

Induction of TR4 Orphan Receptor by Retinoic Acid in Human HaCaT Keratinocytes

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Human TR4 orphan receptor (TR4) can modulate the transcriptional activity of the reporter gene containing an AGGTCA direct repeat-hormone response element. Here we studied the potential role of TR4 in human HaCaT keratinocytes. Using a chloramphenicol acetyltransferase reporter gene assay, it was shown that TR4 can suppress retinoic acid-induced transactivation by 47.3% in human HaCaT keratinocytes. Electrophoretic mobility shift assay indicated that this suppression may be due to TR4 binding with higher affinity to the retinoic acid response element than retinoid receptors. Western blot analysis further suggested that retinoic

acid can increase the expression of TR4 protein in human HaCaT keratinocytes, indicating that TR4 acts as a negative feedback modulator for retinoic acid action. Interestingly, TR4 expression is increased in normal human keratinocytes when substituting a low calcium medium with a high calcium medium. Together, our data suggested, for the first time, that an orphan receptor, such as TR4, may play an important part in retinoid-mediated signaling pathways in human keratinocytes, providing a new insight into keratinocyte biology. Keywords: differentiation/TR4/vitamin A. *J Invest Dermatol* 112:426-431, 1999

Members of the steroid/thyroid hormone receptor superfamily are transcription factors that regulate the expression of target genes by binding to their hormone response elements (Evans, 1988; Tsai and O'Malley, 1994). Some members of this family are putative receptors, designated as orphan receptors, having unidentified ligands (O'Malley and Conneely, 1992; Enmark and Gustafsson, 1996). The human TR4 orphan receptor (TR4) was isolated by our laboratory from human prostate and testis libraries, using degenerative polymerase chain reaction cloning (Chang *et al*, 1994). From cDNA sequence analysis, human TR4 consists of 615 amino acid residues and its calculated molecular weight is 67.3 kDa. The amino acid sequence of TR4 is closely related to that of TR2, one of the first identified orphan receptors (Chang *et al*, 1989), suggesting that TR2 and TR4 orphan receptors form a unique subclass among the steroid/thyroid hormone receptor superfamily. Based on the sequence of P box, which is three amino acid residues in the first zinc-finger of the DNA-binding domain, it has been speculated that TR4 belongs to the estrogen receptor/thyroid hormone receptor subfamily, which can bind to AGGTCA direct repeats (direct repeats; AGGTCA (n)_x AGGTCA, x = 0-6). We have identified a TR4 response element containing an imperfect direct repeat 2 in the SV40 major late promoter (Lee *et al*, 1995). Using a chloramphenicol acetyltransferase (CAT) reporter gene assay, we found that TR4 can induce the transcriptional activity of

the reporter gene containing a direct repeat four hormone response element and the intronic enhancer of the human ciliary neurotrophic factor receptor gene (Lee *et al*, 1997; Young *et al*, 1997). TR4, therefore, could modulate the ligand-mediated steroid/thyroid hormone receptor action.

Among the ligands for steroid/thyroid receptor superfamily, retinoic acid (RA) and vitamin D are especially important modulators for human keratinocyte growth and differentiation (Bikle and Pillai, 1993; Fisher and Voorhees, 1996). RA inhibits differentiation of cultured human keratinocytes through suppression of expression of differentiation-markers, such as keratins 1 and 10 (Fuchs and Green, 1981) and filaggrin (Asselineau *et al*, 1990). Vitamin D at concentrations higher than 10⁻⁸ M markedly inhibits keratinocyte growth and enhances differentiation (Itin *et al*, 1994).

Considering these findings, it is of interest to study the expression and function of TR4 in human keratinocytes. In this study, we used human HaCaT keratinocytes as well as normal primary human keratinocytes.

Using a CAT reporter gene assay through their endogenous cognate receptors, we demonstrated here that TR4 suppresses RA-mediated, but not vitamin D mediated transcriptional activity in human HaCaT keratinocytes. We also demonstrated that the expression level of TR4 is increased by RA in HaCaT keratinocytes, indicating that TR4 acts as a negative feedback modulator for RA action in human keratinocytes. Finally, we were able to show that TR4 expression is increased in normal human keratinocytes by switching from a low calcium medium to a high calcium medium.

