Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 545-552

NUCLEAR ORPHAN RECEPTOR-BINDING RETINOIC ACID RESPONSE ELEMENTS IN KERATINOCYTES

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Received July 13, 1994

Summary: Keratinocytes are responsive cells for retinoic acid (RA) mediated signal transduction. In this study, we demonstrate binding of some important orphan receptors to previously identified retinoic acid response elements (RAREs) regulated by RA in keratinocytes. Using electrophoretic mobility shift assays (EMSAs) we show that in vitro translated ARP1 (apolipoprotein AI regulatory protein1), EAR3 and EAR2 (v-erb A related genes) bind two RAREs. The RAREs investigated were previously identified in the VL30 retrotransposon (termed VLRRE2) and retinoic acid receptorβ2 (termed RAREβ) genes, respectively. Furthermore, using an anti-ARP antibody that recognizes both ARP1 and EAR3 we show that these ARP subfamily member(s) present in keratinocytes bind to both RAREs. To our knowledge, this is the first demonstration of the binding of these proteins in keratinocytes to response elements regulated by RA in these cells. Our data suggest that ARP subfamily member(s) may modulate RA mediated transcription in epidermis.

Retinoids have important biological functions in growth, differentiation, development and tumorigenesis (1-4). Epidermis constitutes one of the major target tissues where both deficiency and excess of vitamin A produce pathological changes (2). Differentiation of cultured keratinocytes is suppressed by all-trans-retinoic acid (RA) inhibiting differentiation-specific markers such as keratins 1 and 10 (1), filaggrin (5) and epidermal type I transglutaminase (6).

We have was previously demonstrated that a member of the retrotransposon family (VL30), which is expressed in mouse epidermis (7), is induced by RA in cultured mouse keratinocytes through two retinoic acid response elements (RAREs termed VLRRE1 and VLRRE2). The VLRREs bound recombinant vaccinia virus expressed retinoic acid receptor (RARs either α, β or γ) and retinoid X receptor (RXRα)

0006-291X/94 $5.00

heterodimers (8). In addition, the RAREβ present in the RARβ2 gene (9) mediates RA induction in these cells when coupled to a heterologous promoter (8).

Interestingly, the VLRREs showed significant homology to the RARE identified in the site A of the apolipoprotein AI gene (apoAIRARE) (10). ARP1 (apolipoprotein AI regulatory protein1) a novel member of the orphan receptor family binds to site A present in the apoAI gene and modulates the transcription of the apoAI gene (11). Two other orphan receptors identified as homologs of the v-erbA gene products (EAR3 and EAR2) (12) show 98.5% and 89.4% homology in their DNA binding domain to ARP1, respectively. These proteins appear to belong to the ARP subfamily of nuclear receptors (11). Moreover, EAR3 has been shown to be identical to the COUP transcription factor (COUP-TF) which interacts with the chicken ovalbumin upstream promoter and regulates its transcription (13).

To assess the potential role of these nuclear orphan receptors in RA regulated transcription in epidermis, it is essential to study their binding to RAREs regulated by RA in keratinocytes. In this study, we have investigated the binding of ARP1, EAR3 and EAR2 to VLRRE2 and RAREβ both in vitro and in crude keratinocytes nuclear extracts employing electrophoretic mobility shift assay (EMSA). We show that both VLRRE2 and RAREβ constitute targets for in vitro translated ARP1, EAR3 and EAR2. In addition, an anti-ARP antibody recognizes endogenous VLRRE2 and RAREβ-binding activity present in crude nuclear keratinocyte extracts. These data provide evidence that member(s) within the ARP subfamily of nuclear orphan receptors may regulate RA mediated transcription in epidermis.

MATERIALS AND METHODS

Cell culture conditions

All chemicals, media and growth factors were purchased from Sigma, unless stated otherwise. Balb/MK cells were grown in MCDB153 medium supplemented with 50 μM $CaCl_2$, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5% Chelex-treated fetal calf serum (FCS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml fungizone.

