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**ANALYSIS OF THE VITAMIN D RECEPTOR RESIDUES 305 AND 397 IN
1,25(OH)₂D₃ BINDING**

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Alejandra Acevedo

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ABSTRACT

ANALYSIS OF THE VITAMIN D RECEPTOR RESIDUES 305 AND 397 IN 1,25(OH)₂D₃ BINDING

by Alejandra Acevedo

The hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), mediates its actions via the vitamin D receptor (VDR). The mechanism by which 1,25(OH)₂D₃ and VDR bind includes an interaction between two histidine residues in VDR and the 25-hydroxyl group of 1,25(OH)₂D₃. Site-directed mutagenesis was used to synthesize single mutants, Histidine305Alanine (H305A) and Histidine397Phenylalanine (H397F), and the double mutant H305A/H397F. The cDNA encoding for VDR (wt) or mutants, expressed in Cos-1 cells, displayed the following binding affinities for [³H]-1,25(OH)₂D₃: wtVDR= 0.67±0.18nM, H305A=1.49±0.66nM, H397F=1.03±0.06nM, H305A/H397F=1.92±0.74nM. Data suggest that H305A and the double mutant are significantly different than wild-type. Cotransfection of the cDNA encoding for VDR with a cDNA encoding for secreted alkaline phosphatase containing a vitamin D response element was performed. The mutants activated 50% maximal gene transcription at the following concentrations: wtVDR= 2.77±0.74nM: H305A=215±160nM, H397F=139±88nM, H305A/H397F=362±330nM. All mutants were significantly different from wild-type suggesting that the histidines are critical for trans-activation.

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LIST OF ABBREVIATIONS

AF-2	Transactivation Function- 2
ATP	Adenosine Triphosphate
Ci	Curies
cDNA	Complementary Deoxyribonucleic Acid
DBP	Vitamin D Binding Protein
DBD	DNA Binding Domain
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic Acid
DPM	Disintegrations per Minute
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
H305A	Histidine 305 Alanine
H397F	Histidine 397 Phenylalanine
H305A/H397F	Histidine 305 Alanine/ Histidine 397 Phenylalanine
HVDRR	Hereditary 1,25-Dihydroxyvitamin D-Resistant Rickets, Type II
LBD	Ligand Binding Domain
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCS	Newborn Calf Serum
dNTP	Deoxy- Nucleoside Triphosphate
PBS	Phosphate Buffered Saline

PTH	Parathyroid Hormone
RLU	Relative Light Units
RXR	Retinoid X Receptor
TED	Tris(hydroxymethyl)aminomethane; EDTA; dithiothreitol
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

INTRODUCTION

Vitamin D compounds do not fit the classical definition of a vitamin since they may be photochemically synthesized in the body. The active metabolite of vitamin D is 1,25-dihydroxyvitamin D₃ (see Synthesis below). Many of the biological actions of 1,25-dihydroxyvitamin D₃ are mediated through its interaction with the vitamin D receptor (VDR). Although 1,25-dihydroxyvitamin D₃ is a powerful anti-rachitic agent, its primary effect on bone is the stimulation of bone resorption leading to an increase of calcium and phosphorous levels in the serum (Underwood and DeLuca, 1984). Maintaining constant levels of serum calcium is crucial, whether calcium is available from the diet or not (Collins, 2000). If the serum ion product is not maintained at the appropriate level for normal bone mineralization, disease states such as rickets may develop in children, or osteomalacia may develop in adults. Historically, most cases of rickets, since it is a deficiency disease of vitamin D, became virtually extinct by treating patients with small concentrations of vitamin D (O'Riordan, 1997) or by exposing them to sunlight. However, other cases of rickets were attributed to the resistance of the action of vitamin D, and thus, were termed vitamin D- resistant rickets, some of which are due to genetic defects in the VDR. The actions of vitamin D as well as vitamin D related diseases will be discussed later. First, an understanding of the biosynthetic pathway of vitamin D is necessary.

Synthesis of 1 α ,25-Dihydroxyvitamin D₃

Vitamin D compounds are derived from the cyclopentanoperhydrophenanthrene ring system of cholesterol (Collins, 2000), which is a common substrate of steroid synthesis. Cholesterol is oxidized to 7-dehydrocholesterol. In the malpighian skin layer (Collins and Norman, 2001), 7-dehydrocholesterol is converted to previtamin D₃ upon the skin's exposure to sunlight. During this conversion, the bond between carbons 9 and 10 is broken, thus producing the seco-steroid. After a few days, previtamin D₃ isomerizes to vitamin D₃ (Holick, 1981) via a reversible antarafacial [1,7]-sigmatropic hydrogen shift (Okamura et al., 2000). The compound may be stored in fat and muscle tissues until it is needed (Glorieux, 1997). Vitamin D₃ is biologically inert and must be metabolized into a biologically active form (Norman, 1980) in order to initiate its biological functions. Vitamin D₃ is therefore removed from the skin to the circulatory system via the vitamin D-binding protein (DBP), a blood transport protein specific for vitamin D metabolites. The DBP carries vitamin D₃ to the liver where a hydroxyl group is added to the 25-carbon position of vitamin D₃ forming 25(OH)D₃, the major circulating form of vitamin D (Collins, 2000). The enzyme, vitamin D₃ 25-hydroxylase, catalyzes this reaction (Tucker et al., 1973). Since the reaction is poorly regulated, circulating levels of 25(OH)D₃ bound to DBP reflect the body quantity of vitamin D₃ (Mawer, 1980). From the liver, 25(OH)D₃ is returned to the blood stream and is carried by DBP to the kidney, where a second hydroxyl group is added. The enzyme, 25-hydroxyvitamin D₃-1- α -hydroxylase, adds a hydroxyl group to the carbon-1 position of 25(OH)D₃ (Henry

and Norman, 1974), thus producing $1\alpha, 25$ -dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the most biologically active metabolite of vitamin D_3 (Figure 1).