MATERIALS AND METHODS

Cell culture Human HaCaT keratinocytes (originated in Dr. Norbert Fusenig's laboratory), an immortalized cell line, were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 100 U penicillin/streptomycin per ml (Boukamp *et al*, 1988; Pincelli *et al*, 1997). For RA and vitamin D treatment, we used Dulbecco's modified Eagle's medium containing charcoal-stripped fetal bovine serum. MCF-7 cells, a breast cancer cell line, were also cultured in Dulbecco's modified Eagle's medium

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Abbreviations: TR4, TR4 orphan receptor; CAT, chloramphenicol acetyltransferase; RA, retinoic acid; RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; atRA, all-*trans* retinoic acid; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

plus 10% fetal bovine serum and 100 U penicillin/streptomycin per ml. Neonatal foreskins were obtained with informed consent according to the regulations of the University of Rochester Human Subjects Review Board. Keratinocyte cultures were established as described previously (Haake and Cooklis, 1997), and cultured in keratinocyte growth medium (KGM) containing 30 µg bovine pituitary extract per ml, 0.1 ng human epidermal growth factor per ml, 5.0 µg insulin per ml, and 0.5 µg hydrocortisone per ml (KGM; Clonetics, Palo Alto, CA). For each experiment, we used the third passage of foreskin keratinocytes. When we examined TR4 expression change with the Ca²⁺ switch in the medium, we used KGM without bovine pituitary extract, human epidermal growth factor, insulin, and hydrocortisone.

Polyclonal antibodies against TR4 To obtain a large amount of TR4 antigen, the *Escherichia coli* pET expression system was utilized. The expression of the full-length TR4 was performed according to the manufacturer's instruction (Novagen, Madison, WI) with the addition of six consecutive histidine residues at the N-terminal TR4. The lysates were centrifuged at 20,800 × *g* for 20 min at 4°C. The extract was analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie Blue staining, or purified by a one-step metal chelating chromatography (Novagen). The antigen was prepared by directly cutting from the sodium dodecyl sulfate–polyacrylamide gel electrophoresis, emulsified with Freund's complete adjuvant, and then injected into rabbits.

Plasmids The pCMX-TR4 plasmid was constructed as described previously (Lee *et al.*, 1995). To construct pRARβ-CAT, we synthesized the double-stranded oligonucleotides of RA response element (RARE) (GGTTCACCGAAAGTTCA) from the promoter region of RA receptor β (RARβ) (Sucov *et al.*, 1990) and ligated it into the *Bgl*II site of pCAT promoter vector (Promega, Madison, WI). The reporter plasmid P450cc24-CAT, which contains 5'-flanking region (–2200 to +188) of the rat vitamin D₃ 24-hydroxylase gene, was kindly provided by Dr. Y. Kato (Ohyama *et al.*, 1994). The expression plasmids, pSG5-RARα and pCMX-retinoid X receptor α (RXRα), were kindly provided by Dr. Ronald M. Evans (The Salk Institute for Biological Studies, Howard Hughes Medical Institute, La Jolla, California).

Nuclear extracts Nuclear extracts were prepared following a mini-extract procedure (Lee *et al.*, 1988). Briefly, the cells were harvested and lysed by pushing through a 25-gauge hypodermic needle. The nuclear pellet was resuspended in buffer C [500 mM NaCl, 20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM dithiothreitol] and incubated on a rotating wheel for 30 min at 4°C. The nuclear debris was eliminated by centrifugation for 30 min and supernatants were dialyzed for 2 h against buffer D (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol). Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA).

Coupled *in vitro* transcription and translation The expression plasmids, pCMX-TR4, pSG5-RARα, and pCMX-RXRα, were *in vitro* transcribed and translated by the rabbit reticulocyte-based transcription/translation kit (TNT coupled reticulocyte lysate system) (Promega) as described previously (Lee and Chang, 1995).

Transient transfection Polybrene with dimethylsulfoxide shock was used for transient transfection into HaCaT keratinocytes, as reported previously (Jiang *et al.*, 1991). Four micrograms of CAT constructs were cotransfected with 1–2 µg of pCMX-TR4. To normalize the transfection efficiency, 1 µg of β-galactosidase expression vector, pCMVβ (Clontech), was cotransfected in all experiments. MCF-7 cells were transfected using a modified calcium phosphate precipitation method (Mizokami and Chang, 1994).

CAT assay Twenty-four hours after transfection, we treated cells with 10^{–6} or 10^{–8} M all-*trans* retinoic acid (atRA), 10^{–7} or 10^{–10} M 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], or ethanol as a vehicle. At 48 h after transfection, the cells were washed twice with phosphate-buffered saline (–) and harvested. The cells were lysed by three cycles of freeze-thawing. The lysates were centrifuged for 5 min at 20,800 × *g*. To inactivate the internal inhibitory fraction for CAT activity, the supernatants were incubated at 57°C for 10 min and used for the CAT reaction. The reaction products were dissolved with ethyl acetate and applied to thin-layer chromatography using a solvent of 95% chloroform and 5% methanol. CAT activity was measured by PhosphorImager (Molecular Dynamics, Sunny Vale, CA).

