Published in final edited form as:

J Steroid Biochem Mol Biol. 2007 March ; 103(3-5): 440–445.

1,25-DIHYDROXYVITAMIN D₃ INDUCES EXPRESSION OF THE WNT SIGNALING CO-REGULATOR LRP5 VIA REGULATORY ELEMENTS LOCATED SIGNIFICANTLY DOWNSTREAM OF THE GENE'S TRANSCRIPTIONAL START SITE

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Abstract

Canonical Wnt signaling is essential for bone formation. Activation involves binding of secreted members of the Wnt family of proteins with a membrane receptor Frizzled on osteoblasts, an interaction that is facilitated by LRP5/LRP6 co-receptors. LRP5 is known to play a particularly important role in bone formation such that the loss of this protein results in a reduction in osteoblast number, a delay in mineralization and a reduction in peak BMD. During the course of a VDR ChIP-chip analysis we found that 1,25(OH)₂D₃ could induce binding of the VDR to sites within the *Lrp5* gene locus. Importantly, this interaction between 1,25(OH)₂D₃-activated VDR and the *Lrp5* gene led to both a modification in chromatin structure within the *Lrp5* locus and the induction of LRP5 mRNA transcripts *in vivo* as well as *in vitro*. One site within *Lrp5* was discovered to confer 1,25(OH)₂D₃ response to a heterologous promoter in osteoblastic cells, permitting both the identification and characterization of the component VDRE. While the regulatory region in *Lrp5* was highly conserved in the human genome, the VDRE was not. Our studies show that 1,25(OH)₂D₃ can enhance the expression of a critical component of the Wnt signaling pathway which is known to impact osteogenesis.

Keywords

osteoblasts; Wnt signaling pathway; vitamin D response element; ChIP on chip; vitamin D receptor; coregulators

1. Introduction

The actions of 1,25(OH)₂D₃ are vital to the maintenance of calcium and phosphorus homeostasis, and are critical to the integrity of the vertebrate skeleton [1–3]. Central to these actions is the ability of 1,25(OH)₂D₃ to diffuse into cells and to interact specifically with the vitamin D receptor (VDR). In turn this protein binds directly to regulatory regions of target genes altering their transcriptional output. Selective VDR DNA binding, therefore, enables the cell to direct its transcriptional machinery to that subset of genes whose products orchestrate the specific biological response to 1,25(OH)₂D₃.

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Early studies have shown that $1,25(\text{OH})_2\text{D}_3$ plays a significant role in controlling mature osteoblast function, particularly as it relates to the secretion of bone matrix and the capacity to promote bone mineralization [4–6]. These actions are complex and impact such events as early osteoblast precursor cell progression as well as osteoblast proliferation and differentiation. Furthermore, these effects are highly cell stage-specific, and have produced an array of diverse and sometimes conflicting observations with respect to osteoblast biology. While $1,25(\text{OH})_2\text{D}_3$ modulates the expression of functional genes involved in osteoblast differentiation the hormone is also able to influence the expression of 1) secreted factors that activate cellular differentiation pathways, 2) components that represent key transducing factors within those pathways, and 3) the transcription factor targets that serve as mediators of these pathways at the level of gene expression. Therefore, the hormone is likely capable of triggering a broad range of effects on osteoblast proliferation, differentiation and function [5].

The canonical Wnt signaling pathway also plays a crucial role in osteoblast proliferation and differentiation, and appears to exert a significant developmental role in bone formation [7–9]. This pathway is initiated through the interaction of members of the Wnt family with the cell surface receptor Frizzled and co-receptor LRP5 [10]. Stimulation leads to inhibition of the activity of glycogen synthase kinase-3 β (GSK3 β) which results in stabilization and redistribution of the transcription factor β -catenin to the nucleus where it activates LEF/TCF mediated gene transcription (Fig. 1a). Activation of Frizzled and its co-receptor (LRP5 & 6) is also regulated by numerous Wnt antagonists several of which involve additional membrane proteins [7].

Many of the osteogenic effects of Wnt signaling were identified through discovery of the biological consequences associated with aberrant expression of LRP5; hypermorphic alleles of LRP5 were associated with high bone mass [11,12] and loss-of-function mutations were associated with osteoporosis-pseudoglioma syndrome [13]. The *Lrp5* gene has subsequently been inactivated in mice resulting in a phenotype of low bone mass similar to that seen in humans [14]. Importantly, a thorough investigation of the phenotype of this mouse model revealed that the primary effect of Wnt activation was to influence both osteoblast proliferation and function, particularly as it relates to both the secretion of bone matrix and subsequent timely mineralization.

