Modulation of Keratinocyte Gene Expression and Differentiation by PPAR-Selective Ligands and Tetradecylthioacetic Acid

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Peroxisome proliferator-activated receptors (PPARs) are pleiotropic regulators of growth and differentiation of many cell types. We have performed a comprehensive analysis of the expression of PPARs, transcriptional cofactors, and marker genes during differentiation of normal human keratinocytes using a combination of reverse transcriptase polymerase chain reaction, Northern and Western blotting, and immunohistochemistry. PPARδ was the predominant PPAR subtype in human keratinocytes and highly expressed in basal cells and suprabasal cells. Induction of PPARα and PPARγ expression was linked to differentiation, and accordingly, expression of PPARα and PPARγ was in essence confined to suprabasal cells. Differentiation was not accompanied by significant changes in the expression of the coactivators CREB-binding protein, p300, steroid receptor coactivator 1, or the corepressors nuclear receptor corepressor and silence mediator for retinoid and thyroid hormone receptors. We critically evaluated the effects of selective PPAR ligands and a synthetic fatty acid analog, tetradecylthioacetic acid. Tetradecylthioacetic acid activated all human PPAR subtypes in the ranking order PPARδ >> PPARα > PPARγ. All selective PPAR ligands marginally induced transglutaminase-1 expression with the PPARδ-selective ligand L165041 being the most potent. The PPARα- and PPARγ-selective ligands Wy14643 and BRL49653 had negligible effect on involucrin expression, whereas a dose-dependent induction was observed with L165041. Simultaneous addition of L165041 and BRL49653 synergistically induced strong involucrin expression. Additionally, L165041 potently induced CD36 mRNA expression. Administration of tetradecylthioacetic acid resulted in a dramatic decrease in proliferation and a robust upregulation of the expression of involucrin and transglutaminase. Our results indicate that tetradecylthioacetic acid may affect keratinocyte gene expression and differentiation via PPAR-dependent and PPAR-independent pathways, and that the latter play an important role. *Key words: CD36/FAT/involucrin/MAPK/PPARs/transglutaminase-1. J Invest Dermatol 116:702–712, 2001*

The epidermis is a stratified squamous epithelium in which the basal layer is composed of progenitor cells that undergo a highly sequential differentiation program as they leave the basal membrane and rise through the suprabasal layers. Each step of differentiation is characterized by the expression of specific marker genes (Fuchs, 1990). The transition from the spinous to the granular layer is accompanied by upregulation of genes encoding structural proteins of the cornified envelope such as involucrin (Inv) and later transglutaminase-1 (Tg-1) (Rice and Green, 1979; Thacher and Rice, 1985). Although the stages of differentiation with their concomitant changes in marker gene expression are well characterized, relatively little is known about differentiation-specific transcription factors that determine the expression of specific marker genes during differentiation.

Substantial evidence has accumulated implicating classical nuclear hormone receptors such as the retinoid and vitamin D receptors in the regulation of epidermal differentiation and function (Pillai and Bikle, 1991; Itin *et al*, 1994; Fisher and Voorhees, 1996). Studies have shown that the retinoid receptors are important for epidermal development. Thus, ligands of the retinoic acid receptor (RAR) and the vitamin D receptor modulate epidermal differentiation and proliferation (Hosomi *et al*, 1983; Floyd and Jetten, 1989; Pillai and Bikle, 1991), and overexpression of a dominant negative mutant of RARα in the basal layer of epidermis resulted in a thin epidermis and scaly skin (Saitou *et al*, 1995). In addition,

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Abbreviations: ALBP, adipocyte lipid-binding protein; CBP, CREB-binding protein; E-FABP, epidermal fatty acid binding protein; ERK, extracellular signal-regulated kinase; Inv, involucrin; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, thiazolyl blue; NCoR, nuclear receptor corepressor; OPD, o-phenylenediamine; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RAR, retinoic acid receptor; RXR, retinoid × receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SRC-1, steroid receptor coactivator 1; TBP, TATA binding protein; Tg-1, transglutaminase-1; TTA, tetradecylthioacetic acid.

targeting of the dominant negative RARα to the suprabasal layer led to impaired formation of lamellar membranes, and the extracellular stratum corneum membranes appeared thinner and more loosely packed (Imakado *et al*, 1995). Recently, members of the peroxisome proliferator-activated receptor (PPAR) subfamily and the oxysterol-responsive liver x receptor have been implicated in the control of epidermal differentiation and barrier formation (Hanley *et al*, 1999; Komuves *et al*, 1998, 2000a, b).

The PPAR family comprises three subtypes PPARα, PPARγ, and PPARδ. All three subtypes are expressed in human keratinocytes (Rivier *et al*, 1998). The PPARs serve as pleiotropic regulators of cellular proliferation, differentiation, and homeostasis, and numerous studies have revealed the importance of PPARα and PPARγ in the control of lipid homeostasis (for reviews, see Kersten *et al*, 2000; Willson *et al*, 2000). In contrast, the biologic significance of PPARδ has remained more elusive. PPARδ has been associated with embryo implantation (Lim *et al*, 1999), colonocyte growth (He *et al*, 1999), and cholesterol metabolism (Leibowitz *et al*, 2000), and recently the phenotype of PPARδ-deficient mice was reported (Peters *et al*, 2000). Interestingly, PPARδ-deficient mice exhibit an exacerbated hyperplastic response to topical application of 12-O-tetradecanoyl-phorbol-13-acetate on the skin, suggesting that lack of PPARδ influences control of keratinocyte proliferation and/or differentiation (Peters *et al*, 2000).

The epidermis represents a tissue with high rates of fatty acid and cholesterol metabolism where accumulation and deposition of cholesterol, fatty acids, and sphingolipids constitute an integral part of the terminal epidermal differentiation program culminating in the formation of a competent epidermal barrier (Hanley *et al*, 1997, 1999). Recent work has emphasized the importance of PPARα in keratinocyte differentiation and barrier formation. Administration of known PPARα agonists was found to stimulate rodent keratinocyte differentiation *ex vivo* (Hanley *et al*, 1998) and to accelerate development of the fetal epidermal permeability barrier (Hanley *et al*, 1997, 1999). PPARα activators profoundly influenced lipid metabolism in reconstructed epidermis (Rivier *et al*, 2000), and recently it was demonstrated that topical treatment with PPAR ligands promoted differentiation and reversed induced hyperproliferation of murine epidermis (Komuves *et al*, 2000a, b). Finally, it should be noted that the farnesoid X receptor ligands farnesol and juvenile hormone III were shown to stimulate keratinocyte differentiation in a PPARα-dependent manner (Hanley *et al*, 2000a). These findings point to PPARα as an important regulator of epidermal differentiation and homeostasis. From the analysis of PPARα-deficient mice it is well documented that the effects of PPARα agonists on proliferation and expression of marker genes requires PPARα (Komuves *et al*, 2000a). No (Lee *et al*, 1995) or only minor (Komuves *et al*, 2000a) defects in epidermis development and function have been reported in PPARα-deficient mice, however. Of interest, systemic administration of the PPARγ-selective ligand troglitazone was reported to ameliorate the clinical symptoms of chronic psoriasis and reverse the abnormal phenotype of transplanted psoriatic skin (Ellis *et al*, 2000). Yet, administration of PPARγ ligands to keratinocytes *ex vivo* has consistently been reported not to influence differentiation (Hanley *et al*, 1997, 1998). Taken together, these findings suggest that a critical evaluation of the role of the PPAR family in keratinocyte differentiation and function would be warranted.

In this study we have analyzed in detail the expression of the PPARs during *ex vivo* differentiation of human keratinocytes, in isolated basal and suprabasal cells, and in sections of human skin. Using concentrations of PPAR subtype selective ligands that exclusively targeted the appropriate PPAR subtype we present evidence that PPARα- and PPARγ-selective ligands have negligible effect on keratinocyte marker gene expression. Interestingly, the PPARδ-selective ligand L165041 induced expression of Inv in a dose-dependent manner. All three PPAR subtype selective ligands either alone or in combination only modestly affected keratinocyte proliferation. In contrast, the thiasubstituted fatty acid tetradecylthioacetic acid (TTA) strongly induced expression of keratinocyte differentiation marker genes and exerted a profound antiproliferative action.

Administration of TTA to rats has been shown to induce marked changes in the profile of liver and plasma lipids (Frøyland *et al*, 1997; Madsen *et al*, 1997). In this report we show that TTA is a potent activator of human PPARδ and in addition a weak activator of human PPARα and PPARγ. The potency of TTA as an antiproliferative agent and inducer of keratinocyte marker gene expression, however, by far exceeded its efficacy and potency as a pan PPAR ligand and could not be recapitulated by combined addition of PPAR-selective ligands. We suggest that the previously observed effects on keratinocyte differentiation and function associated with the administration of high concentrations of PPAR ligands in part relate to changes in lipid metabolism and/or accumulation of secondary lipid mediators rather than direct activation of PPAR-mediated transactivation. Furthermore, our results suggest that TTA may hold promise as an interesting compound for the treatment of various epidermal disorders characterized by hyperproliferation and aberrant differentiation.

MATERIALS AND METHODS

Cell culture and differentiation Keratinocytes from normal adult human skin specimens were isolated after plastic surgery. First passage keratinocytes were grown in keratinocyte serum-free medium (Keratinocyte-SFM; Gibco BRL/Life Technologies, Gaithersburg, MD) supplemented with 50 μg per ml bovine pituitary extract, 5 ng per ml human recombinant epidermal growth factor, and 5 μg per ml gentamycin and replated in 75 cm^2 culture flasks (Nunc, Roskilde, Denmark) or at a density of 3500 cells per well in 96-well microtiter plates (Nunc) preheated at 37°C. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. When cells reached 30%–40% confluency they were treated with Keratinocyte-SFM containing selective PPAR ligands (either alone or in combination as indicated), TTA, or 1.2 mM $CaCl_2$. Wy14643 was obtained from Calbiochem, BRL49653 was kindly provided by J. Fleckner (Novo Nordisk, Bagsværd, Denmark), L165041 was kindly provided by D.E. Moller (Merck Research Laboratories, Rahway, NJ), and TTA was prepared as previously described (Spydevold and Bremer, 1989). Cells not treated with PPAR ligands or TTA received a similar volume of vehicle [0.1% dimethylsulfoxide (DMSO)]. Medium was changed every day. To induce differentiation, keratinocytes at 30%–40% confluency (day 0) were treated with 1.2 mM $CaCl_2$. HaCaT cells were obtained from L. Aarenstrup (Danish Cancer Society, Denmark). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL/Life Technologies) with 10% fetal bovine serum (FBS) and antibiotics (62.5 μg per ml penicillin, 100 μg per ml streptomycin sulfate) in a humidified atmosphere of 5% CO_2 at 37°C.