Electrophoretic mobility shift assay Electrophoretic mobility shift assay analysis was performed as described previously (Cooney *et al.*, 1993). The oligonucleotides were 5' end labeled with a γ-³²P adenosine triphosphate (specific activity = 6000 Ci per mmol) (New England Nuclear, Beverly, MA) by T4 polynucleotide kinase to 2–8 × 10⁸ cpm per µg. Labeled oligonucleotides (0.1 or 0.001 ng) were incubated with *in vitro* translated proteins, in the presence or absence of unlabeled oligonucleotide competitor, for 15 min at room temperature. The binding buffer contained 25 mM Tris-HCl (pH 8.0), 10% glycerol, 50 mM KCl, 2 mM EDTA, 0.05% TritonX-100, 5 mM dithiothreitol, 1% β-mercaptoethanol, 5 ng per ml bovine serum albumin, 1.5 units per ml aprotinin, and 1 µg poly(dI.dC) (Pharmacia, Piscataway, NJ). DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel at 4°C in 0.5 × TBE buffer (1 × TBE = 0.09 M Tris-borate and 0.002 M EDTA). Gels were fixed in 50% ethanol and 10% acetic acid for 30 min, followed by drying. The radioactive gels were analyzed by autoradiography. As a probe, we used the double-stranded oligonucleotides of RARE (GGTTCACCGAAAGTTCA) from the promoter region of RARβ (Sucov *et al.*, 1990).

Western blot analysis Before treatment with atRA and 1,25-(OH)₂D₃, we maintained the cells for 1 wk in Dulbecco's modified Eagle's medium containing 10% charcoal-stripped fetal bovine serum. Then 10^{–6} and 10^{–8} M atRA or 10^{–7} and 10^{–10} M 1,25-(OH)₂D₃ were added to subconfluent HaCaT keratinocytes cultured on 100 mm dishes. At the indicated time after treatment, keratinocytes were washed twice with ice-cold phosphate-buffered saline (–) and extracted with an extraction buffer (1% NP-40, 1 µg per ml aprotinin, and 400 mM NaCl in 20 mM HEPES, pH 7.2). Protein concentration was determined using Bradford reagent (Bio-Rad). Twenty micrograms per lane of nuclear extract and cell lysate protein from keratinocytes were loaded on to 10% polyacrylamide gel, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was soaked overnight in 5% skimmed milk in phosphate-buffered saline (–)/0.05% Tween at 4°C, and then incubated with anti-TR4 polyclonal antibodies at a 1:5000 dilution in 5% skimmed milk in phosphate-buffered saline (–)/0.05% Tween for 2 h at room temperature. After being washed three times at intervals of 10 min with phosphate-buffered saline (–)/0.05% Tween, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) at a 1:2000 dilution for 1 h at room temperature. Detection was performed with Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). Western blot analysis for MCF-7 cells was performed using the same procedure. For analysis of the expression level of TR4 protein after Ca²⁺ switch, normal human keratinocytes were first cultured in KGM with 0.15 mM Ca²⁺. When the keratinocyte cultures reached subconfluency, the Ca²⁺ concentration was increased to 1.8 mM. The cells were harvested at 0, 24, 48, and 72 h after increasing Ca²⁺ concentration. The cells were pelleted by centrifugation, and lysed in 2% sodium dodecyl sulfate. The lysate was sonicated and the protein concentration was measured (Gibson *et al.*, 1996). Twenty micrograms of protein were subjected to western blot analysis as described above. The expression amounts of TR4 were quantitated by collage image analysis software (Fotodyne, Hartland, WI).

RESULTS

TR4 protein is expressed in HaCaT keratinocytes Western blot analysis was performed to examine whether TR4 is expressed in human HaCaT keratinocytes. *In vitro* translated TR4 protein was used as a positive control (**Fig 1a, lane 2**). The TR4 protein with the molecular weight of 67 kDa was detected with polyclonal antibodies against TR4 in the cell lysates (**Fig 1a, lane 1**) as well as in the nuclear extracts (**Fig 1b**). With preimmune serum, these bands were not detected (data not shown). The specificity of this immunoblot with polyclonal antibodies against TR4 was confirmed in western blot analysis of MCF-7 cells transiently transfected with pCMX-TR4 or pCMX (mock). The 67 kDa band was detected only in the cells transfected with pCMX-TR4 (**Fig 1c**).

