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PROSPECT

Vitamin D Regulated Keratinocyte Differentiation

Daniel D. Bikle*

Medicine and Dermatology Endocrine Research Unit, VA Medical Center, University of California, San Francisco, California

Abstract The epidermis is the largest organ in the body. It is comprised primarily of keratinocytes which are arranged in layers that recapitulates their programmed life cycle. Proliferating keratinocytes are on the bottom—the stratum basale. As keratinocytes leave the stratum basale they begin to differentiate, culminating in the enucleated stratum corneum which has the major role of permeability barrier. Calcium and the active metabolite of vitamin D, $1,25(OH)_2D_3$, play important roles in this differentiation process. The epidermis has a gardient of calcium with lowest concentrations in the stratum basale, and highest concentrations in the stratum granulosum where proteins critical for barrier function are produced. Vitamin D is made in different layers of the epidermis, but $1,25(OH)_2D_3$ is made primarily in the stratum basale. Together calcium and $1,25(OH)_2D_3$ regulate the ordered differentiation process by the sequential turning on and off the genes producing the elements required for differentiation as well as activating those enzymes involved in differentiation. Animal models in which the sensing mechanism for calcium, the receptor for $1,25(OH)_2D_3$, or the enzyme producing $1,25(OH)_2D_3$ have been rendered inoperative demonstrate the importance of these mechanisms for the differentiation process, although each animal model has its own phenotype. This review will examine the mechanisms by which calcium and $1,25(OH)_2D_3$ interact to control epidermal differentiation. J. Cell. Biochem. 92: 436–444, 2004.

Key words: epidermis; calcium; phospholipase C; coactivators; 25OHD-1 alpha hydroxylase

The epidermis is the largest organ in the body, critical for life. It keeps what we need inside, and keeps what we don't need outside—the barrier function. The primary cell in the epidermis responsible for this barrier function is the keratinocyte. One of environmental insults to which the epidermis is constantly exposed is ultraviolet (UV) radiation. UV radiation in excess is harmful—it causes DNA damage that can result in cancer. However, from the perspective of this review, UV is beneficial in that the epidermis uses the energy in UV to convert 7-dehydrocholesterol (7-DHC) to pre vitamin D_3 which then isomerizes to vitamin D_3. The epidermis is self-renewing. Proliferating keratinocytes are found only in the base of the epidermis, in the layer known as the stratum basale. Daughter cells leave the stratum basale and differentiate on their journey to the surface, where as the enucleated cells of stratum corneum they form the permeability barrier. Along the way genes are sequentially turned on and off to produce proteins which contribute to the fully differentiated keratinocyte. For example, basal keratinocytes produce keratins 5 and 14. As they enter the stratum spinosum, the production of keratins 1 and 10 replaces that of keratins 5 and 14. Involucrin, an important component of the cornified envelope, and transglutaminase-K, the enzyme which cross links involucrin and other substrates to form the cornified envelope, are also made in the keratinocytes of the stratum spinosum. The next higher level, the stratum granulosum, is marked by the presence of keratohyalin granules. These granules contain loricrin, a major component of the cornified envelope, and profilaggrin, a precursor of filaggrin which serves as a bundling protein for the keratin filaments. The

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*Correspondence to: Daniel D. Bikle, MD, PhD, Professor of Medicine and Dermatology, Endocrine Research Unit, VA Medical Center, University of California, San Francisco, CA. E-mail: doctor@itsa.ucsf.edu

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stratum granulosum also contains lamellar bodies whose contents of lipids and lipid processing enzymes are secreted into the junction between the stratum granulosum and stratum corneum to provide the "mortar" between the "bricks" which are the corneocytes of the stratum corneum.

This sequence of events is tightly regulated. A number of agents can impact the differentiation process. Our focus has been on calcium and $1,25(OH)_2D_3$. In vitro, calcium is one of the most potent means of stimulating epidermal differentiation. In vivo, a calcium gradient forms in the epidermis (lowest concentrations in the stratum basale, highest concentrations in the stratum granulosum). This gradient appears important for the differentiation process in vivo. The keratinocytes of the stratum basale contain vitamin D receptors and produce their own supply of $1,25(OH)_2D_3$. Thus the keratinocyte, and only the keratinocyte, has the entire metabolic machinery to produce $1,25(OH)_2D_3$ from 7-dehydrocholesterol (7-DHC), and is a target for that hormone as well. Given that $1,25(OH)_2D_3$ is a calcium regulating hormone, it should come as no surprise that the effects of calcium and $1,25(OH)_2D_3$ on keratinocyte differentiation are interacting.