Separation of keratinocytes into basal and suprabasal cells Normal adult skin specimens obtained from plastic surgery were cleaned from fat and cut through the epidermal side using a scapula. The skin specimens were incubated overnight on ice with 25 U per ml dispase II (Roche, Mannheim, Germany) in Hanks' buffered saline solution. The epidermis was peeled off using a pincer and the epidermal sheets were incubated in 0.05 mg per ml trypsin (Gibco BRL/Life Technologies) at 37°C until single cells were released. Trypsin activity was inhibited by the addition of serum-containing medium. The cells were centrifuged at 1200*g and* resuspended in preheated Keratinocyte-SFM. The cell suspension was added to tissue culture flasks coated with rat tail collagen (Macklis *et al*, 1985). After 1 h nonattached cells were collected as the suprabasal fraction and attaching basal cells were collected using a rubber policeman. Cell pellets were frozen at –70°C until use.

Determination of viability and proliferation Viability/proliferation was measured using a modified thiazolyl blue (MTT) assay (Mosmann, 1983). Twenty-five microliters of 5 mg per ml MTT in phosphate-buffered saline (PBS) (NaCl 8 g per liter, KCl 0.2 g per liter, $Na_2HPO_4 \cdot 2H_2O$ 1.44 g per liter, KH_2PO_4 0.2 g per liter, pH 7.4) were added to each well, and plates were placed in an incubator until the growing crystals penetrated the cell walls, typically after 3–4 h. Plates were flicked to remove medium and freeze thawed twice before formazan crystals were solubilized in ethanol:acetone (60:40 wt/wt) by gentle shaking for 30 min at 4°C. The amount of formazan was

quantified in an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. Background values at 650 nm were subtracted.

Determination of Tg-1 expression by ELISA Cells were subjected to two freeze–thaw cycles and Tg-1 was determined by ELISA (Michel *et al*, 1991). Each well was blocked with 200 μl 1% bovine serum albumin in PBS for 1 h at 37°C and then incubated for 1 h at 37°C with 100 μl Tg-1-specific monoclonal antibody B.C1 (Biomedical Technologies, Stoughton, MA) diluted 1:1000 in PBS containing 1% bovine serum albumin. Wells were washed three times for 5 min in PBS containing 0.05% Tween 20 and incubated for 1 h with 100 μl secondary horseradish peroxidase conjugated goat-antimouse antibody (DAKO, Glostrup, Denmark) diluted 1:2500 in PBS containing 1% bovine serum albumin. The wells were washed three times with PBS containing 0.05% Tween 20, and once with PBS. A hundred microliters of o-phenylenediamine (OPD) substrate was added, and after 30 min in the dark, reactions were stopped with 100 μl 2 N sulfuric acid. The amount of Tg-1 was measured by quantifying the OPD reaction with an ELISA reader at 490 nm and subtracting background at 650 nm. All values were normalized to the results of the viability assays to correct for variation in cell number (Svendsen *et al*, 1997).

Plasmids The hPPARα cDNA was subcloned from pCR2.1hPPARα (kindly provided by J. Fleckner, Novo Nordisk, Denmark) by partial digestion with *Xba*I and *BamH*I and inserted into the *Xba*I/*BamH*I sites of the pcDNA3.1(+) mammalian expression vector (Invitrogen, San Diego, CA), generating pcDNA3.1(+)hPPARα. The hPPARδ and hPPARγ1 cDNAs were excised from pCR2.1hPPARδ and pCR2.1hPPARγ1 (kindly provided by J. Fleckner) using *Xba*I/*Xho*I and inserted into the *Xba*I/*Xho*I sites of pcDNA3.1(+), generating pcDNA3.1(+)hPPARδ and pcDNA3.1(+)hPPARγ1. The pcDNA3.1(+)hPPARγ2 plasmid was constructed by amplification of the hPPARγ2 cDNA from pSG5 hPPARγ2 (kindly provided by A. Elbrecht, Merck Research Laboratories, Rahway, NJ) by polymerase chain reaction (PCR) using *Asp*718/*Xba*I tagged primers (upstream primer 5′-AATTGGTACCACCATGGGTG-AAACTCTGGGAGATTCTCC-3′ and downstream primer 5′-CTA-GTCTAGACTGCTAGTACAAGTCCTTG-3′) and insertion into the *Asp*718/*Xba*I sites of pcDNA3.1(+). The PPREx3-tk-luc reporter contains three copies of the peroxisome proliferator-activated receptor response element (PPRE) from the acyl-CoA oxidase promoter and was kindly provided by R.M. Evans. A CMV-β-galactosidase vector was used for normalization (Clontech, Palo Alto, CA).

Transactivation assays HaCaT cells were seeded in 12-well plates (Nunc) in DMEM containing 10% FBS. After 18 h medium was changed to DMEM containing 10% stripped bovine serum and transfections were performed using the BES-$CaCl_2$ method (Chen and Okayama, 1987). Each well received 35 μl BES reagent (pH 6.95), 35 μl 0.25 M $CaCl_2$, and 0.75 μg PPREx3-tk-luc reporter construct in combination with expression vectors for either hPPARα (0.01 μg), hPPARδ (0.05 μg), hPPARγ1 (0.05 μg), or hPPARγ2 (0.05 μg), and CMV-β-galactosidase (0.1 μg) for normalization. Empty expression vector was added to ensure equal promoter load and pBluescript(ks +) was added to a total of 1.2 μg DNA. Cells were incubated with the transfection mixture at 35°C in an atmosphere of 3% CO_2 for 10 h and washed twice in PBS. Cells were subsequently incubated for ~16 h in serum-free DMEM containing ligands as indicated or vehicle (0.1% DMSO). The luciferase and β-galactosidase activities in cell lysates were determined by standard techniques. Luciferase activities were normalized to β-galactosidase activities. Transfections were performed in duplicate, measured in duplicate, and repeated four to six times.

RNA purification Total RNA was purified according to a modified procedure of Chomczynski and Sacchi (1987). The integrity of all RNA samples was confirmed by electrophoresis in denaturing formaldehyde-containing gels.

Multiplex reverse transcriptase PCR (RT-PCR) Multiplex RT-PCR was performed essentially as described previously (Jensen *et al*, 1996; Hansen *et al*, 1999). All reactions contained the TATA binding protein (TBP) primer set as an internal standard together with one additional primer set. The following primer sets (with number of cycles and amplicon length in parentheses) were used: hPPARα upstream primer, 5′-CACAACCAGCACCATCTGGTC-3′; hPPARα downstream primer, 5′-CCCTTGCAGCCTTCACACGCGTG-3′ (28 cycles, 395 bp); hPPARδ upstream primer, 5′-GCTATGACTGGGCCTGC-AGG-3′; hPPARδ downstream primer, 5′-CCCTTGCACCCCTCA-CATGCATG-3′ (25 cycles, 331 bp); hPPARγ1 upstream primer, 5′-CCTCGAGGACACCGGAGAG-3′; hPPARγ1 downstream primer, 5′-CCCTTGCATCCTTCACAAGCATG-3′ (28 cycles, 444 bp); hPPARγ2 upstream primer, 5′-GGGTGAAACTCTGGGAGATTCTC-3′; hPPARγ2 downstream primer, 5′-CCCTTGCATCCTTCACAA-GCATG-3′ (28 cycles, 480 bp); CREB-binding protein (CBP) upstream primer, 5′-GACATGACTGTCCTGTTTGCC-3′; CBP downstream primer, 5′-GTTGTTATTCTTCCTGCTGG-3′ (25 cycles, 343 bp); p300 upstream primer, 5′-GCCTAAACTCTCATCTCCGG-3′; p300 downstream primer, 5′-TAGTGCCCATCCCCATGTTGG-3′ (25 cycles, 377 bp); nuclear receptor corepressor (NCoR) upstream primer, 5′-GGTACACAGATTACTTTCCC-3′; NCoR downstream primer, 5′-CCAAAGGTGTGATTACACTGG-3′ (25 cycles, 316 bp); silencing mediator for retinoid and thyroid hormone receptors (SMRT) upstream primer, 5′-TTCTTCAGCAAGCTGACCGAG-3′; SMRT downstream primer, 5′-AGGTGAGTGTGTGGTCACTC-3′ (28 cycles, 376 bp); steroid receptor coactivator 1 (SRC-1) upstream primer, 5′-CCTCTT-CTTTTGGAGGCTTTGG-3′; SRC-1 downstream primer, 5′-ACT-TCATAACGCTGGCAAGC-3′ (28 cycles, 320 bp); epidermal fatty acid binding protein (E-FABP) upstream primer, 5′-CCATGGCCACAG-TTCAGCAGC-3′; E-FABP downstream primer, 5′-CCAATGCACCA-TCTGTAAAGTTGCAG-3′ (25 cycles, 285 bp); CD36 upstream primer, 5′-ATTGGTGCTGTCCTGGCTGTG-3′; CD36 downstream primer, 5′-CACCATTGGGCTGCAGGAAAG-3′ (28 cycles, 316 bp); Inv upstream primer, 5′-CTCCTCAAGACTGTTCCTCC-3′; Inv downstream primer, 5′-GCAGTCATGTGCTTTTCCTCTTGC-3′ (20 cycles, 143 bp); Tg-1 upstream primer, 5′-GCGGCAGGAGTATGT-TCTTA-3′; Tg-1 downstream primer, 5′-AGGGATGTGTCTGTG-TCGTG-3′ (25 cycles, 444 bp); adipocyte lipid-binding protein (ALBP) upstream primer, 5′-GGTACCTGGCTTGTCTCC-3′; ALBP downstream primer, 5′-CCCATTTCTGCACATGTACC-3′ (20 cycles, 325 bp); TBP upstream primer, 5′-CTTCGGAGAGTTCTGGGA-TTGTACC-3′; TBP downstream primer, 5′-AATCAGTGCCGTGGT-TCGTG-3′ (176 bp). Ten microliters of each reaction was dried down and resuspended in formamide dye mix and loaded onto 0.3 mm 0.8 M urea, 1 × TBE, 6% polyacrylamide gels. Electrophoresis was for 3 h at 40 W. Gels were dried and exposed overnight to PhosphorImager storage screens. Screens were scanned in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Northern blotting Total RNA (5 μg per sample) was separated on 1.2% formaldehyde-agarose gels and transferred to Hybond N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) by capillary blotting. RNA integrity was assessed by methylene blue staining of the membrane. ^{32}P-dCMP labeled probes were generated using human PPARα, PPARδ, PPARγ, Inv, keratin 14, and γ-actin gene fragments using the Prime-It labeling Kit (Stratagene, La Jolla, CA). The probes were purified using microspin G-25 columns (Pharmacia Biotech). Hybridization with the ^{32}P-labeled probes was carried out by standard methods. Blots were exposed overnight to PhosphorImager storage screens. Screens were scanned in a PhosphorImager (Molecular Dynamics).