TR4 suppresses RA-induced transactivation in HaCaT keratinocytes To study the potential roles of TR4 in keratinocytes, we used CAT reporter gene assays to examine the effect of TR4 on RA-induced and vitamin D-induced transactivation in HaCaT keratinocytes. Previously, it was reported that keratinocytes express endogenous retinoid receptors capable of specifically activating RARE but not retinoid X response element (Xiao *et al.*, 1995). Therefore, to assay the RA-induced transactivation, we used the pRARβ-CAT reporter plasmid, which contains the synthetic

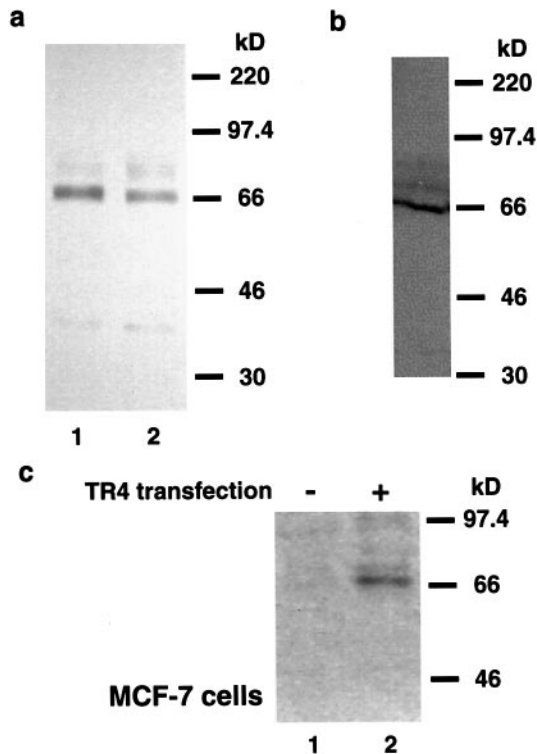


Figure 1. TR4 protein is expressed in HaCaT keratinocytes. (a) Twenty micrograms of cell lysate (lane 1) from subconfluent HaCaT keratinocytes, and 5 μ l of TNT-translated TR4 (lane 2) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting, followed by immunoblotting with polyclonal antibodies against TR4. (b) Twenty micrograms of nuclear extracts from HaCaT keratinocytes were treated as (a). (c) Twenty micrograms of cell lysates from MCF-7 cells transiently transfected with pCMV (lane 1) or pCMV-TR4 (lane 2) were treated as (a). Bars on the left margin represent the molecular mass marker in kilodaltons.

RARE (GGTTCAccgaaAGTTCA) sequence obtained from the promoter region of RAR β (Sucov *et al*, 1990). The reporter plasmid for a vitamin D-induced transactivation assay was P450cc24-CAT, which contains the 5'-flanking region (-2200 to +188) of the rat vitamin D₃ 24-hydroxylase gene (Ohyama *et al*, 1994). Without transfection of exogenous RA receptors and vitamin D receptor, we analyzed ligand-induced transactivation of CAT reporter plasmid via endogenous cognate receptors in HaCaT keratinocytes. We observed a 9.9-fold induction with a pharmacologic concentration (10^{-6} M) of atRA (Rollman and Vahlquist, 1983) in the absence of TR4 expression vector (Fig 2, lane 1 vs 2). Co-transfection of 1 and 2 μ g of pCMX-TR4 decreased this CAT activity by 23.3% and 47.3%, respectively (Fig 2, lanes 3 and 4). Although we detected a 38.8-fold induction with a supraphysiologic concentration (10^{-7} M) of 1,25-(OH)₂D₃ (Itin *et al*, 1994) without exogenous TR4, cotransfection of 2 μ g of pCMX-TR4 did not have a significant effect on CAT activity (Fig 2, lanes 9-11). In addition, we examined the effect of TR4 on these CAT activities induced by the physiologic concentration of atRA (10^{-8} M) (De Leenheer *et al*, 1982) and 1,25-(OH)₂D₃ (10^{-10} M) (Bouillon, 1983). Likewise, cotransfection of 1 and 2 μ g of pCMX-TR4 reduced the 10^{-8} M atRA-induced CAT activity by 45.0% and 59.7%, respectively (Fig 2, lanes 5-8); however, 10^{-10} M 1,25-(OH)₂D₃-induced transactivation could not be significantly changed by the transfection with 2 μ g of pCMX-TR4 (Fig 2, lanes 12-14). Co-transfection of pCMX-TR4 had no effect on the basal (ligand-independent) CAT activity (data not shown). These results indicated that TR4 can suppress RA-induced, but not vitamin D-induced transactivation in human HaCaT keratinocytes.