In this article we will briefly review the mechanisms by which calcium and $1,25(OH)_2D_3$ regulate the differentiation process of the keratinocyte. Much of our information comes from in vitro studies, but the recent availability of mice lacking the calcium receptor (CaR), VDR

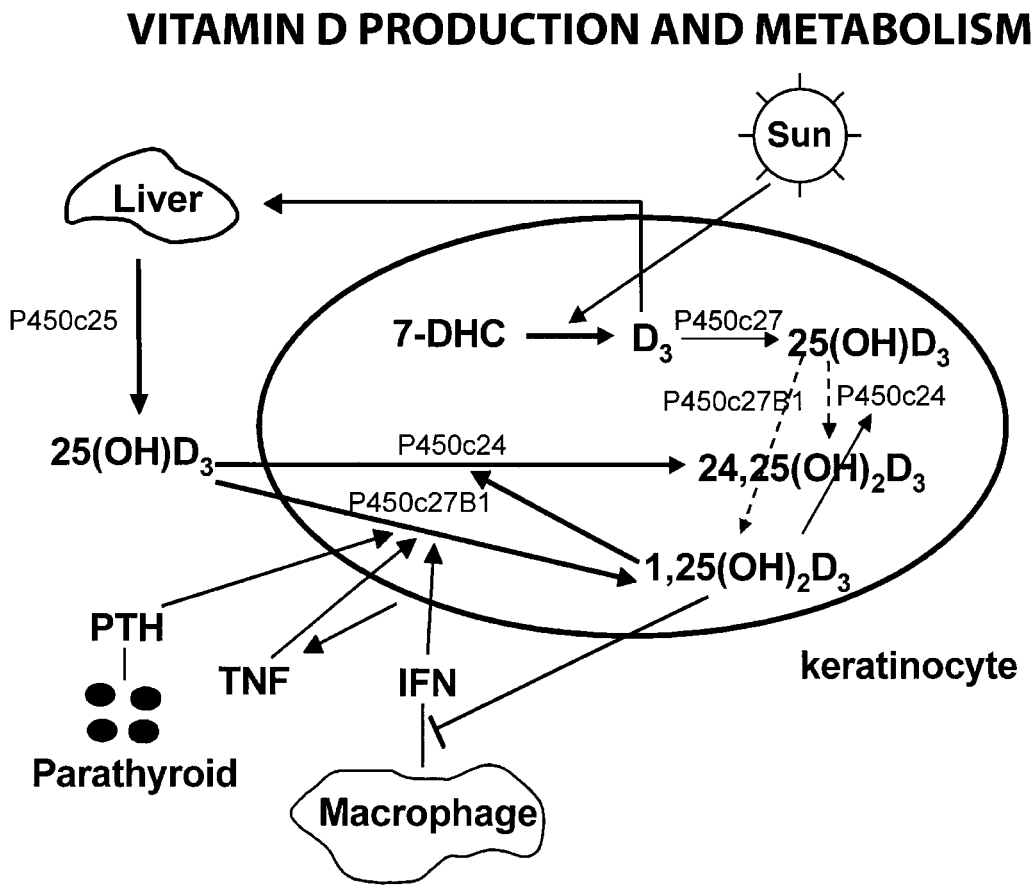

Fig. 1. Vitamin D production and conversion to $1,25(OH)_2D_3$ in the keratinocyte. 7-Dehydrocholesterol (7-DHC) is converted to vitamin D_3 by a photochemical reaction. The vitamin D_3 produced is either transported out of the keratinocyte to the liver, where it is converted to $25OHD_3$, or metabolized directly to $25OHD_3$ in the keratinocyte by the enzyme 25-hydroxylase (CYP27). $25OHD_3$ is metabolized either to $24,25(OH)_2D_3$ or to $1,25(OH)_2D_3$ by the enzymes 24-hydroxylase (CYP24) and 1α-hydroxylase (CYP27B1), respectively. Parathyroid hormone (PTH) secreted by the parathyroid gland (PTG) stimulates the production of $1,25(OH)_2D_3$, as does tumor necrosis factor-α (TNF) secreted by keratinocytes and interferon-γ (IFN) secreted by macrophages. $1,25(OH)_2D_3$ promotes its own catabolism by inducing the 24-hydroxylase [also responsible for $24,25(OH)_2D_3$ production], and decreasing IFN secretion by macrophages.

or the 25OHD 1α-hydroxylase (1OHase) (the enzyme responsible for producing $1,25(OH)_2D_3$) is helping to refine the concepts derived from in vitro studies.