Figure 1. Synthesis of $1,25(OH)_2D_3$. The carbon atoms are numbered based on standard steroid notation.

The kidney enzyme, 25 -hydroxyvitamin D_3 - 1α -hydroxylase (1 -hydroxylase), and the liver enzyme, vitamin D_3 - 25 -hydroxylase, are cytochrome P450 enzymes found in mitochondria. All cytochrome P450 enzymes function as mixed oxidases using electrons

from NADPH and molecular oxygen (Black and Coon, 1987). The 1-hydroxylase is expressed in the proximal convoluted tubules of the kidney, and circulating concentrations of $1,25(\text{OH})_2\text{D}_3$ primarily reflect its renal synthesis (Miller and Portale, 2000). Renal 1-hydroxylase activity is tightly regulated by parathyroid hormone (PTH), calcium, phosphorous and $1,25(\text{OH})_2\text{D}_3$. The most important determinant of 1-hydroxylase activity is the vitamin D status of the organism.

When circulating concentrations of $1,25(\text{OH})_2\text{D}_3$ are low, the kidney, by the action of 1-hydroxylase, produces high levels of $1,25(\text{OH})_2\text{D}_3$. When circulating concentrations of $1,25(\text{OH})_2\text{D}_3$ are high, synthesis of $1,25(\text{OH})_2\text{D}_3$ by the kidney is reduced (Henry, 1992). Mutations in the enzyme 1-hydroxylase cause vitamin D-dependent rickets type I (Kitanaka et al., 1998; Wang et al., 1998), which is an autosomal recessive disorder. Administration of $1,25(\text{OH})_2\text{D}_3$ can completely reverse the effects of the disease.

Another regulator of $1,25(\text{OH})_2\text{D}_3$ production is parathyroid hormone (PTH) (Collins, 2000). PTH is released when serum calcium levels are low, thus stimulating the activity of 1-hydroxylase and producing $1,25(\text{OH})_2\text{D}_3$ (Henry et al., 1992). $1,25(\text{OH})_2\text{D}_3$ operates in a feedback mechanism to modulate and/ or reduce the secretion of PTH (Henry et al., 1992). High circulating levels of $1,25(\text{OH})_2\text{D}_3$ have been shown to decrease the levels of PTH by a direct and an indirect mechanism (Collins, 2000). The indirect mechanism is due to the increase of serum calcium levels which inhibits PTH production. The direct mechanism involves the interaction of $1,25(\text{OH})_2\text{D}_3$ with its receptor to suppress the expression of the PTH gene (Collins, 2000).

In vitamin D deficiency diseases, the concentration of $25(\text{OH})\text{D}_3$ is low and the measurement of this compound may give the best estimate of the vitamin D status of the body (Preece et al., 1975). The concentration of $1,25(\text{OH})_2\text{D}_3$ is also low; however, this low concentration may be reversed by the addition of small supplements of vitamin D, which can produce dramatic and sustained improvements (O'Riordan, 1997).

To reiterate, $1,25(\text{OH})_2\text{D}_3$ is the biologically active metabolite of vitamin D and its production is affected by the tight regulation of the activity of the enzyme 1-hydroxylase. $1,25(\text{OH})_2\text{D}_3$ is a highly dynamic molecule and can adopt a number of conformations around the A-ring, seco B- ring and side chain. Its structure will be discussed next.

1 α ,25-Dihydroxyvitamin D₃ Structure

Various vitamin D compounds are distinguished from other steroids in that the B ring is broken and are thus termed seco-steroids. When the bond between carbons 9 and 10 is broken, the seco-steroid undergoes a [1,7]-sigmatropic hydrogen shift isomerization (Okamura et al., 2000) (Figure 2).

Figure 2. Seco-steroid of vitamin D.

The isomerization of the conjugated triene system of double bonds allows the A-ring to rapidly interconvert between two chair conformations and allows the compound itself to interconvert between cis or trans conformations and other intermediate rotations. In chair conformer A, the hydroxyl at carbon-1 is axial whereas the hydroxyl at carbon-3 is equatorial. In chair conformer B, the hydroxyl at carbon-1 is equatorial, whereas the hydroxyl at carbon-3 is axial (Bouillon et al., 1995) (Figure 3).

Figure 3. Chair conformations of the A –ring (Collins and Norman, 2001; Bouillon et al., 1995).