Production of anti-PPARδ antibodies A tagged cDNA encoding mouse PPARδ was generated by PCR and inserted into the *Bam*HI and *Sal*I sites of the bacterial expression vector pQE-11 (Qiagen, Valencia, CA). The PPARδ expression vector and a vector expressing the *Escherichia coli* chaperones GroEL and GroES were transformed into *E. coli* strain BL21 (Novagen, Madison, WI). The details of expression and purification of active PPARδ will be described elsewhere. In short, PPARδ expression was induced by addition of IPTG, and the His-tagged PPARδ was purified by cobalt-affinity chromatography. The His-tagged PPARδ was eluted with 150 mM imidazole and dialyzed against PBS (pH 7.3) overnight at 4°C. The purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the protein concentration was measured by using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

For production of polyclonal antibodies, 500 μg purified His-tagged PPARδ were mixed with 250 μl incomplete Freund's adjuvant and injected subcutaneously into a rabbit. Injections were repeated six times with intervals of 14 d. Anti-PPARδ antibodies were affinity purified from the antiserum. In short, immune serum was absorbed against GST immobilized on glutathione-sepharose beads, and anti-PPARδ antibodies were subsequently affinity purified by binding to a GST–PPARδ–A/B domain fusion protein immobilized on CNBr-activated sepharose. Bound antibodies were eluted with 0.1 mM glycine-HCl (pH 2.8) and immediately neutralized in PBS (pH 7.6). The specificity of the affinity-purified anti-PPARδ antibodies was assessed by Western blotting.

Western blotting Preparation of whole cell extracts, blotting, enhanced chemiluminescence, and stripping of membranes was performed as described previously (Hansen *et al*, 1999). Protein

concentrations were determined using the Bradford method (Bio-Rad) and confirmed by Coomassie staining of control gels. Forty micrograms of protein were separated in standard minigels and equal loading/transfer was confirmed by amidoblack staining of membranes. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20. Incubation with primary and secondary antibodies was performed in TBS containing 5% nonfat dry milk for 1.5 h. After incubation with antibodies, membranes were washed in TBS containing 0.1% Tween 20. Primary antibodies used were: mouse antihuman Tg-1 (1:500) (Biomedical Technologies), mouse antihuman Inv (1:1000) (Biogenesis, U.K.), mouse anti-PPARα (kindly provided by J. L. Su, Glaxo Wellcome, NC; Su *et al*, 1998) (1:8000), mouse anti-PPARγ (E-8; Santa Cruz Biotechnology, Santa Cruz, CA) (1:1000), rabbit anti-TBP and rabbit anti-TFIIB (C-18 and SI-1; Santa Cruz Biotechnology) (1:2000), p44/42 mitogen-activated protein kinase (MAPK) (1:1000), P-p44/42 MAPK (1:1000), Jun amino-terminal kinase (JNK) (1:500), P-JNK (1:500), p38 MAPK (1:1000), P-p38 MAPK (1:1000) (all New England Biolabs, Beverly, MA). Secondary antibodies were horseradish peroxidase conjugated antimouse or antirabbit IgG antibodies (DAKO, Glostrup, Denmark), both diluted 1:2000. Protein extracts from 293 overexpressing PPARγ2 or PPARδ were used as positive controls. 293 cells were transiently transfected in 60 mm dishes by the calcium phosphate coprecipitation technique using 10 μg of PPARγ2 or PPARδ per dish. The cells were harvested 24 h after transfection.

Immunostaining Normal human keratinocytes were grown on glass cover slips in Keratinocyte-SFM. When cells reached 20% confluency they were treated with Keratinocyte-SFM containing selective PPAR ligands as indicated, TTA, 1.2 mM $CaCl_2$, or a similar volume of vehicle (0.1% DMSO). Medium was changed every day. On day 3 cells were fixed in 4% neutral buffered formalin and incubated in blocking buffer (0.5% Blocking Reagent, Roche, 0.2% Triton) for 20 min. The permeabilized cells were incubated at room temperature for 1.5 h with either Alexa 594 (Molecular Probes, Eugene, OR), conjugated anti-Inv antibody (SY-5, 10 μg per ml) (kindly provided by F.M. Watt, ICRF, London, U.K.), or Tg-1-specific monoclonal antibody (1:50) (Biomedical Technologies). Cells incubated with Tg-1 antibody were subsequently incubated for 1 h with Alexa 488 conjugated goat antimouse IgG antibody (Molecular Probes) 1:400 in blocking buffer. Nuclei were visualized by staining with Hoechst 33258 (Hoechst, Germany). Cells were covered with mowiol (Hoechst) based mounting medium containing 2.5% DABCO (1,4-diazabicyclo-(2,2,2)-octan; Fluka, Germany) as an antifade reagent. Cells were photographed with a Photometrics Series 200 cooled CCD camera mounted on a Leica DM upright microscope using the application IPLab (Scanalytics, Fairfax, VA). The same exposure time was used and files were normalized to the same standard to enable comparison.

Immunohistochemistry Tissue was fixed in 3.7% paraformaldehyde and embedded in paraffin, and 4–6 μm thick sections were prepared. Sections were microwaved for 15 min in TEG for detection of PPARα and PPARγ or in 10 mM citrate acetate (pH 6.0) for detection of PPARδ. Blocking was performed overnight and sections were incubated with antibodies specific for PPARδ (1:500), PPARα (1:1000) (Su *et al*, 1998), or PPARγ (1:160) (E8; Santa Cruz Biotechnology) at 4°C overnight. Slides were subsequently incubated with a biotinylated goat antirabbit or antimouse antibody, and visualized using a standard streptavidin biotin (LSAB) horseradish peroxidase technique with AEC (1-amino-9-ethylcarbazol) as chromogen. The slides were counterstained in hematoxylin and mounted.

RESULTS

Expression of PPARs and cofactors in human keratinocytes Normal adult human epidermal keratinocytes were isolated from human skin obtained after plastic surgery and grown in serum-free medium. When the cells reached 30%–40% confluency, differentiation was induced by raising the concentration of $CaCl_2$ to 1.2 mM. Total RNA was isolated as indicated during the following 5 d, and the expression of the three PPAR subtypes (α, δ, and γ), cofactors involved in PPAR-mediated transactivation, and differentiation markers was determined by a combination of semiquantitative RT-PCR, Northern blotting, and Western blotting (**Fig 1**). Using RT-PCR, PPARα and PPARδ were clearly detected on day 0, whereas PPARγ was barely detectable. In agreement with previous reports (Rivier *et al*, 1998), expression of

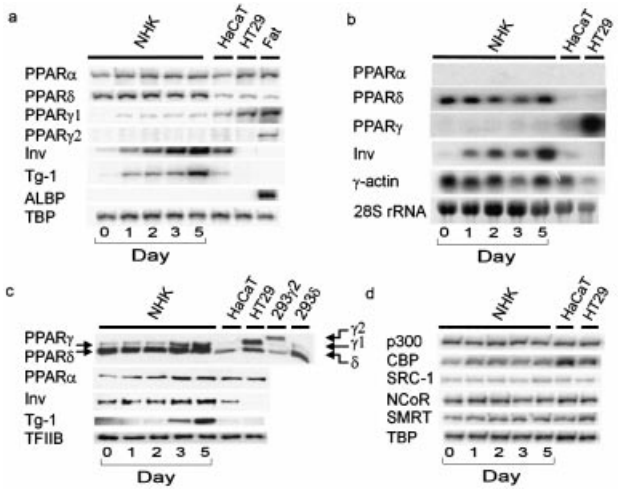

Figure 1. Expression of PPAR subtypes and PPAR cofactors during differentiation of human keratinocytes. Pre-confluent normal human keratinocytes were incubated in Keratinocyte-SFM containing high calcium (1.2 mM) and harvested on days 0, 1, 2, 3, and 5 for preparation of total RNA and whole cell extracts. Note that, generally, the intensity of signals in the individual panels can only be compared horizontally. (*a*) Multiplex RT-PCR analysis of expression of PPARα, PPARδ, PPARγ1, PPARγ2, Inv, Tg-1, aP2/ALBP, and TBP. (*b*) Northern blot analysis of total RNA (5 μg) hybridized with PPARα, PPARδ, PPARγ, Inv, and γ-actin specific probes. Equal loading, transfer, and integrity of the RNA were verified by methylene blue staining of 28S rRNA. (*c*) Whole cell protein extracts were prepared on the indicated day. 40 μg of protein were loaded in each lane. Expression of PPARα, PPARδ, PPARγ1, PPARγ2, Inv, and Tg-1 was analyzed by Western blotting. Equal loading/transfer was confirmed by amidoblack staining of membranes and by incubation with anti-TFIIB antibody. (*d*) Multiplex RT-PCR analysis of expression of p300, CBP, SRC-1, NCoR, and SMRT.

PPARα and PPARγ1 increased in concert with the increase in Inv and Tg-1 expression. Expression of PPARδ was hardly affected during differentiation. If anything, the expression of PPARδ was slightly decreased. The PPARγ2 isoform was not detectable, and this together with the fact that the lipid-binding protein aP2/ALBP (a marker for fat) was undetectable provided evidence that the cultured normal human keratinocytes were not contaminated with fat (**Fig 1*a***).

