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Endogenous Retinoic Acid Receptor (RAR)-Retinoid X Receptor (RXR) Heterodimers Are the Major Functional Forms Regulating Retinoid-responsive Elements in Adult Human Keratinocytes

BINDING OF LIGANDS TO RAR ONLY IS SUFFICIENT FOR RAR·RXR HETERODIMERS TO CONFER LIGAND-DEPENDENT ACTIVATION OF hRARβ2/RARE (DR5)*

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We have examined how retinoic acid receptors (RARs) and retinoid X receptors (RXRs) at physiological concentrations regulate distinct retinoid-responsive elements, hRARβ2/βRARE (DR5) and rCRBPII/RXRE (DR1), in keratinocytes from human skin, a major retinoid target. *In vitro*, endogenous RARγ and RXRs bound to these elements as heterodimers (RAR·RXR) but not homodimers (RAR·RAR or RXR·RXR). In cultured keratinocytes, all-*trans* retinoic acid, 9-*cis* retinoic acid, and CD367 activated βRARE but not RXRE via endogenous RAR·RXR (ED_{50} = 2.3, 3.8, and 0.3 nM, respectively) whereas SR11237 showed no significant effect. All-*trans* retinoic acid, 9-*cis* retinoic acid, and SR11237 activated RXRE via overexpressed RXR·RXR (ED_{50} = 110, 120, and 11 nM, respectively), indicating interconversion between retinoic acid isomers, whereas co-overexpression of RARα or RARγ suppressed this activation. Unlike 9cRA, CD367 neither induced formation of nor activated RXR·RXR. Overexpression of RAR or RXR mutated in transactivation domain AF-2 suppressed endogenous receptor activity over βRARE. Our data suggest that 1) in keratinocytes, RAR·RXR-mediated pathway dominates over that mediated by RXR·RXR; 2) RAR-selective CD367 and RXR-selective SR11237 can be used to identify these two distinct pathways, respectively; 3) βRARE is mainly regulated by RAR·RXR, in which RAR alone confers ligand inducibility whereas AF-2 of unliganded RXR is required for transactivation by liganded RAR AF-2; 4) lack of RXRE activity in keratinocytes is due to low endogenous levels of RXR·RXR and inhibition by RAR·RXR; and 5) interaction among RXRs is much lower than that between RAR and RXR.

Retinoic acid (RA)[1] is an important regulator of normal epidermal cell homeostasis (1). For many years, RA and synthetic retinoids have been used to treat cystic acne, psoriasis, and certain epithelial malignancies. Recent studies in mice and humans have demonstrated that topical RA improves the wrinkled appearance of sun-damaged skin (2–4), as well as post-inflammatory hyperpigmentation (5). *In vitro*, RA inhibits differentiation of keratinocytes (KCs) (6, 7). The biological effects produced by RA are believed to be mediated all or in part by two families of nuclear receptors, retinoic acid (RARs) (8–11) and retinoid X (RXRs) receptors (12–14).

RARs and RXRs are ligand-dependent transcription factors that bind to *cis*-acting DNA sequences called RAREs, which are composed of directly repeated hexameric half-sites with consensus sequences (5′-PuG(G/T)TCA-3′), within the transcriptional regulatory regions of target genes and thereby regulate the rate of transcription of these genes (for review see Refs. 15 and 16 and references therein). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily, each of which consists of three major functional domains, an N-terminal transactivation domain (AF-1), a central DNA binding/dimerization domain, and a C-terminal multi-functional domain, which is involved in ligand binding, dimerization, and transactivation function (AF-2) (13, 17–26). The RAR family is composed of RARα, β, and γ and their isoforms (27–29), which recognize two natural stereoisomers of RA, all-*trans* RA (tRA) and *9-cis* RA (9cRA) (8, 30, 31). The RXR family consists of three members, α, β and γ (13, 14), which are activated exclusively by 9cRA (30, 31). Recently, synthetic retinoids such as SR11237 and SR11217 have also been identified as RXR-selective ligands (32).

In cell-free systems, RARs form heterodimers with RXRs (13, 33, 34). On the other hand, RXRs are able to form not only homodimers but also heterodimers with other members of the steroid/thyroid hormone receptor superfamily including peroxisome proliferator-activated receptor, thyroid hormone receptor, vitamin D receptor, *apo*AI regulatory protein, and chicken ovalbumin upstream transcription factor (15, 18, 19, 35–38). Interestingly, RAREs with different spacing between half-sites have been found to be selectively activated by heterodimers and/or homodimers formed among overexpressed RARs and RXRs. For example, *in vitro*, βRARE present in both human and mouse RARβ2 gene promoters (39, 40), which is a DR5 core motif composed of two half-sites separated by 5 base pairs,

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[1] The abbreviations used are: RA, retinoic acid; 9cRA, 9-*cis* retinoic acid; Ab, antibody; CAT, chloramphenicol acetyltransferase; DR1, directly repeated consensus hexameric half-sites separated by one base pair; DR5, directly repeated consensus hexameric half-sites separated by five base pairs; hRARβ2, human retinoic acid β2; KC, keratinocyte; KGM, keratinocyte growth medium; m, mutant; rCRBPII, rat cellular retinol binding protein II; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; RXRE, retinoid X response element; tk, thymidine kinase; tRA, all-*trans* retinoic acid; wt, wild type; HPLC, high pressure liquid chromatography.

binds RAR·RXR heterodimers efficiently in a ligand-independent manner (13, 33, 34, 41) and *in vitro* translation-derived and 9cRA-activated RXR homodimers to a certain degree (35). In contrast, retinoid X response element (RXRE) in the rCRBPII gene promoter (42), which is a DR1 core motif consisting of five consensus half-sites spaced from each other by one nucleotide, interacts efficiently with not only RAR·RXR heterodimers in a ligand-independent way (35, 38) but also *in vitro* translation-derived and 9cRA-activated RXR homodimers (26, 35). A synthetic reporter gene tk-CAT containing βRARE was activated by overexpressed RARs and/or RXRs in various cell lines (34, 41), whereas the same reporter gene containing RXRE was transactivated by only RXRs but not RARs in certain cell types (34, 38, 42, 43).

However, in living tissues, endogenous levels of the RAR and RXR members and their isoforms are tightly controlled (14, 27–29). Recently, Dollé *et al.* (44–47) and others (14, 48, 49) have shown that developing mouse embryos display distinct spatio-temporal expression patterns for the transcripts of each RAR and RXR member. The transactivation properties and differential expression pattern of these receptors suggest that the pleiotropic effects of retinoids may be in part due to combinatorial dimerization of various RAR and RXR members whose expression is ultimately controlled in a cell type-specific way (50). In addition, receptor activity may be further regulated by cell-specific levels of natural retinoids such as tRA and 9cRA, which have been shown recently to be interconverted in mammalian cell lines (30, 31, 51).

In adult human epidermis and cultured KCs, mRNAs for RARα and γ and RXRα and β were found, with RARγ and RXRα being the predominant species (14, 52–55). To date, very little is known about the DNA binding and transactivation properties of these endogenous RAR and RXR proteins in adult human epidermal KCs, although induction of βRARE activity by retinoids was observed in human foreskin KCs (56) and transformed epidermal KCs (57). In this study, we took advantage of the special features of two distinct RAREs mentioned above, βRARE (DR5) and RXRE (DR1), to determine how at physiological concentrations RARs and RXRs act to regulate transcription in cultured normal KCs from adult human skin in response to various natural and synthetic retinoids.

EXPERIMENTAL PROCEDURES

Ligands—All-*trans* retinoic acid was purchased from Sigma. 9-*cis* retinoic acid was generously provided by P. F. Sorter, J. F. Grippo, and A. A. Levin (Hoffmann-La Roche, Nutley, NJ) (30). CD367 was kindly given by Dr. B. Shroot (CIRD, Sophia Antipolis, Valbonne, France) (58). SR11237 (81104-BASF) was provided by B. Janssen (BASF-Aktiengesellschaft, D6700, Ludwigshafen, Germany). SR11237 was originally prepared by M. I. Dawson (32).