Because of the presence of the two sp^2 centers, it is difficult to determine whether the A ring is truly chair-like as opposed to half-chair-like as in cyclohexene (Bouillon et al., 1995). The X-ray (Trinh-Toan et al, 1976) and solution structural studies (Eguchi and Ikekawa, 1990; Wing et al., 1974; Wing et al., 1975) suggest that there is about a fifty degree out of plane twist angle between the carbons 5, 6 and carbons 10, 19 double bonds, which imparts a chair-like shape to the A ring (Bouillon et al., 1995). This chair-like nature of the A ring is confirmed via X-ray crystallographic studies and suggests that

free 1,25(OH)₂D₃ crystallizes with its A ring in the B-chair conformation and that its 6,7-*s-trans* single bond is 3° out of plane (Suwinska and Kutner, 1996).

In solution, 1,25(OH)₂D₃ is believed to exist approximately 99% as 6-*s-trans*-1,25(OH)₂D₃ whereas the 6-*s-cis*-1,25(OH)₂D₃ is spectroscopically undetectable. Nevertheless, the latter must be present since it mediates the known equilibration of 6-*s-trans*-1,25(OH)₂D₃ with 1,25-previtamin D₃ (Okamura et al., 2000) (Figure 4).

Figure 4. The dynamic changes of the *seco*-B ring. All of the carbon atoms of the 6-*s-trans* conformer are numbered using standard steroid notation (Collins and Norman, 2001; Bouillon et al., 1995).

Due to the rapid conformational isomerism about single bonds, it is possible that a significant boat-like (or twist boat) A ring conformation might exist in solution and be important in receptor binding (Okamura et al., 1974). It was later known from solution NMR studies of free 1,25(OH)₂D₃ that the A ring is partitioned between near equimolar amounts of the chair conformations (55% B-conformation: 45% A-conformation) in nonpolar solvents (Wing et al., 1975). Regarding the *seco*-B ring, the 6,7-single bond orientation is 6-*s-trans* and planar within experimental error of NMR measurements

(Okamura et al., 2000). In addition, the side chain may assume an innumerable array of conformations by carbon-carbon single bond rotations.

The structure of $1,25(\text{OH})_2\text{D}_3$ has been studied extensively to understand the mechanism by which it binds to its receptor to mediate its biological functions. It is important to understand this mechanism as well as to clearly define which conformer of $1,25(\text{OH})_2\text{D}_3$ is bound to its nuclear receptor and other proteins in order to design and develop possible therapeutic analogs (Okamura et al., 2001). Next, the functions of $1,25(\text{OH})_2\text{D}_3$ will be discussed, followed by its mode of action with the nuclear receptor.

Functions of $1\alpha, 25$ -dihydroxyvitamin D_3

The biological actions of $1\alpha, 25$ -dihydroxyvitamin D_3 ($1,25(\text{OH})_2\text{D}_3$) may be categorized under classical functions and non-classical functions. The classical functions include maintaining calcium and phosphorous homeostasis. In humans, serum calcium levels are normally maintained between 9.5 and 10.5 mg/100mL, whereas the phosphorous concentration is between 2.5 and 4.3 mg/100mL (Norman, 1979). Coupled with PTH, $1,25(\text{OH})_2\text{D}_3$ maintains serum calcium and phosphate levels by its actions on the intestine, kidney, bone, and parathyroid gland (Collins and Norman, 2001). In the intestine, $1,25(\text{OH})_2\text{D}_3$ is involved in the absorption of calcium by stimulating the expression of calbindin D, a calcium binding protein. In the kidney, $1,25(\text{OH})_2\text{D}_3$ inhibits 1-hydroxylase activity, decreasing the synthesis of $1,25(\text{OH})_2\text{D}_3$; however, the actions of vitamin D on calcium and phosphorous metabolism in the kidney is unclear (Collins and Norman, 2001). In the bone, $1,25(\text{OH})_2\text{D}_3$ is involved in bone resorption to

maintain serum calcium levels. Under normal physiological conditions, bone formation and bone resorption are tightly balanced (Norman and Hurwitz, 1993). Also, $1,25\text{-(OH)}_2\text{D}_3$ stimulates bone growth and mineralization through an indirect effect of providing minerals for bone matrix incorporation through an increase of intestinal calcium and phosphorous absorption (Collins, 2000). In the parathyroid gland, PTH reduction is regulated by $1,25\text{-(OH)}_2\text{D}_3$ directly or indirectly as mentioned above.

The non-classical actions of $1,25\text{-(OH)}_2\text{D}_3$ have been shown in tissues not related to calcium homeostasis (Collins, 2000). For example, $1,25\text{-(OH)}_2\text{D}_3$ is involved in regulating the growth and differentiation of a variety of cell types, including those of hematopoietic and immune systems (Lemire, 1992). Also, in the skin and in malignant cell types (Colston et al., 1981; Roodman et al. 1985), $1,25\text{-(OH)}_2\text{D}_3$ appears to exert affects on cellular growth and differentiation. In the pancreas, $1,25\text{-(OH)}_2\text{D}_3$ has been found to be essential for normal insulin secretion (Collins and Norman, 2001). In addition, $1,25\text{-(OH)}_2\text{D}_3$ has a direct effect on Ca^{2+} transport in cultured myoblasts and skeletal muscle tissue (Collins and Norman, 2001) and was shown to be important for cardiac muscle function (Walters et al., 1986; Rabie et al., 1986; Boland et al., 1992).