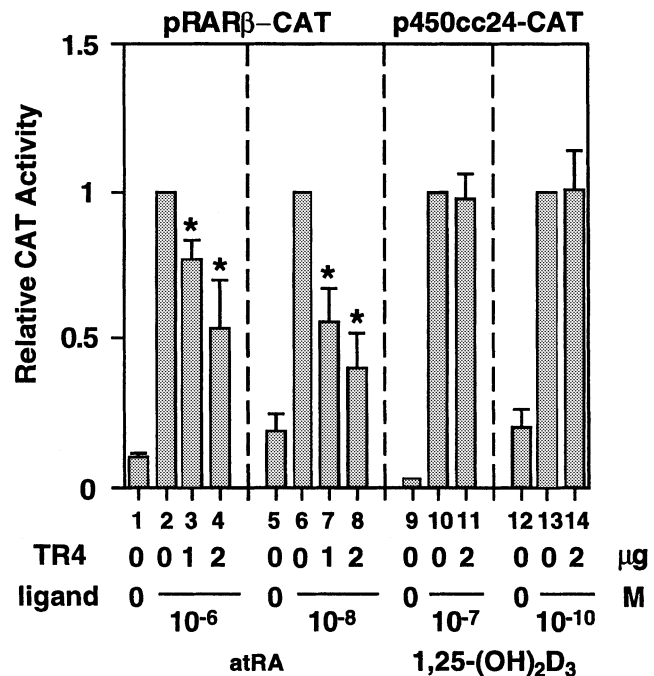


Figure 2. TR4 can suppress RA-induced pRAR β -CAT, but not vitamin D-induced P450cc24-CAT reporter activity. The reporter plasmids (4 μ g of pRAR β -CAT and P450cc24-CAT) were transfected into HaCaT keratinocytes in the presence of the indicated amount of pCMX-TR4 plasmid. The cells were treated with 10^{-6} and 10^{-8} M atRA, 10^{-7} and 10^{-10} M 1,25-(OH)₂D₃, or ethanol only, as indicated. CAT activities were determined and expressed as relative activity of each reporter in the presence of atRA or 1,25-(OH)₂D₃ but in the absence of pCMX-TR4 plasmid. Error bars, mean \pm SD from three independent experiments. *Significant difference from the control (lanes 2 or 6), $p < 0.01$.

TR4 binds to RARE with higher affinity than RAR/RXR To test whether heterodimer formation between TR4 and RAR or RXR could be the possible mechanism for TR4 suppression of RA-induced transactivation, we performed electrophoretic mobility shift assays. The RARE sequence, GGTTCAccgaaAGTTCA, obtained from the promoter region of RAR β (Sucov *et al*, 1990) was used as a probe. *In vitro* translated TR4 orphan receptor binds specifically to RARE with one clear band shift (Fig 3a, lane 3 vs lane 9; open triangle). This shifted band could be competed out by the further addition of a 100-fold excess of unlabeled RARE (data not shown). When *in vitro* translated TR4 was replaced by either *in vitro* translated RAR α (Fig 3a, lane 1) or RXR α (Fig 3a, lane 2), no specific band shift was observed. A clear band shift appeared, however, when we added both *in vitro* translated RAR α and RXR α (Fig 3a, lane 4; closed arrow). This band was quite different from that of the TR4-RARE complex, which migrates at a much higher position (Fig 3a, lane 3 vs lane 4). When *in vitro* translated TR4 was incubated together with *in vitro* translated RAR α /RXR α , two distinct shift bands could be observed, one for TR4 and another for RAR α /RXR α . There was no third shift band of heterodimer between TR4 and RAR α /RXR α (Fig 3a, lanes 5-8). These data indicated no significant heterodimer formation between TR4 and RAR α and/or RXR α . Next, we compared the binding affinity of TR4 and RAR/RXR to RARE. Consistent with Fig 3a, both *in vitro* translated TR4 and RAR α /RXR α can bind to 0.1 ng RARE (Fig 3b, lanes 1 and 2). When 0.001 ng RARE probe was incubated with *in vitro* translated TR4 and RAR α /RXR α , however, only TR4 can bind to RARE (Fig 3b, lane 3). These results suggest that TR4 can suppress RAR/RXR activity through binding to RARE with a higher affinity than RAR/RXR.

