lower case letters indicate random nucleotides):

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58
forward: 5’-ttrcttgcggatcctagttgattcggagacatggacaccc-3’
85
reverse: 5’-agagcactttagggctggaaagaaacagctgcggtgccagcagc-3’
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The amplified region of RARX was isolated and subcloned into Bluescript plasmid and sequenced by dye-deoxy chain termination [28] using modified T7 polymerase (Sequenase, U.S. Biochemical) and synthetic oligonucleotides. The sequence of the PCR product was found to be identical to the published RAR sequence [10]. 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 [24]. The keratin 5 probe was isolated from a human skin cDNA library and its identity was confirmed by DNA sequencing. The vimentin cDNA probe p4F1 (ATCC 59517) has been described [29].

**RESULTS**

**Differential Effects of RA on CRABP-2 mRNA In Vivo and In Vitro** We have reported that CRABP-II mRNA is markedly and significantly induced in adult human keratinocytes cultures at confluence [23]. Figure 1 demonstrates that the same behavior is observed in neonatal human KC. As is the case in epidermal keratome biopsy specimens, CRABPI mRNA is undetectable by these methods in cultured adult and neonatal keratinocytes (data not shown). As reported previously [23], prolonged (48-h) treatment of confluent adult keratinocytes with high concentrations of RA (3 X

![Figure 1](image-url)
The effectiveness of the separation procedure was demonstrated morphologically by light microscopy of paraffin sections (data not shown) and by comparing the expression of vimentin (middle panel) and keratin 5 (lower panel) transcripts in dermal specimens and keratome biopsies. As expected, the vimentin probe hybridized strongly to the dermal samples, whereas the keratin 5 probe hybridized strongly to the keratome samples.

Expression of RAR-γ Isoforms and RXR in RA-Treated Skin: Isoform-specific probes were prepared by PCR amplification using primers specific for of the 5′ ends of RAR-γ1 and RAR-γ2 mRNA [24], and used to compare the expression of isoform transcripts in vehicle-treated and RA-treated human skin. In Fig 4, RNA blots derived from vehicle-treated or RA-treated human skin were probed with a 3′ terminal 400 bp Nal-I EcoRI fragment common to both isoforms of RAR-γ [7], then stripped and sequentially reprobed with the isoform-specific probes. In each experiment, genomic DNA (Southern) blots were hybridized in parallel as a control for probe specificity and sensitivity (Fig 4, right panels). Each DNA blot hybridization reveals a different pattern, as each probe is derived from a different exon of the RAR-γ gene [24]. However, the RNA bands detected by each probe are indistinguishable in size (Fig 4, left panels). As demonstrated by the two representative individuals shown in Fig 4, no induction of RAR-γ2 transcripts has been ob-

Figure 3. Expression of CRABP-II in human dermis. Five samples of heat-separated dermis (left panels) are compared to five unpaired keratome biopsy samples (right panel). The blots were sequentially hybridized against CRABP-II (upper panels), vimentin (middle panels), and keratin five cDNA probes (lower panels). Mobilities of 28 and 18S ribosomal RNA are indicated to the left.
Figure 4. Expression of RAR-γ isoforms in RA treated skin. Left panels, RNA blots (40 μg total RNA) of samples prepared from keratome biopsies after 4 d of treatment of two volunteers (1 and 2) with 0.1% RA cream (RA) or its vehicle (Veh) under continuous plastic wrap occlusion. The blots were hybridized against the RAR-γ 3' end probe, which detects both isoforms, then re-hybridized sequentially against the RAR-γ1, RAR-γ2, and cyclophilin probes. Right panels, parallel hybridizations of genomic DNA blots (10 μg DNA per lane) to control for probe sensitivity and specificity [8]. Enzymes used to digest the genomic DNA are given above the lanes. Hybridization probes are indicated to the right of the autoradiograms. In order to avoid overexposure, the RAR-γ 3' RNA blot hybridization shown in the upper left panel was exposed for only one-fourth as long as the corresponding DNA blot.