Reporter Gene Plasmids, Expression Vectors for RARα and γ, RXRα, and Dominant Negative RARα and RXRα Mutants—Reporter genes, βRARE-tk-CAT and RXRE-tk-CAT, were constructed by inserting double-stranded oligodeoxyribonucleotides, 5′-*tcgac*taAG**GGGTTCA**C-CGAA**AGTTCA**CTCGCA-3′ (consensus hexameric half-sites shown in bold capital letters, random nucleotides in lower case letters, and cloning sites in italics) containing βRARE (DR5) from hRARβ2 gene promoter (39) and 5′-*tcga***CTGTCACAGGTCACAGGTCACAGGTCA**C-**AGTTCA**-3′ containing RXRE(DR1) from the rat CRBPII gene promoter (42), respectively, into the polycloning sites located upstream of synthetic reporter gene tk-CAT in pBS-tk-CAT. To construct pBS-tk-CAT, a *Eco*O190I-*Nde*I fragment (379 base pairs) containing a putative AP-1 binding site (59) was removed from plasmid pBS(−) (Stratagene) by digestion with appropriate restriction enzymes, and the plasmid was recircularized using T4 DNA ligase after the *Eco*O190I and *Nde*I sites were filled in with nucleotides by DNA polymerase I Klenow fragment according to Sambrook *et al.* (60), resulting in plasmid pBS-1. Then, the *Hin*dIII-*Eco*RI fragment (1.8 kilobase pairs), which contains a minimal promoter from the herpes simplex virus tk gene linked to the bacterial CAT gene, was isolated from pBLCAT8+ (61) and inserted into pBS-1 between sites *Hin*dIII and *Eco*RI to give pBS-tk-CAT. Reporter gene, $\beta RARE_3$-tk-CAT, contains three head-to-tail copies of βRARE located upstream of tk-CAT in pBLCAT8+. Expression vectors used for expressing RARα, RARγ, and RXRα are pSG5-hRARα1, pSG5-hRARγ1, and pSG5-mRXRα, respectively. Expression vectors for the RAR and RXR dominant negative mutants, which contain limited deletions of the AF-2 domain at the C terminus (Δ(412–462) for RAR and Δ(449–467) for RXR), are pSG5-mRARαdn and pSG5-mRXRαdn (62), respectively, and are referred to as dnRAR and dnRXR, respectively, thereafter.

Human Skin Epidermal Biopsies—Keratome biopsies were obtained from buttock skin of healthy adult human volunteers as described (63). Blade depth was set as 0.2 mm to cut near the epidermis-dermis junction. Thus, biopsies contained primarily epidermis with residual amounts of dermis. Typically, cells in the epidermal biopsies are composed of 95% KCs and 5% other cell types, which are primarily epidermal melanocytes and Langerhans cells. In addition, biopsies contain a few endothelial cells, fibroblasts, and dendritic cells. Therefore, the data regarding human epidermis in this study predominantly pertain to retinoid receptors in epidermal KCs. Biopsies taken were immediately kept in ice-cold Hank's balanced salt solution (Sigma) prior to preparation of nuclear extracts or KC culture. All procedures involved in handling human subjects received prior approval by the University of Michigan Institutional Review Board, and each individual provided written consent.

Keratinocyte Culture—Primary cultures of normal human KCs were prepared as described (64). Subcultures were expanded in a defined serum-free medium, KGM (Clonetics, San Diego, CA).

Preparation of Nuclear Extracts—Keratome biopsies were treated in Dulbecco's phosphate-buffered saline containing 0.25% (w/v) trypsin, 0.1% (w/v) EDTA at 37 °C for 30 min, and trypsinization was stopped by adding fetal bovine serum (Life Technologies, Inc.). KCs were released from tissues by scraping. For cultured KCs, cells at stage of the third passage were collected in Dulbecco's phosphate-buffered saline by scraping culture dishes with a rubber policeman. Nuclear extracts were prepared as described (65).

Overexpression of RARγ and RXRα in Cultured Keratinocytes—Cultured KCs at the stage of the second passage were seeded into ϕ 100-mm dishes. At 80–90% confluence, 15 dishes of cells were transfected by lipofection with a total of 12.5 μg/dish of expression vector DNA for RARγ and/or RXRα (at 1:1 ratio in the case with two receptors co-transfected). Cells were fed with fresh KGM 18 h after transfection and harvested 48 h thereafter. Nuclear extracts were prepared as described above.

Gel Mobility Shift Assays—Regular and immunological gel mobility shift assays were performed according to Rochette-Egly *et al.* (66) with modifications. Double-stranded oligodeoxyribonucleotides, which contain the wild type (wt) or mutated (m) retinoic acid-responsive elements from the hRARβ2 gene promoter (βRARE) and the rCRBPII gene promoter (RXRE), were labeled using [γ-^{32}P]ATP (6000 Ci/mM, DuPont NEN) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Sequences of these 5′-γ-^{32}P-labeled probes are 5′-tcgactaAG**GGGTTCA**CCGAA**AGTTCA**CTCGCA-3′ for βRAREwt (consensus hexameric half-sites shown in bold capital letters and random nucleotides in small case letters); 5′-tcgactaAG***TT*TTCA**CCGAA**AGTTCA**CTCGCA-3′ for βRAREm1 (mutations underlined); 5′-tcgactaAG***TT*TTCA**CCGAA**AGTTGT***C**TCGCA-3′ for βRAREm2; 5′-tcga**CTGTCACAGGTCACAGGTCACAGGTCACAGTTCA**-3′ for RXRwt; and 5′-tcga**CTGTCACAAGTCACAAGTCACAAGTCACAATTCA**-3′ for RXREm. Mouse monoclonal antibodies used in immunological gel mobility shift assays were 4RX (19), which is directed against the E region of all three RXR subtypes (α, β, and γ), and Ab4γ(hF) (66), which is specific to the F region of RARγ. Amounts equivalent to undiluted antibody fluids used in most cases were 0.8 μl for 4RX and 1.2 μl for Ab4γ(hF). Rabbit polyclonal antibodies RPα(F) and RPβ(F) were used for analyzing RARα and β, respectively. Radioactive DNA·protein complexes were resolved at room temperature on non-denaturing 5% polyacrylamide (acrylamide:bisacrylamide = 30:1 (w/w)) gels containing 5% glycerol, 22.5 mM Tris borate, and 0.5 mM EDTA and then visualized by autoradiography at −70 °C using Hyperfilm-MP films (Amersham Corp.) with two intensifying screens. For quantifying these complexes, PhosphorImager was used (Molecular Dynamics).

Transfection of Keratinocytes and CAT Assay—KCs at stage of the second passage were passed and seeded into ϕ 60-mm dishes. When cell density reached about 80% confluence, 2 or 3 μg of reporter gene plasmids and/or 400 ng of expression vectors for receptors were introduced into the cells by lipofection. Lipofection was performed using either Lipofectin or LipofectAce as transfection reagents according the manufacturer (Life Technologies, Inc.). Each experiment was repeated with cells prepared from a different individual, which represents a

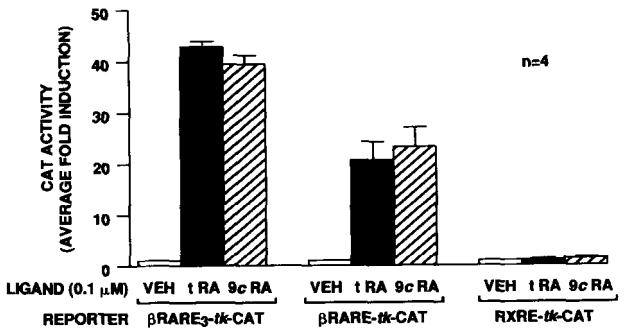

FIG. 1. **Ligand-dependent transactivation of retinoid-responsive reporter genes, βRARE$_3$-tk-CAT and βRARE-tk-CAT, but not RXRE-tk-CAT, by endogenous retinoid receptors in KCs.** The *y* axis represents relative CAT activity, which is the average value from four independent experiments (n = 4), and the *x* axis shows types of ligands used to treat cells and reporter genes transfected. Standard errors are shown as *vertical bars*. Cultured KCs were transfected with reporter gene DNA, 3 μg for βRARE$_3$-tk-CAT and 2 μg for βRARE-tk-CAT and RXRE-tk-CAT. After transfection, cells were treated with vehicle (0.1% ethanol) or 0.1 μM tRA or 0.1 μM 9cRA for 48 h.

sample number (n) of one. 18 h after transfection, cells were treated with KGM containing appropriate ligands for 48 h before harvesting. Prior to CAT measurement, variations in transfection efficiencies were normalized based on β-galactosidase activity generated from a co-transfected constitutive expression vector (2 μg) for bacterial *LacZ* gene, pXJ40-*LacZ*, as described (67).