Expression vectors

Expression vectors used for in vitro translation of ARP1, EAR3 and EAR2 were kindly provided by Dr. John A. A. Ladias (ARP1 and EAR3) and Dr. Tadashi Yamamoto (EAR2). The ARP1, EAR3 and EAR2 proteins were synthesized in vitro using TNT rabbit reticulocyte lysate kit (Promega) according to the description of the supplier.

Antibodies used in EMSAs

The anti-ARP antibody was kindly provided by Dr. Sotirios K. Karathanasis. The anti-EAR2 antibody was a genorous gift from Dr. Ronald M. Evans and Dr. Thomas Perlmann.

Preparation of nuclear extracts and EMSAs

Complementary oligonucleotides were synthesized on an Applied Biosystems oligonucleotides synthesizer (Foster City, Ca). The sequences of these are shown in Table I (VLRRE2 and RAREβ) except the oligonucleotide used as nonspecific competitor which is a PEA3 binding sequence (TTAAGCAGGAAGTGACC) present in the polyoma virus enhancer (14). The corresponding double-stranded oligonucleotides used as probes were labeled with [α-^{32}P] dATP by klenow polymerase. Balb/MK cells were grown to confluence and then extracts were prepared in the presence of leupeptin 10μg/ml, pepstatin 5μM (Boehringer) and aprotinin 100 KIE/ml (BAYER Inc., Germany) as described previously (15). BALB/MK extracts or in vitro rabbit reticulocyte lysate translated proteins were incubated in a binding buffer containing 5% glycerol, 10 mM Hepes, 100 mM KCl, 1.5 units/ml aprotinin, 1 μg poly(dI.dC) and O.5 μg poly (dA.dT) (Pharmacia). Antibodies were added to the binding reaction 30 minutes prior to addition of the probe wherever indicated. The protein-DNA complexes formed during the band shift reactions were separated on precooled, pre-electrophoresed (2h, 200V) 5% polyacrylamide gels (29:1) with 0.5 X TBE (1 X TBE: 90 mM Tris borate and 1mM EDTA) as the running buffer at 4°C at 200V.

RESULTS

ARP1, EAR3 and EAR2 bind VLRRE2 and RAREβ

Table I shows a sequence alignment of the two RAREs both of which are functional in keratinocytes (VLRRE2 and RAREβ) and sequence motifs known to bind members within the ARP subfamily of nuclear receptors

Table I

Sequence comparison of VLRRE2 and RAREβ to two closely related binding sequences

RAREβ	TGAACTttcggTGAACC
VLRRE2	TAAACTttTCACCC
ApoAIRARE	TGAACCctTGACCC
cOV	TGACCttTGACA

RARE identified in the VL30 gene termed VLRRE2 (8) and RAREβ identified in the RARβ2 promoter (9) are aligned to closely related apolipoprotein AI (apoAIRARE) (10) and the chicken ovalbumin (cOV) sequence (13). The half site sequences are represented in capital letters and the nucleotide space sequences in small letters.