These non-classical actions of $1,25\text{-(OH)}_2\text{D}_3$ have been the basis for the development of potential therapeutic agents that lack the calcemic activity of $1,25\text{-(OH)}_2\text{D}_3$, yet maintain the ability to inhibit cell proliferation and promote cell differentiation. This is an important characteristic in that using therapeutic doses of $1,25\text{-(OH)}_2\text{D}_3$ to treat certain cancers and skin diseases may provoke hypercalcemia, a

chronic condition in which excessive amounts of calcium is found in the blood.

Hypercalcemia can result in the calcification of soft tissues, leading to death.

1,25(OH)₂D₃ is believed to produce most of its actions by a mechanism mediated by an interaction with the nuclear vitamin D receptor. Since the VDR is ligand-dependent, it requires 1,25(OH)₂D₃ binding to become active. Such a mechanism is discussed below.

Mode of Action

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), acts via two major signal transduction pathways: nongenomic and genomic. The nongenomic actions of 1,25(OH)₂D₃ are rapid and have been shown to occur at the cellular (calcium transport across a tissue) and subcellular level (membrane calcium transport, changes in intracellular second messengers) (Bouillon et al., 1995). The rapid transport of calcium mediated by 1,25(OH)₂D₃ in the intestine is called transcaltachia (Collins, 2000). A study done by Nemere and coworkers suggests that such a response may occur within 1-5 minutes (Nemere et al., 1984; Wali et al., 2003). It has been proposed that a membrane receptor for 1,25(OH)₂D₃ exists (Nemere et al., 1998). In addition, it was suggested that the ligand-receptor complex mediates the signal transduction of the hormone via the opening of voltage-gated Ca²⁺ channels so as to initiate the biological response (Zhou et al., 1992).

The genomic pathway involves the nuclear vitamin D receptor (VDR) located within the target tissue. When 1,25(OH)₂D₃ binds to its cognate receptor, it follows a

mechanism similar to that of steroid hormones such as testosterone, aldosterone, progesterone and estrogen (Lowe et al., 1992). A model for steroid hormone action is shown in Figure 5.

Figure 5. Mode of action of VDR. VDR= vitamin D Receptor; RXR= Retinoid X Receptor; D= $1,25(\text{OH})_2\text{D}_3$.

$1,25(\text{OH})_2\text{D}_3$ can passively enter the cellular membranes and the nucleus to bind to VDR. The binding of the $1,25(\text{OH})_2\text{D}_3$ ligand promotes the heterodimerization of VDR with the retinoid x receptor (RXR), a member of the nuclear receptor superfamily. The complex

then binds to DNA in target tissues by recognizing a vitamin D response element (VDRE), which consists of direct hexanucleotide repeats with a spacer of three nucleotides (Haussler et al., 1997) that is located in the promoter regions of target genes (Hannah and Norman, 1994). The $1,25(\text{OH})_2\text{D}_3$ ligand promotes VDR-RXR heterodimerization and specific, high affinity VDRE binding, whereas the RXR ligand, 9-*cis* retinoic acid, suppresses $1,25(\text{OH})_2\text{D}_3$ –stimulated transcription by diverting RXR to form homodimers (Haussler et al., 1997). Binding $1,25(\text{OH})_2\text{D}_3$ activates VDR by changing the position of an amphiphilic α -helix referred to as the (trans)activation function 2 (AF-2) domain (Danielian et al., 1992). This AF-2 domain provides an interface in which cell-specific co-activators may interact and mediate contacts to the basal transcription machinery (Nayeri and Carlberg, 1997). As a result, activation of the nuclear receptor stimulates the activity of RNA polymerase II, a process called transcriptional activation (trans-activation) (Cavaillès et al., 1995).

In summary, in VDR-mediated transcriptional activation, $1,25(\text{OH})_2\text{D}_3$ binding to VDR alters the conformation of the ligand binding domain such that it allows heterodimerization with RXR, facilitates VDRE binding by inhibiting RXR binding to its cognate ligand, thereby activating the complex to recruit coactivators to its AF-2 domain (Haussler et al., 1997). Thus, transcription is initiated to induce biological responses.

The Vitamin D Receptor

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily which includes receptors for glucocorticoids (GR), progesterone (PR), estrogen (ER),

hormonal forms of vitamins A (RAR, RXR) and D (VDR) and many orphan receptors (Collins, 2000). These receptors have a common structural organization consisting of five domains (Krust et al., 1986) (Figure 6). The A/B domain is a poorly conserved region among all nuclear receptors. In VDR, the function of the A/B region is not clearly defined. The DNA binding domain (DBD), region C, is the most conserved region throughout the members of the superfamily. It extends from amino acid residues 25 to 90, it is basic, and it is rich in cysteine residues (Lin et al., 1996). The amino acid numbering system for the VDR used in this report is in accord with the sequence described by Baker and coworkers, and the sequence is shown in Appendix A (Baker et al., 1988). These DBD residues fold to form two zinc-finger regions. The first zinc finger region, which is composed of four cysteine residues coordinated to zinc in a tetrahedral arrangement and several hydrophobic amino acids, recognizes the DNA response element (Collins, 2000) on target genes. The second zinc-finger region, composed of five cysteine residues and many basic amino acids, is also involved in DNA binding and plays a role in receptor dimerization. Region D is a flexible hinge region, with no specific function except to bend in such a way that allows the DBD and the ligand binding domain to be in closer proximity. Region E is the ligand binding domain (LBD) and the second most conserved region within the family of nuclear receptors.

