Lymphoid Enhancer-binding Factor-1 (LEF1) Interacts with the DNA-binding Domain of the Vitamin D Receptor*

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The vitamin D receptor (VDR) is an important regulator of mineral ion homeostasis. Humans with mutations in the VDR develop hereditary vitamin D-resistant rickets, which is often associated with normal mineral ion homeostasis; however, these studies demonstrate impaired regulation of the canonical Wnt signaling pathway in primary keratinocytes lacking the VDR. To identify the key effector of canonical Wnt signaling that interacts with the VDR, GST pull-down studies were performed. A novel interaction between the VDR and LEF1 (lymphoid enhancer-binding factor-1) that is independent of β-catenin was identified. This interaction is dependent upon sequences within the N-terminal region of the VDR, a domain required for VDR-DNA interactions and normal hair cycling in mice. Mutation of specific residues within the N-terminal region of the VDR not only abrogated interactions between the VDR and LEF1 but also impaired the ability of the VDR to enhance Wnt signaling in vdr−/− primary keratinocytes. Thus, this study demonstrates a novel interaction between the VDR and LEF1 that is mediated by the DNA-binding domain of the VDR and that is required for normal canonical Wnt signaling in keratinocytes.

The vitamin D receptor (VDR) is an important regulator of mineral ion homeostasis. Humans with mutations in the VDR develop hereditary vitamin D-resistant rickets, which is often accompanied by alopecia totalis (Ref. 1; reviewed in Ref. 2). Mice lacking the VDR (vdr−/−) phenocopy the human disease (3). The skeletal changes observed in vdr−/− mice can be prevented by normalizing mineral ion homeostasis; however, alopecia persists, suggesting a unique role for the VDR in skin (4). Alopecia is not observed in the absence of 25-hydroxyvitamin D 1α-hydroxylase (5, 6) or in vitamin D-deficient humans; therefore, the VDR has 1,25-dihydroxyvitamin D-independent functions in the epidermis: the absence of receptor results in alopecia, whereas the absence of ligand does not (7). Morphogenesis of the pilosebaceous unit is not impaired in the absence of the VDR; however, vdr−/− mice are unable to initiate postnatal hair cycles due to a defect intrinsic to the keratinocyte component of the hair follicle (7, 8). vdr−/− mice also exhibit defects in self-renewal and lineage commitment of keratinocyte stem cells (KSCs), multipotent progenitor cells that can give rise to all lineages of the pilosebaceous unit (9). Although these studies demonstrate that the VDR is required for normal KSC function, the molecular mechanism by which the VDR exerts these effects is not clear.

Hair follicle morphogenesis and the regulation of postnatal hair cycling are dependent upon reciprocal interactions between the epithelial component of the hair follicle, the keratinocyte, and the mesodermal component, the dermal papilla. Postnatally, the lower part of the epidermal component of the hair follicle undergoes cycles of growth (anagen) during which KSCs from the bulge area of the hair follicle give rise to the lower part of the hair follicle that produces a new hair shaft. These cells then undergo apoptosis, marking the beginning of the catagen phase of the hair cycle, resulting in approximation of the dermal papilla to the bulge area of the hair follicle. During the telogen phase, signals from the dermal papilla communicate with the KSCs in the bulge area, initiating a new hair cycle. Initiation of anagen is thought to be dependent upon activation of KSCs by the canonical Wnt signaling pathway, resulting in induction of Sonic Hedgehog (Shh) and a proliferative response in keratinocytes that regenerate the hair follicle. Studies in genetically engineered mouse models have established the critical importance of these two pathways in the hair follicle. Keratinocyte-specific deletion of β-catenin leads to abnormal hair follicle morphogenesis and impairs hair follicle regeneration, whereas activating mutations of β-catenin result in de novo hair follicle formation and the appearance of hair follicle tumors (10). Two transcriptional effectors of the canonical Wnt signaling pathway, TCF3 (T cell factor-3) and LEF1 (lymphoid enhancer-binding factor-1), are important for maintaining epidermal homeostasis. TCF3 is thought to act primarily in maintaining KSC populations in mice, whereas LEF1 is thought to direct KSC differentiation along the hair follicle lineage (11).

