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Two distinct coactivators, DRIP/mediator and SRC/p160, are differentially involved in VDR transactivation during keratinocyte differentiation☆

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Abstract

Cell programs such as proliferation and differentiation involve the sequential activation and repression of gene expression. Vitamin D, via its active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), controls the proliferation and differentiation of a number of cell types, including keratinocytes, by directly regulating transcription. Two classes of coactivators, the Vitamin D receptor (VDR) interacting proteins (DRIP/mediator) and the p160 steroid receptor coactivator family (SRC/p160), control the actions of nuclear hormone receptors, including the Vitamin D receptor. However, the relationship between these two classes of coactivators is not clear. Using GST–VDR affinity beads, we have identified the DRIP/mediator complex as the major VDR binding complex in proliferating keratinocytes. After the cells differentiated, members of the SRC/p160 family were identified in the complex but not major DRIP subunits. Both DRIP205 and SRC-3 potentiated Vitamin D-induced transcription in proliferating cells, but during differentiation, DRIP205 was no longer effective. These results indicate that these two distinct coactivators are differentially involved in Vitamin D regulation of gene transcription during keratinocyte differentiation, suggesting that these coactivators are part of the means by which the temporal sequence of gene expression is regulated during the differentiation process.

Keywords: Vitamin D receptor; Transcription; Coactivator; Keratinocyte; Proteomics

1. Introduction

Epidermal keratinocytes provide an excellent model for the study of epithelial cell proliferation and differentiation. Keratinocytes undergo differentiation as they migrate to the upper layers of the skin. In cell culture, this process can be reproduced by maintaining epidermal keratinocytes in different calcium concentrations [1]. The active metabolite of Vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), has profound effects upon this process by potentiating the action of calcium [2]. Binding of $1,25(OH)_2D_3$ to the Vitamin D receptor (VDR) is believed to exert this effect by modulating the transcription of target genes. Targeted disruption of VDR alters this process both in epidermal and hair follicle keratinocytes [3–5]. VDR, like other nuclear hormone receptors, is modulated in its activity by coactivators. In particular, a key complex called Vitamin D receptor interacting proteins (DRIP) (also known as TRAP/SMCC, PBP, ARC or human mediator) has been isolated and shown to be required for VDR transactivation [6]. One subunit, DRIP205/TRAP220, directly binds to VDR and other nuclear receptors through its second NR box, having a conserved LxxLL nuclear receptor binding motif, and is thought to be the main anchor for the complex to VDR [7]. A second group of coactivators is the p160 coactivator or the steroid receptor coactivator (SRC) family (reviewed in [8]). The SRC/p160 family recruits other coactivators such as CREB binding protein (CBP), its homolog p300, and pCAF proteins [8] which likely facilitate transcription through their HAT activity. Importantly, in biochemical purifications, DRIP/mediator and SRC/p160 exist as distinct complexes [7]. The co-existence of these two distinct coactivator complexes raises the question of whether they are competitively or co-operatively functioning in transcriptional activation. In this study, we examined their respective roles during keratinocyte differentiation.

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2. Materials and methods

GST or GST fusion proteins were prepared using the bulk GST purification module (Amersham Pharmacia Biotech, Piscataway NJ) according to the manufacturer's protocol. Nuclear extracts were prepared from keratinocytes as described [9]. VDR binding proteins were purified using GST–VDR affinity beads as described [7]. The protein was identified by using MALDI MS as described [10]. Human keratinocytes at two different stages in the differentiation were used for transfection: Both cell types were transfected by a promotor reporter construct of either VDREx2 E1B luciferase [7] or the full-length rat 24-hydroxylase promotor containing multiple VDREs [11]. Transfected cells were treated either with vehicle (EtOH) or 1×10^{-8} M $1,25(OH)_2D_3$ for 24 h. Cells were lysed, and both firefly and renilla luciferase activities were measured with a dual luciferase reporter assay system (Promega). The results were expressed as the fold induction by $1,25(OH)_2D_3$ over the vehicle control.

3. Results

In order to isolate cofactors interacting with VDR, we utilized a GST fusion protein containing the ligand-binding domain (LBD) of VDR including the activation domain AF-2 essential for binding to the coactivators. Nuclear extracts were prepared from proliferating and differentiated epidermal keratinocytes. They were incubated with GST–VDR (LBD) affinity beads in the presence or absence of ligand (1×10^{-7} M $1,25(OH)_2D_3$), using a similar purification scheme described in the original isolation of the DRIP complex [12]. The bound proteins were eluted and analyzed by SDS-PAGE. The protein complex from proliferating keratinocytes included different proteins ranging in molecular weight from 33 to 250 kDa, which were purified specifically with the ligand (Fig. 1, lanes 1,2). The protein bands were excised from the SDS gel, in-gel digested by trypsin and subsequently analyzed by mass spectrometry (MS). The proteins were identified by matrix-assisted laser desorption and ionization (MALDI) MS peptide mass mapping and/or peptide sequencing by liquid chromatography–electrospray ionization (nanoLC–ESI) tandem MS [10]. The proteins p250-p77 were identified as DRIP250, 240, 205, 150, 130, 100, 92, and 77, respectively (Table 1). The low molecular weight proteins included mammalian homologs of mediator proteins such as mediator 6 and mediator 8 which can assemble with RNA Pol II and Elongin complex capable of stimulating the rate of elongation by RNA Pol II [13]. Members of the SRC/p160 family were not observed in the VDR binding complex isolated from proliferating keratinocytes (Table 1). The DRIP/mediator complex in proliferating keratinocytes contains major DRIP subunits including a DRIP205 which