The RT-PCR analysis indicated that PPARδ is by far the most abundantly expressed PPAR subtype in normal human keratinocytes in that additional cycles of PCR were required to obtain signals from the PPARα and PPARγ mRNAs. To substantiate this notion, Northern blotting was carried out. As seen in **Fig 1(*b*)**, PPARδ is highly expressed, and in accordance with the results obtained by the RT-PCR analysis its expression level was not significantly altered during differentiation of normal human keratinocytes. In contrast, expression of both PPARα and PPARγ was barely detectable by northern blotting. PPARα mRNA was clearly detectable in cells transfected with the PPARα expression vector showing that the lack of PPARα hybridization signals reflected the low abundance of PPARα mRNA in the analyzed human cells (results not shown). Expression of the PPARδ gene is initiated from several distinct promoters giving rise to a series of different PPARδ mRNAs that vary significantly with respect to translational efficiency (L.K. Larsen, personal communication). Consequently, we examined PPAR subtype expression during differentiation of normal human keratinocytes at the protein level by Western blotting. As shown in **Fig 1(*c*)** and paralleling the results obtained by analysis of PPAR subtype mRNA levels, the PPARδ protein remained rather constant during differentiation, whereas the levels of PPARα and PPARγ1 protein increased.

As it is well established that ligand-dependent transactivation of PPARs is modulated by interactions with both coactivators and corepressors (for reviews, see Xu *et al*, 1999; Glass and Rosenfeld, 2000) we examined the expression of a panel of known PPAR coactivators (p300, CBP, and SRC-1) and corepressors (SMRT and NCoR) to determine whether their expression was altered in response to epidermal differentiation. As shown in **Fig 1**(***d***), expression of these cofactors remained remarkably constant during the course of keratinocyte differentiation.

To compare the results obtained from differentiation *ex vivo* with the *in vivo* situation, we isolated basal and suprabasal cells directly from human epidermis. Using RT-PCR (**Fig 2***a*) and northern blotting (**Fig 2***b*) we showed that PPARδ was highly expressed in both the basal and the suprabasal cell fraction with basal cells exhibiting the strongest signal. Expression of PPARα and PPARγ mRNA was close to the detection limit in basal cells, but upregulated in suprabasal cells. As it is known that the separation procedure only results in a crude separation of basal cells from suprabasal cells, expression markers specific for basal cells (keratin 14) and suprabasal cells (Inv) were used to determine the purity of each cell population. The almost complete lack of Inv expression in the basal cell fraction suggests that this fraction only had a limited contamination by suprabasal cells, whereas the clearly detectable signal from keratin 14 in the suprabasal fraction indicated contamination by basal cells. The fact that the PPARδ signal was strongest in the basal cell fraction thus indicates that basal cells exhibit the highest level of PPARδ expression. We next compared the level of expression of the panel of PPAR cofactors at the RNA level in basal and suprabasal cells. As shown in **Fig 2**(*c*) no significant differences were observed. In summary, using two different approaches we showed that PPARδ is the predominant PPAR subtype in keratinocytes with a high level of expression in basal cells. The expression of PPARα and PPARγ is considerably lower, and the pattern of expression is the reverse of that of PPARδ, i.e., low in basal cells and higher in suprabasal cells. *Ex vivo* differentiation of normal human keratinocytes largely recapitulated these findings. PPARδ expression was significant, and at most declined only marginally during differentiation, whereas expression of PPARα and PPARγ initially was at the detection limit but increased considerably during the course of differentiation.

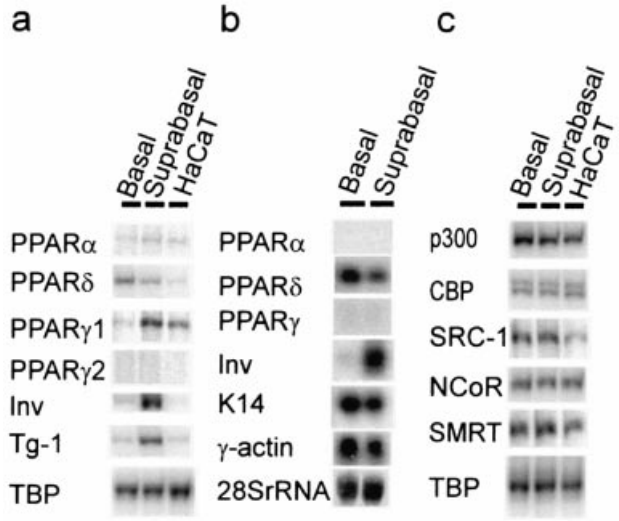

Figure 2. Expression of PPAR subtypes and PPAR cofactors in basal and suprabasal layers of human epidermis. Total RNA was purified from populations of enriched basal and suprabasal normal human keratinocytes isolated from human skin. Note that, generally, the intensity of signals in the individual panels can only be compared horizontally. (*a*) Multiplex RT-PCR analysis of expression of PPARα, PPARδ, PPARγ1, PPARγ2, Inv, Tg-1, and TBP. (*b*) Northern blot analysis of total RNA (5 μg) hybridized with PPARα, PPARδ, PPARγ, Inv, keratin 14 (K14), and γ-actin specific probes. Equal loading, transfer, and the integrity of the RNA were verified by methylene blue staining of 28S rRNA. (*c*) Multiplex RT-PCR analysis of expression of p300, CBP, SRC-1, NCoR, and SMRT.

Immunohistochemical analysis of PPARs in human epidermis Using *in situ* hybridization Matsuura *et al* (1999) reported that PPARδ mRNA was confined to the suprabasal layer of normal human epidermis. This finding contrasts with our observation that PPARδ is abundantly expressed in basal cells, and consequently we decided to perform an immunohistochemical analysis of PPAR subtype expression in human epidermis (**Fig 3**). Sections of human paraformaldehyde fixed skin were probed with antibodies specific for the individual PPAR subtypes. In keeping with the results obtained on fractionated human epidermis, strong PPARδ immunoreactivity was apparent in the basal as well as the suprabasal layers. Interestingly, PPARδ appeared to localize both to nuclei and cytoplasm in the basal cells. In contrast PPARδ immunoreactivity was confined to the nuclei in the suprabasal cells. No PPARα or PPARγ immunoreactivity was detectable in basal cells. In accordance with the differentiation-induced expression of PPARγ, expression of PPARγ was detectable in the suprabasal layer. As PPARδ, PPARγ exhibited an exclusively nuclear localization in the suprabasal layer. Surprisingly, very few cells stained positive for PPARα. The monoclonal anti-PPARα antibody used is generally considered as a potent antibody for immunohistochemistry (Su *et al*, 1998), and using several different tissues known to express PPARα (liver, kidney, intestine, and muscle) we detected strong signals of PPARα immunoreactivity demonstrating the functionality of the antibody (results not shown). Taken together, our results indicate that expression of PPARα is very low in human epidermis.

Analysis of the efficacy and potency of PPAR-selective ligands and TTA in transactivation mediated via human PPAR subtypes Several recent reports have focused on the involvement of PPARα in epidermal processes such as barrier development, proliferation, and differentiation; however, little

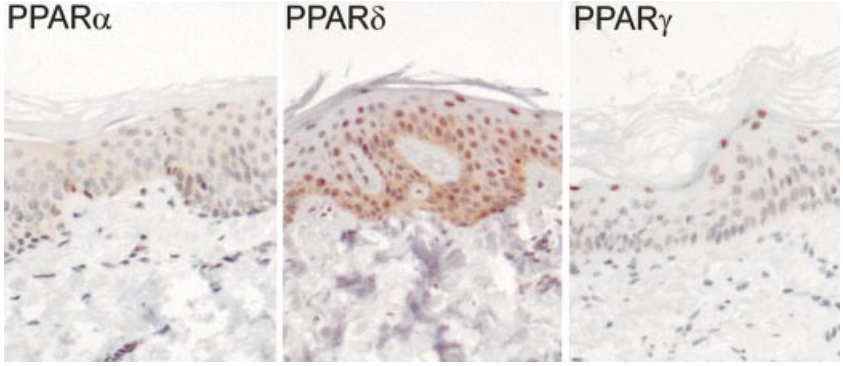

Figure 3. Expression of PPAR subtypes in normal human epidermis. Immunohistochemical analyses of PPARα, PPARδ, and PPARγ expression and localization in normal human epidermis. Paraffin-embedded sections of normal human epidermis sections were incubated with purified polyclonal or monoclonal antibody for PPARδ, PPARα, or PPARγ at 4°C overnight. Slides were subsequently incubated with a biotinylated goat antirabbit or antimouse antibody, and visualized using a standard streptavidin biotin (LSAB) horseradish peroxidase technique. The slides were counterstained in hematoxylin.

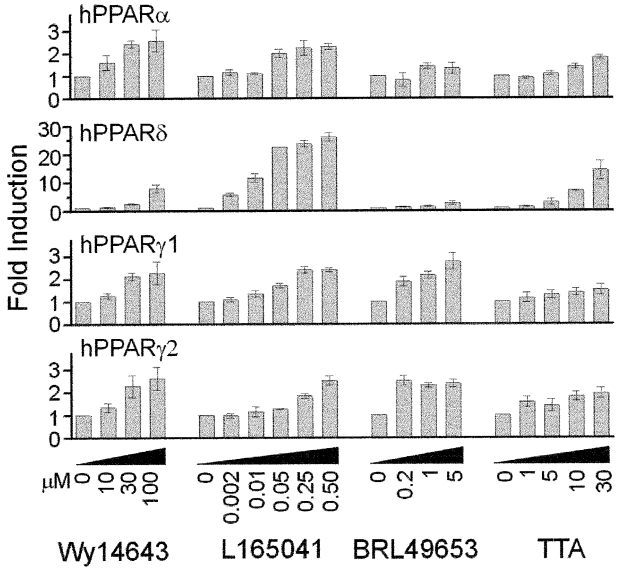

Figure 4. Differential activation of PPARα, PPARδ, and PPARγ by selective ligands and TTA. HaCaT cells were transiently transfected with the PPREx3-tk-luc reporter (0.75 μg) and CMV-β-galactosidase (0.1 μg) in combination with either hPPARα (0.01 μg), hPPARδ (0.05 μg), hPPARγ1 (0.05 μg), or hPPARγ2 (0.05 μg). In all transfections empty expression vector was added to ensure equal promoter load. Cells were incubated with media containing varying concentrations of Wy14643, L165041, BRL49653, TTA, or vehicle (0.1% DMSO) for approximately 16 h prior to harvest. Reporter activity was normalized to β-galactosidase values. All transfections were performed independently a minimum of four times in duplicate. In each experiment the transactivation observed with no addition of ligand was set equal to one to obtain a robust measure of ligand-induced transactivation. Ligand-dependent fold induction is presented as the mean ± SD.