lefl knock-out mice are born with few hair follicles and develop alopecia by 12 days of age (12). Of note, keratinocyte-specific expression of a dominant-negative LEF1 transgene results in alopecia accompanied by lipid-filled dermal cysts and sebaceous tumors, a phenotype analogous to that observed in the vdr−/− mice (13). Shh contributes to morphogenesis of the pilosebaceous unit and is critical for anagen induction and progression (14–16). Treatment of vdr−/− mice with a Shh agonist transiently restores hair cycling, suggesting that defective Hedgehog signaling contributes to the hair follicle defects in these mice (17). Cross-talk between the Hedgehog and canonical Wnt signaling pathways has been observed in many organ systems, including the epidermis, where activation of the...
canonical Wnt pathway induces Hedgehog signaling (10, 18, 19), whereas the absence or inhibition of Wnt signals impairs Hedgehog expression (20, 21). Because the unliganded VDR modulates the canonical Wnt signaling pathway in primary keratinocytes and co-immunoprecipitates with LEF1 and β-catenin (9), studies were undertaken to examine the molecular interactions of the VDR with effectors of this pathway and to determine the consequences of VDR ablation on the expression of shh and its downstream effector gli1 (22).

**EXPERIMENTAL PROCEDURES**

**Engineering of GST Fusion Proteins**—The human VDR lacking the initiator ATG codon was subcloned in frame with the GST tag into pGEX5x.1. Deletion mutants were engineered from wild-type pGEX5x.1-VDR by PCR. DNA sequencing was performed to ensure the fidelity of replication.3

**GST Fusion Protein Expression**—BL21 bacteria (GE Healthcare) were transformed with GST-VDR fusion genes. Large-scale cultures were grown and induced according to the manufacturer’s instructions. After induction of protein expression, bacteria were pelleted by centrifugation at 1000 × g for 20 min and frozen at −80 °C. Pellets were thawed on ice and lysed in 5 ml of bacterial lysis buffer (25 mM Hepes (pH 7.9), 20% glycerol, 1 mM MgCl₂, 0.1% Triton X-100, 3 mg/ml lysozyme, 1 μg/ml RNase, 10 μg/ml DNase, and one Roche Complete mini protease inhibitor tablet/10 ml). Lysates were centrifuged at 20,000 × g for 40 min and used immediately or stored at −80 °C. For GST pulldown assays, equimolar amounts of GST-VDR lysates were bound to 40 μl of 50% GST-Sepharose beads in a final volume of 400 μl of bacterial binding buffer (25 mM Hepes (pH 7.9), 20% glycerol, and 1 mM MgCl₂ (pH 7.4)) for 30 min at 4 °C. The beads were then washed three times with bacterial binding buffer at 4 °C. Equimolar amounts of COS-7 lysates expressing LEF1, β-catenin, or TCF3 were added in a final volume of 400 μl of COS binding buffer (25 mM Hepes (pH 7.4), 10% glycerol, and 50 mM KCl) to GST-VDR-bound beads for 1 h at 4 °C and then washed three times with COS binding buffer at 4 °C. Protein complexes were eluted with 40 μl of GST elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione (pH 8.0)) for 15 min at room temperature. Input (10%) and eluted (25%) fractions were loaded onto SDS-polyacrylamide gels and then Western-blotted to detect proteins.

**Antibodies**—The following antibodies were used to detect the VDR: 9A-7 (Abcam, Cambridge, MA) and H-81, N-20, and C-20 (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-β-catenin antibody (BD Biosciences) was used to detect short deletion mutants. HA-tagged LEF1 was detected using an anti-HA antibody (Cell Signaling Technology, Danvers, MA), whereas endogenous LEF1 was detected using anti-LEF1 antibody C12A5 (Cell Signaling Technology). TCF3 was detected using anti-TCF3 antibody M-20 (Santa Cruz Biotechnology). Endogenous and constitutively active β-catenin were detected using mouse anti-β-catenin antibody (BD Biosciences). Retinoid X receptor (RXR)-α was detected using anti-RXRα antibody F-1 (Santa Cruz Biotechnology). SRC3 (steroid receptor coactivator-3) was detected using anti-SRC3 antibody 5E11 (Cell Signaling Technology). VDR-interacting protein (DRIP)/Mediator was detected using anti-TRAP80 antibody ab70125 (Abcam).