Figure 6. Vitamin D receptor domain structure. The N-terminal domain, residues 1-24, is the A/B domain. The C domain, residues 25-90, is the DNA binding domain. The D domain, residues 91-135, is a flexible hinge region. The E domain, residues 136-427, is the ligand binding domain.

In VDR, this region consists of about 290 amino acid residues. It contains a hydrophobic pocket for ligand binding. In addition to functioning as an LBD, this region possesses the hormone-dependent transactivation function 2 (AF-2) that can interact with receptor coactivators. When VDR binds to its cognate ligand, $1,25(\text{OH})_2\text{D}_3$, the receptor-ligand complex undergoes a conformational change which increases the receptor's ability to activate gene transcription. In its simplest terms, the LBD can be thought of as a molecular switch that, upon ligand binding, shifts into a transcriptionally active state (Mangelsdorf et al., 1995).

Ligand and VDR Binding

Thus far, crystallographic structures of sixteen nuclear receptor-ligand binding domains have been determined (Yamada et al., 2003). These receptors include the holo and apo forms of the retinoid X receptor (Bourguet et al., 1995; Egea et al., 2000), retinoic acid receptor (Renaud et al., 1995), androgen receptor (Matias et al., 2000; Sacks

et al., 2001), progesterone receptor (Williams and Sigler, 1998), the peroxisome proliferator-activated receptor (Nolte et al., 1998; Xu et al., 1999), and the estrogen receptor (Brzozowski et al., 1997; Shiau et al., 1998; Pike et al., 1999), complexes with the natural ligand, synthetic agonists and antagonists, and ternary complexes with the ligand and coactivator. Recently, the crystallographic structure for VDR-LBD bound to its natural ligand was solved (Rochel et al., 2000). The VDR was engineered as to eliminate the amino acid residues between 165 and 215 because these residues made crystallization difficult and were believed to have no biological significance. Rochel and coworkers determined the active conformation of the bound $1,25(\text{OH})_2\text{D}_3$ to VDR (Rochel et al., 2000). Their experimental work showed that both bound and free $1,25(\text{OH})_2\text{D}_3$ crystallize in the chair - B conformation (Rochel et al., 2000). Recall that the chair B-conformation has the carbon 1-OH and carbon 3-OH groups oriented equatorially and axially, respectively (Rochel et al., 2000; Okamura et al., 2000). The most noticeable difference between the two structures was that the 6,7-s-trans single bond of receptor bound $1,25(\text{OH})_2\text{D}_3$ was twisted approximately 30° out of plane (Rochel et al., 2000), compared to the 3° in the free ligand (Suwinska and Kutner, 1996) resulting in the curved shape of the ligand bound to the receptor (Rochel et al., 2000). The crystal structure also revealed the amino acid residues in the VDR that come into contact with the three hydroxyl groups of $1,25(\text{OH})_2\text{D}_3$ (Figure 7). The contact points are as follows: the 1-carbon hydroxyl hydrogen bonds with serine-237 and arginine-274, the 3-hydroxyl group forms hydrogen bonds with serine-278 and tyrosine-143, and the 25-hydroxyl group is hydrogen bonded to histidine-305 (H305) and histidine-397 (H397).

Figure 7. Contact points of 1,25(OH)₂D₃ in the ligand binding pocket. Carbon atoms are colored in gray, oxygen atoms are colored in red, nitrogen atoms are colored in blue.

A hydrogen bond network around the histidine residues indicates that H305 is a hydrogen bond acceptor and H397 is a hydrogen bond donor (Rochel et al., 2000). The residues mentioned above are believed to be important in ligand binding and possibly, trans-activation. It may be concluded that a mutation found in any of the above residues, as well as some mutations in residues of the DNA binding domain (DBD), may result in a decrease in ligand binding and trans-activation. Naturally occurring mutations in the gene encoding the VDR have resulted in hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR), also known as vitamin D-dependent rickets, Type II, which is an autosomal recessive disorder (Feldman and Malloy, 1990; Hughes et al., 1991), characterized by early onset rickets, hypocalcemia, elevated 1,25(OH)₂D₃ levels, and, unlike HVDRR-type I, resistance to 1,25(OH)₂D₃ treatment (Brooks et al., 1978; Rosen

et al., 1979). Most of the mutations in HVDRR have been found in the DBD, a location that does not affect the ligand-binding properties of the receptor, but interferes with the high affinity binding of VDR to DNA (Malloy et al., 1997). However, a few mutations of HVDRR have been found in the LBD, one of the mutations being a conversion of a histidine to a glutamine residue at the 305 amino acid position. This mutation occurs in an amino acid that is critical for the normal functioning of the VDR. In the present study, the importance of the histidine residue at the 305 position, as well as the importance of the histidine residue at the 397 position was evaluated.

