P- 4  Mechanism of Action of 1,25-Dihydroxyvitamin D on Target Gene Expression

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I. INTRODUCTION

The final active form of vitamin D is 1α,25-dihydroxyvitamin D (1,25-(OH)2D). It is this substance released from the proximal convoluted tubule cells in normal animals and man that stimulates the intestine to absorb calcium and phosphorus, the osteoblasts of bone to secrete certain matrix proteins, and to facilitate the mobilization of calcium from bone, and to stimulate distal renal tubule cells to reabsorb calcium [1-3]. In addition to these classical functions of vitamin D, it is now well established that this hormone plays an important role in cellular differentiation [4,5]. It can, therefore, be regarded as a developmental hormone as well as one responsible for calcium homeostasis and normal bone metabolism.

This hormone, in carrying out these functions, localizes in the nuclei of target cells [1-3]. Its actions are generally blocked by transcriptional and translational inhibitors, especially in well-defined organ culture systems [6,7]. Since this compound is a steroid, it is expected to function as all other steroid hormones i.e., through a cytoplasmic/nuclear receptor. A receptor for 1,25-(OH)2D3 was discovered in 1974-1976 [8,9], is now well characterized [10], has been cloned [11,12], and much is known in terms of structure and function of its domains [13,14]. Fig. 1 illustrates the expected molecular mode of action of 1,25-(OH)2D3 in eliciting its cellular responses. The subsequent sections will deal with what is known concerning the receptor(s) for 1,25-(OH)2D3 and how it interacts with the genome to bring about expression of specific target genes.

Fig. 1. Mode of action of 1,25-(OH)2D in intestinal epithelial cells.
II. STRUCTURE OF 1,25-(OH)2D3 RECEPTOR

With the generation of monoclonal antibodies directed against the 1,25-(OH)2D3 receptor, it became possible to clone the receptor cDNA [11,12]. By screening a rat kidney lambda GT11 cDNA expression library, using three monoclonal antibodies directed to different epitopes on the 1,25-(OH)2D3 receptor, a clone was obtained which encoded for 367 amino acids of the receptor structure [11]. This was sequenced and by means of a primer extension procedure, the full length sequence of the cDNA encoding for the 1,25-(OH)2D3 receptor was obtained [13]. Similarly, using monoclonal antibodies, a partial cDNA was obtained from a chick intestinal library [12], and a full length cDNA encoding the human receptor was obtained [14]. The rat receptor is shorter than the human receptor by four amino acids, and the two receptors show 100% homology in the DNA binding domain, approximately 55% homology in the hinge region and as much as 94% in the ligand binding domain. Pike and colleagues, by means of translation experiments, could find evidence for only one 1,25-(OH)2D3 receptor [15]. Thus far there have been no reports of 1,25-(OH)2D3 receptor subtypes. However, there is always the possibility that receptor subtypes may be found in the future, especially because 1,25-(OH)2D3 analogs exist that cause differential expression of target genes [16, 17].

At least three groups have chemically synthesized analogs of 1,25-(OH)2D3 which will induce differentiation of HL-60 cells to monocytes, while having little or no effect on calcium metabolism in vivo [16, 17]. Thus, some of the genes expressed in response to 1,25-(OH)2D3 can be expressed in response to analogs whereas others appear to be expressed only when 1,25-(OH)2D3 is provided. This led to the possibility that the receptor found in HL-60 cells might be different from the receptor found in the intestine. Characterization of the receptor in HL-60 cells did not support this conclusion [18]. Furthermore, we have recently cloned the cDNA encoding the receptor from HL-60 cells and found it to encode for a receptor identical to that reported by Baker et al. [14] in normal human fibroblasts (Goto, H., Chen, K.-S. and DeLuca, H. F., in preparation). It is of some interest, however, that the cDNA encoding the receptor has never been obtained from the intestine which might be considered more likely to be a calcium metabolism organ. Thus, the conclusion that there are no receptor subtypes relating to the function of 1,25-(OH)2D3 in calcium versus differentiation is premature.

By means of deletion analyses and analogy to other steroid hormone receptors, the various domains of the 1,25-(OH)2D3 receptor have been deduced [13, 19]. This receptor has a very small "A" domain which is on the N terminus prior to the DNA binding or "B" domain. The DNA binding domain shows evidence for two zinc fingers strictly by analogy to the glucocorticoid receptor [19]. The C domain appears to have less homology between man and rat, whereas the D domain being the ligand binding domain, is approximately 94% homologous between the two species. The E domain is also small in this receptor. The 1,25-(OH)2D3 receptor appears to be the smallest in the superfamily of transcriptionally active steroid hormone receptors [13, 19]. Further deductions regarding structure must await the isolation of adequate amounts of pure receptor and the use of physical tools such as nuclear magnetic resonance spectroscopy and X-ray crystallography. This will require the production of large amounts of receptor not available on a natural product basis. The richest source of receptor found in the natural products is the 2,000 fmol of binding activity per mg protein found in the porcine intestine [20]. This is still far below the amount that would be required to obtain sufficient amounts for these physical studies. Thus, there have been efforts made by Sone et al. and Ross et al. to express the cDNA encoding the receptor in an over-expression system. Sone et al. has been able to produce approximately 100 pmol of binding activity per mg protein using a yeast expression system [21]. As shown in Table 1, using a
recombinant baculovirus expression system in insect cells, we have been able to produce as much as 5-13% of the total cellular protein as receptor giving rise to receptor levels of approximately 2,000 pmol/mg protein [22]. This system is now being developed further to obtain sufficient amounts of receptor for physical studies.