During a ChIP-chip screen of genes capable of influencing osteoblast function, we found potential binding sites for VDR on the *Lrp5* gene. $1,25(\text{OH})_2\text{D}_3$ activation and VDR binding was correlated directly to subsequent modifications that occurred on chromatin within this locus as well as to increased levels of expression of LRP5 both *in vitro* and *in vivo*. Cloning of each of these potential regulatory regions revealed that the major regulatory site could confer $1,25(\text{OH})_2\text{D}_3$ response to a heterologous promoter, allowing for identification and characterization of the active VDRE. Interestingly, this VDRE was not conserved in the human gene despite significant conservation in the surrounding region. Our studies identify mouse *Lrp5* as a target gene for $1,25(\text{OH})_2\text{D}_3$ and provide a mechanism whereby this hormone can influence the expression of a regulatory component influential in modulating bone formation.

2. Materials and Methods

2.1 Tiled oligonucleotide microarray analysis

ChIP-chip analysis was carried out as described by others [15]. In brief, DNA was isolated by ChIP and then subjected to ligation mediated PCR. The resulting amplicon pools were labeled with Cy3 or Cy5 dyes through indirect labeling and then mixed in the presence of CoT-1 DNA, denatured, and co-hybridized to custom oligonucleotide microarray (Nimblegen Systems Inc, Madison, WI). The microarrays were scanned using an Axon 4000B scanner. Custom arrays included maskless *in situ*-synthesized 50-mers at 2 bp intervals representing a 132 kb screen

of the mouse *Lrp5* gene from 20 kb upstream of the gene's TSS to 10 kb downstream of the final 3' non-coding exon. After sample co-hybridization, the logarithmic enrichment ratio of Cy5 to Cy3 intensities (log2) were plotted as a function of chromosome nucleotide position (Dec.2004 Assembly) and scored with a peak finding algorithm [16].

2.2 Animal studies

Eight week-old C57BL6 wildtype mice were dosed by intraperitoneal injection of 1,25(OH)₂D₃ (10 ng/g body weight). Groups of animals (n = 3) were sacrificed at 0–6 hours and calvarial tissue was collected for RNA isolation. Experimental protocols were reviewed and approved by the Research Animal Resources Center (University of Wisconsin-Madison, Madison, WI).

2.3 RNA isolation and analysis

Total RNA was isolated from homogenized animal tissue or cells using Triazol reagent obtained from MRC (Cincinnati, OH). The isolated RNA was reverse transcribed using the Superscript III RNase H Reverse Transcriptase kit (Invitrogen) and then subjected to PCR analysis using Real Time or standard PCR methods.

2.4 Transfection analysis

MC3T3-E1, ST2, mOB and/or MG63 cells were seeded into 24-well plates (5.0×10^4 cells/well) in the appropriate complete media. Cells were transfected 24 hrs later with Lipofectamine Plus in serum and antibiotic-free media. Cells were harvested 24 hours after stimulation and the lysates were assayed for luciferase and β -galactosidase activities as previously described [17].

2.5 Statistical Analysis

All values are expressed as mean $\pm$ SEM. We evaluated differences between groups through one way analysis of variance (ANOVA) or student's one tailed t-test.

3. Results and Discussion

Our investigation was initiated by exploring the capacity of 1,25(OH)₂D₃ to promote VDR and RXR binding to genes with the potential to influence the osteoblast. *Lrp5* was one such candidate. We utilized a scanning method wherein DNA sequences across genes of interest were tiled on a DNA microarray at 50 bp resolution and then screened with DNA labeled fragments obtained from ChIP using antibodies to either VDR or RXR. This approach yielded three potential binding sites for the VDR/RXR on *Lrp5* located both proximal to the TSS and at extended distances (Fig 2). Independent ChIP analysis confirmed variable VDR/RXR binding at these intronic sites in ST2, MC3T3-E1, and primary mOB cells, although the *mLrp5*-P2 region was the most consistent in all target cells examined. The locations of the vitamin D regulatory regions within *Lrp5* introns were surprising, since the bulk of the VDREs thus far identified have been located within the first kilobase or so upstream of the TSS. Other studies, however, have identified other hormone regulatory elements within downstream introns [18,19], suggesting that our results do not establish a new paradigm.