Goals of the Project

The goal of this project was to determine how the histidines at 305 and 397 of VDR affect the binding affinity of $1,25(\text{OH})_2\text{D}_3$, and subsequent receptor-mediated events such as the induction of gene expression. In order to achieve this goal, a comparative analysis was done. In this case, wild-type VDR and mutated VDR were compared based on their binding affinity for $1,25(\text{OH})_2\text{D}_3$ and based on the concentration of $1,25(\text{OH})_2\text{D}_3$ required for each to obtain a half-maximal response of induction of gene transcription.

The initial work involved substituting the two key histidine residues with other residues so as to disrupt hydrogen bonding with the ligand. In addition to disrupting hydrogen bonding, it was interesting to determine whether adding space in the ligand binding pocket plays a role in ligand binding and transactivation. To determine this, the two key histidine residues were substituted differently. In order to obtain substitutions,

specific mutations in the cDNA encoding the VDR were incorporated. Three mutations were introduced using a technique known as site-directed mutagenesis. One mutation consisted of changing the histidine at the 305 position to alanine (H305A), another mutation consisted of changing the histidine at the 397 position to phenylalanine (H397F), and the third mutation incorporated both of the aforementioned mutations (H305A/H397F). The phenylalanine residue is similar to histidine with respect to size, while the alanine residue is smaller in size. Both substitutions eliminate the ability of the replaced residue to form hydrogen bonds by replacing the charged polar side chain of histidine with a nonpolar side chain possessed by both alanine and phenylalanine. In addition, substitution to alanine results in a voided space within the ligand binding pocket, while substitution to phenylalanine may possibly retain the same space as that occupied by the histidine residue.

After site-directed mutagenesis was completed, the presence of the desired mutations was confirmed using a method of nucleic acid sequencing called the chain-terminator method. This technique utilizes a nucleoside triphosphate along with its dideoxy analog to extend a labeled primer and terminate it in order to generate truncated chains that end with that particular nucleoside triphosphate. Gel electrophoresis separates the chains according to their lengths and the sequence of the DNA may be read.

Once the presence of the desired mutations was confirmed, the comparative experiments were performed. Such experiments consisted of binding assays, which allowed the binding affinities of VDR bound to $1,25(\text{OH})_2\text{D}_3$ to be determined, and

transcriptional activation assays, which employed a secreted alkaline phosphatase enzyme as a reporter gene.

MATERIALS AND METHODS

Composition of Buffers and Solutions

Bacterial Cell Culture

Ampicillin Solution (100mg/mL): 1g Ampicillin, Sodium salt (Mediatech, Inc.) in 10mL millipore water. Filter sterilize using 0.45 μ M syring filter.

NZY+ Broth (1L): 10g Bacto Casein Digest, 1g Bacto Casamino Acids, 5g Bacto Yeast Extract, 5g Sodium Chloride, 0.98g Magnesium Sulfate, anhydrous.

LB Broth (1L): 10g Bacto tryptone, 5g Yeast Extract, 5g Sodium Chloride.

LB Agar (1L): 10g Bacto Tryptone, 15g Bacto Agar, 5g Bacto Yeast Extract, 5g Sodium Chloride.

Eukaryotic Cell Solutions

Chloroquine Solution (8mM): 0.041g chloroquine diphosphate (Fluka), 10mL millipore water. Filter sterilize using 0.45 μ M syring filter.

DEAE Solution (10mg/mL): 1g DEAE-dextran hydrochloride (Fluka) per 100mL phosphate buffered saline (10mM). Filter sterilize using 0.22 μ M filter.

Dulbecco's Modification of Eagle's Medium (DMEM): 13.38g/ L DMEM powder (Cellgro), 1.4g/L sodium bicarbonate, 10mL/ L antibiotic solution (Cellgro 3C-004-C1 100X: 100,000 U/mL Penicillin, 25 μ g/mL amphotericin, 10,000 μ g/ mL streptomycin) 10% newborn calf serum (Biowhittaker). Filter sterilize using 0.22 μ M filter.

PBS (10mM) pH 7.4 (1L): 9.55g PBS powder (Cellgro). Dissolve up to 1L millipore water adjusting to appropriate pH. Filter sterilize using 0.22 μ M filter.

TED Buffer (1L): 10mM tris (hydroxymethyl) amino-methane; 1mM ethylene diaminetetraacetic acid; 0.5mM dithiothreitol; pH 7.5.