III. FUNCTION OF THE RECEPTOR IN GENE EXPRESSION

Among the steroid hormone superfamily of receptors, specific hormone response elements (HREs) are found usually in the promoter region of the responsive gene to which the steroid hormone receptor binds. This binding is essential for the hormone to stimulate expression of the subsequent gene. It is clear, however, that these response elements do not have to be at any particular position in the promoter region and may also be found in other regions of the gene [23]. Using the chloramphenicol transacyetylase (CAT) reporter gene not found in mammalian cells, it has been possible to place response elements upstream of this reporter gene with the intervening presence of a viral thymidine kinase promoter. This construction imparts hormonal responsiveness to this CAT system. Using the reporter gene system, deletion analysis and receptor binding studies, four 1,25-(OH)2D3 response elements have been isolated and their structures determined. These are shown in Fig. 2 in a manner used by Umesono et al. [23]. It is clear that these response elements bear some resemblance to each other but show certain repeat sequences either in the same direction or in alternate directions separated by an intervening sequence. Recently, Umesono et al. [23] has deduced a consensus sequence shown in Fig. 2 and demonstrated that a 3 bP sequence must interrupt the two arms of the domains that bind the 1,25-(OH)2D3 receptor. We have recently deduced the DRE of the rat intestinal calbindin D-9k gene as shown in Fig. 2 [28]. Three bases shown by the arrows appear to be needed with other less specific base requirements present. Thus, although related, the DREs are not identical.

Fig. 2 Vitamin D Response Elements. *Umesono et al. Cell 65, 1255-1266, 1991.

Nevertheless, the presence of a response element is required for the steroid hormone to stimulate a target gene. This stimulation requires the presence of an endogenous hormone receptor as in the case of T47D cells or the cotransfection of a cDNA encoding for the receptor in an expression vector as will be illustrated below.

Additional evidence for the essentiality of a response element has been provided by gel shift experiments. In these experiments, a radioactively labeled, double-stranded DNA segment containing a response element is incubated with the 1,25-(OH)2D3 receptor. The mixture is then electrophoresed on a non-denaturing
polyacrylamide gel and subjected to autoradiography. Specific binding of the
response element by the receptor is illustrated by a shift in radioactivity to a
higher molecular weight range corresponding to the presence of the receptor. The
addition of a 10-fold excess of unlabeled response element DNA fragment will
compete successfully against this binding, and the addition of a monoclonal
antibody directed against the receptor will cause a further shift in the labeled
response element band. As illustrated in Fig. 3, the gel shift requires not only
the presence of the receptor but also a nuclear accessory factor(s). In our
experiments we have used the recombinant rat receptor produced in the baculovirus
system described above, and found that it is unable to bind to the rat osteocalcin
response element unless another factor(s) found in nuclear extract is present.
Once this accessory factor(s) is provided, specific gel shift of the response
element is noted. The presence of ligand is not required for the gel shifts to
occur. Purified porcine intestinal nuclear 1,25-(OH)2D3 receptor will also not
bind to the response element unless the accessory factor is provided. The
accessory factor is provided by the target tissue nuclear extract which has been
stripped of receptor by monoclonal antibody immunoprecipitation. Nuclear extracts
of non-target tissues, such as spleen and skeletal muscle, do not have the
accessory factor. Liver appears to have an accessory factor, but whether it is
identical to that provided by intestine and kidney remains to be determined. These
results illustrate that the receptor does not require ligand to bind to the
response element, and secondly, an accessory factor is required for binding to the
response element. We have purified the accessory factor and find it to be a
protein of approximately 59-64,000 molecular weight (manuscript in preparation).
Further work is continuing on this important accessory factor. Liao et al. also
have noted the necessity of a nuclear accessory factor in their gel shift
experiments involving the 1,25-(OH)2D3 receptor [28].