EPIDERMAL PRODUCTION OF $1,25(OH)_2D_3$

As previously mentioned keratinocytes are not only capable of producing D_3 but of metabolizing D_3 via the vitamin D-25 hydroxylase and the 25OHD-1OHase to form the active metabolite $1,25(OH)_2D_3$ [Bikle et al., 1986a,b; Matsumoto et al., 1991; Lehmann et al., 2001] (Fig. 1). The vitamin D-25 hydroxylase in keratinocytes is the same mitochondrial enzyme (CYP27) that converts vitamin D_3 to $25OHD_3$ in the liver [Masumoto et al., 1988; Lehmann et al., 1999]. Its expression is increased by vitamin D and UVB irradiation [Masumoto et al., 1988; Lehmann et al., 1999]. Similarly 1OHase in the epidermis is the same enzyme (CYP27B1) as that found in the kidney [Fu et al., 1997]. Its expression and enzymatic activity are tightly regulated and coupled to the differentiation of these cells [Pillai et al., 1988]. Enzymatic activity is greatest in the undifferentiated cells. Growing the cells in 0.1 mM calcium, which retards differentiation, permits the cells to maintain higher 1OHase activity than when they are grown in 1.2 mM calcium [Bikle et al., 1989], although acute changes in calcium have little effect on $1,25(OH)_2D_3$ production. These observations in vitro are consistent with the finding that 1OHase expression is highest in the stratum basale of the epidermis in vivo [Zehnder et al., 2001].

Parathyroid hormone (PTH) exerts a modest stimulation of $1,25(OH)_2D$ production by keratinocytes. However, this involves a different mechanism than that resulting in stimulation of $1,25(OH)_2D_3$ production by PTH in the kidney. The keratinocyte does not have a classic PTH receptor coupled to adenylate cyclase. Furthermore, these effects of PTH are not reproduced by cAMP or its membrane-permeable derivatives, suggesting that the actions of PTH may be operating through a mechanism independent of cAMP [Bikle et al., 1986a]. $1,25(OH)_2D_3$ negatively regulates its own levels within the keratinocyte. This negative feedback loop is similar to that observed in the kidney. In the keratinocyte, this feedback inhibition is not mediated by an effect on $1,25(OH)_2D_3$ production but is due solely to stimulation of $1,25(OH)_2D_3$ catabolism through induction of the enzyme 25OHD 24-hydroxylase. This enzyme metabolizes $25OHD_3$ and $1,25(OH)_2D_3$ to $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, respectively [Xie et al., 2002b]. The exquisite responsiveness of the 25OHD-24-hydroxylase to $1,25(OH)_2D_3$ in keratinocytes may explain why so little $1,25(OH)_2D_3$ appears to enter the circulation from the skin when renal production of $1,25(OH)_2D_3$ is intact. Both tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) stimulate $1,25(OH)_2D_3$ production by keratinocytes [Morhenn and Wood, 1988; Bikle et al., 1989, 1991b; Pillai et al., 1989]. Although IFNγ is not made in keratinocytes, TNFα is produced by these cells, and its synthesis is stimulated by UV [Trefzer et al., 1993] and barrier disruption [Wood et al., 1994]. Thus, environmental perturbations could enhance $1,25(OH)_2D_3$ production in the skin, and the increased levels of $1,25(OH)_2D_3$ could play a role in the recovery from UV damage and/or barrier disruption. We will return to this point when we discuss recent results with the 1OHase null mouse.

