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Vitamin D Regulated Keratinocyte Differentiation: Role of Coactivators

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Abstract 1,25 Dihydroxyvitamin D (1,25$(OH)_2$D) regulates the differentiation of keratinocytes. 1,25$(OH)_2$D raises intracellular free calcium (Cai) as a necessary early step toward stimulating differentiation. 1,25$(OH)_2$D induces the calcium sensing receptor (CaR) in keratinocytes and enhances the calcium response of these cells. Activation of the CaR by calcium increases intracellular free calcium by a mechanism involving phospholipase C (PLC) cleavage of phosphatidylinositolbisphosphate into inositoltrisphosphate (IP_3) and diacylglycerol (DG). 1,25$(OH)_2$D induces the family of PLCs. PLC-γ1 has a DR6 VDRE in its promoter which binds and is activated by VDR/RAR rather than VDR/RXR. The involucrin gene, which encodes a critical component of the cornified envelope, contains a DR3 VDRE in its promoter that acts in conjunction with a nearby AP-1 site. The sequential regulation of these genes is critical for the differentiation process. In undifferentiated keratinocytes, the VDR binds preferentially to the DRIP complex of coactivators. However, with differentiation DRIP 205 is no longer produced, and the VDR switches partners to the SRC family (SRC2 and 3). These studies suggest that at least part of the sequential activation of genes required during keratinocyte differentiation is regulated by the change (availability) of these different coactivator complexes. J. Cell. Biochem. 88: 290–295, 2003.

Key words: involucrin; transglutaminase; vitamin D receptor; steroid receptor coactivator; phospholipase C; calcium receptor

The observation that 1,25$(OH)_2$D induces keratinocyte differentiation was first made by Hosomi et al. [1983] and provided a rationale for the previous and unexpected finding of 1,25$(OH)_2$D receptors in the epidermis [Stumpf et al., 1979]. 1,25$(OH)_2$D is likely to be an autocrine or paracrine factor for epidermal differentiation since it is produced by the keratinocyte, but under normal circumstances keratinocyte production of 1,25$(OH)_2$D does not appear to contribute to circulating levels [Bikle et al., 1986]. Recent evidence that the keratinocyte also converts vitamin D to 25OHD indicates that the keratinocyte is unique in its possession of the complete pathway from 7-dehydrocholesterol to 1,25$(OH)_2$D [Lehmann et al., 1999]. The receptors for and the production of 1,25$(OH)_2$D vary with differentiation [Pillai et al., 1988] in a manner which suggests feedback regulation; both are reduced in the later stages of differentiation. The differentiation pathway that we have studied most directly is the formation of the cornified envelope (CE). 1,25$(OH)_2$D increases gene expression and the mRNA and protein levels for involucrin, a precursor for the CE, and transglutaminase, the enzyme which crosslinks involucrin and other substrates to form the CE. Thus, 1,25$(OH)_2$D promotes CE formation at subnanomolar concentrations in preconfluent keratinocytes [Hosomi et al., 1983; Smith et al., 1986; McLane et al., 1990; Pillai and Bikle, 1991; Su et al., 1994]. At these same concentrations, 1,25$(OH)_2$D may either promote or inhibit proliferation [Itin et al., 1994; Bollag et al., 1995; Gniadecki, 1996] indicating

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that differentiation is not dependent on the antiproliferative actions of $1,25(OH)_2D$. Concentrations of $1,25(OH)_2D$ above $10^{-9}M$ are generally found to have only an antiproliferative effect. The mechanisms underlying the proproliferative actions are not known. The antiproliferative effects are accompanied by an increase in TGF-β [Kim et al., 1992] and a reduction in c-myc and the EGF receptor mRNA levels [Matsumoto et al., 1990]. In other cell lines, the antiproliferative actions are associated with an arrest at G1 with an increase in the inhibitors of G1 progression including $p21^{waf1}$, $p27^{kip1}$, and ink4 family members (p15, p16, p18) [Liu et al., 1996], and a decrease in the phosphorylation of the retinoblastoma protein pRB.

INTERACTION OF $1,25(OH)_2D$ AND CALCIUM

Calcium clearly affects the ability of $1,25(OH)_2D$ to stimulate keratinocyte differentiation, and vice versa. We [Su et al., 1994] observed that both calcium in the absence of $1,25(OH)_2D$ and $1,25(OH)_2D$ at low (0.03 mM) calcium raise the mRNA levels for involucrin and transglutaminase in a dose dependent fashion by stimulating gene expression. The stimulation of mRNA levels by calcium and $1,25(OH)_2D$ is synergistic at early time points, 4 h after addition of calcium and 28 h after addition of $1,25(OH)_2D$, but longer periods of incubation lead to a paradoxical fall in the mRNA levels for these proteins. This is due to the fact that although transcription is increased by calcium and $1,25(OH)_2D$, stability of the mRNA is reduced in cells incubated with calcium and $1,25(OH)_2D$ also in a synergistic fashion. The mechanism by which mRNA stability is regulated by calcium and $1,25(OH)_2D$ remains unexplored.