**Cell Culture and Reporter Assays**—COS-7 cells were maintained DMEM (Invitrogen) with 10% FBS (HyClone, Logan, UT) and penicillin/streptomycin (Invitrogen). For generation of cell lysates expressing pcDNA3.1-LEF1, pcDNA3.1-β-catenin, and pcDNA3.1-TCF3, cells were transfected using Lipofectamine reagent (Invitrogen). Cells were harvested after 48 h in cell lysis buffer (25 mM Hepes (pH 7.4), 10% glycerol, 50 mM KCl, and Roche Complete mini protease inhibitor tablets). Lysates were centrifuged at 16,000 × g for 10 min at 4 °C and used immediately or stored at −80 °C. For reporter assays, cells were plated at a density of 150,000 cells/12-well plate overnight and then transfected as described above. Cells were lysed in 1× passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined using a Dual-Luciferase reporter assay system (Promega) as directed by the manufacturer using a PerkinElmer EnVision 2104 multilabel reader. Transfection efficiency was normalized by correcting firefly luciferase for cotransfected Renilla luciferase activity in each sample.

**Primary Keratinocyte Culture**—Primary keratinocytes were isolated from neonatal vdr−/− mice as described previously (8). Briefly, skin was harvested from neonatal mice (postnatal days 0–4) and digested overnight at 4 °C in 0.25% trypsin (Invitrogen). The epidermis was separated from the dermis, minced, and stirred in modified minimal essential medium for 1.5 h at 4 °C. Cell suspensions were strained and plated onto collagen-coated dishes. Keratinocytes were maintained in modified minimal essential medium containing 0.1% glucose, 0.001% phenol red, and 0.045 mM CaCl₂ supplemented with 4% Chelex-treated FBS, antibiotic/antimycotic (Invitrogen), 2 mM l-glutamine (BioWhittaker), and 10 ng/ml EGF (BD Biosciences). Keratinocyte differentiation was induced by culturing cells in medium supplemented with 2 mM CaCl₂ for 7 days. Keratinocytes were transfected using Lipofectamine LTX and Plus reagents (Invitrogen). Luciferase assays were performed as described above.

**Immunoprecipitation**—Cells were rinsed with PBS and then lysed in immunoprecipitation buffer (1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris (pH 7.4)) for 10 min on ice. Lysates were precleared for 1 h at 4 °C with 50% protein G-Sepharose beads (GE Healthcare). Immunoprecipitation was performed overnight at 4 °C with 5 μg of anti-VDR antibody 9A-7 or control antibody CD49f (BD Biosciences). Immunoprecipitated lysates were incubated with protein G-Sepharose beads for 1 h at 4 °C, after which protein complexes were dissociated by boiling for 10 min in 1× reducing SDS loading buffer. Samples were loaded onto SDS-polyacrylamide gels and Western-blotted to detect proteins.

**Real-time PCR**—Total RNA was extracted from mouse skin and cultured keratinocytes using the RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the DNA Engine Opticon system (MJ Research, Waltham, MA). Primers were designed to span introns, and the absence of contaminating DNA was confirmed in all samples. The levels of mRNA encoding each gene of inter-

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3 Oligonucleotide sequences are available upon request.
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**RESULTS**