RESULTS

Activation of the hRARβ2 Retinoic Acid Response Element (βRARE) but not the rCRBPII Retinoid X Response Element (RXRE) by both tRA and 9cRA via Endogenous Retinoid Receptors in Adult Human Epidermal Keratinocytes—To determine transcriptional properties of endogenous nuclear retinoid receptors, three retinoid-responsive reporter genes, βRARE-tk-CAT, βRARE$_3$-tk-CAT, and RXRE-tk-CAT were introduced by lipofection into adult human KCs cultured in a defined serum-free medium, KGM (see "Materials and Methods"). As shown in Fig. 1, in comparison with vehicle, 0.1 μM tRA or 9cRA significantly induced βRARE-tk-CAT activity by about 20-fold. When βRARE$_3$-tk-CAT, which contains three head-to-tail copies of βRARE, was used as a reporter, tRA and 9cRA induced activity by about 40-fold. In contrast, when RXRE-tk-CAT was introduced into KCs, neither tRA nor 9cRA significantly induced its activity. These results indicate that KCs contain endogenous retinoid receptors capable of specifically activating βRARE but not RXRE.

Keratinocyte Endogenous RARγ and RXRs Bind in Vitro to βRARE as RAR·RXR Heterodimers but not Homodimers—mRNAs for RARα and γ and RXRα and β were found in cultured adult human KCs with RARγ and RXRα being the major forms (14, 52–55). To know how these receptor proteins interact with βRARE, we have examined nuclear extracts prepared from cultured KCs by gel mobility shift assays. As shown in Fig. 2*A*, incubation of ^{32}P-labeled oligodeoxyribonucleotide probes (20 fmol) containing a wild type βRARE (βRAREwt) with KC nuclear extracts (8 μg) resulted in A complexes (*lane 2*), which were abolished by mutations in both half-sites of βRARE (βRAREm2 in *lane 1*). Formation of the A complexes was significantly reduced by increasing amounts (0.05–2 pmol) of unlabeled competitor DNA, βRAREwt (*lanes 3–6*). However, βRAREm1 (*lanes 7–10*) containing mutations in one of the two half-sites in βRARE competed only slightly the A complexes at 100-fold excess (*lane 10*). As expected, βRAREm2 did not compete these complexes (*lanes 11–14*). No differences were observed among experiments performed in the presence of vehicle (10% ethanol) or 1 μM tRA or 9cRA (data not shown).

The protein content of the A complexes was further examined by immunological gel mobility shift assays (Fig. 2*B*). Postincubation of binding reaction mixtures with mouse monoclonal antibodies specific to either RARγ (RARγ Ab) or that recognize all three members of the RXR family (α, β, and γ) (RXR Ab) resulted in formation of supershifted complexes B (*lane 8*) and C (*lane 9*), respectively, and reduction of fast migrating complexes A1 but not complexes A2 in the A complexes (in comparison with *lane 7*), indicating that the A1 complexes contain RARγ and/or RXR. The A1 and A2 complexes are too close to be completely separated from each other under our experimental conditions. Complexes B and C were also obtained with KC nuclear extracts (2 μg) containing co-overexpressed RARγ and RXRα, respectively (*lanes 13* and *14*), using the same antibodies. The nature of the A2 complexes, which also specifically recognize βRARE, is not known. Note that under the assay conditions (lower amounts of extracts and shorter exposure time) the A2 complexes were absent or less visible with nuclear extracts from KCs transfected with RARγ and/or RXRα. Interestingly, addition of RARγ Ab to the RXR Ab-containing reaction mixture (*lane 9*) further supershifted a portion of the C complexes while resulting in double-supershifted D complexes (*lane 10*) whose amount is approximately equal to that of B in *lane 8*. Thus, formation of the D complexes identifies RARγ-RXR heterodimers, which were also observed with co-overexpressed RARγ and RXRα (*lane 15*). Interestingly, the amount of the B complexes (*lane 8*) is less than that of C (*lane 9*). Furthermore, the sum of the amount of double-supershifted D complexes and that of single-supershifted C complexes migrating faster than D in *lane 10* almost equals that of the C complexes in *lane 9*. These observations suggest that the C complexes in *lane 10* may possibly be heterodimers formed between RXRs and unidentified dimerization partners, which were supershifted by RXR Ab but not RARγ Ab. Similar supershift patterns were obtained with nuclear extracts (8 μg) prepared from human epidermis (*lanes 1–5*), although levels of RARγ and RXRs bound to βRARE in epidermis (*lanes 1′–5′*) are relatively higher than those in cultured KCs (*lanes 5′–10′*). Complexes analogous to A2 were not detectable in epidermis (*lanes 1–5*). No significant differences were observed among experiments performed in the presence of vehicle (ethanol) or 1 μM 9cRA or tRA (data not shown).

To exclude the possibility that both A1 (*lane 3*) and C (*lane 5*) complexes not supershifted by RARγ Ab result from insufficient amounts of this antibody used, an antibody titration experiment was performed with nuclear extracts from epidermis that contain higher levels of the endogenous receptors than those from cultured KCs (Fig. 2*C*). As little as 0.4 μl of RXR Ab completely supershifted the A1 complexes (*lane 4*). On the other hand, increasing amounts of RARγ Ab above and beyond 1.2 μl did not significantly increase the amount of the B complexes nor reduce that of A1 (compare *lane 9* with *lane 12*). The simultaneous decreases of the C and D complexes in *lanes 10* and *14*, respectively, were most likely caused by an excessive quantity of protein contained within the combined RARγ and RXR antibody fluids. Similar titration data were obtained with nuclear extracts from cultured KCs.

RARα and β were not detected in nuclear extracts from cultured KCs by the same assays using appropriate antibodies (data not shown), although under the same conditions RARα present at very low levels but not RARβ was readily detectable in extracts from epidermis (68). No complexes corresponding to endogenous RARγ or RXR homodimers were observed with both extracts, which would have been double-supershifted by monoclonal RARγ Ab or RXR Ab alone (Fig. 2*B*, *lanes 3*, *4*, *8*, and *9*).