Site-Directed Mutagenesis

The human VDR cloned into the expression vector pcDNA1.1 (Invitrogen) containing an ampicillin resistant gene was used as a template for site-directed mutagenesis. Two clones of mutated hVDRs, (H305A and H397F), were produced by changing the cDNA coding for the amino acid residue to alanine or phenylalanine according to the instructions given in the QuikChange Site-Directed Mutagenesis kit (Stratagene). Two complimentary oligonucleotides for each mutant, twenty-eight bases in length, containing the desired mutation were obtained (from Sigma-Genosys) to serve as primers for the 305 mutant and for the 397 mutant (see Table 1). For the double mutant hVDR (H305A/H397F), the 397 mutant pcDNA1.1-hVDR expression vector was used as a template with the 305 primers. The sample reaction consisted of mixing 125ng of forward primer, 125ng of reverse primer, 10ng of pcDNA1.1-hVDR template, 5 μ l of 10X reaction buffer, 1 μ l of dNTP mix, and nuclease-free water to a final volume of 50 μ l. Then, 1 μ l of *Pfu Turbo* DNA polymerase (2.5 U/ μ l) was added. The reaction was placed in an MJ Research Minicycler, using a heated lid, under the following steps which consisted of the following parameters: "Step 1" 95°C for 30 seconds; "Step 2" 95°C for

30 seconds; “Step 3” 72°C for 1 minute; “Step 4” 72°C for 12 minutes; Steps 2 through 4 were repeated 14 times. After the cycle program was completed, the sample reaction was treated with 1µl of the *Dpn* I restriction enzyme (10 U/µl) by thoroughly mixing the enzyme and sample contents, micro-centrifugating the contents for 1 minute, and immediately incubating the reaction at 37°C for 1 hour.

Table 1. Site-Directed Mutagenesis Primers (aqua nucleotides indicate desired mutation)

Mutant	Primer	Oligonucleotide (5'-3' direction)
H305A	Forward	GACCAAAGCCGGAGGCCAGCCTGGAGCTG
	Reverse	CAGCTCCAGGCTGGCTCCGGCTTTGGTC
H397F	Forward	CCTCAATGAGGAGTTCTCCAAGCAGTACC
	Reverse	GGTACTGCTTGGAGAACTCCTCATTGAGG

Transforming into XL1-Blue Supercompetent Cells

A tube of Epicurian Coli XL1- Blue Supercompetent cells (Stratagene) was thawed on ice. For each transformation, 50µl of supercompetent cells was transferred to a pre-chilled Fisher 2059 polypropylene tube. To one tube, either 1µl or 4 µl of *Dpn* I-treated sample was added. The tubes were swirled to mix the transformation reaction and then incubated on ice for 30 minutes. The tubes were then heat shocked at 42°C for 45 seconds in a Precision 183 water bath. The reactions were then placed on ice for 2 minutes before 0.5mL of NZY⁺ broth (preheated to 42°C) was added to each tube. The

transformation reactions were incubated at 37°C for 1 hour with shaking at 225 rpm in a Lab-Line Orbit Environ Shaker. Following 1 hour of incubation, the contents in each tube were spread onto an LB agar plate containing 100µg/mL ampicillin. The plates were incubated at 37°C for 18 hours in a Thermodyne Type 37900 Culture Incubator.

Plasmid Mini Preparations

Bacterial colonies were picked from agar plates with a sterilized wooden stick and used to inoculate in 3mL of NZY + broth containing 100µg/mL ampicillin. The broth was allowed to incubate at 37°C for 16- 20 hours, shaking at 250 rpm. The plasmid was purified according to the instructions provided in the Eppendorf Perfect Prep® Plasmid Mini kit. The purified DNA was resuspended in 70µl of TE (Tris, EDTA, pH 8.0) buffer (preheated to 70°C).

DNA Sequencing

Primers were end-labeled using Promega's end-labeled primer protocol. Various primers were used (Table 2). The protocol calls for the use of 10pmol of primer, 10 µCi/µl of [γ -³³P] ATP (Amersham Pharmaceuticals: 2500 Ci/mmol specific activity), 1µl of T4 polynucleotide kinase 10X Buffer, 10U/µl of T4 polynucleotide kinase, and nuclease-free water to a final volume of 10µl. Once these reagents were combined in a 0.5mL microcentrifuge tube, the tube was incubated at 37°C for 30 minutes, then at 90°C for 2 minutes, to inactivate the kinase, using a MJ Research Minicycler.

The template DNA was sequenced using the *fmol* DNA Cycle Sequencing System (Promega), which utilizes the dideoxy method. For each set of sequencing reactions, four 0.5 microcentrifuge tubes were labeled with G, A, T, or C. To the appropriately labeled tube, 2µl of d/ddNTP mix was added and placed at 4°C until it was needed. In a microcentrifuge tube, the following were combined for each template/ primer combination: 0.1µg of template DNA, 5µl of *fmol* Sequencing 5X Buffer, 1.5pmol of end-labeled primer, and nuclease-free water to a final volume of 16µl. Finally, 1µl of Sequencing Grade *Taq* DNA Polymerase (5U/µl) was added. The contents were briefly mixed by pipetting and were briefly centrifuged. Then, the set of tubes that were previously labeled as G, A, T, or C, were placed on ice and 4µl of the enzyme/primer/ template mix was added to each tube. The reaction tubes were briefly mixed and centrifuged, and were quickly placed in a thermal cycler, preheated to 95°C, using a heated lid. The thermal cycler parameters for cycle sequencing were as follow: "Step 1" 2 minutes at 95°C; "Step 2" denaturation for 30 seconds at 95°C; "Step 3" primer annealing for 30 seconds at 42°C; "Step 4" extension of primers for 1 minute at 70°C; Steps 2 through 4 were repeated 29 times. After the cycle program was completed, 3µl of *fmol*® Sequencing Stop Solution was added to terminate the reaction. The samples were stored at -20°C. Prior to gel run, the samples were heated to 70°C for 5 minutes. Then, 3µl of each sample were loaded onto a 6% polyacrylamide, 7M urea sequencing gel (80mL SequaGel-6, 20mL SeguaGel-6 Buffer (National Diagnostics), containing 800µl 10%-ammonium persulfate. The gel was electrophoresed at 70 Watts for 4.5 to 5 hours on a Sequencing Gel Apparatus Model S2 (Life Technologies, Inc). The gel was