ROLE OF CALCIUM AND $1,25(OH)_2D_3$ IN EPIDERMAL DIFFERENTIATION

Response to the Calcium Switch

As mentioned in the "Introduction," calcium is a potent means to induce keratinocyte differentiation in vitro [Hennings and Holbrook, 1983]. The effects of calcium can be seen within minutes as the rapid development of cell to cell contact, desmosome formation, and realignment of actin and keratin bundles near the cell membrane at the point of intercellular contacts. Desmoplakin (a component of desmosomes), fodrin (an actin and calmodulin binding spectrin-like protein), and calmodulin are redistributed to the membrane shortly after the calcium switch by a mechanism that is blocked by cytochalasin, an agent that disrupts microfilament reorganization [Inohara et al., 1990; Yoneda et al., 1990; Zamansky et al., 1991]. Within hours of the calcium switch, the cells begin to make involucrin [Rubin et al., 1989; Pillai et al., 1990; Su et al., 1994], loricrin [Hohl et al., 1991], transglutaminase [Rubin et al., 1989; Pillai et al., 1990; Su et al., 1994], keratins K1 and K10 [Yuspa et al., 1989], and filaggrin [Yuspa et al., 1989], and they start to form cornified envelopes [Yuspa et al., 1989; Pillai et al., 1990]. The mRNA levels for these proteins

increase following the calcium switch [Yuspa et al., 1989; Hohl et al., 1991; Su et al., 1994], indicating that these effects of calcium represent genomic actions, a conclusion confirmed by nuclear run on and promoter construct experiments for many of these genes. Calcium response regions have been identified in the promoters of the *involucrin* [Ng et al., 1996] and *Kl* [Huff et al., 1993] genes.

Regulation of Intracellular Calcium

Keratinocyte differentiation is tightly linked to a rise in intracellular free calcium ($[Ca^{2+}]_i$) [Pillai and Bikle, 1991]. Raising the extracellular calcium concentration ($[Ca^{2+}]_0$) increases $[Ca^{2+}]_i$ [Yuspa et al., 1989; Yoneda et al., 1990; Pillai and Bikle, 1991; Huff et al., 1993; Su et al., 1994; Ng et al., 1996]. The initial rise is mediated by the CaR [Oda et al., 1998, 2000; Tu et al., 2001], the same protein initially cloned and sequenced from the parathyroid gland (PTG) [Garrett et al., 1995]. Cells lacking the CaR do not respond to $[Ca^{2+}]_0$ acutely [Tu et al., 2001], and the epidermis of mice lacking the CaR does not differentiate normally [Komuves et al., 2002]. As cells differentiate, they lose their acute response to calcium [Pillai et al., 1990]. This coincides with a switch from producing the full length CaR to an alternatively spliced form lacking exon 5 [Oda et al., 1998]. Only the full length CaR is capable of responding acutely to $[Ca^{2+}]_0$ with an increase in $[Ca^{2+}]_i$ [Oda et al., 1998]. Following the initial rise, the increase in $[Ca^{2+}]_i$ is sustained. Agents such as ATP, which stimulate only a transient increase in $[Ca^{2+}]_i$, do not promote differentiation. Several channels have been identified in the keratinocyte membrane that are candidates for mediating calcium induced calcium influx [Mauro et al., 1990, 1993; Galietta et al., 1991; Grando et al., 1996; Oda et al., 1997]. Recent studies from our laboratory (Tu and Bikle, unpublished) suggest that the trp channels 1 and 4 may carry much of this calcium influx at least following store depletion.

The Role of Phospholipases

Calcium both induces and activates the phospholipase C family, whose members provide additional second messengers for mediating the effects of calcium on the keratinocyte [Jaken and Yuspa, 1988; Xie and Bikle, 1999]. The principal enzymes involved are phospholipase C (PLC) β and γ1. These enzymes hydrolyze phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). As for the response of $[Ca^{2+}]_i$ to $[Ca^{2+}]_0$, the rise in IP_3 and DAG is both immediate and prolonged following the calcium switch. The prolonged increase in IPs appears to be due to calcium activation of PLC-γ1, although the initial increase in IP_3 and $[Ca^{2+}]_i$ after the calcium switch appears to be mediated by PLC-β. Activation of PLC-γ1 is mediated by a calcium induced increase in src family tyrosine kinases [Carpenter and Ji, 1999]. Keratinocytes lacking PLC-γ1 fail to differentiate in response to calcium [Xie and Bikle, 1999].