Another important aspect of the hormone’s receptor function is its
phosphorylation. Phosphorylation of the 1,25-(OH)2D3 receptor has been reported
in 3T6 cells and in COS-1 cells cotransfected with the rat 1,25-(OH)2D3 receptor
cDNA in an expression vector [29, 30]. The most important experiments however have
been those carried out with chick intestinal organ cultures [31]. In these
experiments when the organ cultures are incubated in the absence of 1,25-(OH)2D3

![Fig. 3. Polyacrylamide gel shifts of radioactively-labeled DRE induced by 1,25-(OH)2D receptor and requirement for accessory protein. Lane 1: DRE; lane 2: pig intestinal nuclear extract + DRE; lane 3: recombinant receptor + DRE; lane 4: receptor depleted pig intestinal nuclear extract + DRE; lane 5: recombinant receptor + DRE + pig intestinal nuclear extract depleted of receptor. Detection was by autoradiography.](image-url)
but in the presence of P-32, essentially no labeling of the receptor occurs. Upon addition of 1,25-(OH)2D3, within 15-30 minutes, the receptor becomes phosphorylated. The phosphorylation occurs long before the target organ response of increased cellular calcium uptake and prior to the appearance of mRNA encoding for the calcium binding protein. These results illustrate that in a functional organ culture system, the receptor becomes phosphorylated before a target organ response occurs and this phosphorylation requires the presence of ligand. We have, therefore, continued our experiments to determine the site of this phosphorylation and whether it is essential for the hormone to influence gene expression. By means of P-32 labeling of the receptor in pig kidney LLC-PK1 cells, we have been able to determine that all of the phosphorylation occurs on a serine [30], and furthermore, cleavage of the phosphorylated receptor using S. aureus strain V8 protease on Cleveland gels reveals that the phosphorylation is found in a 23,000 molecular weight segment which spans part of the hinge region and approximately one-half of the ligand binding domain [30].

Using the CAT reporter gene assay system in CV-1 cells that have been cotransfected with the expression plasmid containing the cDNA encoding for the receptor, a 5-10-fold stimulation of CAT activity containing 1,25-(OH)2D3 at 10 nM concentration can be obtained with the human osteocalcin DRE (Darwish, H., Moss, V., and DeLuca, H. F., submitted). A protein kinase A activator, 8-bromocyclic AMP, will stimulate CAT activity in a vitamin D receptor dependent fashion in the absence of ligand. The presence of both the ligand and the protein kinase A activator causes an even further increase in CAT activity. Inhibitors of protein kinase A, i.e. PK1 and H9, will inhibit the 1,25-(OH)2D3 stimulation of CAT activity in this system (Fig. 4). Furthermore, an inhibitor of protein phosphatase, namely Okadaic acid, will also stimulate CAT activity in a receptor-dependent fashion. These results provide strong if not conclusive proof that phosphorylation of the receptor is required for stimulation of gene expression by 1,25-(OH)2D3. The requirement for phosphorylation can only be established by determining the exact site of phosphorylation and site-specific mutation of the involved amino acid(s).

![Figure 4. CAT responses to 1,25-(OH)2D3 in the presence of protein kinase inhibitors, pK1 and H9. (A) Betagen analysis (B) TLC analysis](image_url)
IV. A MODEL FOR THE MOLECULAR MECHANISM OF ACTION OF 1,25-(OH)2D3 ON TARGET GENE EXPRESSION

To explain the results we have obtained to date, we propose a model for the role of the receptor and 1,25-(OH)2D3 in eliciting a target gene response (Fig. 5). Our results indicate that when an accessory factor characteristic of a target tissue is present, the 1,25-(OH)2D3 receptor can bind to the vitamin D response element in the promoter region of a gene. The ligand is not a requirement for this binding to occur. It is likely that the next step is the interaction of ligand (1,25-(OH)2D3) with the receptor/accessory protein/response element/target gene complex. This complex can then become the substrate for a protein kinase that phosphorylates a specific serine(s) in the ligand binding domain of the receptor molecule. This phosphorylation is required for the subsequent binding and/or activation of the RNA polymerase, together with other possible factors, to elicit transcription of the particular target gene. At this time it is not clear whether the ligand must remain with the complex or whether the ligand does not have to be present after phosphorylation has occurred. Whether the receptor binds to the response element as a monomer, dimer, or a tetramer is unknown, but based on homology to other steroid hormones and unpublished work (Darwish, H., and DeLuca, H. F.) at least a dimer is anticipated as is illustrated in Fig. 5.

V. OTHER POTENTIAL MECHANISM

With the chemical synthesis of a large number of 1,25-(OH)2D3 analogs has come the discovery that the differentiative action of 1,25-(OH)2D3 can be separated from its calcium mobilizing actions [17]. Thus, 24-homologs of 1,25-(OH)2D3, 22-oxa-1,25-(OH)2D3, 19-nor-1,25-(OH)2Ds, and the Leo Pharmaceutical MC-903, are compounds which can carry out the differentiative action without the calcemic action [17]. These compounds must carry out this function through modulation by receptor. However, it is clear that the 24-dihomo-1,25-(OH)2D3 can stimulate the production of the mRNA encoding for the calcium binding protein in intestinal cells while not stimulating intestinal calcium transport [16]. This raises the question of whether there may be some other way in which the 1,25-(OH)2D3 receptor functions on calcium other than through a nuclear mechanism or that there may be a different form of the receptor that functions in calcium versus differentiative actions, or that the receptor responds differentially to these analogs. Because of the differential activity of the analogs, therefore, consideration must be given to possible alternative mechanisms of action of 1,25-(OH)2D3 in addition to nuclear mechanisms.
VI. REFERENCES