RA upregulates the expression level of TR4 protein in HaCaT keratinocytes In order to investigate whether TR4 can

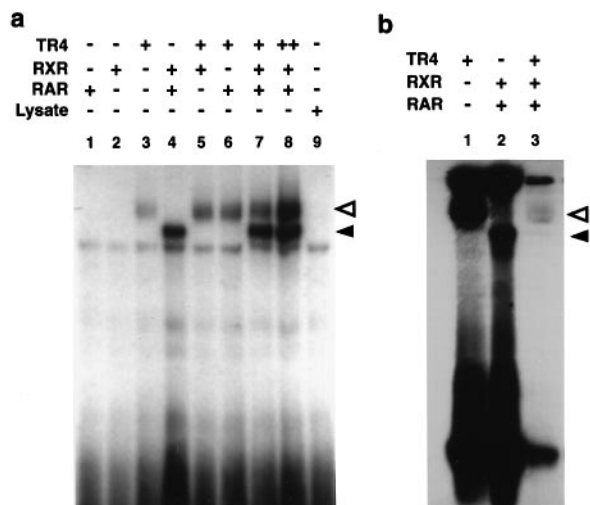


Figure 3. TR4 does not form a heterodimer with RAR/RXR but binds to RARE-RAR β with higher affinity than RAR/RXR. (a) *In vitro* translated TR4 (2 μ l), RAR α (2 μ l), and RXR α (1 μ l) were incubated with 0.1 ng 32 P-labeled RARE-RAR β oligomer and analyzed by an electrophoretic mobility shift assay. The amount of TR4 was increased to 4 μ l in lane 8. Rabbit reticulocyte lysate (2 μ g) was used as a control (lane 9). The closed triangle indicates the RARE-protein complex formed by RAR/RXR heterodimer, and the open triangle, by TR4. The gel was exposed to a X-ray film overnight. (b) *In vitro* translated TR4 (2 μ l), RAR α (2 μ l), and RXR α (1 μ l) were incubated with 0.1 ng 32 P-labeled RARE-RAR β oligomer and analyzed by an electrophoretic mobility shift assay (lanes 1 and 2). The amount of 32 P-labeled RARE-RAR β oligomer was decreased to 0.001 ng in lane 3. The closed and open triangles indicate the RARE-protein complex formed by RAR/RXR heterodimer and TR4, respectively. The gel was exposed to a X-ray film for 5 d to detect the band shift in lane 3.

act as a negative feedback regulator, we tested the effect of RA on the expression level of TR4 protein using western blot analysis. Following treatment with atRA at a pharmacologic concentration (10^{-6} M), TR4 protein expression increased ≈ 2.03 -fold and 2.94-fold at 12 and 24 h, respectively, suggesting that TR4 can act as a strong negative feedback modulator for RA in human HaCaT keratinocytes (Fig 4a). At a physiologic concentration of atRA (10^{-8} M), the TR4 protein expression level in HaCaT keratinocytes was increased to 2.56-fold and 3.15-fold at 6 and 12 h, respectively. TR4 protein level returned to the basal level at 24 h, however (Fig 4b). These results suggest that TR4 can function as a relatively short-acting regulator at the physiologic concentration (10^{-8} M), as compared with the pharmacologic concentration (10^{-6} M). For the control, we also tested the effect of vitamin D. Results showed that TR4 protein expression was not changed by treatment of HaCaT keratinocytes with either the supraphysiologic or physiologic concentrations (10^{-7} or 10^{-10} M) of 1,25-(OH) $_2$ D $_3$ (data not shown).

TR4 protein level in normal human keratinocytes is increased after switching from low to high Ca $^{2+}$ medium

As our data above indicated that TR4 could act as a negative feedback regulator for RA signaling in keratinocytes and RA has been implicated in major roles in cell differentiation, it was of interest to determine whether there is any change of TR4 protein expression in normal human keratinocytes by switching from low to high Ca $^{2+}$ medium. At first, normal human keratinocytes were cultured in KGM with 0.15 mM Ca $^{2+}$, which favors proliferation. When the keratinocyte cultures reached subconfluency, the Ca $^{2+}$ concentration was increased to 1.8 mM. The cells were harvested at 0, 24, 48, and 72 h after increasing Ca $^{2+}$ concentration, followed by cell lysis and western blot analysis. At 24 and 48 h, TR4 protein expression increased to 2.56-fold and 2.04-fold, respectively, relative to low Ca $^{2+}$ cultures, indicating that TR4 in normal human