Figure 5. RXR transcripts in normal human skin. Blots of 40 μg total RNA per lane (samples 1–4) or approximately 2 μg poly (A)* RNA (samples 5–8) extracted from keratome biopsies are shown. Volunteers were treated topically with RA vehicle (lanes 1 and 3) or 0.1% RA cream (lanes 2 and 4) for 4 d prior to biopsy, or received no treatment (lanes 5–8). Hybridization probes used are indicated at the top of the figure. Mobilities of the 5 kb RXR and 3.3 kb RAR-γ transcripts are indicated to the right, and mobilities of 28S and 18S ribosomal RNA are indicated to the left.

Comparison of CRABP-II, RAR-γ, and RXR mRNA Levels in Psoriasis Figure 6 compares the expression of CRABP-II, RAR-γ, and cyclophilin in keratome biopsies of normal and involved psoriatic epidermis. Note that CRABP-II transcripts are markedly overexpressed in psoriatic lesions relative to normal skin, whereas RAR-γ transcripts are expressed at the same relative levels as the reference gene, cyclophilin. In other experiments we have also shown that RXR transcripts are expressed at the same levels in

Figure 6. Comparison of CRABP-II and RAR-γ mRNA levels in normal versus psoriatic human skin. Forty micrograms total RNA derived from unpaired keratome biopsies of normal or lesional psoriatic epidermis from three different individuals each are shown. Hybridization probes used are indicated to the right. For cyclophilin (lower panel), only the relevant hybridizing bands are shown.
normal and psoriatic epidermis (data not shown). Moreover, the RAR-γ1 probe yielded a hybridization pattern identical to that obtained using the RAR-γ 3′-terminal NarI-EcoRI probe, whereas RAR-γ2 transcripts were undetectable upon rehybridization of the same blot (data not shown).

**DISCUSSION**

In a recent report [8], we demonstrated a specific 45-kDa–specific RA binding activity in normal adult human epidermis. Very low levels of RAR-α, and high levels of RAR-γ, transcripts were found in this tissue, whereas RAR-β transcripts were undetectable [8]. These results suggest that the bulk of the 45 kDa RA binding activity represents RAR-γ, although it remains possible that the abundance of the translated proteins may differ from those of the cognate RNA. RAR-γ was also the principal form of RAR expressed in cultured dermal and lung fibroblasts, which differed from keratinocytes in that RAR-β was inducible by RA only in fibroblasts [8]. RAR expression was otherwise not affected by treatment with RA or agents that activate protein kinase C and other intracellular signal transduction pathways. In addition, RAR transcript levels were not elevated in psoriatic lesions relative to normal skin [8]. These observations suggested that mechanisms other than regulation of receptor expression might regulate retinoid responsiveness in the skin.