**GST-VDR Interacts with LEF1, but Not with β-Catenin or TCF3**—Immunoprecipitation of LEF1 results in coprecipitation of the VDR and β-catenin (9). Because LEF1 and β-catenin interact directly and β-catenin has been shown to interact with the VDR in the presence of ligand (25), studies were performed to determine whether the unliganded VDR interacts with LEF1, β-catenin, or both using GST pulldown assays. An N-terminal GST fusion of the VDR was expressed in bacteria. Pulldown experiments were performed in the absence of ligand using COS-7 cell extracts expressing HA-LEF1 and constitutively active or endogenous β-catenin (Fig. 1A and data not shown). Although LEF1 coeluted with GST-VDR, β-catenin did not, demonstrating that the VDR interacts with LEF1 in the absence of ligand, independently of β-catenin. GST pulldown assays were also performed with COS-7 cell lysates expressing TCF3 (Fig. 1B). No interaction was detected between GST-VDR and TCF3. GST pulldown experiments performed using GST alone or HA-Gαs demonstrated that HA-Gαs did not coelute with GST-VDR and that HA-LEF1 did not coelute with GST alone (data not shown). Therefore, the interaction between LEF1 and the VDR is not dependent upon the GST or HA tag.

**The Endogenous VDR and LEF1 Co-immunoprecipitate in Keratinocytes**—The GST pulldown studies demonstrated an interaction between the bacteriologically expressed VDR and LEF1. To determine whether the endogenous VDR and LEF1 interact, the VDR was immunoprecipitated from wild-type primary keratinocytes. Although basal levels of LEF1 were very low, it co-immunoprecipitated with the VDR in both proliferating and differentiating keratinocytes. γ-Catenin was well expressed but did not co-immunoprecipitate with the VDR in these assays. RXRα, which is known to heterodimerize with the VDR, although it is expressed only at very low levels, also co-immunoprecipitated with the VDR in both proliferating and differentiating keratinocytes (Fig. 2). The VDR has been shown to interact in a ligand-dependent fashion with DRIP/Mediator and SRC2 and SRC3 complexes during keratinocyte proliferation and differentiation, respectively (26–29). Interactions between the VDR and these coactivators were not detected presumably because the keratinocytes were cultured in the absence of ligand (Fig. 2).

**Sequences within the N-terminal Region of the VDR Are Required for Interaction with LEF1**—The VDR is a 427-amino acid protein that contains multiple functional domains preserved among nuclear receptors (Fig. 3A). Two short activation function domains, AF1 and AF2, important for nuclear coactivator recruitment are found at the N and C termini of the VDR, respectively. The ligand-binding domain is required for 1,25-dihydroxyvitamin D binding. The DNA-binding domain (DBD) contains two zinc fingers that mediate VDR-DNA interactions. Between the DBD and the ligand-binding domain lies a flexible hinge region required for RXR heterodimerization.

To identify the region of the VDR responsible for mediating interactions with LEF1, deletion mutants of the VDR were engineered as GST fusions. Pulldown experiments were performed to evaluate LEF1 binding. Although deletion of either the AF1

![FIGURE 1. GST-VDR interacts with LEF1.](image1)

![FIGURE 2. The endogenous VDR and LEF1 co-immunoprecipitate in primary keratinocytes.](image2)
or AF2 domain did not affect VDR-LEF1 interactions, deletion of the N-terminal sequences containing both the AF1 domain and the DBD abrogated these interactions (Fig. 3A). To determine whether the sequences containing the AF1 domain and the DBD are sufficient for VDR-LEF1 interaction, a deletion mutant of the VDR expressing only these domains was engineered (Fig. 3B). Pulldown experiments demonstrated that this GST-AF1DBD-VDR interacted with LEF1. Mice lacking the first zinc finger of the VDR express a truncated protein that employs a surrogate ATG codon located between the two zinc fingers of the VDR (M52). Expression of this truncated VDR in mice results in rickets with alopecia, a phenotype identical to mice with total absence of the VDR (30). To determine whether the M52-VDR interacts with LEF1, it was engineered as an N-terminal GST fusion. Pulldown studies demonstrated that GST-M52-VDR did not interact with LEF1 (Fig. 3B), confirming that an intact DBD is required for interaction with LEF1. Because deletion of the AF1 domain in the context of full-length GST-VDR did not impair LEF1 interactions, a GST-DBD fusion was engineered. Pulldown studies demonstrated that the sequences in the DBD are sufficient to mediate interactions with LEF1. Thus, sequences within the DBD of the VDR are both necessary and sufficient for mediating interactions with LEF1.