We extended our findings by demonstrating that 1,25(OH)₂D₃ activation and VDR/RXR binding was associated with functional consequences to the chromatin located within these regions of the *Lrp5* gene. As expected, both VDR as well as RXR bound to the P1-P3 regions of the *Lrp5* gene, although binding at P2 was clearly predominant (Fig 3). The ChIP analysis depicted also shows both a strong residual as well as 1,25(OH)₂D₃-inducible histone 4 acetylation, which was particularly evident at *mLrp5*-P1 and *mLrp5*-P2. Since histone 4

modification is also seen in the Ctrl region, it suggests that the acetylation pattern on the *Lrp5* gene is potentially very broad. Perhaps most interesting is the observation that RNA pol II levels are enhanced in response to $1,25(\text{OH})_2\text{D}_3$ at all three regions. While the *mLrp5*-P1 region is located proximal to the TSS, and therefore likely to represent RNA pol II recruitment to a *bona fide* pre-initiation complex, the appearance of RNA pol II at *mLrp5*-P2 and *mLrp5*-P3 was surprising. Taken together, these results suggest that *mLrp5*-P2 and *mLrp5*-P3, and perhaps *mLrp5*-P1 represent regulatory enhancer regions capable of both modifying chromatin in response to $1,25(\text{OH})_2\text{D}_3$ induction and VDR/RXR binding, and acting as potential recruitment centers for increasing the RNA pol II levels necessary for enhanced transcription.

Although all three regions of *Lrp5* bound VDR and RXR, *mLrp5*-P2 was the only fragment active in transcriptional activation *in vitro* and therefore was the only fragment for which direct mapping studies could be conducted. These studies identified a functional VDRE located 19,241/19,255 bp downstream of the TSS (Fig. 4a). Interestingly, while there is conservation of a large region of *Lrp5* P2 between species, the VDRE is not maintained in the human genome due to a 3 bp substitution in the downstream halfsite.

Having established a $1,25(\text{OH})_2\text{D}_3$ -stimulated interaction between the VDR and the *Lrp5* gene, we next examined whether this interaction was capable of inducing *Lrp5* expression. $1,25(\text{OH})_2\text{D}_3$ induced *Lrp5* not only *in vitro*, but also *in vivo*. A single dose of $1,25(\text{OH})_2\text{D}_3$ (10 ng/g bw) for periods up to 6 hours displayed a time-dependent upregulation in LRP5 mRNA from wildtype calvarial tissue (Fig. 5). This suggests that $1,25(\text{OH})_2\text{D}_3$ -mediated induction of *Lrp5* expression is mediated by an interaction of VDR and RXR with the P1–P3 regions of *Lrp5*.

Vitamin D is a bone remodeling agent. Thus, its overall effects on bone formation as well as bone resorption via direct regulation of gene expression are well established [5,20]. That many of these effects of $1,25(\text{OH})_2\text{D}_3$ in bone are redundant with other regulatory factors is also well recognized and highlighted most recently by the finding that the dramatic skeletal phenotype observed in the VDR null mouse can be rescued through a diet rich in calcium and phosphorous [21]. It is now believed, however, that high levels of calcium and phosphorus cannot fully normalize the skeletal defects associated with genetic ablation of the VDR or $1,25(\text{OH})_2\text{D}_3$ deficiency, manifest as reductions in osteoblast numbers, mineral apposition rate, and bone volume compared to that seen in wildtype mice. The potential anabolic effects of $1,25(\text{OH})_2\text{D}_3$ both on osteoblast differentiation as well as osteoblastic bone formation are consistent with that of many of the activities induced by the Wnt family of regulators. Whether the upregulation of *Lrp5* by $1,25(\text{OH})_2\text{D}_3$ contributes significantly to the overall actions of $1,25(\text{OH})_2\text{D}_3$ on bone remains to be determined. If so, the effects of $1,25(\text{OH})_2\text{D}_3$ would be similar to that observed with the BMPs, which induce a similar rapid and sustained upregulation of *Lrp5* during the first 6 days or so of osteoblast proliferation and differentiation [13].

Interestingly, studies in other systems have suggested that vitamin D might inhibit Wnt signaling. Accordingly, recent observations have shown that VDR, and other nuclear receptors, can bind directly to β -catenin and repress wnt/ β -catenin/LEF-TCF signaling [22]. The bulk of this activity, however, has been observed in colon cancer cells, wherein the actions of Wnt signaling are largely proliferative and where β -catenin activation has been dis-regulated. The exact relationships connecting each of these pathways in different tissues and cells are not fully understood at this stage and remain to be resolved.