Interactions of $1,25(OH)_2D_3$ and Calcium

The mechanisms by which $1,25(OH)_2D_3$ alters keratinocyte differentiation are multiple and overlap with the mechanisms by which calcium regulates differentiation (Fig. 2). Some studies have shown an acute increase in $[Ca^{2+}]_i$ associated with an acute increase in phosphoinositide turnover (producing a rise in both IP_3 and DAG) following $1,25(OH)_2D_3$ administration [Tang et al., 1987; Yada et al., 1989; McLaughlin et al., 1990; Tang and Ziboh, 1991], but we [Bikle et al., 1996] and others have been unable to reproduce these acute effects. The rise in $[Ca^{2+}]_i$, IP_3, and DAG is accompanied by translocation of PKC to the membrane [Yada et al., 1989]. Down-regulation of PKC and inhibition of its activity have been reported to block the ability of $1,25(OH)_2D_3$ to stimulate cornified envelope formation [Yada et al., 1989]. However, the role of PKC in mediating or interacting with $1,25(OH)_2D_3$ in its effects on keratinocyte differentiation remains virtually unexplored.

Calcium and $1,25(OH)_2D_3$ interact in their ability to inhibit proliferation and stimulate involucrin and trans-glutaminase gene expression [Su et al., 1994]. The higher the $[Ca^{2+}]_0$, the more sensitive is the keratinocyte to the antiproliferative effect of $1,25(OH)_2D_3$ (and vice versa) [McLane et al., 1990]. Part of this synergism can be explained by the ability of $1,25(OH)_2D_3$ to induce the CaR [Ratnam et al., 1996b] and so make the keratinocyte more sensitive to $[Ca^{2+}]_0$ [Ratnam et al., 1996b]. Secondly, both calcium and $1,25(OH)_2D_3$ induce the PLC family of enzymes [Pillai et al., 1995; Xie and Bikle, 1997], and calcium can further activate these enzymes by non genomic means. The mechanism by which calcium induces PLC