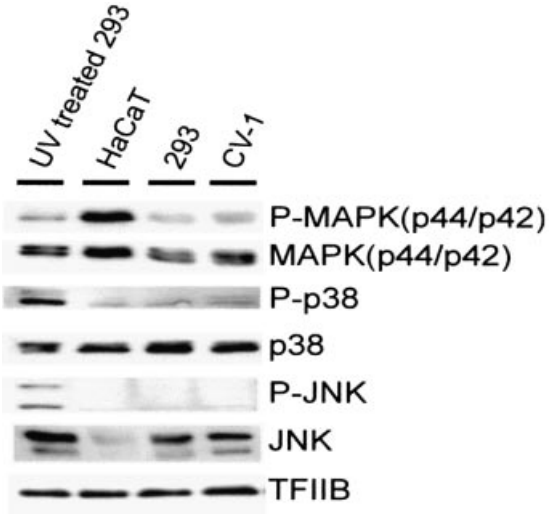

Figure 5. HaCaT cells exhibit high basal levels of activated p44/p42 (ERK1 and ERK2) MAPK. Whole cell extracts were prepared from HaCaT, 293, and CV-1 cells. 40 μg of protein were separated in sodium dodecyl sulfate polyacrylamide gels and analyzed for the expression of total p44/p42 MAPK, JNK, and p38 MAPK, or the activated form of the kinases. A whole cell extract from ultraviolet-irradiated 293 cells was used as a positive control (*lane 1*). Equal loading/transfer was confirmed by amidoblack staining of membranes and by incubation with anti-TFIIB antibody.

attention has been given to the other PPAR subtypes (Hanley *et al*, 1997, 1998, 1999; Rivier *et al*, 1998, 2000; Matsuura *et al*, 1999; Komuves *et al*, 2000a, b). To extend earlier studies, we therefore decided critically to investigate the possible involvement of the different PPARs in epidermal differentiation using subtype-selective ligands. Ligand selectivity and efficacy may exhibit cell-type specificity, and furthermore, even though PPARα, PPARδ, and PPARγ possess distinct ligand-binding specificities, few if any ligands exhibit strict subtype specificity (for review, see Willson *et al*, 2000). Consequently, we undertook a comprehensive analysis of ligand-dependent PPAR-mediated transactivation in the spontaneously transformed human keratinocyte cell line HaCaT. HaCaT cells exhibit a differentiation profile comparable with normal human keratinocytes (Boukamp *et al*, 1988; Ryle *et al*, 1989) and were chosen to avoid donor-to-donor variability and ensure consistency in transfection analyses. HaCaT cells were transfected with a PPAR-responsive reporter construct and PPAR expression vectors, and then treated with Wy14643, L165041, and BRL49653, which are selective for PPARα, PPARδ, and PPARγ, respectively (Kliewer *et al*, 1994, 1995; Forman *et al*, 1995; Lehmann *et al*, 1995; Berger *et al*, 1999). In addition, we included the thiasubstituted fatty acid analog TTA that in a concentration-dependent manner has been shown to activate all three murine PPAR subtypes (Helledie *et al*, 2000).[1] **Figure 4** illustrates the selectivity, efficacy, and potency of the different ligands. As described previously, BRL49653 exhibited very high specificity towards PPARγ; only at a concentration of 5 μM was PPARδ-mediated transactivation slightly enhanced. Similarly, 10 μM Wy14643 selectively targeted PPARα. The PPARδ-selective ligand L165041 clearly is a very potent PPARδ activator, which at concentrations lower than 50 nM targeted PPARδ with high selectivity. TTA was seen to be a very potent PPARδ ligand, and, in keeping with the previous studies using mouse PPARs, it also induced PPARα- and PPARγ-dependent transactivation. It should be noted that the ligand-induced PPARγ-mediated transactivation was consistently found to be rather modest in HaCaT cells even though systematic attempts to optimize the transfection protocol were carried out. PPARγ-dependent transactivation is negatively regulated by MAPK- or JNK-mediated phosphorylation of one serine residue in the A/B domain (Hu *et al*, 1996; Camp and Tafuri, 1997; Camp *et al*, 1999). We have noticed that normal human keratinocyte cells exhibited high basal activity of extracellular signal-regulated kinase 1 (ERK1) and ERK2 MAPK, and the same phenomenon was observed in HaCaT cells (**Fig 5**). Thus, the blunted ligand-induced transactivation of PPARγ may be related to this high basal MAPK activity, and accordingly addition of an inhibitor of ERKII activation enhanced ligand-induced transactivation (results not shown).

The effects of PPAR-selective ligands and TTA on epidermal marker gene expression and cell morphology To evaluate how the different PPAR activators affected expression of a known PPAR-responsive gene and differentiation of keratinocytes, we first measured the mRNA levels of the fatty acid transporter CD36/FAT and the two differentiation markers Inv and Tg-1 in normal human keratinocytes treated with PPAR-selective activators over a period of 3 d (**Fig 6*a***). The proximal promoter of the CD36/FAT gene has been shown to harbor a PPAR-responsive element, and treatment with PPARα or PPARγ agonists has been reported to induce expression of the CD36/FAT gene in a tissue-specific manner (Motojima *et al*, 1998; Tontonoz *et al*, 1998; Feng *et al*, 2000). In keeping with this, administration of BRL49653 or Wy14643 induced expression of CD36/FAT. Interestingly, the PPARδ-selective ligand L165041 induced a dose-dependent expression of CD36 suggesting that CD36/FAT is also a PPARδ-responsive gene. This is in accordance with the recent finding that PPARδ also targets a PPAR-responsive element of the CD36/FAT promoter

[1]Madsen L, Guerre-Millo M, Berge E, *et al*: Tetradecylthioacetic acid induces expression of PPARα target genes, improves insulin sensitivity and reduces adiposity in two animal models of insulin resistance (submitted).

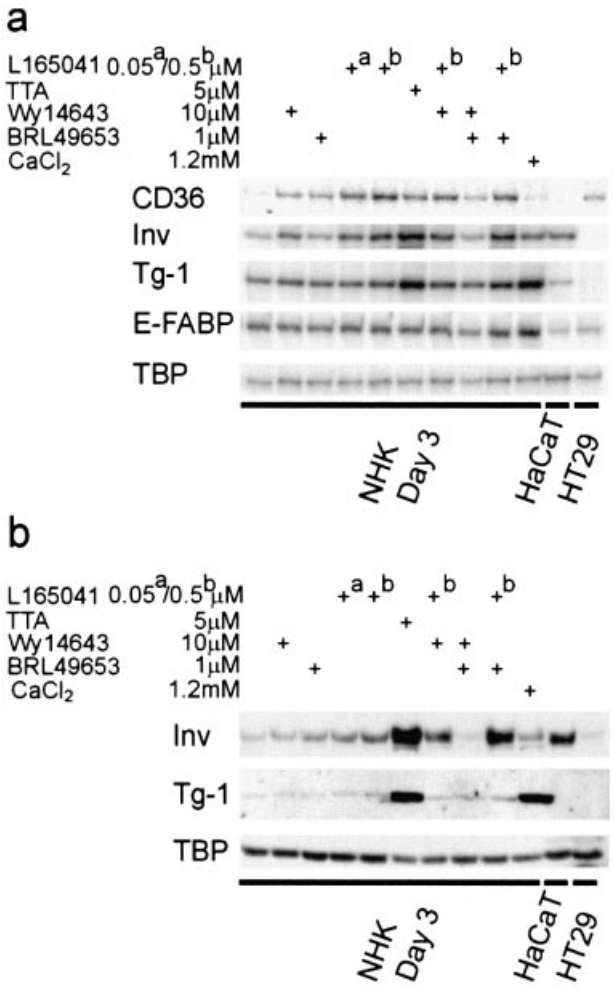

Figure 6. Induction of epidermal differentiation-specific genes and CD36 in keratinocytes treated with selective PPAR ligands and TTA. Normal human keratinocytes were incubated for 3 d in Keratinocyte-SFM containing low calcium (0.09 mM) in the presence of vehicle (0.1% DMSO), 10 μM Wy14643, 1 μM BRL49653, 5 μM TTA, and 0.05 μM/0.5 μM L165041 either alone or in combination. Normal human keratinocytes incubated in high calcium (1.2 mM) served as a control for differentiation. (*a*) Total RNA was harvested and expression of CD36, Inv, Tg-1, E-FABP, and TBP was analyzed by multiplex RT-PCR. TBP served as internal standard. (*b*) Whole cell extracts were prepared and 30 μg of protein were loaded in each lane. Expression of the differentiation-specific genes Inv and Tg-1 was analyzed by Western blotting. Equal loading/transfer was confirmed by amidoblack staining of membranes and by incubation with anti-TBP antibody.

(L. Teboul and P.A. Grimaldi, personal communication). The PPARα-selective agonist Wy14643 induced CD36/FAT expression to a level comparable to that observed with BRL49653. Finally, addition of TTA induced expression of CD36/FAT mRNA to a level slightly higher than those observed with Wy14643 or BRL49653. Treatment with $CaCl_2$, a well-established and potent inducer of keratinocyte differentiation, did not induce expression of CD36/FAT. A number of FABP have been found to be regulated by PPARs (for review, see Schoonjans *et al*, 1996) and it has been discussed whether the E-FABP is also regulated by the PPARs. Here we show that the expression level of E-FABP mRNA is unchanged upon treatment of normal human keratinocytes with specific PPAR activators or TTA (**Fig 6*a***), suggesting that PPARs do not regulate E-FABP expression. This observation is consistent with the observations of Rivier *et al* (2000) showing that E-FABP is not regulated by PPARα agonists in reconstructed epidermis. The expression pattern of E-FABP contrasts with that of CD36/FAT. CD36/FAT expression was upregulated by TTA and PPARα-, PPARγ-, or PPARδ-selective ligands, but unaffected by $CaCl_2$ treatment, whereas the opposite pattern was found regarding E-FABP expression.