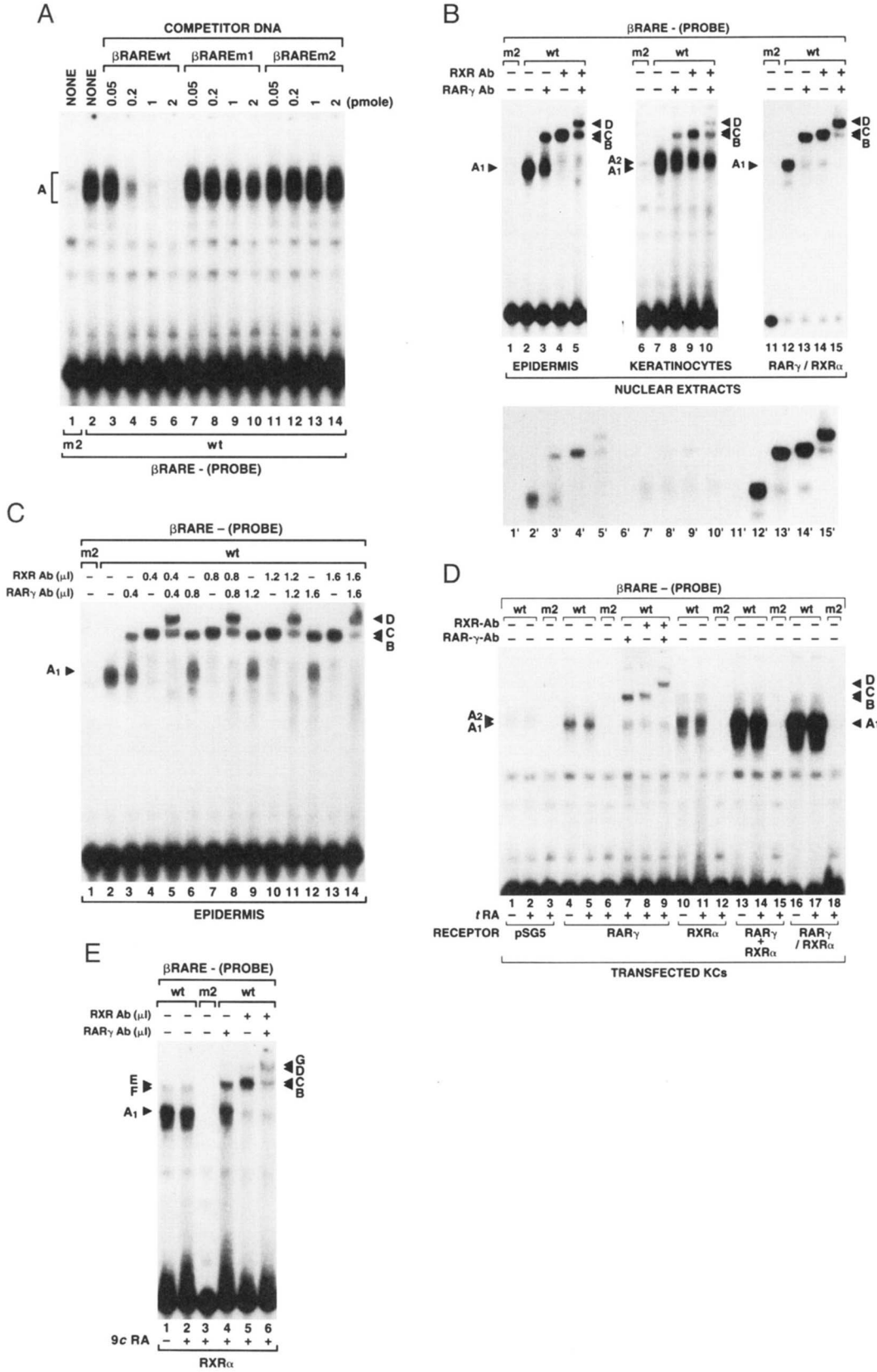

FIG. 2. **Gel electrophoretic mobility shift analysis of endogenous RARγ and RXRs from human epidermis and cultured KCs and of RARγ and RXRα overexpressed in KCs using βRARE.** Resolved complexes are indicated by *labeled triangles* along both *sides* of the gels. Antibodies used (1.2 μl for RARγ Ab and 0.8 μl for RXR Ab unless indicated differently) in postincubation are shown immediately *above* the gels. 1 μM 9cRA was included in all binding reactions unless indicated differently immediately *below* gels. *A*, competition for formation of specific βRARE-bound A complexes by wild type βRARE but not that containing mutations in half-sites. Types of ^{32}P-labeled probes are shown *below* the gel, and types and amounts of unlabeled competitor DNA (equivalent to 2.5-, 10-, 50-, and 100-fold excess) are shown on the *top*. *In vitro* binding reactions were performed with 8 μg of nuclear extracts from cultured KCs. Similar or identical results were obtained in the absence of 9cRA (data not shown). *B*, endogenous RARγ and RXRs in nuclear extracts from human epidermis and cultured KCs bind to βRARE as heterodimers but not

To know whether RARγ at higher concentrations is able to bind to βRARE as RARγ homodimers, nuclear extracts from KCs transfected with RARγ and/or RXRα were analyzed by gel mobility shift assays (Fig. 2*D*). In comparison with KCs transfected with parental expression vector pSG5 (*lanes 1–3*), transfection of KCs with RARγ alone increased levels of βRARE-bound A1 complexes in the absence (*lane 4*) or presence of tRA (*lane 5*). Mutations affecting both half-sites in βRARE abolished formation of these complexes (*lane 6*). The increased A1 complexes apparently correspond to RARγ-RXR heterodimers formed between overexpressed RARγ and endogenous RXRs, since these complexes were completely supershifted by RARγ Ab and/or RXR Ab (*lanes 7–9*). Similar binding took place with KCs transfected with RXRα alone (*lanes 10–12*). No complexes corresponding to RARγ homodimers were observed, which would have been double-supershifted by RARγ Ab alone. Combining the extracts containing overexpressed RARγ (*lanes 4* and *5*) with those containing overexpressed RXRα (*lanes 10* and *11*) synergistically increased the amount of the A1 complexes (*lanes 13* and *14*), similar to the case with extracts containing co-overexpressed RARγ and RXRα (*lanes 16* and *17*), indicating that KCs transfected with single receptors did contain high levels of RARγ or RXRα, respectively. Thus, RXR is required for RARγ to efficiently bind to βRARE, confirming the previous finding (13, 33, 34).

Taken together, our experiments demonstrate that endogenous RARγ and RXR proteins in human epidermis and cultured KCs bind to βRARE *in vitro* almost exclusively as RARγ-RXR heterodimers but not RARγ homodimers nor RXR homodimers. Similar binding can occur when RARγ is overexpressed alone or co-overexpressed with RXRα. In addition, more RXR proteins *versus* RARγ most likely bind to βRARE as heterodimers formed between RXR and as yet unidentified dimerization partners. In the case with the extracts from epidermis, RARα proteins most likely contribute in part to the formation of such heterodimers (68).

Differential Regulation of RXRE by Overexpressed RARα, RARγ, and RXRα—As shown by the *in vitro* binding study, RXR proteins are clearly present in KCs at significant levels. Therefore, lack of RXRE activity in these cells may be due to 1) lack of transcriptional intermediary factors (69) required for RXRs to transactivate RXRE, 2) lack of RXR homodimers *in vivo*, or 3) presence of inhibitory factors such as RARs (42). To distinguish among these possibilities, we have transfected KCs with 2 μg of RXRE-tk-CAT together with 400 ng of expression vectors for RXRα, RARα, and RARγ and determined their ability to regulate RXRE. As shown in Fig. 3, in the presence of 0.1 μM 9cRA, pSG5, a parental expression vector for the receptors, did not significantly affect reporter activity. Overexpression of either RARα or RARγ did not result in significant increase of RXRE activity. However, when RXRα was overexpressed alone, 9cRA strongly induced reporter activity, indicating that KCs contain transcriptional intermediary factors necessary for RXRs to transactivate RXRE. Co-overexpression of either RARα or RARγ together with RXRα resulted in suppression of RXRα-mediated activation of RXRE by about 80 and 90%, respectively. Similar results were obtained when either RARα or RARγ was co-overexpressed together with RXRβ (data not shown). These data indicate that in KCs, RAR·RXR heterodimers are weaker activators of RXRE. In other words, RARs inhibit RXR activity over RXRE via forming the RAR·RXR heterodimers.

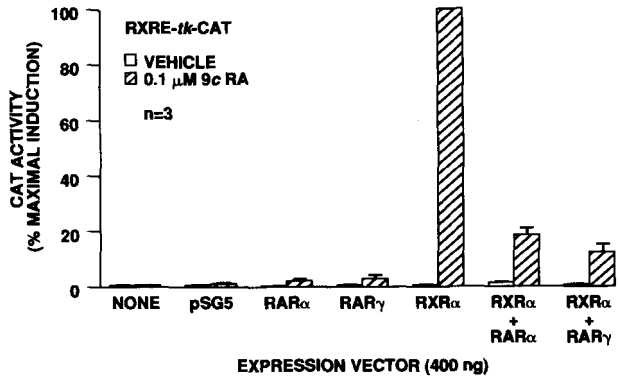

FIG. 3. **Differential regulation of reporter gene RXRE-tk-CAT by overexpressed RXRα, RARα, and RARγ in KCs.** The *y* axis shows average CAT activity expressed as % maximal induction, and the *x* axis shows types of expression vectors cotransfected. Data are presented as average values derived from three independent experiments ($n = 3$). The *open* and *filled boxes* represent cells treated with vehicle (0.1% ethanol) or 0.1 μM 9cRA, respectively. KCs were transfected with 2 μg of reporter RXRE-tk-CAT alone or together with parental expression vector pSG5 or expression vectors for RXRα, RARα, and RARγ.