1,25(OH)2D RAISES CAI BY INDUCING THE CALCIUM RECEPTOR

The ability of $1,25(OH)_2D$ to increase intracellular free calcium levels (Cai) [Pillai and Bikle, 1991] accounts for at least part of the ability of $1,25(OH)_2D$ to induce differentiation. The keratinocyte responds to extracellular calcium via the same calcium receptor (CaR) found in the parathyroid gland and other tissues [Bikle et al., 1996; Oda et al., 1998; Tu et al., 1999]. This CaR is critical for calcium induced differentiation [Oda et al., 2000; Tu et al., 2001] in that knocking out the CaR in mice [Ho et al., 1995; Oda et al., 2000] or in human keratinocytes by antisense constructs [Tu et al., 2001] leads to keratinocytes which fail to respond to calcium with respect to increases in Cai and/or increases in the differentiation markers. $1,25(OH)_2D$ increases the mRNA levels of the CaR and enhances the responsiveness of the keratinocyte to calcium [Ratnam et al., 1999]. One interesting twist in this story is that keratinocytes make both a full length form of the CaR and an alternatively spliced form in which exon 5 is spliced out [Oda et al., 1998]. The alteratively spliced CaR does not mediate the acute response to calcium (by increases in either Cai or IP_3), and its function is unclear [Oda et al., 1998; Oda et al., 2000].

1,25(OH)2D RAISES CAI BY INDUCING THE PHOSPHOLIPASE C (PLC) FAMILY

A second mechanism by which $1,25(OH)_2D$ increases Cai involves the induction of the family of PLCs [Pillai et al., 1995]. As a result, $1,25(OH)_2D$ was found to increase both the basal and hormone (calcium, ATP, bradykinin, histamine) stimulated levels of IP_3 and Cai [Pillai et al., 1995]. We [Xie and Bikle, 1997] then investigated the mechanism by which $1,25(OH)_2D$ induced the PLC family, focusing on PLC-γ1 because it is the most abundant PLC in keratinocytes [Punnonen et al., 1993] and potentially the most important with respect to mediating the effects of $1,25(OH)_2D$ and calcium on proliferation and differentiation. The 5′-flanking region of the human PLC-γ1 gene was isolated from a human P1 genomic DNA library. Deletion and mutation studies of this fragment demonstrated a VDRE that contains a motif arranged as two direct repeats separated by six bases (DR6) between −786 and −803 base pairs (Fig. 1). Incubation of the oligonucleotide containing the DR6 with keratinocyte nuclear extracts produced a specific protein–DNA complex that shifted to a higher molecular weight form upon the addition of an antibody to the vitamin D receptor (VDR) and to the retinoic acid receptor (RAR) but not to the retinoid X receptor (RXR). These results indicate that for this VDRE, the VDR couples to RAR [Xie and Bikle, 1998]. All trans retinoic acid (tRA) potentiated the ability of $1,25(OH)_2D$ to stimulate not only the PLC-γ1 VDRE/luciferase

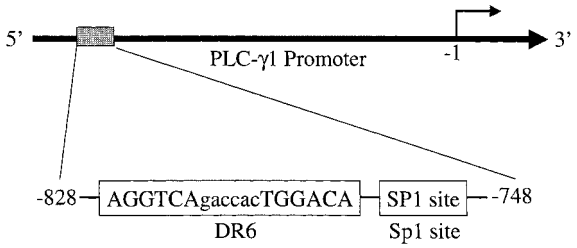

Fig. 1. The sequence of the vitamin D response element (VDRE) in the phospholipase C-γ1 promoter. Unlike most VDREs, this VDRE has six nucleotides between the two half sites (DR6). This VDRE is also unusual in that it recognizes VDR/RAR heterodimers rather than VDR/RXR heterodimers.

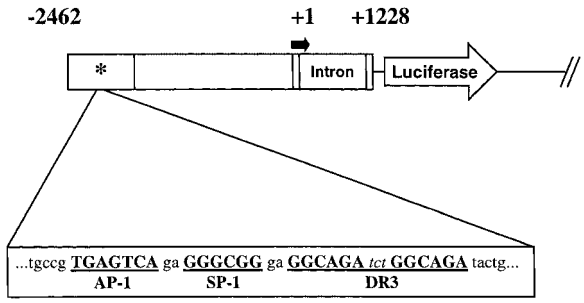

Fig. 2. The sequence of the calcium response element (CaRE) in the involucrin promoter. The CaRE contains an AP-1 site which is essential for basal activity as well as the ability of both calcium and 1,25(OH)$_2$D to stimulate involucrin gene expression. In contrast, the DR3 appears to mediate only 1,25(OH)$_2$D regulated expression of the involucrin gene. The functions of the SP-1 site are less clearly established.

construct in transfected keratinocytes but also the mRNA levels for PLC-γ1 in the normal keratinocytes [Xie and Bikle, 1998].