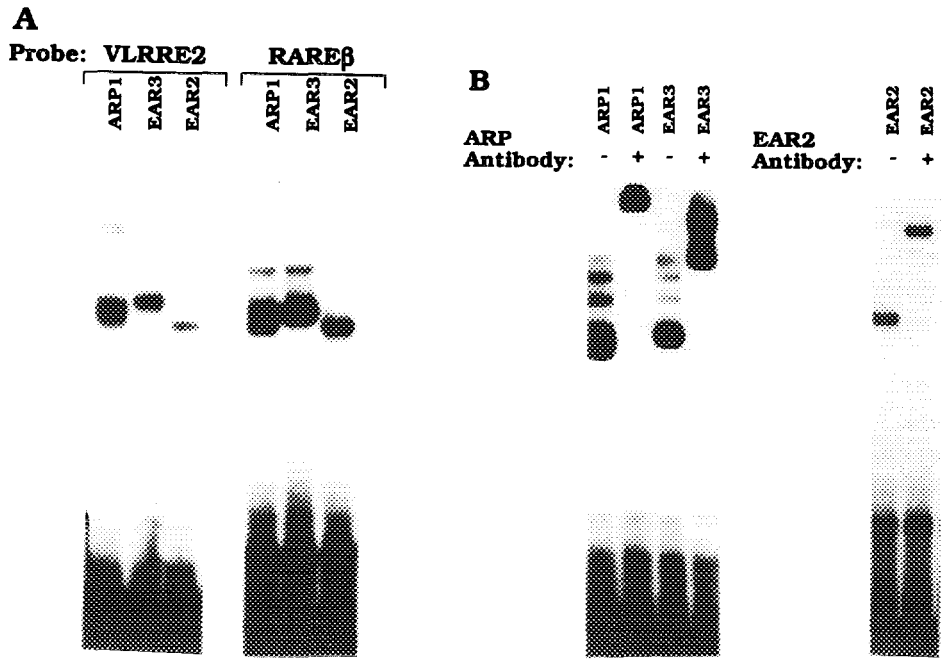


Figure 1. In vitro rabbit reticulocyte lysate-translated ARPI, EAR3 and EAR2 bind VLRRE2 and RAREβ. A, autoradiograph of a gel shift experiment where [^{32}P] labeled VLRRE2 and RAREβ were incubated with in vitro translated ARPI, EAR3 and EAR2 as shown. B, autoradiograph of a gel shift experiment where anti-ARP antibody (1:2 dilution) was added to in vitro translated ARP1 and EAR3 while anti-EAR2 antibody (1:5 dilution) was added to in vitro translated EAR2 as shown. The minus sign (-) indicates that no antibody was added in that reaction. The reaction was incubated with [^{32}P] labeled RAREβ as probe. Specific recognition by the antibody is indicated by the supershift.

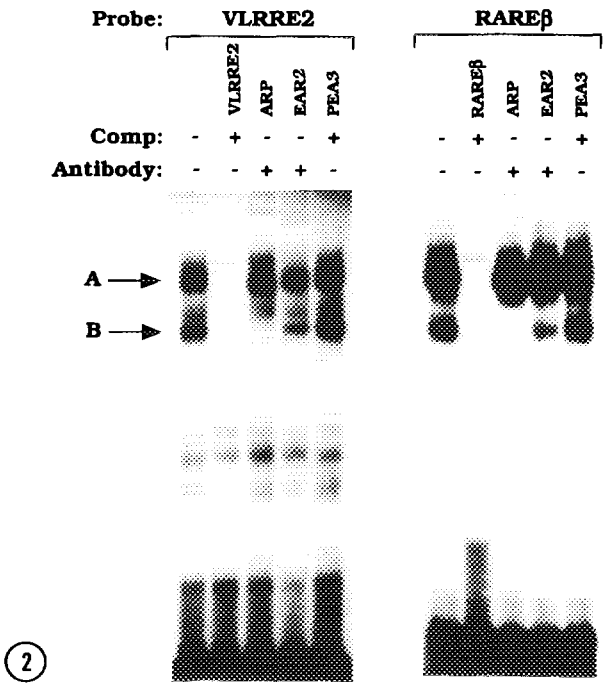
(apoAIRARE and chicken ovalbumin (cOV) sequence). The similarities observed between these sequences prompted us to investigate the possibility that VLRRE2 and RAREβ could bind nuclear orphan receptors within the ARP subfamily. We therefore performed EMSAs using in vitro reticulocyte lysate-translated ARP1, EAR3 and EAR2 and oligonucleotides corresponding to VLRRE2 and RAREβ as probes. As shown in Fig. 1A, protein-DNA complexes were obtained using ARP1, EAR3 and EAR2 indicating their ability to bind both VLRRE2 and RAREβ. The ARP1 and EAR3 complexes were supershifted when an anti-ARP antibody was included in the binding reaction suggesting that the anti-ARP antibody recognized both proteins. In addition, The EAR2 complex was supershifted when an anti-EAR2 antibody (Fig.1B) was included in the binding reaction indicating that the antibody recognized EAR2. Furthermore, the anti-ARP antibody did not recognize in vitro translated

EAR2 while the anti-EAR2 antibody did not recognize in vitro translated ARP1 or EAR3 (data not shown).