As shown in **Fig 6**(***a***), treatment with Wy14643 resulted in a modest induction of Inv mRNA expression. Addition of L165041 led to a dose-dependent induction of Inv mRNA expression, whereas BRL49653 alone had no significant effect on Inv mRNA expression. Simultaneous addition of L165041 and Wy14643 did not increase the level of Inv mRNA expression above that observed with L165041 alone. Similarly, combined treatment with Wy14643 and BRL49653 did not induce Inv mRNA expression above the level observed with Wy14643 alone. Interestingly, simultaneous addition of L165041 and BRL49653 strongly induced Inv mRNA expression, indicating synergy between PPARδ and PPARγ. Western blotting essentially recapitulated the results obtained by RT-PCR showing a dose-dependent induction of Inv protein by L165041 and a strong synergy between L165041 and BRL49653 (**Fig 6*b***). Each PPAR-selective ligand induced expression of Tg-1 mRNA expression. Of interest, addition of TTA induced expression of Inv and Tg-1 mRNA to levels that significantly exceeded those obtained by treatment with the PPAR-selective ligands. The potency of TTA as an inducer of Inv and Tg-1 expression was even more dramatic when expression was analyzed at the protein level by Western blotting (**Fig 6*b***). It should be noted that TTA induced Inv and Tg-1 mRNA and protein expression to levels equal to or higher than those observed upon treatment with $CaCl_2$. Taken together these results suggest that TTA, apart from possibly inducing Inv and Tg-1 expression via PPAR-dependent pathways, exerted a pronounced effect on Inv and Tg-1 expression by mechanisms unrelated to the function of TTA as a PPAR ligand and activator.

It has been reported that the morphologic changes accompanying $CaCl_2$-induced *ex vivo* differentiation of normal human keratinocytes grown on tissue culture plastic are relatively modest (Gandarillas and Watt, 1997). Most notably, addition of $CaCl_2$ allows the formation of desmosomes leading to the typical pattern of cells growing in clusters. As the PPAR-selective ligands to variable degrees, and particularly TTA, upregulated expression of keratinocyte differentiation markers, we examined whether treatment with TTA or the different subtype-selective PPAR activators affected normal human keratinocyte morphology during the early period of the differentiation process. In parallel, expression of Inv and Tg-1 was examined by indirect immunofluorescence microscopy (**Fig 7**). As evaluated by phase contrast microscopy, the morphology of normal human keratinocytes treated for 3 d with ligands or 1.2 mM $CaCl_2$ exhibited notable differences. $CaCl_2$-treated cells clearly grew in clusters, whereas none of the cells treated with PPAR ligands or TTA exhibited cluster-like growth. It appeared that dishes treated with PPAR ligands or TTA contained more strongly refractile cells with a rounded-up appearance. Moreover, even though it is not apparent from the micrographs, the density of cells in dishes treated with TTA was much lower than vehicle-treated cells or cells treated with PPAR-selective ligands, indicating that TTA apart from inducing keratinocyte-specific markers also led to growth arrest or a decrease in the rate of proliferation. This result parallels the decrease in cellular proliferation and growth arrest associated with induction of keratinocyte differentiation by treatment with calcium, 1,25-dihydroxyvitamin D_3, oxysteroles, or various PPARα activators (Pillai and Bikle, 1991; Itin *et al*, 1994; Hanley *et al*, 1998, 1999, 2000a, b). *Ex vivo* differentiation of keratinocytes has been reported to be associated with changes in cell size (Teumer *et al*, 1994). Interestingly, careful comparison of the morphology of $CaCl_2$- and TTA-treated cells indicated that the cytoplasmic to nuclear ratio of the TTA-treated cells tended to be smaller than that of $CaCl_2$-treated cells. Corroborating the results obtained by Western blotting, indirect immunofluorescence microscopy revealed comparable levels of expression of Inv and Tg-1 in the $CaCl_2$- and

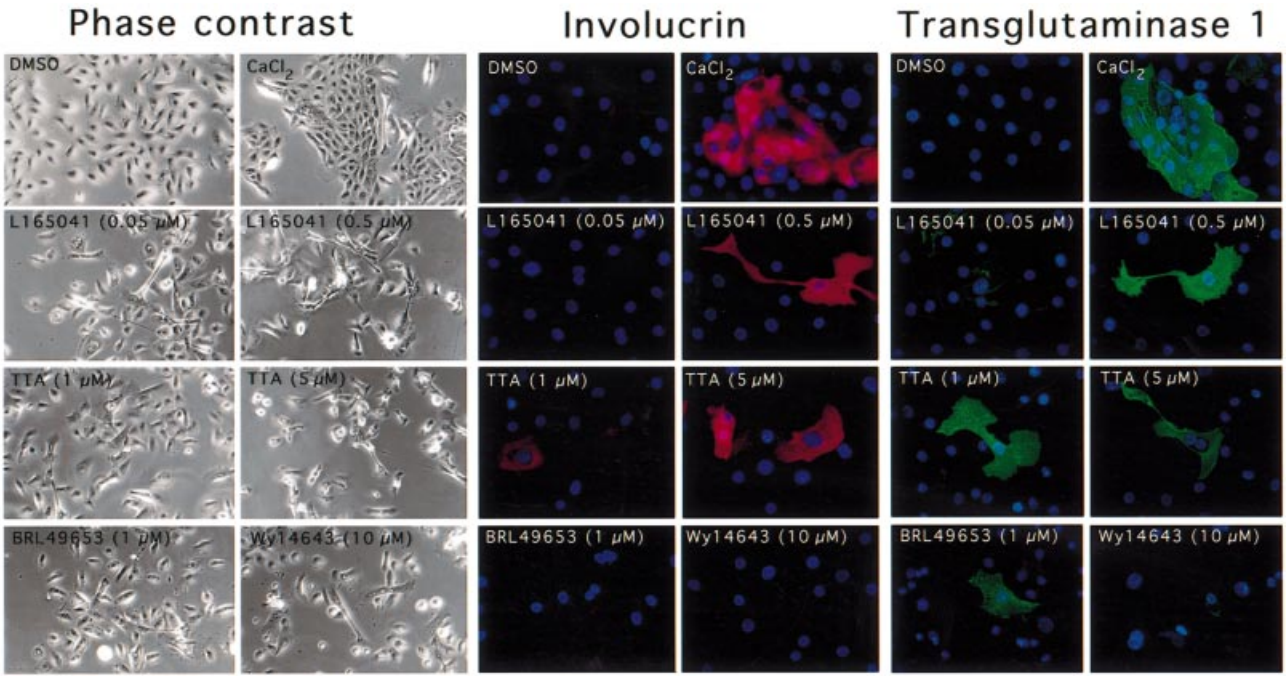

Figure 7. Morphology and expression of differentiation-specific genes in keratinocytes treated with selective PPAR ligands and TTA. Normal human keratinocytes were incubated for 3 d in Keratinocyte-SFM containing low calcium (0.09 mM) in the presence of TTA or selective PPAR ligands either in combination or alone as indicated. Normal human keratinocytes incubated in high calcium (1.2 mM) served as a control for differentiation. Representative flasks were photographed on day 3 (phase contrast). Expression of Inv and Tg-1 was determined by immunofluorescence microscopy using Inv (Alexa 594, red) or Tg-1 (Alexa 488, green) specific antibodies. Nuclei were visualized by staining with Hoechst 33258 (blue).

TTA-treated cells. Furthermore, this analysis also showed that the PPARδ-selective ligand L165041 induced Inv expression in a dose-dependent manner. Thus, even though treatment with $CaCl_2$ and TTA induced overlapping sets of keratinocyte marker genes, this observation suggests that TTA and $CaCl_2$ exert differential effects on keratinocyte differentiation.

The effect of TTA on keratinocyte proliferation and differentiation To examine in a more quantitative manner the effect of PPAR-selective ligands and TTA on keratinocyte proliferation, normal human keratinocytes were treated with PPAR ligands, TTA, and $CaCl_2$ as indicated in **Fig 8**, and proliferation was assessed using a modification of the MTT assay as described in *Materials and Methods*. In parallel the expression of Tg-1 was monitored using an ELISA-based assay (**Fig 8**). In agreement with the previously published results showing a growth inhibitory action of clofibrate (Hanley *et al*, 1998), administration of Wy14643 led to a dose-dependent decrease in normal human keratinocyte proliferation. BRL49653 also inhibited proliferation, whereas the effect of L165041 was less pronounced. Combinations of the different PPAR-subtype-selective ligands did not result in an inhibition of cellular proliferation exceeding those obtained with individual ligands. Noteworthy, TTA exerted a strong and dose-dependent inhibition of normal human keratinocyte proliferation that by far exceeded that observed for the PPAR-selective ligands. The antiproliferative action of $CaCl_2$ was only marginal in these experiments. Recapitulating the results obtained by RT-PCR and Western blotting, only TTA and $CaCl_2$ significantly induced Tg-1 expression. Thus, the results obtained using the MTT assay clearly distinguished TTA from PPAR-selective ligands, and substantiated the notion that TTA strongly inhibited normal human keratinocyte proliferation and potently induced expression of keratinocyte differentiation marker genes.

DISCUSSION

In this study we have examined the expression of the individual PPAR subtypes and cofactors involved in PPAR-mediated transactivation in human epidermis and cultured keratinocytes. Combining analyses at the mRNA and protein levels with immunohistochemistry, we present conclusive evidence that PPARδ is the predominant PPAR subtype in human epidermis. PPARδ is highly expressed in basal cells, and is also abundantly expressed in the suprabasal layer. During differentiation of human keratinocytes *ex vivo*, the level of PPARδ expression remained at a high level. In contrast, expression of PPARα and PPARγ1 was low in undifferentiated keratinocytes, but increased significantly during differentiation. It was recently reported that the PPARγ2 transcript could be detected in human keratinocytes using RT-PCR (Ellis *et al*, 2000). We have been unable to detect PPARγ2 mRNA using RT-PCR, however. In agreement with the analyses of keratinocyte cultures induced to differentiate *ex vivo* and isolated epidermal fractions, immunohistochemical analyses of sections of human skin revealed strong PPARδ expression in basal as well as in suprabasal cells. PPARα and PPARγ immunoreactivity could not be detected in the basal cells, but PPARγ was clearly present in the suprabasal layer. This pattern of expression is in accordance with previous results assaying PPAR subtype expression at the RNA level by RT-PCR (Rivier *et al*, 1998), but contrasts with results obtained by *in situ* hybridization showing that PPARδ expression was confined to the suprabasal level (Matsuura *et al*, 1999). Considering our consistent detection of PPARδ mRNA and protein in basal cells, however, it is conceivable that the lack of *in situ* PPARδ mRNA signals in basal cells might be ascribed to problems associated with the *in situ* hybridization technique.