Although the induction of PLC-γ1 by 1,25(OH)2D could explain part of the ability of 1,25(OH)$_2$D to raise Cai, it was not clear how important this mechanism was to 1,25(OH)$_2$D regulated differentiation. Accordingly, we [Xie and Bikle, 1999] blocked expression of PLC-γ1 in human keratinocytes by transfecting cells with an antisense human PLC-γ1 cDNA construct. These cells demonstrated a specific reduction in PLC-γ1 protein levels compared to the empty vector-transfected cells and a marked reduction in the mRNA and protein levels of the differentiation markers, involucrin and transglutaminase, following administration of 1,25(OH)$_2$D [Xie and Bikle, 2001].

INVOLUCRIN PROMOTER VITAMIN D RESPONSE ELEMENT

We [Ng et al., 2000] mapped the calcium responsive region of the involucrin promoter to a region ~2,100 bases 5′ of the translation start site, and called this the calcium response element (CaRE) (Fig. 2). This region contains an AP-1 site which is critical for basal as well as calcium and 1,25(OH)$_2$D stimulation of involucrin gene expression in that mutations of this AP-1 site lower basal expression and block calcium and 1,25(OH)$_2$D stimulation. Separated from this AP-1 site by an SP-1 site is a vitamin D response element (VDRE) with a DR3 configuration. This VDRE binds VDR/RXR complexes. Mutation of the VDRE blocks 1,25(OH)$_2$D stimulation of involucrin gene expression, but does not alter basal activity or calcium stimulation [Bikle et al., 2002].

SEQUENTIAL ACTIVATION OF THE VITAMIN D RECEPTOR BY DRIP AND SRC COACTIVATOR COMPLEXES

The control of keratinocyte proliferation and differentiation by 1,25(OH)$_2$D appears to involve a sequential turning on and off of genes as the cells progress from the proliferating basal cell to the fully differentiated corneocyte. As a first step to determine whether this transition involved a change in the regulation of the VDR function itself, we [Oda et al., submitted] examined the ligand dependent binding of proteins to the VDR at different stages of differentiation. A GST-VDR construct (gift from Dr. Paul McDonald) was expressed and used to bind proteins from nuclear extracts of undifferentiated and differentiated (by calcium) keratinocytes. The bound proteins were eluted from the GST-VDR column, separated by SDS-PAGE, and the proteins identified by mass spectroscopy. The complex from the proliferating cells was nearly identical to that previously identified in other cells by other groups and named vitamin D receptor interacting proteins (DRIP) [Rachez et al., 1999], thyroid receptor activating proteins (TRAP) [Yuan et al., 1998], or activator receptor complex (ARC) [Naar et al., 1999]. When nuclear extracts were obtained from differentiated keratinocytes, the complex was quite different. The major anchoring protein of the DRIP complex, namely DRIP205, was no longer present. Instead, two members of the steroid receptor coactivator (SRC) family were present, SRC 2 and 3, along with several smaller members of the DRIP complex. Western analysis of cellular extracts from keratinocytes

at different stages of differentiation showed a decrease in DRIP 205 with differentiation, but no change in SRC 2 or 3. Transfection of undifferentiated keratinocytes with DRIP205 or SRC 3 potentiated the ability of $1,25(OH)_2D$ to stimulate expression of a VDRE/luciferase construct. However, when the same experiment was performed in differentiated keratinocytes, only transfection with SRC3 enhanced the response to $1,25(OH)_2D$. These results suggest that DRIP may be more important in mediating the effects of $1,25(OH)_2D$ on the earliest stages of differentiation, but SRC 2/3 may be more important during the latter stages of differentiation (Fig. 3). We are currently testing this hypothesis.