In summary, although the similarity of effects of vitamin D could be fortuitous and entirely independent of key extracellular signaling pathway activity, it is also possible that the related effects arise through a direct regulatory action by the steroid hormone on one or more components of these key control pathways. Indeed, vitamin D is known to regulate both signal

pathway components as well as many of the transcription factors they target, making this hypothesis a plausible one. In support of this type of activity by vitamin D, we show that 1,25(OH)₂D₃ is able to upregulate the expression of *Lrp5* transcripts in osteoblasts. Mechanistically, *Lrp5* upregulation is due to the presence of three control regions located within the gene, at least one of which contains a specific VDRE. Interestingly, while the *Lrp5* P2 region was generally conserved within the human locus, critical differences were apparent within the VDRE sequence itself. Despite this, our results in the mouse provide a possible secondary mechanism whereby 1,25(OH)₂D₃ may impact osteoblast biology.

Acknowledgements

We thank the members of the Pike Laboratory for their individual contributions to this work. We thank Adam Steinberg for preparation of the second figure. This work was supported by NIH grants to J.W.P.

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Fig. 1. Canonical Wnt Signaling Pathway. A, Binding of Wnt to Frizzled and LRP5 results in stabilization of β -catenin and consequently activation of TCF/LEF-responsive gene transcription. B, Inhibition of Wnt signaling occurs when LRP5 is shunted away through interactions with antagonistic cytokines.

Fig. 2.

ChIP-Chip analysis identifies VDR-binding regions in the mouse *Lrp5* locus. ChIP-chip analysis of the mouse *Lrp5* candidate target gene. Upper panel: schematic diagram of the mouse *Lrp5* gene and its 23 exons. Base pair numbering indicates nucleotide location on chromosome 19 (December 2004 Assembly). The arrow indicates the direction of transcription on the reverse strand. Lower panel: Individual data tracks representing the enrichment ratios of Cy5 to Cy3 of hybridization intensity (log₂) for VDR plus hormone versus input DNA and RXR plus hormone versus input DNA. Base pair numbering indicates nucleotide location on chromosome 19 (December 2004 Assembly). The highlighted areas indicate peaks of interest that are designated *mLrp5*-P1, *mLrp5*-P2, and *mLrp5*-P3.

Fig. 3.

VDR/RXR heterodimer binding to the *mLrp5*-P1- *mLrp5*-P3 regions of *Lrp5* enhances chromatin modification at histone 4 and induces the recruitment of RNA polymerase II. A, ChIP analysis of 1,25(OH)₂D₃ induced VDR and RXR binding, histone acetylation and RNA pol II presence at the P1-P3 and Ctrl regions of the *Lrp5* gene. MC3T3-E1 cells were treated with either vehicle or 1,25(OH)₂D₃ (10⁻⁷ M) for 3 hrs and then subjected to ChIP using antibodies specific to VDR, RXR, tetra-acetylated histone 4, RNA pol II or an irrelevant IgG antibody. DNA precipitates were isolated and then subjected to PCR.

Fig. 4. The mouse *mLrp5*-P2 region contains a functional VDRE. A, Conservation of P2 within the mouse, human and rat *Lrp5* genes. Upper panel: The diagram depicts the boxed position of the *Lrp5*-P2 region located within the intron between exons 1 and 2. The nucleotide and chromosomal position of an analogous region in the human and rat genes are indicated. Lower panel: Nucleotide sequence of the P2 region with the mouse, human and rat *Lrp5* gene wherein half-sites of a putative VDRE identified by the CONSITE algorithm is indicated. B, The panel illustrates the VDRE constructs used to evaluate the transcriptional activity of the potential VDRE. C, Transcriptional activity of wildtype and mutant forms of *mLrp5*-P2. MC3T3-E1 cells were co-transfected with pCH110- β gal, pcDNA-hVDR, and either ptk-luc control vector, ptk-*mLrp5*-P2 or the mutant version of ptk- *mLrp5*-P2. Cells were treated with either vehicle or increasing concentrations of 1,25(OH) $_2$ D $_3$ (10^{-7} M) for 24 hrs and then evaluated for both luciferase and β -gal activity. Each point represents the normalized RLU average $\pm$ SEM for a triplicate set of transfections. * indicates $p < 0.01$ compared to vehicle treated sample (one way ANOVA).

Fig. 5.

1,25(OH)₂D₃ induces *Lrp5* mRNA levels *in vivo* via a VDR-dependent mechanism. Female C57Bl6 mice (n = 3/group) were treated with a single dose of 1,25(OH)₂D₃ (10 ng/g bw) via IP injection. The calvaria were then harvested after 0, 1, 3 and 6 hr and the isolated RNA subjected to qRT-PCR analysis using β-actin and *Lrp5* primers as described in Materials and Methods. *Lrp5* measurements were normalized to β-actin levels. Each point represents the mean ± SEM. Differences were determined by student's one tailed t-test (*, p<0.05 in comparison to vehicle).