The lack of significant PPARα immunoreactivity in skin sections was unexpected as a previous study had reported that PPARα immunoreactivity was associated with nuclei of suprabasal cells in reconstructed human epidermis (Rivier *et al*, 2000). RT-PCR revealed that PPARα mRNA is expressed in suprabasal cells, but as shown by northern blotting the level of PPARα mRNA was extremely low. Similarly, even though Western blotting demonstrated the presence of PPARα in suprabasal cells, the potency of the antibody used would allow detection of PPARα at the picogram level. Taken together our findings indicate that PPARα

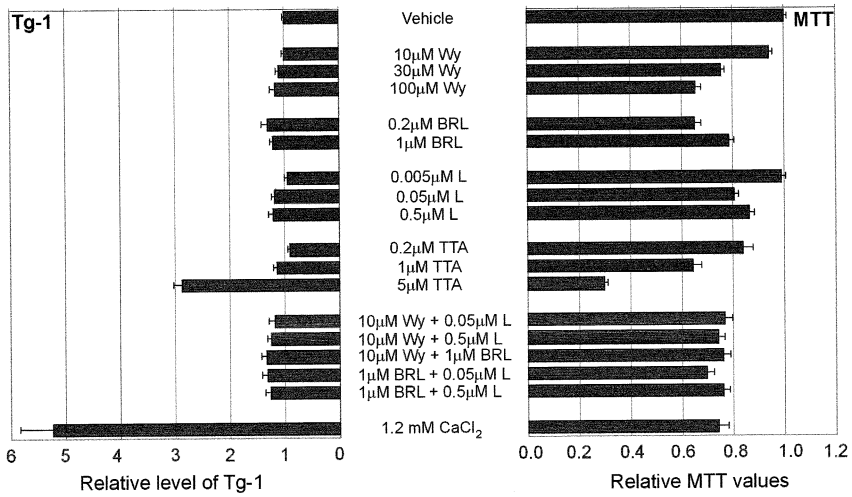

Figure 8. TTA induces the late epidermal differentiation marker Tg-1 and inhibits proliferation of keratinocytes. Normal human keratinocytes were seeded into 96-well plates and incubated for 3 d in Keratinocyte-SFM containing low calcium (0.09 mM) in the presence of vehicle (0,1% DMSO), 5 μM TTA, 10 μM Wy14643, 1 μM BRL49653, and 0.05 μM/0.5 μM L (L165041) either alone or in combination as indicated. Normal human keratinocytes incubated in high calcium (1.2 mM) served as a control for differentiation. MTT assay was performed as described in *Materials and Methods*. Tg-1 expression was determined using ELISA. The values are normalized to MTT viability values as described in *Materials and Methods*. Vehicle is set to 1, and the relative values are normalized relative to vehicle. Both assays were performed three times in triplicate. Results are presented as mean ± SD.

is expressed to very low levels in human epidermis. Of interest, PPARδ was found to localize both to the cytoplasm and the nuclei in basal cells. In contrast, both PPARδ and PPARγ exhibited solely nuclear localization in the suprabasal layer. It remains to be established whether the intracellular localization of PPARδ in basal cells is influenced by the availability of ligands in analogy with the ligand-dependent nuclear translocation observed for PPARs in other cell types (Chinetti *et al*, 1998; Bishop-Bailey and Hla, 1999; Kawahito *et al*, 2000).

The abundant expression of PPARδ in basal cells and the distribution of PPARδ between nuclei and cytoplasm make it tempting to speculate that PPARδ may play a role in the proliferation of keratinocytes, and in a ligand-dependent manner serve as a molecular switch involved in the initiation of keratinocyte differentiation. Along this line, it is intriguing that an analysis of PPAR subtype expression during rat embryonic development showed that PPARδ expression became strongly upregulated in the epidermis by day 18.5 (Braissant and Wahli, 1998), a time point that coincides with the acceleration of barrier formation (Hanley *et al*, 1997).

PPAR-mediated transactivation depends on and is regulated by a complex interplay of coactivators and corepressors. We analyzed the expression of prominent cofactors implicated in the control of PPAR-dependent transactivation during *ex vivo* differentiation of keratinocytes and in cells of the basal and suprabasal layer. No significant changes in the expression of CBP, p300, SRC-1, NCoR, and SMRT were observed during *ex vivo* differentiation, and accordingly basal and suprabasal cells exhibited identical profiles of expression of these cofactors.

Previous studies of the role of PPARs in keratinocyte differentiation and function have employed rather high concentrations of PPAR ligands. To critically evaluate the effect of PPAR-selective ligands on keratinocyte differentiation and expression of marker genes, we performed an extensive characterization of efficacy and potency of selective PPAR ligands in the human keratinocyte cell line HaCaT. In addition, we investigated the capability of the thiasubstituted fatty acid analog TTA to activate individual PPAR subtypes. As expected, BRL49653 at concentrations at or below 1 μM targeted only PPARγ. Wy14643 at concentrations at or below 10 μM exhibited PPARα selectivity, and L165041 at concentrations below 50 nM was a potent and highly specific activator of PPARδ. In transactivation experiments using mouse PPARs, we found that TTA most efficaciously activated PPARα (Helledie *et al*, 2000).[1] In this study using human PPARs we observed that TTA more efficaciously and potently activated PPARδ, whereas TTA-dependent activation of PPARα and PPARγ was more modest. Using appropriate concentrations of ligands that secured subtype specificity we somewhat unexpectedly found that PPARα- and PPARγ-selective ligands alone had a negligible effect on Inv gene expression. The PPARδ-selective ligand L165041 induced expression of Inv in a dose-dependent manner, however, and interestingly, simultaneous addition of L165041 and the PPARγ-selective ligand BRL49653 resulted in a strong synergistic induction of Inv mRNA and protein. It has been described that PPARδ induces PPARγ expression in preadipocytes (Bastie *et al*, 1999; Hansen *et al*, 2001). Whether a similar mechanism is involved in the synergistic activation of Inv expression in keratinocytes remains to be established.

Activators of PPARα were reported to accelerate epidermal differentiation and barrier formation (Hanley *et al*, 1997, 1998, 1999; Rivier *et al*, 1998; Matsuura *et al*, 1999; Komuves *et al*, 2000a, b), and treatment with PPARγ-selective ligands was reported to normalize psoriasis lesions (Ellis *et al*, 2000). These results would seem to directly implicate PPARα and PPARγ in the control of keratinocyte differentiation and function. Indeed, the effect of topical application of the PPARα agonist clofibrate on epidermal differentiation and marker gene expression was shown to be dependent on PPARα expression (Komuves *et al*, 2000a). The fact that the epidermis of PPARα-deficient mice appears grossly normal with only minor detectable differences in comparison with wild type mice, however, indicates that PPARα does not play a decisive role in the control of epidermal proliferation and differentiation, or that other transcription factors may compensate for the lack of PPARα expression (Komuves *et al*, 2000a). Furthermore, a number of observations would seem to argue against PPARα and PPARγ being critical proximal mediators or executors controlling keratinocyte differentiation and function. Addition of PPARγ ligands to keratinocytes *ex vivo* does not influence differentiation (Hanley *et al*, 1997, 1998). Synergistic activation of the functional PPARα/retinoid x receptor (RXR) heterodimer by simultaneous administration of PPARα and RXR ligands is a characteristic of PPARα-mediated transactivation (Kliewer *et al*, 1992; Mukherjee *et al*, 1998). Yet, no synergistic effect of PPARα and RXR ligands on keratinocyte differentiation *per se* was observed (Hanley *et al*, 1997). It is known that PPARα activators profoundly influenced lipid metabolism in reconstructed epidermis, and in this context synergy between RXR and PPARα ligands was observed (Rivier *et al*, 2000). Taken together these findings indicate that the impact of PPAR activators on differentiation to a large extent may be related to subtle alterations in lipid metabolism and/or accumulation of lipid mediators, and that these alterations may rely directly and indirectly on PPAR-mediated processes. This view is also consistent with the strong effect of TTA on keratinocyte proliferation and marker gene expression. TTA profoundly affects lipid metabolism in target tissue (Frøyland *et al*, 1997; Madsen *et al*, 1997). TTA is a bona fide PPAR ligand that

preferentially activates human PPARδ, and to a lesser extent also human PPARα and PPARγ. The potency of TTA is lower than the synthetic PPARδ-selective ligand L165041, however, and combinations of the potent synthetic PPAR-selective ligands did not exert effects on proliferation and marker gene expression of the same magnitude as those elicited by TTA. In conclusion, we suggest that the potency of TTA in regulating keratinocyte differentiation and function results from the unique metabolic properties of TTA as a relatively potent pan PPAR agonist, and additionally, TTA is able to markedly alter lipid metabolism in a PPAR-independent manner. We further propose that alterations in lipid metabolism possibly leading to the production of lipid mediators constitute a major pathway involved in the control of keratinocyte differentiation and function. Finally, the dual functionality of TTA and the fact that it is well tolerated with no reported adverse side-effects makes TTA an interesting compound for the treatment of epidermal disorders characterized by hyperproliferation and aberrant differentiation.