INTEGRATION MODEL

Our current working model for the mechanisms by which $1,25(OH)_2D$ regulates keratinocyte differentiation is shown in Figure 4. The keratinocyte expresses a CaR which by coupling to and activating PLC controls the production of two important second messengers, IP_3 and DAG. IP_3 stimulates the release of calcium from intracellular stores thus raising Cai. The rise in DAG and Cai activate PKC. The initial rise in Cai may also trigger the influx of calcium through calcium channels, maintaining an elevation in Cai for a prolonged period of time. Activated PKC leads to the induction and activation of AP-1 transcription factors which regulate the transcription of a number of genes including keratin 1, transglutaminase, involucrin, loricrin, and profilaggrin which are required for the differentiation process. $1,25(OH)_2D$, which is produced by the keratinocyte in a highly regulated fashion, modulates calcium regulated differentiation at several steps. First, $1,25(OH)_2D$ increases CaR expression, thus making the cell more responsive to calcium. Secondly, $1,25(OH)_2D$ induces all the PLCs

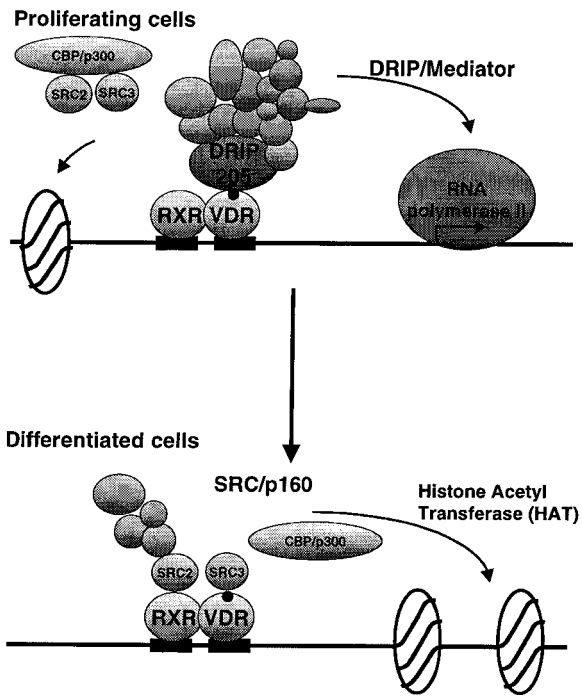

Fig. 3. Model showing selective utilization of two distinct VDR coactivators, DRIP/mediator and SRC/p160, in transcriptional activation during keratinocyte differentiation. Both DRIP/mediator and SRC/p160 may be involved in vitamin D regulated transcription of undifferentiated proliferating cells (upper). At this stage, transcriptional activation may require primarily the linking of the VDR complex to the general transcription machinery including RNA Pol II. After the cells are differentiated, DRIP205 apparently is no longer required, and SRC/p160 family members take the dominant role in transcriptional control (lower).

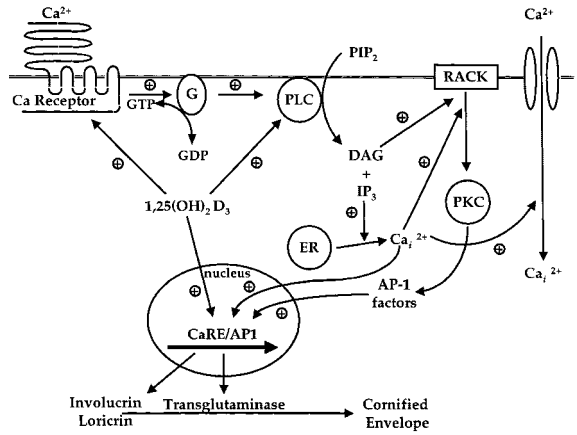

Fig. 4. A model of the interactions between calcium and $1,25(OH)_2D$ in regulating keratinocyte differentiation. The essential features of this model include the G protein coupled calcium sensing receptor (CaR) which when activated by calcium leads to the activation of phospholipase C (PLC), thus producing two important second messengers from the hydrolysis of phosphatidylinositol bisphosphate (PIP_2)—diacyl glycerol (DAG) and inositol tris phosphate (IP_3). IP_3 stimulates the release of calcium from intracellular stores, thus increasing intracellular free calcium concentrations (Cai). The increase in DAG and Cai activate protein kinases (PKC). PKC in turn induces and/or activates the AP-1 family of transcription factors which regulate the expression of the genes encoding the proteins such as involucrin, transglutaminase (TG) and loricrin involved in cornified envelope (CE) formation. The increase in Cai may also stimulate the influx of calcium through calcium channels thus prolonging the Cai signal. $1,25(OH)_2D$ increases the expression of both the CaR and PLC, enhancing the cellular response to calcium. Both calcium and $1,25(OH)_2D$ may regulate the transcription of involucrin, TG, and loricrin by mechanisms other than through AP-1.

again increasing the responsiveness of the cell to calcium. Finally, $1,25(OH)_2D$ may have a direct effect on the transcription of the genes such as involucrin in addition to promoting their expression by increasing Cai. Differentiation requires a carefully orchestrated turning on and off of genes critical for the differentiation process. Sequential activation of the VDR by the DRIP and SRC coactivator complexes may provide such control.

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