The Minimal Region Required for VDR-LEF1 Interaction Is in the First Zinc Finger of the VDR—Because the DBD is both necessary and sufficient for VDR interactions with LEF1, C-terminal truncations of the DBD were engineered as N-terminal GST fusions to identify the minimal domain of the VDR necessary for interaction with LEF1 (Table 1). Although deletion of

| A | GST-VDR | GST-ΔAF1-VDR | GST-ΔAF1DBD-VDR | GST-AF2-VDR |
|---|---------|-------------|----------------|-------------|
| COS7 LEF1 | + | + | + | + | + | + |
| GST-VDR | + | + |
| GST-ΔAF1-VDR | + | + |
| GST-ΔAF1DBD-VDR | + | + |
| GST-AF2-VDR | + | + |
| GST-AF2-VDR | | |

| B | GST-AF1DBD-VDR | GST-M52-VDR | GST-DBD-VDR |
|---|----------------|-------------|-------------|
| COS7 LEF1 | + | + | + |
| GST-AF1DBD-VDR | + | + |
| GST-M52-VDR | + | + |
| GST-DBD-VDR | | | |

**FIGURE 3.** Sequences within the N-terminal region of the VDR are required for interaction with LEF1. Wild-type or mutant GST-VDR bound to glutathione-Sepharose was incubated with COS-7 cell extracts overexpressing HA-LEF1. Input (i; 10%) and eluted (e; 25%) fractions were subjected to Western blotting to detect wild-type or mutant GST-VDR and HA-LEF1. A, mutant VDRs lacking the domains indicated were tested for their ability to interact with LEF1. B, N- and C-terminal truncations of the VDR were tested for their ability to interact with LEF1. The asterisks denote alternative translation start sites at Met-52. Data are representative of those obtained in at least three independent GST pulldown assays. HD, hinge domain; LBD, ligand-binding domain.
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**TABLE 1**
The region of the VDR required for interaction with LEF1 resides within the first zinc finger. GST-VDR truncation mutants bound to glutathione-Sepharose were tested for their ability to interact with LEF1. The results of these studies are summarized below. The name, domain structure, amino acid range, and ability to interact with LEF1 are indicated for each mutant. The GST tag is shown in the white boxes, the AF1 domain in the medium grey boxes, and the two zinc fingers of the DBD in light grey boxes. The asterisks denote Met-52, the alternative VDR start site in a line of vdr<sup>−/−</sup> mice with alopecia (30).

| Deletion Mutant Name | Domain Structure | AA Range | LEF1 Interaction |
|----------------------|------------------|----------|------------------|
| GST-AF1DBD-VDR       | AF1 | Zn1 | Zn2 | 8-114 | + |
| GST-Zn1Zn2-VDR       | | | | 8-92 | + |
| GST-DBbx-VDR         | | | | 8-67 | - |
| GST-Zn1-VDR          | | | | 8-57 | + |
| GST-AF1DBD-VDR       | AF1 | | | 21-114 | + |
| GST-AF1Zn1-VDR       | AF1 | Zn1 | | 21-57 | + |
| GST-AF1Zn1-53-VDR    | AF1 | Zn1 | | 21-53 | - |
| GST-AF1Zn1-46-VDR    | AF1 | Zn1 | | 21-46 | - |
| GST-AF1Zn1-40-VDR    | AF1 | Zn1 | | 21-40 | - |
| GST-AF1Zn1-32-VDR    | AF1 | Zn1 | | 21-32 | - |

Amino acids 58–114 did not affect VDR-LEF1 interactions, further C-terminal truncations abrogated these interactions. GST-ΔAF1Zn1-VDR, consisting of residues 21–57, is the shortest deletion mutant that retained the ability to interact with LEF1, demonstrating that residues in the first zinc finger of the VDR are required for interactions with LEF1.