Endogenous and Co-overexpressed RARγ and RXR Bind in Vitro to RXRE as Heterodimers but not Homodimers—To know how endogenous RARs and RXRs in KCs interact with RXRE, gel mobility shift assays were performed using ^{32}P-labeled probes containing a wild type RXRE (RXREwt). As shown in Fig. 4*A*, incubation of RXREwt with KC nuclear extracts resulted in two closely migrating complexes A1 and A3 with A1 being the fastest migrating species (*lane 2*). Single point mutations in the consensus half-sites of RXRE (RXREm) abolished formation of the A1 complexes but only reduced that of A3 (*lane 1*). Both A1 and A3 complexes were efficiently competed by increasing amounts of unlabeled competitor DNA, RXREwt (*lanes 3* and *4*). RXREm competed the A3 complexes less efficiently than did RXREwt, whereas the A1 complexes were only slightly reduced by the highest amount of RXREm (*lane 6*). βRAREwt (*lanes 7* and *8*) but not βRAREm2 (*lanes 9* and *10*) also competed significantly these two complexes. No differences were observed among experiments performed in the presence of vehicle (ethanol) or 1 μM 9cRA (data not shown). These data indicate that the A1 and A3 complexes have different DNA sequence preferences for the half-sites in RXRE.

The protein content of the A1 and A3 complexes bound to RXREwt was further analyzed by immunological gel mobility shift assays. As shown in Fig. 4*B*, the A1 complexes (*lane 7*) found with KC nuclear extracts were also formed with co-overexpressed RARγ and RXRα (*lane 20*). Note that the A3 complexes were present at very low levels in nuclear extracts from human epidermis or KCs transfected with RARγ and/or RXRα under the assay conditions (lower amount of extracts

RAR nor RXR homodimers. Types of ^{32}P-labeled probes are indicated at the *top*, and those of nuclear extracts are indicated at the *bottom*. Amount of nuclear extracts used in binding reactions was 8 μg for human epidermis (*lanes 1–5*) and cultured KCs (*lanes 6–10*) and 2 μg for KCs containing co-overexpressed RARγ and RXRα (*lanes 11–15*). Similar or identical results were obtained in the presence of vehicle (10% ethanol) or 1 μM tRA (data not shown). Photographs containing *lanes 1–5*, *6–10*, and *11–15* are derived from autoradiography of the same gel for 90, 180, and 30 min, respectively. The *lower panel* (*lanes 1′–15′*) shows autoradiography of the same gel for 30 min. *C*, antibody titration shows that the A1 complexes are derived from two types of RXR-containing complexes interacting with βRARE. Types of ^{32}P-labeled probes are indicated at the *top*. 8 μg of nuclear extracts from human epidermis were used. *D*, RARγ overexpressed in KCs binds to βRARE preferentially as RARγ-RXR heterodimers but not RAR homodimers, whereas RXRα overexpressed alone binds to βRARE as the RARγ-RXR heterodimers and RXR homodimers with the later being the minor species (*E*). Types of ^{32}P-labeled probes are indicated at the *top*, and those of nuclear extracts from transfected KCs (2 μg) are indicated at the bottom.

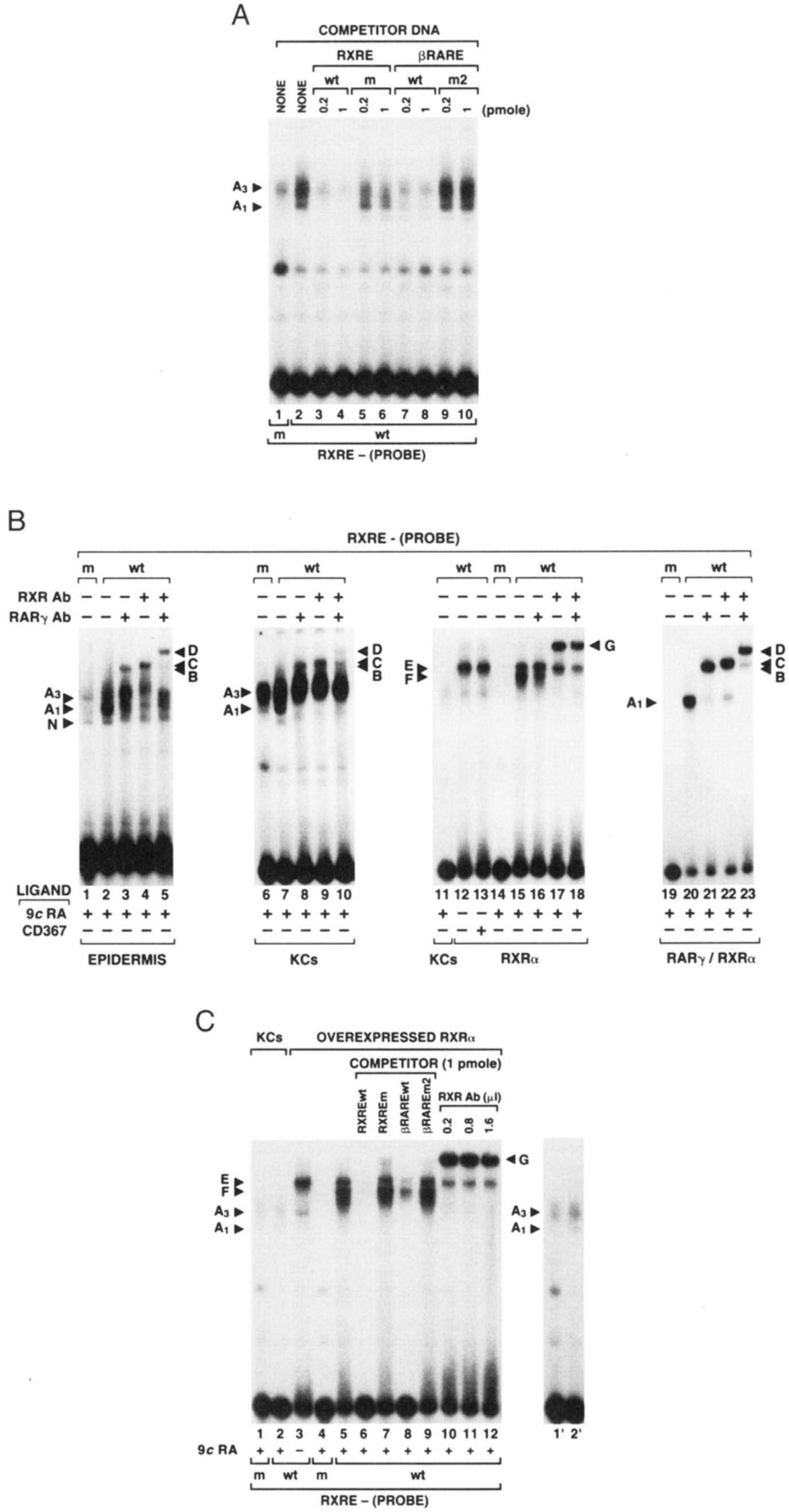

FIG. 4. **Gel electrophoretic mobility shift analysis of endogenous RARγ and RXRs from human epidermis and cultured KCs and of RARγ and RXRα overexpressed in KCs using RXRE.** Resolved complexes are indicated by *labeled triangles* along both sides of the gels. *N*, nonspecific complexes. Antibodies used (1.2 μl for RARγ Ab and 0.8 μl for RXR Ab unless indicated differently) in postincubation are shown immediately *above* the gels. 1 μM 9cRA was included in all binding reactions unless indicated differently immediately *below* gels. *A*, competition for formation of specific RXRE-bound A1 complexes in KC nuclear extracts by wild type RXRE and βRARE but not those containing mutations in half-sites (RXREm and βRAREm2). Types of ^{32}P-labeled probes are shown *below* the gel, and types and amounts in pmoles of unlabeled competitor DNA (equivalent to 10- and 50-fold excess) are shown on the *top*. *In vitro* binding reactions were performed with 8 μg of nuclear extracts from cultured KCs. *B*, endogenous and co-overexpressed RARγ and RXR in KCs bind to RXRE as heterodimers but not RAR homodimers nor RXR homodimers. Types of ^{32}P-labeled probes are indicated at the *top*, and those of nuclear extracts are indicated at the bottom. Amounts of nuclear extracts used in binding reactions were 4 μg for human epidermis (*lanes 1–5*), 8 μg for cultured KCs (*lanes 6–10*), and 2 μg for mock-transfected KCs (*lane 11*) and transfected KCs containing overexpressed RXRα (*lanes 12–18*) or co-overexpressed RARγ and RXRα (*lanes 19–23*). Gels including *lanes 1–10* were subjected to autoradiography for a duration at least four times longer than those comprising *lanes 11–23*, due to relatively lower levels of endogenous RARγ and RXRs *versus* overexpressed receptors. *C*, competition and antibody titration analysis of overexpressed RXRα bound to RXRE. Types of ^{32}P-labeled probes are indicated at the *bottom*, and those of nuclear extracts and competitor DNA are indicated at the *top*. Amounts of nuclear extracts used in binding reactions were 2 μg for both mock-transfected KCs (*lanes 1* and *2*) and transfected KCs containing overexpressed RXRα (*lanes 3–12*). *Lanes 1′* and *2′* on the *left* were obtained from autoradiography of *lanes 1* and *2* for a longer duration.