In keratinocytes, ARP subfamily members bind VLRRE2 and RAREβ

To determine if ARP subfamily member(s) were present among keratinocyte nuclear factors with the ability to bind VLRRE2 and/or RAREβ, EMSAs were performed with nuclear extracts prepared from cultured keratinocytes. Using crude keratinocyte nuclear extracts two complexes, A and B were formed with VLRRE2 as well as RAREβ as shown in Fig. 2. Both complexes formed were specific as judged by the ability of unlabeled VLRRE2 or RAREβ to compete for binding, whereas an unrelated PEA3 oligonucleotide used in the same molar excess (100-fold) did not compete for binding. Additionally, Fig. 2 shows that the formation of complex B was completely inhibited by including the anti-ARP antibody in the binding reaction regardless if VLRRE2 or RAREβ was used as probes. This suggested that either ARP1 and/or EAR3 was present in complex B since this antibody recognized both ARP1 and EAR3 in vitro (Fig. 1B). EAR2 shares significant sequence homology in its DNA binding domain with ARP1 (33) and bound both these RAREs in vitro as shown in Fig. 1A. Interestingly, including an EAR2 specific antibody caused a decrease in the intensity of the complex B indicating that EAR2 is also present in complex B. We have noted that both anti-ARP and anti-EAR2 antibody supershifted the respective complexes in vitro (Fig. 1B). These antibodies did not form supershifts in keratinocyte extracts, but inhibited the formation of complex B (Fig. 2). This may be due to modification of the protein(s) present in crude keratinocyte nuclear extracts. The formation of complex A remains at present unknown as it was not significantly affected by any of the antibodies tested. The formation of complex A and B were similar using keratinocyte nuclear extracts prepared 1 hour after treatment with RA (data not shown).

Next, we wanted to analyze if the complex formed with in vitro translated ARP1 had a similar migrating pattern as complex B formed in crude keratinocyte nuclear extracts and that both proteins were supershifted by the anti-ARP antibody when mixed together. We then perfomed an EMSA where in vitro translated ARP1 and keratinocyte extracts were run in parallel or mixed together as shown in Fig. 3. The in vitro translated ARP1 comigrated with complex B and when both ARP1 and crude nuclear keratinocyte extracts were mixed and incubated with anti-ARP antibody, both complex B and the in vitro translated ARP protein were supershifted. This result indicated that complex B contained protein(s) similar to ARP1.



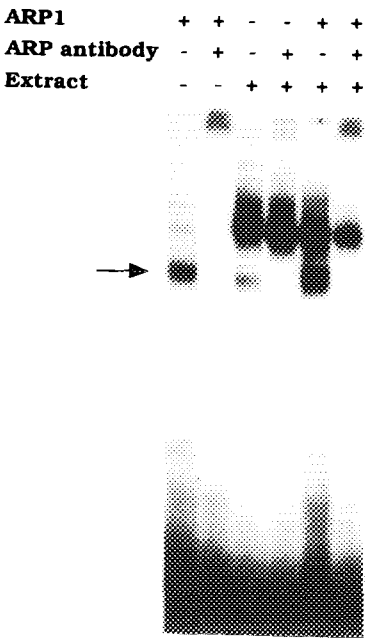


Figure 2. Identification of nuclear orphan receptors belonging to the ARP subfamily that bind VLRRE2 and RAREβ in keratinocytes. Keratinocyte nuclear extracts prepared from BALB/MK cells (2.5 μg) were incubated with [^{32}P] labeled VLRRE2 and RAREβ as probes. The arrows, A and B, represent specific protein-DNA complexes that are discussed in the text. The minus sign (-) indicates that no competitor or antibody was added in that incubation. A 100-fold molar excess of unlabeled oligonucleotides corresponding to VLRRE2, RAREβ and an unrelated PEA3 were used as competitors where indicated by the plus sign (+). Anti-ARP (1:2 dilution) and anti-EAR2 antibody (1:5 dilution) were added to the binding reaction wherever indicated by the plus sign (+).