**Point Mutations in the First Zinc Finger of the VDR Interfere with VDR-LEF1 Interactions**—Sequences in the DBD of nuclear receptor superfamily members are highly conserved. Residues that are divergent mediate interactions with specific cofactors and DNA response elements. Substitution of residues from loops IA and IB of the first zinc finger of the VDR with those of the glucocorticoid receptor (GR) is permissive for DNA binding and activation of VDREs, although to a lesser degree than the wild-type VDR (31). Because the LEF1 interaction domain of the VDR resides within this region, these point mutations were engineered as N-terminal GST fusions lacking the AF1 domain. Pulldown studies demonstrated that both mutant VDRs retained the ability to interact with LEF1 (Fig. 4A). Two additional mutants were engineered by substituting non-conserved residues from thyroid receptor-β (TRβ) and the GR. GST pulldown studies demonstrated that the GST-ΔAF1GRZn1-VDR mutant retained the ability to interact with LEF1, whereas the GST-ΔAF1TRβZn1-VDR mutant did not (Fig. 4B).

**The TRβ-VDR Is Unable to Enhance Canonical Wnt Signaling in vdr<sup>−/−</sup> Keratinocytes**—The VDR is required for synergistic activation of a Wnt reporter gene by β-catenin and LEF1 in primary keratinocytes (9). To determine whether the VDR modulates canonical Wnt signaling in keratinocytes through an interaction with LEF1, full-length GST-TRβ-VDR was subcloned into a mammalian expression vector and tested for its ability to restore canonical Wnt signaling in the absence of the VDR. Wnt reporter assays (TOPflash) were performed in primary keratinocytes isolated from vdr<sup>−/−</sup> mice. Although the wild-type VDR enhanced TOPflash expression by >2-fold in the presence of β-catenin and LEF1 (<i>p</i> < 0.005), the TRβ-VDR was unable to enhance reporter gene expression relative to the empty vector pcDNA3.1 (Fig. 5A), suggesting that the VDR-LEF1 interaction is required for the actions of the VDR on the canonical Wnt signaling pathway. To determine whether the TRβ-VDR is able to activate ligand-dependent VDR transactivation, COS-7 cells were cotransfected with a VDRE-luciferase reporter and the wild-type or TRβ-VDR. In the presence of ligand, the wild-type VDR significantly enhanced VDRE reporter activity compared with untreated cells (<i>p</i> < 0.005 for 10<sup>−8</sup> M and <i>p</i> < 0.05 for 10<sup>−6</sup> M); however, the TRβ-VDR did not. These data suggest that the mutated residues are required not only for canonical Wnt signaling but also for transactivation in response to 1,25-dihydroxyvitamin D (Fig. 5B).

**Expression of Canonical Wnt Target Genes Is Altered in the Absence of the VDR**—To determine whether canonical Wnt target genes are misregulated in the epidermis of vdr<sup>−/−</sup> mice, quantitative real-time PCR was performed on skin isolated from wild-type or vdr<sup>−/−</sup> mice at 28 days of age, the beginning of the first postnatal anagen in mice. These analyses demonstrated that expression of c-myc, a classical canonical Wnt target gene, was suppressed in the absence of the VDR. Furthermore, the expression of shh and gli1, canonical Wnt target genes critical for hair follicle response to canonical Wnt signaling during anagen, was also significantly down-regulated in vdr<sup>−/−</sup> skin compared with wild-type controls (Fig. 6A). Additional quantitative real-time PCR studies were performed in primary keratinocytes isolated from neonatal vdr<sup>−/−</sup> and wild-type mice. Although shh mRNA was not detectable in these cells, significant down-regulation of c-myc and gli1 was
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Figure 5. The TRβ-VDR does not rescue impaired canonical Wnt signaling in vdr−/− primary keratinocytes. A, primary vdr−/− keratinocytes were cotransfected with Renilla luciferase, TOPflash luciferase, β-catenin, and LEF1 along with pcDNA, the wild-type VDR, or the TRβ-VDR. After 24 h, Wnt reporter activity (TOPflash) was quantitated. Firefly luciferase was normalized for cotransfected Renilla luciferase. B, COS-7 cells were cotransfected with Renilla luciferase, VDRE-luciferase, and the wild-type VDR or TRβ-VDR. After 18 h of treatment with 10−8 or 10−9 M vitamin D, VDRE-luciferase reporter activity was quantitated. Firefly luciferase was normalized for cotransfected Renilla luciferase. Results are an average of at least three independent transfections. *, p ≤ 0.05; **, p ≤ 0.005. Panels to the right are Western analyses demonstrating expression of the VDR and TRβ-VDR proteins.