and/or shorter exposure time). Postincubation of binding reaction mixtures with antibodies specific to either RARγ or RXRs significantly supershifted the A1 but not the A3 complexes, resulting in complexes B (*lane 8*) and C (*lane 9*), respectively. Note that the amount of the C complexes is higher than that of B, similar to the case with βRARE. Formation of the B complexes identifies RAR-containing heterodimers and that of the C complexes, RXR-containing heterodimers. Thus, the A1 complexes apparently correspond to RXREwt-bound endogenous RARγ and/or RXRs. Complexes B and C were also obtained

with co-overexpressed RARγ and RXRα using the same antibodies (*lanes 21* and *22*). During postincubation, addition of RARγ Ab into binding reaction together with RXR Ab further supershifted a portion of the C complexes, causing formation of D complexes (*lane 10*), which were also obtained with co-overexpressed RARγ and RXRα (*lane 23*). Formation of the D complexes identifies RARγ-RXR heterodimers. The remaining C complexes (*lane 10*) not double-supershifted by RARγ Ab may possibly correspond to RXR Ab-associated heterodimers formed by RXRs and unidentified dimerization partners other than RARγ. Binding similar to that with cultured KCs occurred with nuclear extracts prepared from epidermis (*lanes 1–5*) although unlike cultured KCs, the C complexes in human epidermis were almost completely double-supershifted by RARγ Ab (*lane 5*). In these three types of extracts, no complexes corresponding to the RARγ and RXR homodimers were found, which would have been double-supershifted by RARγ Ab or RXR Ab alone. Similar results were obtained in the presence of vehicle (ethanol) or 1 μM 9cRA. The nature of the A3 complexes is not known. Whether the A3 complexes are formed by nuclear proteins involved in formation of the A2 complexes over βRARE remains to be determined.

To know whether, at relatively higher levels *versus* RARs and other dimerization partners, RXRs are able to form RXR homodimers, RXRα overexpressed alone in KCs was analyzed (Fig. 4*B*, *lanes 11–18*). In the presence of vehicle, incubation of nuclear extracts containing overexpressed RXRα with RXREwt gave rise to E complexes (*lane 12*), which is absent in a control reaction performed with the same amount of nuclear extracts from mock-transfected KCs (*lane 11*). Addition of 9cRA into the binding reaction resulted in formation of F complexes (*lane 15*) whose mobility is lower than that of the RARγ-RXR heterodimers (A1 in *lane 20*). Mutations in the half-sites of RXRE (RXREm) abolished both E and F complexes (*lane 14*). The specificity of these two complexes were confirmed by the competition experiment shown in Fig. 4*C*, since RXREwt (*lane 6*) but not RXREm (*lane 7*) specifically competed both complexes. As shown in Fig. 4*B*, the F complexes apparently correspond to the RXR homodimers bound to RXRE, since RXRα Ab (*lane 17*) but not RARγ Ab (*lane 16*) completely supershifted the F complexes while resulting in formation of G complexes. The mobility of the G complexes is close to that of the double-supershifted RARγ-RXR heterodimers (D in *lane 23*). The observation that the RXRα proteins overexpressed in KCs form homodimers in a 9cRA-dependent way is consistent with the previous finding with *in vitro* transcribed-translated RXRα (35). The nature of the E complexes is yet unknown. These complexes found only in KCs transfected with RXRα alone are not related to RARγ because they were not supershifted by RARγ Ab (*lane 16*). However, as shown in Fig. 4*C*, although RXR Ab reduced the intensity of E to a certain extent, adding excessive amounts of RXR Ab did not completely supershift the E complexes (*lanes 10–12*) while the F complexes were completely double-supershifted by the lowest amount (0.2 μl) of the antibody (*lane 10*).

To determine whether RXRs overexpressed alone in KCs are also capable of binding to βRARE as homodimers in addition to RARγ-RXRα heterodimers, nuclear extracts from KCs transfected with RXRα alone were analyzed. As shown in Fig. 2*D*, overexpression of RXRα increased the amount of the A1 complexes (compare *lanes 10* and *11* with *lanes 1* and *2*) and resulted in E complexes (*lane 10* in Fig. 2*D* and *lane 1* in Fig. 2*E*) when βRAREwt was used as a probe. The presence of tRA (Fig. 2*D*, *lane 11*) or 9cRA (Fig. 2*E*, *lane 2*) did not significantly alter the binding patterns. As shown in Fig. 2*E*, mutations in both half-sites of βRARE (βRAREm2) abolished formation of both A1 and E complexes (*lane 3*). RARγ Ab supershifted only a portion of the A1 complexes resulting in B complexes (*lane 4*), while RXR Ab almost completely supershifted the A1 complexes to give C complexes (*lane 5*). In addition, G complexes corresponding to RXR homodimers double-supershifted by RXR Ab were also detected with βRARE (*lane 5*), as in the case with RXRE. The fact that the quantity of the G complexes is very low suggests that complexes analogous to F (RXR·RXR) free of RXR Ab may be masked by the E complexes and/or background signals (*lane 2*). Addition of RARγ Ab together with RXR Ab into binding reaction further supershifted a portion of the C complexes to give D complexes (RARγ-RXR) (*lane 6*), which co-migrate with G (RXR·RXR) (*lane 5*). In this case, C complexes not double-supershifted by RARγ Ab were found again as expected (*lane 6*). These data indicate that RXRs bind to βRARE as homodimers only when its levels are much higher than other dimerization partners and that RAR·RXR and other RXR-containing heterodimers have higher affinity for this element than the RXR homodimers do. The idea that the RXR homodimers have lower affinity for βRARE than for RXRE was further confirmed by the competition experiment shown in Fig. 4*C*. βRAREwt (*lane 8*) but not βRAREm2 (*lane 9*) competed specifically but less efficiently for formation of the F complexes than did RXRE (*lane 6*).

Thus, results from these experiments clearly indicate that 1) endogenous RXRs bind to RXRE as RAR·RXR heterodimers but not homodimers due to the presence of RARγ and other unidentified dimerization partners (at least in the case of cultured KCs), 2) RXRs are able to form RXRE-bound homodimers only when their concentrations are much higher than other dimerization partners and in the presence of 9cRA, and 3) βRARE has much lower affinity for RXR homodimers than RXRE does.

Specificity and Potency of tRA, 9cRA, CD367, and SR11237 in Regulation of Transactivation of RXRE by Overexpressed RXRα in KCs—9cRA binds to and activates both RXRs and RARs (30, 31, 51). tRA binds to and activates RARs but not RXRs except that, in certain cell lines, it activates RXRs as a result of its conversion to 9cRA (30, 31, 51). SR11237 has been shown to be a specific ligand for RXRs but not RARs (32). Although CD367 binds and activates RARs with high affinity (58, 70), its biological activity on RXRs has not been reported. We have compared the effect of CD367 on RXRα-mediated activation of RXRE-tk-CAT with that of tRA, 9cRA, and SR11237. KCs were co-transfected with 2 μg of RXRE-tk-CAT and 400 ng of expression vector for RXRα. As shown in Fig. 5*A*, tRA, 9cRA, and SR11237 significantly induced reporter activity via overexpressed RXRα in a dose-dependent manner. SR11237 was the most potent ligand for RXRα with an ED_{50} value (concentration required to give 50% of maximal induction) of 11 nM, consistent with its affinity for RXRs (32, 68). However, tRA and 9cRA have similarly much higher ED_{50} values, 110 and 140 nM, respectively. Interestingly, CD367 did not have any effect on RXRα. The biological activity of CD367 over RXRs was further analyzed *in vitro* by gel mobility shift assays. As shown in Fig. 4*B*, unlike 9cRA (*lane 15*), CD367 did not induce formation of RXRα homodimers (*lane 13*), suggesting that this ligand is specific for RARs but not RXRs. In addition, treating cells with 0.1 μM CD367 together with 0.1 μM SR11237 did not significantly alter RXRα-mediated induction of RXRE by SR11237 (data not shown), indicating that CD367 is not an antagonist for RXRs. This result is in agreement with *in vitro* ligand binding studies showing that CD367 binds to RARs but not RXRs (68).