To address this possibility, we have used RNA blot hybridization to assess the steady-state levels of CRABP-I and CRABP-II, RAR-γ isoforms, and RXR in normal, psoriatic, and RA-treated human skin and in keratinocytes cultured under different conditions. Some of these results have been previously published [23]. We have shown that CRABP-II transcripts are rapidly and markedly induced by 0.1% RA treatment of adult human skin [23]. In contrast, RA has little effect on CRABP-I mRNA levels after 4 or 24 h in subcultured, serum-free monolayer cultures of neonatal (Fig. 1) and adult [23] keratinocytes, and marked reduction of CRABP-II transcripts occurs after prolonged (≥ 2 d) exposure to 3 × 10−6 M RA [23]. In order to better understand the different in vivo and in vitro responses of CRABP-II to RA, we made use of a stratifying human skin equivalent (HSE) culture system. HSE cultures consist of a mitotically active, stratifying keratinocyte multilayer overlying a collagen gel contracted by low numbers of dermal fibroblasts [30]. In the HSE system, as in intact skin, CRABP-II transcripts are markedly induced after treatment with low (3 × 10−9 M) or high (3 × 10−6 M) concentrations of RA (Fig 2). Moreover, CRABP-II is induced whether RA is added to the air-exposed surface of the HSE or to the underlying medium. This result is consistent with those of Saurat and co-workers, who demonstrated that CRABP-II is induced to a similar extent in human skin after either systemic [31] or topical [32] RA treatment. There are many possible explanations for the differential RA responsiveness of HSE and subcultured keratinocytes, including exposure to an air-liquid interface, the status of endogenous retinoid stores and metabolic enzymes, the presence of dermal fibroblasts, the extracellular matrix environment, etc. However, as the only cells present in HSE other than keratinocytes are a low number of dermal fibroblasts, the similar RA inducibility of CRABP-II in HSE and in intact skin argues that resident or infiltrating immunocytes, endothelial cells, and circulating blood elements are not required for the induction of CRABP-II by RA in vivo. Although RA is unable to stimulate CRABP-II expression in subcultured keratinocyte cultures, CRABP-II is strongly induced in subcultured neonatal (Fig 1) and adult [23] keratinocytes after exposure to elevated medium calcium concentration [23] and/or growth to confluence ([23], Fig 1). Because these conditions are known to promote expression of a more differentiated keratinocyte phenotype [33,34] as is culture in the stratifying HSE system [30], our results indicate that the differentiation state of keratinocytes may be an important determinant of CRABP-II expression in vivo.

CRABP-I transcripts were not detectable under any of these conditions, strongly suggesting that increased expression of CRABP-II, and not CRABP-I, accounts for the previously reported induction of CRABP upon differentiation of keratinocytes in vitro [33].

Another major difference between traditional submersed keratinocyte culture systems and HSE is the presence of living dermal fibroblasts. Fibroblasts are unlikely to be the source of the CRABP-II induced by RA in HSE, because dermal cells comprise only a very small fraction of the cells in this system. However, it remains to be determined whether dermal cells could play an important role in the induction of CRABP-II in vivo and in the HSE system. For example, the fact that normal human dermis strongly expresses CRABP-II. Although the cellular source of these dermal transcripts is currently unknown, we have shown that CRABP-II is markedly induced by RA in fibroblasts [23]. Thus, the expression of this transcript in human dermis suggests that it is exposed to and responsive to RA in vivo.

Altered expression of RAR isoforms and the low-affinity RXR receptor are two other possible mechanisms by which cellular RA responsiveness might be regulated. Making use of isofrom-specific probes, we found that RAR-γ1 is the predominant isofrom expressed in human skin, although RAR-γ2 is present (Fig 4). Very similar results have been reported in mouse skin [24]. We found no evidence for differential expression of RAR γ1 and γ2 isoforms as a function of RA treatment in vivo (Fig 4).

Using a probe generated by PCR amplification of cDNA prepared from human skin, we demonstrate expression of RXR transcripts in normal, vehicle-treated, and RA-treated human skin (Fig 5). As was the case for the RAR-γ isoforms, we found no evidence for differential expression of RXR as a function of RA treatment. Nevertheless, the evolutionary conservation of RAR-γ isoforms and RXR as well as their presence in skin [7,10,24] suggests that genes that respond specifically to these receptors in the skin will be identified.

Psoriasis is characterized by profound alterations in proliferation and differentiation [11,12]. If RA plays an important role in these processes through actions on nuclear receptors, comparison of normal and psoriatic epidermis offers an opportunity to identify potential modifiers of RA action in human skin. Therefore, we compared the expression of CRABP-II, RAR-γ, and the reference gene, cyclophilin, in normal and psoriatic human epidermis. As shown in Fig 6, CRABP-II was markedly overexpressed in psoriatic compared to normal epidermis, whereas CRABP-I transcripts were undetectable. These results indicate that the increased CRABP protein previously observed in psoriatic lesions [31] is likely to be CRABP-II. Whereas a modest overexpression of RAR-γ transcripts appears to be present in psoriatic lesions (Fig 6, middle panel), similar results were obtained for the reference gene, cyclophilin (Fig 6, lower panel). As reported previously [27], small differences in the expression of multiple reference genes have been observed in comparison of normal and psoriatic epidermis and cannot be considered to be specifically relevant to the psoriatic state.