Table 1
Identification of VDR binding proteins from keratinocytes by mass spectrometry

Band number	Protein identity determined by MALDI peptide mass mapping[a] and/or peptide sequencing[b]	NCBI accession number	Number of peptides sequenced[c]
Proliferating keratinocytes			
p250[d]	DRIP250/TRAP 240/ARC250	4827044	24
p240[d]	DRIP240/TRAP 230/ARC230	4827042	51
p205[d]	DRIP205/TRAP220/p53BP/RB18a/CRSP200	9789555	52
p150[d]	DRIP150/TRAP170/ARC150	4580326	42
p130[d]	DRIP130 /NATp140/ CRSP130/ARC130	7019353	22
p100[d]	DRIP100/TRAP100/ARC100	8699628	14
p92[d]	DRIP92/TRAP95/mediator96b	4868008	13
p77[d]	DRIP77/TRAP80/CRSP77	4838129	15
P60[d]	n.d.		
P42[d]	DRIP33/human mediator 6 (hMed6)	13278645	3
P33[d]	Human mediator 8 (hMed8)	21717644	2
Differentiated keratinocytes			
p205[e]	hsGCN1, translational activator	2282576	2
p180[e]	SRC-3, steroid receptor coactivator/RAC3/AIB1/ACTR/TRAM1	7513298	22
	SRC-2, steroid receptor coactivator/TIF2/GRIP1	5729858	9
p150[e]	DRIP150/TRAP170/ARC150	4580326	9
p100[e]	DRIP100/TRAP100/ARC100	8699628	4
p77[e]	DRIP77/TRAP80/CRSP77	4838129	3
P60[e]	Retinoid X receptor α	4506755	4

n.d. not determined. The p60 band in PK cells was not analyzed but is assumed to be RXRα for differentiated keratinocytes.

[a] Peptide masses were searched against the NCBInr database using MS-Fit (http://prospector.ucsf.edu).

[b] CID spectra were analyzed against the NCBInr database using Mascot (http://www.matrixscience.com) or MS-Tag (http://prospector.ucsf.edu).

[c] Number (#) of peptides sequenced by nanoLC–ESI–MS/MS.

[d] PK gel band number.

[e] DK gel band number.

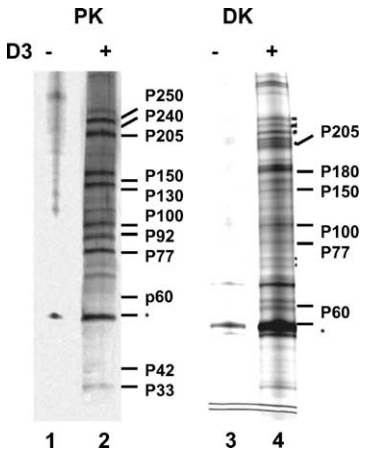

Fig. 1. Purification of VDR binding proteins from keratinocytes. Nuclear extracts were prepared from primary cultures of proliferating (PK) and differentiated keratinocytes (DK). The extracts were incubated with GST–VDR (LBD) affinity beads in the presence or absence of ligand (1×10^{-7} M $1,25(OH)_2D_3$) (D_3). Bound proteins were analyzed by SDS-PAGE,.

directly binds to VDR through its NR boxes. The complex also contains mammalian homologs of yeast mediator proteins [14], indicating that the DRIP/mediator complex may activate transcription [15].

In contrast, differentiated keratinocytes showed a ligand-dependent protein pattern with a lack of major DRIP proteins, and the appearance of a p180 band (a doublet) (Fig. 1 lanes 3, 4). Analysis by mass spectrometry of the p180 band identified tryptic peptides from two proteins, SRC-3 and SRC-2 (data not shown). Protein identification was further confirmed by peptide sequencing using tandem mass spectrometry. The peptides derived from both SRC-3 and SRC-2 were identified by LC MS/MS (Table 1). These results demonstrate that the p180 doublet band is a mixture of the SRC/p160 family members SRC-2 and SRC-3. Major DRIP subunits such as DRIP205, 250, 240, 130, and 92 were not identified, although some DRIP proteins, including DRIP150, 100, and 77, remained (Table 1). These results indicate that the DRIP/mediator complex was the major VDR binding complex in proliferating keratinocytes, similar to that previously reported in Namalwa B [16] and Hela cells [17]. However, upon differentiation, major DRIP subunits decreased and SRC family members became predominant.