Specificity and Potency of tRA, 9cRA, CD367, and SR11237 in Regulation of Endogenous Retinoid Receptor-mediated

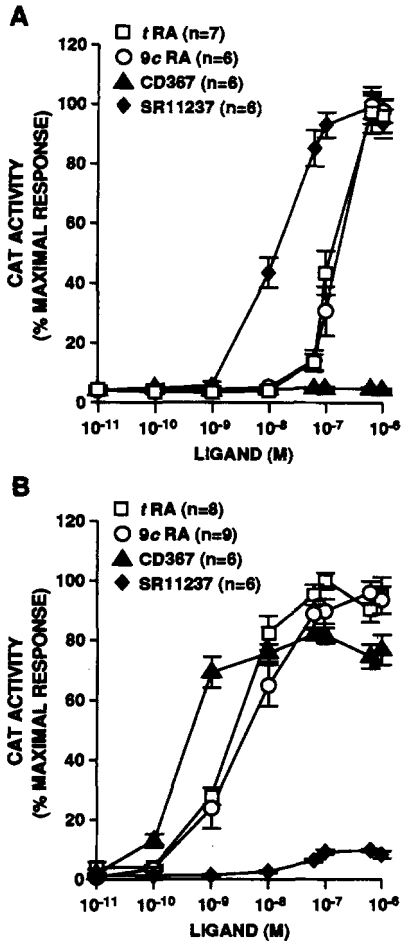

FIG. 5. **Selective and dose-dependent activation of RA-responsive reporter genes, RXRE-tk-CAT via overexpressed RXRα homodimers (*A*) and βRARE$_3$-tk-CAT via endogenous RAR·RXR heterodimers (*B*), by tRA, 9cRA, CD367, and SR11237 in cultured KCs.** The *y* axis represents average CAT activity expressed as % maximal response, and the *x* axis represents concentrations of ligand in a log scale. Labels corresponding to tRA, 9cRA, CD367, and SR11237 are indicated on the *top*. *n* refers to number of human subjects from whom KCs were prepared and analyzed independently. Standard errors are shown as *vertical bars*. KCs were transfected with 2 μg of RXRE-tk-CAT together with 400 ng of the RXRα expression vector (*A*) or 3 μg of βRARE$_3$-tk-CAT alone (*B*).

Transactivation of βRARE in KCs—Specific activation of RXRα by SR11237 but not CD367 provided a means to further determine roles of KC endogenous RARs and RXRs in transactivation of βRARE. KCs were transfected with 3 μg of βRARE$_3$-tk-CAT, and dose responses of endogenous retinoid receptors to tRA, 9cRA, CD367, and SR11237 were measured. As shown in Fig. 5*B*, there was a strong dose-dependent induction of this reporter gene by tRA, 9cRA, and CD367. CD367, the most potent ligand for RARs, activated endogenous retinoid receptors with an ED_{50} value of 0.3 nM, whereas tRA and 9cRA did so with ED_{50} values of 2.3 and 3.8 nM, respectively. In contrast, the RXR-specific ligand, SR11237, did not significantly activate βRARE via endogenous retinoid receptors. These data indicate that tRA, 9cRA, and CD367 induce βRARE activity via endogenous RARs, most probably RARγ, present in the RARγ·RXR heterodimers, whereas binding of RXR-selective SR11237 to RXRs does not activate these heterodimers.

RAR and RXR-derived Dominant Negative Mutants Repressed Transactivation of βRARE$_3$-tk-CAT by Endogenous Retinoid Receptors in KCs—To further ascertain whether the AF-2 function of RXR in endogenous RAR·RXR heterodimers

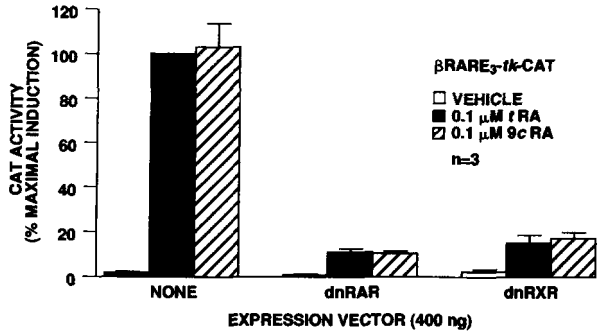

FIG. 6. **Suppression of endogenous RAR·RXR heterodimer-mediated transactivation of βRARE in KCs by the RAR and RXR dominant negative mutants.** The *y* axis shows average CAT activity in % maximal induction, and the *x* axis shows types of expression vectors cotransfected. dnRAR and dnRXR refer to expression vectors for the RAR and RXR dominant negative mutants, respectively. KCs were transfected with 2 μg of βRARE$_3$-tk-CAT alone or together with 400 ng of expression vectors. Results were expressed as average values derived from three independent experiments (n = 3). The *open* and different *filled boxes* represent cells treated with vehicle (0.1% ethanol) or 0.1 μM tRA or 0.1 μM 9cRA, respectively. The standard errors are represented by *vertical bars*.

cooperates with that of RAR in transactivation, KCs were cotransfected with 2 μg of βRARE$_3$-tk-CAT and 400 ng of expression vectors for the dominant negative RAR (dnRAR) or RXR (dnRXR) mutants. These mutants were generated by deleting their C-terminal region leaving their dimerization domain preserved and transactivation function AF-2 impaired (62). Introduction of dnRAR or dnRXR into KCs should result in formation of heterodimers dnRAR·RXR or RAR·dnRXR between dnRAR and endogenous RXR and between dnRXR and endogenous RAR, respectively. In the presence of appropriate ligands at concentrations adequate for activating both RAR and RXR, we expected to see two possible outcomes. 1) If the AF-2 domain of RAR and that of RXR are cooperatively involved in ligand-dependent transactivation of βRARE by RAR·RXR heterodimers, dnRAR·RXR or RAR·dnRXR heterodimers would efficiently bind to but not effectively activate βRARE and at the same time compete endogenous RAR·RXR heterodimers from binding to βRARE. Therefore, we would see a strong reduction (>50%) of endogenous RAR·RXR heterodimer-mediated transactivation of βRARE by either mutant. 2) If the AF-2 domain of RAR and that of RXR are able to independently confer ligand-dependent transactivation of βRARE, through the same binding process we would see a moderate (≤50%) reduction of endogenous RAR·RXR heterodimer-mediated transactivation of βRARE by the transactivation-capable dnRAR·RXR or RAR·dnRXR heterodimers. As shown in Fig. 6, 0.1 μM tRA or 9cRA strongly induced βRARE activity via endogenous RAR·RXR heterodimers in KCs. However, under the same conditions, overexpression of dnRAR or dnRXR drastically reduced endogenous receptor-mediated induction of this reporter by 80–90%. Thus, our results support the idea that regulation of ligand-dependent transactivation over βRARE by the RAR·RXR heterodimers is achieved via the first mechanism.

DISCUSSION

In this study, we demonstrated that natural retinoids including tRA and 9cRA strongly induced βRARE (DR5) activity but not that of RXRE (DR1) in cultured KCs. This result suggests that KCs contain functional endogenous retinoid receptors that specifically activate βRARE but not RXRE. Availability of monoclonal antibodies and synthetic ligands specific to RAR or RXR allowed us to identify roles of endogenous RARs and RXRs in this restricted regulation. Several lines of evidence indicate that RARγ·RXR heterodimers but not RXR homodimers nor

RARγ homodimers are the major regulators of βRARE and RXRE in cultured KCs.