Taken together, our results indicate that cutaneous expression of CRABP-II is highly regulated by RA treatment and in psoriasis, whereas expression of RAR-γ isoforms and RXR appears to be constitutive. However, the biologic significance of increased CRABP-II expression in these settings is presently unclear. Two theories regarding the biologic role of CRABP have been proposed. The “shuttle theory” suggests that CRABP is involved in the transport of RA from the cytosol to nuclear receptors, resulting in enhancement of RA action [35–37]. The “sequestration theory” suggests that CRABP functions to limit the interaction of RA with nuclear receptors [16,38]. If the basic function of CRABP-II is to limit RA action, then genes that are normally suppressed by RA might be precociously expressed in RA-treated and psoriatic epidermis, because CRABP-II is overexpressed in both conditions. Although many alternative theories could be advanced, one speculative model based on the sequestration of RA by CRABP-II is discussed below and shown in Fig 7.

RA suppresses the expression of filaggrin [39], epidermal transglutaminase (ETGase) [40], loricrin [41], and cholesterol sulfotransferase [42] in submersed keratinocyte cultures. However, like
Figure 7. Proposed model for regulation of RA action by CRABP-II in human skin. Width of the triangles depict content of the components given at the top of the figure as a function of epidermal stratification. Chol SO₄ indicates cholesterol sulfate.

CRABP-II, several of these genes are precociously expressed, rather than suppressed, in RA-treated and lesional psoriatic skin. In RA-treated skin, these include ETGase [43] as well as involucrin and filaggrin (D. Rosenthal, C. Griffiths, and J. Vorhees, manuscript submitted). In psoriatic lesions, these include involucrin, ETGase [12,44 - 47], and to a lesser extent, filaggrin [44]. Under normal conditions, CRABP-II is envisioned to accumulate in the cytoplasm as keratinocytes differentiate, perhaps as a function of calcium concentration [33,34]. If CRABP-II acts to limit the nuclear action of RA, either by enhancing its catabolism [48] or by sequestering it in the cytoplasm, accumulation of CRABP would progressively reduce the amount of “free” RA (that is, RA available to activate nuclear receptors). As a result, genes whose expression is normally repressed by RA (e.g., loricrin, filaggrin, transtilamin, etc.) would become derepressed. In psoriatic and RA-treated skin, increased and/or premature expression of CRABP-II would limit access of RA to nuclear receptors, allowing precocious expression of these genes.

Clearly, this model represents only one of many possible scenarios by which the expression of epidermal differentiation markers might be controlled, and is likely to be incomplete in many respects. For instance, it cannot explain the precocious expression of involucrin in psoriasis [12,30,34,46] and in RA-treated skin (D. Rosenthal, C. Griffiths, and J. Vorhees, manuscript submitted), as the expression of involucrin is not suppressed by RA in vitro [46]. Moreover, in RA-treated skin, it is necessary to postulate that a state of “free” intracellular RA deficiency could exist even in the presence of large amounts of exogenous RA. Finally, demonstration that the RNA transcript levels presented in this report accurately reflect the abundance of the cognate proteins (RAR-γ isoforms, RXR, and CRABP) will require development of specific high-afﬁnity antibodies and/or biochemical assays for the proteins involved. In particular, speciﬁc anti-CRABP-II antibodies will allow testing of the model shown in Fig 7. In any case, analysis of the regulatory regions of putative RA target genes for the presence and function of regulatory elements for serum, cAMP, phorbol esters, and other transcriptional regulators in addition to RA should provide important insights into the control of epidermal differentiation.

Note Added in Proof: While this manuscript was under review, Didierjean et al reported overexpression of CRABP-II transcripts in nonlesional and lesional psoriatic skin by in situ hybridization (Biochem Biophys Res Commun 180:204 – 208, 1991).

REFERENCES