The functional requirements for DRIP205 and SRC-3 in the -induced transactivation was significantly potentiated by both DRIP205 and SRC-3 compared to the pcDNA3 vector control in proliferating keratinocytes (Fig. 2A). In contrast, differentiated keratinocytes showed a significant increase in activation by SRC-3 but not by DRIP205 (Fig. 2B), even though both SRC-3 and DRIP205 were over-expressed in both proliferating and differentiated keratinocytes (data not shown). Next, we utilized the dominant negative construct of DRIP205 (aa 527–714; 205 Box Wild), which is derived from the nuclear hormone receptor binding domain containing two LxxLL motifs, to inhibit the function of DRIP205

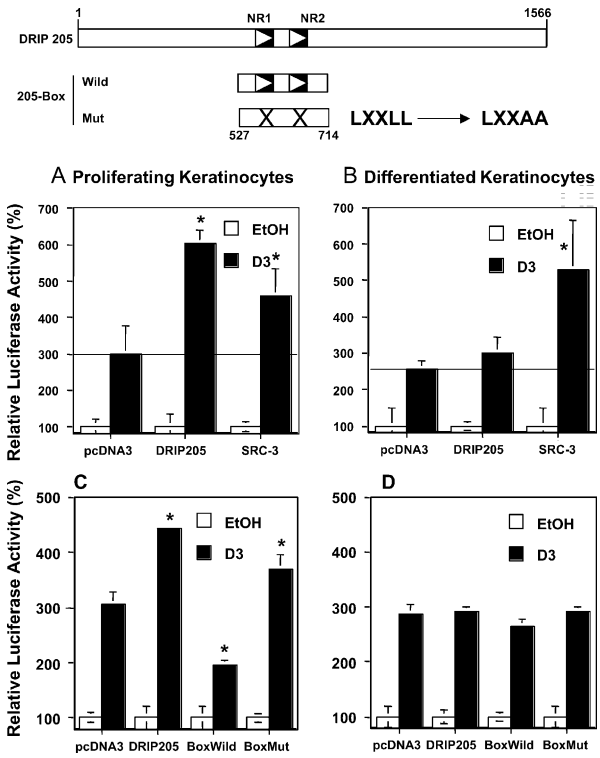

Fig. 2. Differential effects of DRIP205 and SRC-3 on Vitamin D transactivation during keratinocyte differentiation. Both proliferating keratinocytes (A and C) and differentiated keratinocytes (B and D) were transfected with a VDRE construct linked to luciferase. Cells were co-transfected by full length DRIP205 or SRC-3, dominant negative DRIP205 (Box Wild) containing LxxLL motif, or the L to A mutant form of this dominant negative DRIP205 (Box Mut). The $1,25(OH)_2D_3$ (D_3)-induced transactivation was measured, and compared to vector control (pcDNA3).

(Fig. 2) [7]. A construct that contains an L to A mutation in each NR box in the same construct was also used as a control (205Box Mut) (Fig. 2). When proliferating keratinocytes were co-transfected with the dominant negative DRIP205, Vitamin D-induced transactivation was significantly inhibited (Fig. 2C; 205Box Wild), but not when transfected by the mutant (Fig. 2C; 205Box Mut). In contrast, when differentiated keratinocytes were co-transfected by the dominant negative DRIP205, Vitamin D-induced transactivation was not affected (Fig. 2D). These results indicate that both DRIP205 and SRC-3 may be involved in Vitamin D transactivation in proliferating keratinocytes, but only SRC-3 is effective in differentiated cells.

4. Discussion

We propose a model (Fig. 3) in which both DRIP/mediator and SRC/p160 regulate Vitamin D controlled transcription in a sequential process during differentiation. In the early stages of differentiation, direct activation of the general transcriptional machinery through DRIP/mediator and HAT activity through SRC/p160 may be required to initiate gene transcription by VDR and $1,25(OH)_2D_3$. Subsequently,

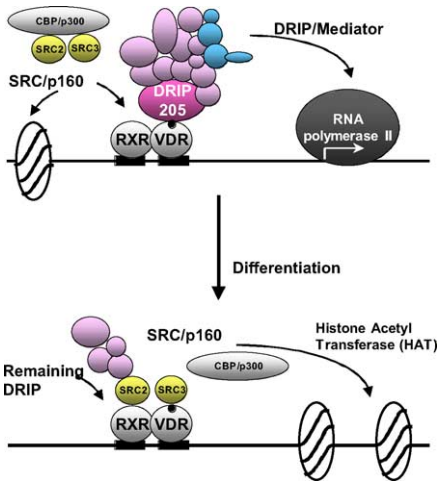

Fig. 3. Model showing selective utilization of two distinct VDR coactivators of DRIP/mediator and SRC/p160 in transcriptional activation during keratinocyte differentiation.

major DRIP components decrease, and the SRC/p160 complex becomes dominant, enabling transcription to occur as the cells differentiate. This model could explain how two distinct coactivators are differentially involved in VDR transcription during the differentiation process, and how they coordinately function in transcription.

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