To determine whether the TRβ-VDR is able to rescue the impaired activation of canonical Wnt target genes, vdr−/− keratinocytes were transfected with the wild-type VDR or TRβ-VDR. Although the wild-type VDR was able to enhance expression of c-myc and gli1, the TRβ-VDR was not (Fig. 6C). These data demonstrate that the expression of canonical Wnt target genes in keratinocytes is modulated by the VDR. Similarly, transfection of vdr−/− keratinocytes with the wild-type VDR, but not with the TRβ-VDR, enhanced expression of cyclin D1 and keratin 10, genes involved in keratinocyte proliferation and differentiation, respectively (Fig. 6D).

The VDR, but Not the TRβ-VDR, Occupies Regulatory Regions of Keratinocyte Genes—Activation of Shh signaling transiently rescues the hair follicle phenotype in vdr−/− mice (17). Furthermore, the regulatory regions of shh and gli1 were reported to have multiple VDREs (18). Consensus VDREs were identified, and studies were performed to determine whether the VDR and TRβ-VDR interact with the regulatory regions of the shh and gli1 genes that contain these sequences. ChIP analyses were performed in vdr−/− primary keratinocytes transfected with the wild-type VDR or TRβ-VDR. Although the VDR was enriched on the sequences of the shh and gli1 genes that contain VDRE consensus motifs, the TRβ-VDR was not (Fig. 7).

Discussion

These investigations identify a novel interaction between the endogenous VDR and LEF1 that is independent of both β-catenin and ligand. Many nuclear receptors have been shown to interact with effectors of the canonical Wnt signaling pathway (32), including the androgen and estrogen receptors, which interact with TCFs/LEFs independently of β-catenin (33, 34). Like the novel VDR-LEF1 interaction identified in this study, the interaction between the androgen receptor and TCF4 is dependent upon the DBD of the androgen receptor (33).

Previous studies have demonstrated an interaction between the VDR and β-catenin that is dependent upon 1,25-dihydroxyvitamin D (18, 25, 35). Although the liganded VDR attenuates canonical Wnt signaling (35, 36), β-catenin has been shown to be a ligand-dependent coactivator of the VDR (18). Traditional nuclear receptor coactivators are recruited to the VDR in response to a conformational change that occurs upon ligand binding, rendering the AF2 domain accessible for interactions with these cofactors (37). Consistent with its role as a VDR coactivator, β-catenin binds to the AF2 region of the VDR. However, the AF2 domain of the VDR is dispensable for VDR-LEF1 interactions, in keeping with our findings that this association occurs in a ligand-independent fashion. Thus, identification of a ligand-independent VDR-LEF1 interaction identifies a potential mechanism by which the unliganded VDR modulates canonical Wnt signaling in keratinocytes.