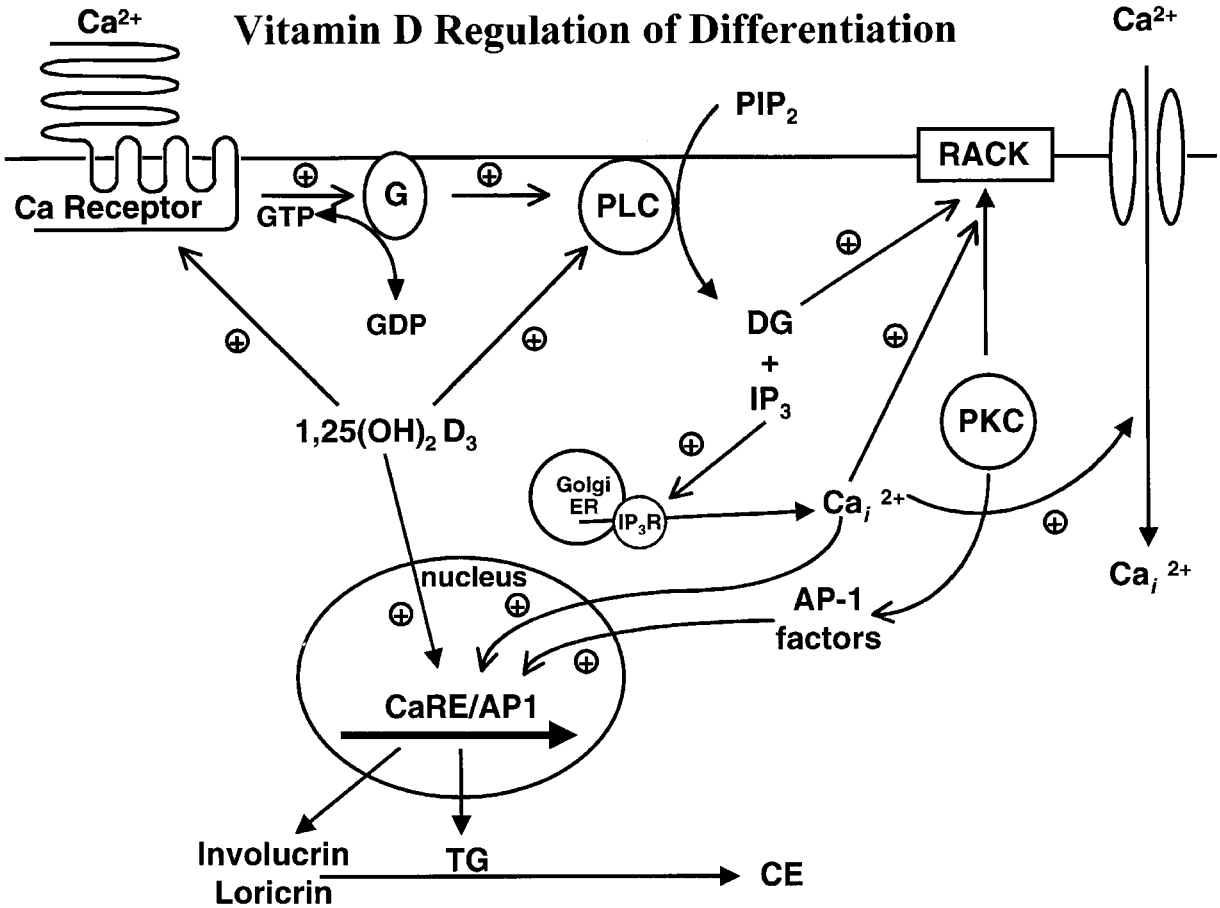

Fig. 2. Regulation by $1,25(OH)_2D_3$ of keratinocyte differentiation. The calcium receptor and the PLCs are induced by $1,25(OH)_2D_3$, which enhances the ability of calcium to raise intracellular calcium, IP_3, and diacylglycerol (DG). The rise in these second messengers leads to protein kinase activation (PKC) in part by translocation of PKCs to their specific membrane receptors (RACK) and opening up of calcium channels in the plasma membrane. $1,25(OH)_2D_3$ in combination with the increase in intracellular calcium and the AP-1 transcription factors activated by PKC stimulates differentiation by inducing the substrates [e.g., involucrin (Inv) and loricrin] for cornified envelope (CE) formation as well as the enzyme transglutaminase (TG), which cross-links these substrates into the CE.

is unclear, but a DR6 type vitamin D response element is present in the promoter of the *PLC-γ1* gene that is critical for its induction by $1,25(OH)_2D_3$. The regulation of gene expression for involucrin and transglutaminase is more complex. Both calcium (in the absence of $1,25(OH)_2D_3$) and $1,25(OH)_2D_3$ (at 0.03 mM Ca^{2+}) raise the mRNA levels for involucrin and transglutaminase in a dose-dependent fashion. The stimulation is synergistic at intermediate concentrations of calcium (0.1 mM) and $1,25(OH)_2D_3$ (10^{-10} M), but not at higher concentrations. The synergism is more apparent at earlier times after the calcium switch (4 h) than later (24–72 h). This result stems from the increased turnover of the transcripts from these genes in the presence of elevated calcium and $1,25(OH)_2D_3$. This provides a protective mechanism by which excess involucrin and transglutaminase production is prevented in the face of increased calcium and/or $1,25(OH)_2D_3$, and may be one mechanism by which the production of such proteins is sequentially turned on and off as the keratinocytes differentiate in vivo. One explanation for the synergism in the induction of involucrin is that the calcium response element (CaRE) and vitamin D response element (VDRE) in the involucrin promoter are quite close spatially [Bikle et al., 2002]. Mutations in the AP-1 site within the CaRE block both calcium and $1,25(OH)_2D_3$ induction of the *involucrin* gene, but mutations within the VDRE block only its response to $1,25(OH)_2D_3$ [Bikle et al., 2002]. The

molecular basis for the synergism with respect to transglutaminase induction has not been elucidated.

In Vivo Models

The recent availability of mice lacking either the VDR or the 1OHase has expanded our understanding of the role of $1,25(OH)_2D_3$ in epidermal differentiation. Although the most striking feature of the VDR null mouse is the development of alopecia (likewise found in many patients with mutations in the VDR), these mice also exhibit a defect in epidermal differentiation as shown by reduced levels of involucrin, profilaggrin, and loricrin and loss of keratohyalin granules [Xie et al., 2002a]. Similarly 1OHase null mice show a reduction in levels of the epidermal differentiation markers [Bikle et al., 2004]. Furthermore, 1OHase null animals have a retarded recovery of barrier function when the barrier is disrupted, which on ultrastructural examination is associated with an impaired reestablishment of the calcium gradient in the epidermis [Bikle et al., 2004]. This has not been observed in VDR null mice. On the other hand 1OHase null animals do not have a defect in hair follicle cycling. The difference in phenotypes between these genotypes is surprising, and points to the possibility that the 1OHase in the epidermis may be doing more than making $1,25(OH)_2D_3$, just as the phenotype in the VDR null animal suggests that the VDR may have functions independent of $1,25(OH)_2D_3$. However, as in the CaR null mouse, differentiation of the epidermis is abnormal in the absence of the VDR or the ability to make $1,25(OH)_2D_3$.