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REFERENCES

Bastie C, Holst D, Gaillard D, Jehl-Pietri C, Grimaldi PA: Expression of peroxisome proliferator-activated receptor PPARδ promotes induction of PPARγ and adipocyte differentiation in 3T3C2 fibroblasts. *J Biol Chem* 274:21920–21925, 1999

Berger J, Leibowitz MD, Doebber TW, *et al:* Novel peroxisome proliferator-activated receptor (PPAR) γ and PPARδ ligands produce distinct biological effects. *J Biol Chem* 274:6718–6725, 1999

Bishop-Bailey D, Hla T: Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-Δ12,14-prostaglandin J2. *J Biol Chem* 274:17042–17048, 1999

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–771, 1988

Braissant O, Wahli W: Differential expression of peroxisome proliferator-activated receptor-α-β, and -γ during rat embryonic development. *Endocrinology* 139:2748–2754, 1998

Camp HS, Tafuri SR: Regulation of peroxisome proliferator-activated receptor γ activity by mitogen-activated protein kinase. *J Biol Chem* 272:10811–10816, 1997

Camp HS, Tafuri SR, Leff T: c-Jun N-terminal kinase phosphorylates peroxisome proliferator-activated receptor-γ1 and negatively regulates its transcriptional activity. *Endocrinology* 140:392–397, 1999

Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752, 1987

Chinetti G, Griglio S, Antonucci M, *et al:* Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 273:25573–25580, 1998

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Ellis CN, Varani J, Fisher GJ, *et al:* Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-γ inhibit keratinocyte proliferation. *Arch Dermatol* 136:609–616, 2000

Feng J, Han J, Pearce SF, Silverstein RL, Gotto AM Jr, Hajjar DP, Nicholson AC: Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ. *J Lipid Res* 41:688–696, 2000

Fisher JG, Voorhees JJ: Molecular mechanisms of retinoid actions in skin. *FASEB J* 10:1002–1013, 1996

Floyd EE, Jetten AM: Regulation of type I (epidermal) transglutaminase mRNA levels during squamous differentiation: down regulation by retinoids. *Mol Cell Biol* 9:4846–4851, 1989

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM: 15-Deoxy-Δ 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83:803–812, 1995

Frøyland L, Madsen L, Sjursen W, *et al:* Effect of 3-thia fatty acids on the lipid composition of rat liver, lipoproteins, and heart. *J Lipid Res* 38:1522–1534, 1997

Fuchs E: Epidermal differentiation: the bare essentials. *J Cell Biol* 111:2807–2814, 1990

Gandarillas A, Watt FM: c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev* 11:2869–2882, 1997

Glass CK, Rosenfeld MG: The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 15:121–141, 2000

Hanley K, Jiang Y, Crumrine D, *et al:* Activators of the nuclear hormone receptors PPARα and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 100:705–712, 1997

Hanley K, Jiang Y, He SS, *et al:* Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARα. *J Invest Dermatol* 110:368–375, 1998

Hanley K, Komuves LG, Bass NM, *et al:* Fetal epidermal differentiation and barrier development *in vivo* is accelerated by nuclear hormone receptor activators. *J Invest Dermatol* 113:788–795, 1999

Hanley K, Komuves LG, Ng DC, *et al:* Farnesol stimulates differentiation in epidermal keratinocytes via PPARalpha. *J Biol Chem* 275:11484–11491, 2000a

Hanley K, Ng DC, He SS, *et al:* Oxysterols induce differentiation in human keratinocytes and increase Ap-1-dependent involucrin transcription. *J Invest Dermatol* 114:545–553, 2000b

Hansen JB, Petersen RK, Larsen BM, Bartkova J, Alsner J, Kristiansen K: Activation of peroxisome proliferator-activated receptor γ bypasses the function of the retinoblastoma protein in adipocyte differentiation. *J Biol Chem* 274:2386–2393, 1999

Hansen JB, Zhang H, Rasmussen TH, Petersen RK, Flindt EN, Kristiansen K: Peroxisome proliferator-activated receptor δ (PPARδ)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J Biol Chem*, 276:3175–3182, 2001

He TC, Chan TA, Vogelstein B, Kinzler KW: PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 99:335–345, 1999

Helledie T, Antonius M, Sørensen RV, *et al:* Lipid-binding proteins modulate ligand-dependent transactivation by peroxisome proliferator-activated receptors and localize to the nucleus as well as the cytoplasm. *J Lipid Res* 41:1740–1751, 2000

Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T: Regulation of terminal differentiation of cultured mouse epidermal cells by 1alpha, 25-dihydroxyvitamin D3. *Endocrinology* 113:1950–1957, 1983

Hu E, Kim JB, Sarraf P, Spiegelman BM: Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science* 274:2100–2103, 1996

Imakado S, Bickenbach JR, Bundman DS, *et al:* Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev* 9:317–329, 1995

Itin PH, Pittelkow MR, Kumar R: Effects of vitamin D metabolites on proliferation and differentiation of cultured human epidermal keratinocytes grown in serum-free or defined culture medium. *Endocrinology* 135:1793–1798, 1994

Jensen J, Serup P, Karlsen C, Nielsen TF, Madsen OD: mRNA profiling of rat islet tumors reveals nkx 6.1 as a beta-cell-specific homeodomain transcription factor. *J Biol Chem* 271:18749–18758, 1996

Kawahito Y, Kondo M, Tsubouchi Y, *et al:* 15-Deoxy-Δ (12,14)-PGJ (2) induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. *J Clin Invest* 106:189–197, 2000

Kersten S, Desvergne B, Wahli W: Roles of PPARs in health and disease. *Nature* 405:421–424, 2000

Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM: Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* 358:771–774, 1992

Kliewer SA, Forman BM, Blumberg B, *et al:* Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA* 91:7355–7359, 1994

Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM: A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* 83:813–819, 1995

Komuves LG, Hanley K, Jiang Y, Elias PM, Williams ML, Feingold KR: Ligands and activators of nuclear hormone receptors regulate epidermal differentiation during fetal rat skin development. *J Invest Dermatol* 111:429–433, 1998

Komuves LG, Hanley K, Lefebvre AM, *et al:* Stimulation of PPARα promotes epidermal keratinocyte differentiation *in vivo*. *J Invest Dermatol* 115:353–360, 2000a

Komuves LG, Hanley K, Man MQ, Elias PM, Williams ML, Feingold KR: Keratinocyte differentiation in hyperproliferative epidermis: topical application of PPARα activators restores tissue homeostasis. *J Invest Dermatol* 115:361–367, 2000b

Lee SS, Pineau T, Drago J, *et al:* Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* 15:3012–3022, 1995

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J Biol Chem* 270:12953–12956, 1995

Leibowitz MD, Fievet C, Hennuyer N, *et al:* Activation of PPARδ alters lipid metabolism in db/db mice. *FEBS Lett* 473:333–336, 2000

Lim H, Gupta RA, Ma WG, *et al:* Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARδ. *Genes Dev* 13:1561–1574, 1999

Macklis JD, Sidman RL, Shine HD: Cross-linked collagen surface for cell culture that is stable, uniform, and optically superior to conventional surfaces. *In Vitro Cell Dev Biol* 21:189–194, 1985

Madsen L, Froyland L, Grav HJ, Berge RK: Upregulated delta 9-desaturase gene

expression by hypolipidemic peroxisome-proliferating fatty acids results in increased oleic acid content in liver and VLDL: accumulation of a delta 9-desaturated metabolite of tetradecylthioacetic acid. *J Lipid Res* 38:554–563, 1997

Matsuura H, Adachi H, Smart RC, Xu X, Arata J, Jetten AM: Correlation between expression of peroxisome proliferator-activated receptor β and squamous differentiation in epidermal and tracheobronchial epithelial cells. *Mol Cell Endocrinol* 147:85–92, 1999

Michel S, Courseaux A, Miquel C, *et al:* Determination of retinoid activity by an enzyme-linked immunosorbent assay. *Anal Biochem* 192:232–236, 1991

Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 65:55–63, 1983

Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N: Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor α and γ activators in a tissue- and inducer-specific manner. *J Biol Chem* 273:16710–16714, 1998

Mukherjee R, Strasser J, Jow L, Hoener P, Paterniti JR Jr, Heyman RA: RXR agonists activate PPARα-inducible genes, lower triglycerides, and raise HDL levels *in vivo*. *Arterioscler Thromb Vasc Biol* 18:272–276, 1998

Peters JM, Lee SS, Li W, *et al:* Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β (δ). *Mol Cell Biol* 20:5119–5128, 2000

Pillai S, Bikle DD: Epidermal vitamin D metabolism, function, and regulation. *Adv Lipid Res* 24:321–341, 1991

Rice RH, Green H: Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 18:681–694, 1979

Rivier M, Safonova I, Lebrun P, Griffiths CE, Ailhaud G, Michel S: Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. *J Invest Dermatol* 111:1116–1121, 1998

Rivier M, Castiel I, Safonova I, Ailhaud G, Michel S: Peroxisome proliferator-activated receptor-α enhances lipid metabolism in a skin equivalent model. *J Invest Dermatol* 114:681–687, 2000

Ryle CM, Breitkreutz D, Stark HJ, Leigh IM, Steinert PM, Roop D, Fusenig NE: Density-dependent modulation of synthesis of keratins 1 and 10 in the human keratinocyte line HACAT and in ras-transfected tumorigenic clones. *Differentiation* 40:42–54, 1989

Saitou M, Sugai S, Tanaka T, Shimouchi K, Fuchs E, Narumiya S, Kakizuka A: Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor. *Nature* 374:159–162, 1995

Schoonjans K, Staels B, Auwerx J: The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* 1302:93–109, 1996

Spydevold O, Bremer J: Induction of peroxisomal beta-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim Biophys Acta* 1003:72–79, 1989

Su JL, Simmons CJ, Wisely B, Ellis B, Winegar DA: Monitoring of PPAR alpha protein expression in human tissue by the use of PPAR alpha-specific MAbs. *Hybridoma* 17:47–53, 1998

Svendsen ML, Daneels G, Geysen J, Binderup L, Kragballe K: Proliferation and differentiation of cultured human keratinocytes is modulated by 1,25 (OH) $_2$D3 and synthetic vitamin D3 analogues in a cell density-, calcium- and serum-dependent manner. *Pharmacol Toxicol* 80:49–56, 1997

Teumer J, Zezulak K, Green H: Measurement of specific mRNA content of keratinocytes of different sizes in relation to growth and differentiation. In: Leigh I, Lane B, Watt F, eds. *The Keratinocyte Handbook*. Cambridge, U.K.: Cambridge University Press, 1994:pp 235–242

Thacher SM, Rice RH: Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* 40:685–695, 1985

Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM: PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 93:241–252, 1998

Willson TM, Brown PJ, Sternbach DD, Henke BR: The PPARs: from orphan receptors to drug discovery. *J Med Chem* 43:527–550, 2000

Xu HE, Lambert MH, Montana VG, *et al:* Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol Cell* 397–403, 1999