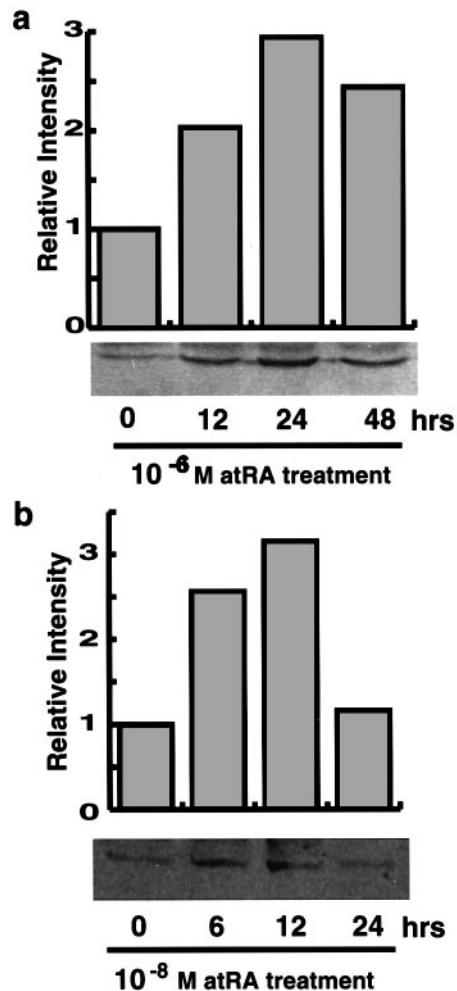


Figure 4. RA upregulates the expression level of TR4 protein. (a) At 0, 12, 24, and 48 h after the addition of 10^{-6} M atRA, HaCaT keratinocytes were harvested, lysed, and 20 μ g of cell lysates were subjected to western blot analysis. (b) At 0, 6, 12, and 24 h after the addition of 10^{-8} M atRA, HaCaT keratinocytes were subjected to western blot analysis same as (a). Each upper graph is the quantitation of each band in the lower immunoblot.

keratinocytes is upregulated by increasing the Ca $^{2+}$ concentration in the medium (Fig 5).

DISCUSSION

RA is essential for normal skin development and function. RA inhibits the induction of differentiation-related markers, such as keratins 1 and 10 (Fuchs and Green, 1981), involucrin (Watt and Green, 1981), filaggrin (Asselineau *et al*, 1990), and transglutaminase (Goldsmith, 1983; Jetten, 1990; Polakowska *et al*, 1991) in human keratinocytes cultured *in vitro*. Transgenic mouse models demonstrated that targeted overexpression of dominant-negative RAR α in suprabasal keratinocytes may result in the defect of lipid deposition and corneal barrier (Imakado *et al*, 1995), and that overexpression of dominant-negative RAR α in basal keratinocytes may also result in dramatic suppression of epidermal maturation (Saitou *et al*, 1995). Fisher and Voorhees, however, argued that these abnormalities could be due to sequestration of RXR, because mutant RAR used in these studies are capable of heterodimerizing with RXR (Fisher and Voorhees, 1996). Thus, much remains to be learned about the physiologic role of RA signaling in the skin.

In this study we proposed a new pathway to modulate RA function in keratinocytes, providing a novel approach for studying RA biology in human skin. The response of keratinocytes to RA is largely different between *in vitro* culture and *in vivo* (Bikle and

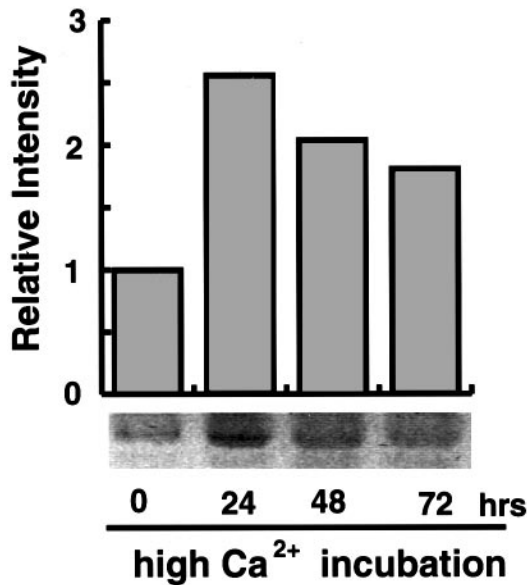


Figure 5. TR4 protein is increased by increasing Ca^{2+} concentration in the medium. At 0, 24, 48, and 72 h after incubating in 1.8 mM Ca^{2+} , normal human keratinocytes were harvested. Twenty micrograms of cell lysates were subjected to western blot analysis. The upper graph is the quantitation of each band in the lower immunoblot.

Pillai, 1993; Fisher and Voorhees, 1996) and modulation of epidermal morphogenesis or differentiation by RA can also be different between the fetal and the neonatal keratinocytes in the physiologic skin equivalent (Haake and Cooklis, 1997). Our finding of bidirectional regulation of TR4 and RA may represent a new pathway, which may help to explain the diversity of RA function in the skin.