Figure 3. Anti-ARP antibody recognizes a complex formed in keratinocytes that comigrates with in vitro translated ARP1. In vitro translated ARP1 and keratinocyte extracts (2.5 μg) prepared from BALB/MK cells were incubated with [^{32}P] labeled RAREβ probe as shown. The anti-ARP antibody (1:2 dilution) was added as indicated. The minus sign (-) indicates that no Balb/MK extract, in vitro translated ARP1 or anti-ARP antibody was added in that incubation. The arrow represents specific protein-DNA complexes that are discussed in the text. Specific recognition by the antibody is demonstrated by the supershift.

DISCUSSION

An important step to the understanding of how RA regulates gene transcription in the target keratinocyte is definition of functional RAREs and the identification of proteins that interact with them in keratinocytes. In this article we report that two RAREs, VLRRE2 and

RAREβ which we have previously shown to be functional in keratinocytes (8) bind members within the ARP subfamily of nuclear orphan receptors both in vitro and in crude keratinocyte nuclear extracts.

VLRRE2 and RAREβ exhibit significant sequence similarities with ApoAIRARE and the cOV sequence (Table I). These sequences bind ARP1 and COUP-TF/EAR3 respectively (11, 13). In addition it has been shown that EAR3 can bind multiple directly repeated elements spaced by 0-5 nucleotides (16). Our results showing that in vitro-translated ARP1, EAR3 and EAR2 were able to bind both RAREs (VLRRE2 and RAREβ) spaced by 2 and 5 nucleotides, respectively, are consistent with these reports.

The finding that ARP subfamily member(s) are present in the endogenous keratinocyte protein-DNA complex (complex B) for both RAREs investigated implicates an important role for this family of nuclear orphan receptors in the regulation of gene expression by RA in epidermal cells. The protein(s) present in complex B could be ARP1 and/or EAR3 since the antibody used recognizes both proteins in vitro. Additionally, we show that EAR2 is also present in complex B. EAR2 may be present as a heterodimer with ARP1 or EAR3 in the complex B. These orphan receptors are able to heterodimerize among themselves in vitro (personal communication, Dr. Sotirios K. Karathanasis). It is tempting to speculate that complex regulation involving intracellular levels of these ARP subfamily members may exist in keratinocytes and control responsiveness to RA. Regulation by these receptors has been well studied in other genes like apoAI, apoAII, apoB and apoCIII in liver and intestinal cells where ARP1, EAR3 and EAR2 function as transcriptional repressors (11, 17). It was also shown that the ARP1 repression of the apoAI promoter was overcome by RXRα in the presence of RA (18). Under our assay conditions we were unable to detect the presence of RARs or RXR in the complexes formed using keratinocyte nuclear extracts (data not shown), which may be due to the masking of these proteins by other factors present in the crude keratinocyte extracts.

In addition, several studies have reported both positive and negative regulation by COUP-TF/EAR3 on different promoter constucts. It was shown to activate transcription from the cOV gene in vitro (13). On the other hand by binding to different GGTCA response elements COUP-TF/EAR3 was able to repress hormonal induction by vitamin D receptor, thyroid hormone receptor and RAR (19). Functional role of the binding of ARP1, EAR3 or EAR2 to RAREs regulated by RA in keratinocytes is presently being investigated.

In conclusion, we have shown that ARP subfamily members bind RAREs, VLRRE2 and RAREβ both in vitro and in crude keratinocyte nuclear

extracts. These observations raise the possibility that this class of orphan receptors may be involved in RA signaling in epidermal cells.

ACKNOWLEDGMENTS

We acknowledge Dr. Staffan Bohm and Anders Berkenstam for helpful discussions. We thank Dr. Sotirios K. Karathanasis, Dr. John A. A. Ladias, Dr. Ronald M. Evans, Dr. Thomas Perlmann, and Dr. Tadashi Yamamoto for their generous gifts of antibodies and cDNAs. This work was supported by the Swedish Cancer Research Society (Project 3161-B93-03XAC).

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