Our *in vitro* binding studies clearly showed that endogenous RARγ and RXR proteins in nuclear extracts from human epidermis and cultured KCs bind to βRARE as RARγ-RXR heterodimers but not RARγ homodimers nor RXR homodimers. In fact, retinoid receptor-related binding activity observed in epidermis was mainly contributed by RARγ and RXRα, which represent 90% of total RAR and RXR proteins, respectively (68), in good correlation with their mRNA levels previously reported (14, 52–54). In cultured KCs, RARα was not detected by specific antibodies in gel mobility shift assays due to both its low levels and limitation on amounts of nuclear extracts that could be loaded on gels (data not shown). However, in human epidermis, low levels of RARα proteins were readily detectable by the same assays (68). RARβ was not detected in human epidermis (68) nor in cultured KCs (data not shown), consistent with the absence of the corresponding mRNA in this tissue (52). On the other hand, RARγ overexpressed in KCs also binds to βRARE exclusively as RAR·RXR heterodimers, and this binding is quantitatively regulated by the levels of RXRs available in KCs. These data further support the notion that RXRs, as cofactors for RARs, are required for RARs to efficiently bind to targets such as βRARE (13, 33, 34, 41).

In both epidermis and cultured KCs, endogenous RXRs, which are present in relative excess *versus* RARs, appear to form heterodimers with unknown dimerization partners besides RARs. In addition to endogenous RAR·RXR heterodimers, overexpressed RARγ proteins bound to βRARE exclusively as RAR·RXR heterodimers but not RAR·RAR homodimers through heterodimerizing with endogenous RXRs (Fig. 2*D*), indicating that the unknown dimerization partners can be dissociated from RXRs by RARs. Recently, Baes *et al.* (71) identified a new orphan receptor, called MB67, which shares high sequence homology with the retinoid receptor families. It binds as MB67·RXR heterodimers to consensus half-sites present in only βRARE (DR5) but not any other direct repeats, and its activity is not regulated by retinoic acid. Their finding suggests that receptors capable of binding to βRARE (DR5) are not limited to receptor dimers formed among the RAR and RXR family members. Whether the endogenous RXR-containing heterodimers other than RAR·RXR observed in this study correspond to dimers formed between RXR and unidentified orphan receptors present in human epidermis and cultured KCs remains to be further characterized.

The ED_{50} values of tRA, 9cRA, and CD367 in induction of βRARE activity via endogenous retinoid receptors correlate well with the affinity of these ligands for RARs but not RXRs, with CD367 being the most potent (51, 58, 70, 72). The fact that RXR-specific ligand SR11237 did not significantly activate βRARE through endogenous RXRs excludes the possibility that RAR-unrelated RXR-containing heterodimers or undetectable endogenous RXR homodimers also contribute to transactivation of βRARE. This result also indicates that binding of ligands to RXR in RAR·RXR heterodimers does not confer ligand-dependent transactivation of βRARE. *In vitro* ligand binding assays have showed that CD367 does not interact with RXRs (68). In this study, we found that this ligand neither induces formation of RXR homodimers *in vitro* nor activates RXRs *in vivo*. Therefore, binding of ligands such as CD367 to only RAR in RAR·RXR heterodimers seems to be sufficient for conferring the ligand inducibility to transactivation of βRARE. In other words, occupation of the E domain of RXR by ligands is most likely not required.

Overexpression of dominant negative mutants, dnRAR and dnRXR, drastically repressed endogenous receptor-mediated induction of βRARE in cultured KCs. These two mutants have been previously shown to be functional in dimerization and DNA binding but not in ligand-dependent transactivation (62). The repression we observed here most likely resulted from formation of transactivation-deficient receptor dimers dnRAR·RXR or RAR·dnRXR involving endogenous wild type RXRs and RARs. These mutant dimers, which contain only one AF-2 domain with transactivation function preserved, most probably competed the remaining endogenous RAR·RXR from binding to βRARE, but their own binding failed to activate this element. Thus, based on the results from both transactivation in cultured KCs and *in vitro* binding studies, we conclude that transactivation of βRARE in KCs is mainly mediated by endogenous RAR·RXR heterodimers, in which RXR is required for RAR to efficiently bind to βRARE, and the RAR ligand binding domain confers ligand inducibility whereas the RXR AF-2 domain without the need for bound ligands cooperates with the AF-2 domain of liganded RAR in transactivation.

Transactivation of either isolated RXRE or the natural RXRE-containing rCRBPII gene promoter was initially observed only under experimental conditions where RXRs were overexpressed alone (34, 42, 43). Recently, Nakshatri and Chambon (38) have further demonstrated that whether RXRE is transactivated by overexpressed RXR homodimers or RAR·RXR heterodimers depends on cell type. In this study, we found that in contrast to βRARE (DR5), RXRE (DR1) was not activated by endogenous retinoid receptors in KCs. Endogenous RAR·RXR heterodimers bound to but failed to transactivate this element in the presence of appropriate ligands. *In vitro*, when RXRE was used as a probe, 9cRA-dependent RXR homodimers were obtained with RXRα overexpressed alone in KCs but not with RXR expressed at endogenous levels in both human epidermis and cultured KCs, similar to the case with *in vitro* transcribed-translated RXRs (26, 35). Under the same conditions, RXRα interacts with βRARE mainly as heterodimers and barely as RXR homodimers. Previous *in vitro* binding studies have shown that RXR homodimers produced by *in vitro* transcription-translation were able to bind to a synthetic RARE, called DR1G, but not RXRE in the absence of 9cRA (38). All of these observations suggest that organization and/or sequences of half-sites in RAREs may also play important roles in ligand-dependent formation of RXR homodimers.

In addition, we found that when RARs were co-overexpressed, high levels of overexpressed RXRs bound to RXRE preferentially as RAR·RXR heterodimers but not RXR homodimers even in the presence of 9cRA. Thus, ligand-independent interaction between RARs and RXRs appears to be much stronger than ligand-dependent interaction among RXRs themselves. In cultured KCs, RARs overexpressed alone did not significantly activate RXRE. Furthermore, overexpressed RARs were able to suppress transactivation of RXRE mediated by overexpressed RXRs, similar to the case with monkey kidney epithelial cells, CV1 (42). These observations together with those from *in vitro* binding studies suggest that in KCs formation of RAR·RXR heterodimers dominate over that of RXR homodimers, and the resulting dominant RAR·RXR heterodimers, which are poor activators for RXRE, may suppress RXR-mediated activation of this element by competing the remaining RXR homodimers from binding to this element, while their own binding does not produce effective transactivation. Recently, a number of groups (36, 38) have shown that in addition to RARs, chicken ovalbumin upstream transcription factor and *apo*AI regulatory protein are able to efficiently form heterodimers with RXRs and bind to RXRE. In this study, we also observed that in cultured KCs, unidentified proteins bound to RXRE as RXR-containing heterodimers. Taken to-

gether, we predict that in KCs, RXRs would form 9cRA-induced homodimers over RXRE only when their concentrations are much higher than those of RARs and of other dimerization partners.

Finally, our finding that in human epidermal KCs the RAR·RXR heterodimer-mediated nuclear retinoid signal transduction pathway dominates over that mediated by RXR homodimers in regulating transcription may be physiologically relevant to the recent finding that in contrast to tRA, SR11237 produced no detectable changes when applied on rhino mouse skin (73). On the other hand, the fact that tRA and 9cRA showed similar potencies in activating RXR homodimers overexpressed in KCs suggests that there is efficient interconversion between these two natural ligands in these cells, similar to that observed with other continuous mammalian cell lines (30, 31, 51). This idea was further supported by results from HPLC analysis of retinoids extracted from KCs treated with 0.1 μM tRA or 9cRA for 48 h, which revealed the presence of both ligands in either case.[2] Unlike natural retinoids tRA and 9cRA, which are subjected to interconversion in living tissues such as human epidermis, synthetic retinoids SR11237 and CD367 are able to independently trigger the RXR homodimer- and RAR·RXR heterodimer-mediated nuclear signal pathways, respectively. Advantages of these synthetic retinoids over natural retinoids may open an avenue leading to not only tissue targeting for therapeutic purposes but can also be used to address the question of whether an RXR homodimer-mediated signal transduction pathway exists in other human tissues.

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