Role of VDR Coactivators

An important problem in understanding the effects of $1,25(OH)_2D_3$ and calcium on keratinocyte differentiation is to explain how genes are sequentially turned on and off during the course of normal differentiation and why such regulation does not occur in transformed keratinocytes. As mentioned previously, the VDR levels and the full length CaR decrease as keratinocytes differentiate. This explains why differentiated keratinocytes are less sensitive to $1,25(OH)_2D_3$ and calcium. Furthermore, as also previously mentioned, calcium and $1,25(OH)_2D_3$ synergistically lead to enhanced turnover of the transcripts for at least some of the genes they induce, again providing a mechanism by which genes are effectively turned on then off. However, these mechanisms do not explain why transformed keratinocytes, which have ample levels of VDR [Ratnam et al., 1996a], produce ample amounts of $1,25(OH)_2D_3$ [Bikle et al., 1991a], and have a robust $[Ca^{2+}]_i$ response to $[Ca^{2+}]_0$ [Pillai et al., 1991] fail to respond to these agents with an increase in transcription of the genes required for differentiation. Oda et al. [2003] recently gained insight into this problem when we examined the coactivator complexes required for VDR function in keratinocytes at different stages of differentiation. The VDR binds to one of two coactivator complexes, Mediator/DRIP (VDR interacting proteins) or p160/SRC (steroid hormone receptor complex), complexes which link the VDR to the RNA polymerase complex. We discovered that the binding of VDR to these complexes is sequential. Binding to Mediator/DRIP occurs in the undifferentiated keratinocyte, but as the cell differentiates, $DRIP_{205}$ (the key protein of the DRIP complex binding to the VDR) levels fall, SRC3 levels increase, and p160/SRC binding to the VDR takes over. This transition from DRIP to p160/SRC does not occur in transformed keratinocytes. The vitamin D response element (VDRE) in the promoter of the *24 hydroxylase* gene, a gene which is induced by $1,25(OH)_2D_3$ in both normal and transformed keratinocytes, binds the VDR whether it is associated with DRIP or SRC. On the other hand the VDREs in the keratin 1 and involucrin promoters bind very little of the VDR/DRIP complex, preferring the VDR/SRC complex. Thus, we hypothesize that this sequential replacement of Mediator/DRIP by p160/SRC is critical for differentiation. Squamous cell carcinomas (SCC) fail to respond to the prodifferentiating actions of $1,25(OH)_2D$ because they fail to downregulate $DRIP_{205}$, and the VDR/SRC complex required for induction of the genes required for differentiation fails to form.

SUMMARY

Keratinocyte differentiation is a tightly controlled process critical for maintenance of the barrier function of skin. Calcium and $1,25(OH)_2D_3$ participate in regulation of keratinocyte differentiation. Keratinocytes contain both CaR and VDR and possess the metabolic machinery to make their own $1,25(OH)_2D_3$ (via the 1OHase). Although most of our information

on the mechanisms by which such regulation occurs comes from in vitro studies, the abnormalities in the epidermis in animals lacking the CaR, the VDR, or the 1OHase attest to the importance of calcium and $1,25(OH)_2D_3$ in regulating keratinocyte differentiation in vivo. Calcium and $1,25(OH)_2D_3$ interact in their regulation of keratinocyte differentiation. A sustained increase in $[Ca]_i$ is required for differentiation to occur. Extracellular calcium acutely increases $[Ca]_i$ via the CaR, but exerts a more prolonged effect on $[Ca]_i$ via activation and induction of PLC and the opening of various calcium channels. $1,25(OH)_2D_3$ potentiates these effects by inducing the CaR and PLC family. Through the sustained rise in $[Ca]_i$, genes producing the proteins required for differentiation are induced in a manner potentiated by $1,25(OH)_2D_3$ acting on these genes through its own VDREs. The sequential turning on and off of genes during the course of differentiation can be understood by the loss of the full length CaR and VDR with differentiation, the synergism between calcium and $1,25(OH)_2D_3$ first in gene induction but subsequently in stimulating mRNA degradation, and the transition from DRIP to SRC coactivator binding to the VDR with differentiation in that the VDRE of genes induced during differentiation appear to be selective for the VDR/SRC complex.

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