Recently, rabbit TR4 was cloned from a rabbit heart cDNA library and the data suggested that rabbit TR4 acts as a repressor in the estrogen receptor-mediated signaling pathway in a bone cell-specific manner (Harada *et al*, 1998). Our results showed that TR4 in keratinocytes can suppress RA, but not vitamin D action. The differential suppression of RA- versus vitamin D-mediated transactivation in keratinocytes is different from our unpublished data (Lee and Chang, submitted, 1999), which suggested that TR4 can repress the vitamin D-mediated transactivation in Chinese hamster ovary cells. In addition, our observation (Lee and Chang, in preparation) suggested that *in vitro* translated TR4 can bind to vitamin D response element in an electrophoretic mobility shift assay. The difference between the results in keratinocytes and Chinese hamster ovary cells may be due to cell type specificity of TR4 function. From recent studies regarding nuclear receptors, it is speculated that the protein environment in the cell, including the interaction with coactivators or corepressors, and basic transcriptional apparatus, greatly influences nuclear receptor function. We have cloned a possible TR4 associated protein gene by yeast two hybrid system (Chang, unpublished data, 1999), which may be useful to clarify this question.

From a clinical aspect, RA is widely used as a drug for many skin diseases, such as psoriasis, hyperkeratosis, sun-damaged skin (skin aging), and acne (Nagpal *et al*, 1996; Cunliffe *et al*, 1997; Gilchrist, 1997). Because of mutual regulation between TR4 and RA signaling, TR4 will be a potential target to study for elucidation of pathologic mechanisms and may allow us to develop new therapies for these skin diseases. For example, the expression level of RAR is not altered in psoriasis (Elder *et al*, 1991), suggesting that a regulator for RA responsiveness in the skin, such as TR4, might be of importance for its pathogenesis.

In this report, we compared the effect of pharmacologic and physiologic concentrations (10^{-6} M and 10^{-8} M) of RA on TR4 protein expression levels. At the physiologic concentration, the

TR4 level returned more quickly to the basal level than that at the pharmacologic concentration. This relatively quick return might be due to the negative autoregulation of atRA by enzymes, such as lecithin:retinol acyltransferase and atRA 4-hydroxylase (Fisher and Voorhees, 1996). Together, these results suggest that TR4 may act as a relatively short-acting negative feedback regulator at the physiologic concentration of atRA, compared with that at the pharmacologic concentration. From our results, 10^{-6} and 10^{-8} M RA differentially modulate TR4 protein expression level. Recently it has been shown that 10^{-6} and 10^{-8} M RA differentially regulate transglutaminase in human keratinocytes (Gibson *et al*, 1998).

Although, among RARs, nearly 90% is RAR γ with 10% RAR α in the epidermis (Fisher *et al*, 1994), we used RAR α /RXR α to compare the binding affinity of TR4 and RAR/RXR with the synthetic RARE, obtained from the promoter region of RAR β (Fig 3). Based on the finding that RAR α , β , and γ bind with similar affinities to this response element (Vasios *et al*, 1991), our results, however, can indicate that TR4 binds to RARE with higher affinity than RAR/RXR.

The increase in the TR4 level by Ca^{2+} switch indicates that TR4 could be involved in keratinocyte terminal differentiation. It is reasonable to speculate that TR4 may influence differentiation by inhibiting RA signaling, as RA causes suppression of terminal differentiation. In addition, TR4 may regulate directly the expression of differentiation-related genes. Alternatively, because TR4 acts as a negative feedback suppressor for RA, TR4 might silently maintain the homeostasis of keratinocytes. The significance of these observations, however, remains to be investigated further. In particular, the identification of the true ligands for TR4 would facilitate new approaches to this question.

In epidermis with differentiation, calcium concentration is low in the basal and spinous layers, but increases to its highest level in the granular layers (Bikle and Pillai, 1993). This gradient *in vivo* indicates an important role of calcium in keratinocyte differentiation. The calcium switch in the medium, however, does not recapitulate the fully differentiated state characteristic of skin *in vivo*. Additionally, as mentioned above, the effect of RA on differentiation markers is different between *in vitro* culture and *in vivo* (Rosenthal *et al*, 1990, 1992; Fisher and Voorhees, 1996), indicating that responses of *in vitro* keratinocytes may not always be true *in vivo*. Therefore, the function and regulation of TR4 during RA treatment and differentiation needs to be further investigated by *in vivo* studies. We, however, believe that our discovery of the novel negative regulation of retinoid action in keratinocytes *in vitro* is unique and significant.

In conclusion, our data demonstrated that an orphan receptor, such as TR4, may play an important part in the signaling pathway in human keratinocytes, possibly providing new insights into keratinocyte biology.

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