The VDR selectively interacts with distinct coactivator complexes at different stages of keratinocyte differentiation (26–29). Although the VDR preferentially interacts with the DRIP complex during the proliferative stage, interactions with SRC2 and SRC3 complexes predominate in differentiating keratinocytes (29). However, these interactions are not observed in the absence of ligand (Fig. 2) (26, 38). In contrast, the interactions between the VDR, RXRα, and LEF1 are seen in both proliferative and differentiated keratinocytes and are independent of ligand. Like the VDR and LEF1, RXRα is an important regulator of postnatal hair cycling. Skin-specific ablation of RXRα, the major RXR isoform expressed in the epidermis, results in alopecia totalis, development of dermal cysts, and an epidermal inflammatory response (39). Mutation or ablation of Hairless (HR) also causes alopecia accompanied by lipid-laden dermal cysts in both humans and mice (40–43). HR is a nuclear receptor co-repressor that directly interacts with the VDR and represses ligand-dependent VDR transactivation of target genes (44). HR also suppresses the expression of two Wnt inhibitors: Soggy and Wise (45, 46). Mutation of the VDR-interacting domain of HR abolishes the repressive actions of HR on Wise, suggesting that interactions with the VDR are
required for the ability of HR to modulate canonical Wnt signaling (45). Consistent with the observation that ligand binding is not required for the VDR-HR interaction (8), VDR-HR interactions are dependent upon sequences within the VDR ligand-binding domain (44), not the AF2 domain. Thus, the interactions of the VDR with HR, LEF1, and RXR/H9251 share common features: notably, they are independent of the AF2 domain and are observed in the absence of ligand. Furthermore, impairing signaling by each of these four transcription factors in keratinocytes in vivo results in a similar phenotype (3, 12, 13, 39, 43).

However, whether this phenotype is a reflection of the VDR, RXRα, and HR converging on the canonical Wnt signaling pathway through interactions with LEF1 in the KSC remains to be determined.

Although the in vivo significance of the VDR-LEF1 interaction is not yet clear, both the VDR and LEF1 clearly play important roles in hair follicle regeneration in the mouse. Studies have identified multiple keratinocyte genes with VDREs and TCF/LEF sites in their promoters (18). Among these are shh and its effector gli1, which is induced 8.76 + 0.86-fold in response to Wnt3a treatment of primary keratinocytes (data not shown). Because one of the early markers of anagen initiation in response to activation of the canonical Wnt signaling pathway is induction of Hedgehog signaling, we examined the expression of these two genes in the skin of vdr null mice at the time of onset of the first postnatal anagen. The reduced expression of these genes is consistent with impaired canonical Wnt signaling in the keratinocytes of the vdr/vdr mice. Our studies also demonstrate occupancy of the VDR on the regulatory regions of both these genes in primary keratinocytes. Expression of shh was not detectable in these cells; however, that of gli1 was markedly impaired in the absence of the VDR.

Although studies in primary keratinocytes are an appropriate model in which to investigate the effects of the unliganded VDR with non-classical co-modulators, they cannot replicate the complex in vivo environment of the pilosebaceous unit. However, the investigations reported herein, along with studies in genetically manipulated mice, have identified novel interactions of the unliganded VDR with the canonical Wnt signaling pathway that are likely to play a critical role in the regulation of

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FIGURE 6. The VDR promotes keratinocyte gene expression. A, skin was isolated from vdr+/− and wild-type mice at 28 days of age for evaluation of mRNA expression by quantitative real-time reverse transcription-PCR. B, RNA isolated from cultured primary keratinocytes from neonatal vdr+/− and wild-type mice was subjected to quantitative real-time reverse transcription-PCR. C and D, RNA isolated from cultured primary vdr+/− keratinocytes transfected with empty vector, the wild-type VDR, or the TRβ-VDR was subjected to quantitative real-time reverse transcription-PCR. Values are expressed as the relative expression of the normalized levels of vdr−/− skin or keratinocyte mRNA compared with wild-type controls. Data shown are based on at least three independent mRNA isolations ± S.E. *, p ≤ 0.05; **, p ≤ 0.005.

FIGURE 7. The VDR, but not the TRβ-VDR, is present on the shh and gli1 promoters. Quantitative ChIP analyses were performed to identify enrichment of the VDR on the shh and gli1 regulatory regions. Values are shown as relative -fold enrichment of the promoter sequences versus coding region sequences compared with that obtained for input samples not subjected to ChIP. Data shown are representative of those obtained with three independent experiments ± S.D. *, p ≤ 0.05; ***, p ≤ 0.001 (by Student’s t test for ChIP samples versus input samples).
postnatal hair cycles. Together, these data suggest that the VDR, through its interactions with LEF1, contributes to regulation of the canonical Wnt and Hedgehog signaling pathways in the epidermis.

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