then soaked in a fixing solution (5% acetic acid, 5% methanol, 90% water solution) for ~ 20 minutes and dried (Savant gel dryer) under vacuum at 80°C for an hour. Once dried, the gel was placed in an autoradiography cassette with BioMax film (Kodak) and allowed to expose for about 5 days. After the film exposure period, the film was developed using the Kodak RP-X –Omat Model M-7B Processor (SJSU Health Building), and the DNA sequence was determined.

Table 2. Primers used for DNA Sequencing.

Primer @ VDR Location	Oligonucleotide (5'-3' direction)
T7	TAATACGACTCACTATAGGG
309	ACT GCC GCA TCA CCA
502	GCG CAT CAT TGC CAT
711	CGT CCA GCT TCT CCA
935	CGC TCC AAT GAG TCC
1164	CGC TGA TTG AGG CCA

Plasmid Isolation and Purification

A large-scale plasmid protocol (QIAGEN) was utilized to isolate and purify plasmid DNA. A single bacterial colony picked from a selected agar plate was used to inoculate 5mL NZY⁺ medium (DIFCO, NZCYM Broth) containing 5µl ampicillin (100µg/ml). The solution was incubated at 37°C, overnight with shaking at 250-300 rpm.

This starter culture was diluted 1/500 into 250mL LB- ampicillin medium (DIFCO, LB Broth Lennox). Cells were incubated at 37°C, overnight with shaking at 250-300 rpm. The bacterial cells were harvested by centrifugation at 6000 rpm for 15min at 4°C using the IEC B-22M centrifuge with an IEC-877 rotor. After the supernatant was poured out, the pellet was resuspended in a buffer solution (50mM Tris(hydroxymethyl) aminomethane, pH 8.0; 10mM ethylenediaminetetraacetic acid (EDTA); 100µg/mL RNase A) and cells were lysed in a lyses buffer solution (200 mM sodium hydroxide, 1% sodium dodecyl sulfate (SDS) (w/v)) by incubation for 5 minutes at room temperature. A neutralization buffer (3.0M potassium acetate, pH 5.5; pre-chilled to 4°C) was then added to the lysate and mixed gently by inversion. The plasmid DNA was then separated from RNA, proteins, and low-molecular-weight impurities with a medium-salt wash (1.0M sodium chloride; 50mM 3-N-morpholinopropanesulfonic acid (MOPS), pH 7.0; 15% isopropanyl (v/v)) through gravity flow on a column. A high-salt buffer (1.6M sodium chloride; 50mM MOPS, pH 7.0; 15% isopropanyl (v/v)) was used to elute the DNA into a 50mL- Falcon polypropylene tube. Finally, isopropanol was added to the eluted DNA to desalt and precipitate it. The tube was centrifuged, as before, for 50 minutes at 7400 rpm at 4°C and the supernatant was removed. The translucent precipitate that resulted was washed with room-temperature 70% ethanol (70% ethanol and 30% endotoxin-free water by volume), by centrifugating, as before, at 7400 rpm at 4°C for 30 minutes. The supernatant was decanted, and the pellet was allowed to air dry, and was resuspended in 0.5mL of TE buffer (10mM Tris-Cl, pH 8.0; 1mM EDTA).

Eco-R1 Restriction Endonuclease

To confirm presence of plasmid DNA after isolation, 1 μ l DNA sample, 1 μ l of 10X buffer H (Promega), 1 μ l of Eco-R1 enzyme (Promega-12 U/ μ L), and 7 μ l of nuclease-free water were mixed in a microcentrifuge tube, and incubated for one hour at 37°C followed by five minutes at 70°C. Blue/orange loading dye (6X) (Promega) was added to each sample, and the samples, along with 0.50 μ g/mL HindIII/ EcoRI cut lambda markers (Promega), were loaded onto a 1% agarose gel in 50mL TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA) supplemented with 5mg/mL ethidium bromide. This electrophoretic system usually ran for one hour at 120 volts in the Fisher Scientific FB-SB-710 gel apparatus. The bands in the gel were observed under ultraviolet light.

Calculating DNA Concentration

DNA concentration was determined using Stratagene Eagle Eye Junior gel documentation/ transilluminator 2020E with version 3.2 Eagle Eye software by comparing the intensity of the band corresponding to the DNA to the intensities of the bands from the lambda markers. The base pairs of the lambda marker band that matched the intensity of the DNA sample was divided by the total number of base pairs in lambda DNA, 48,502 base pairs, multiplied by the lambda marker concentration of 0.5 μ g/ μ l and by the dilution factor.

DNA concentration was also determined using the Hewlet Packard 8452A Diode Array spectrophotometer. In this method, the absorbances at wavelengths 260nm and 280nm were annotated in a spectrum ranging from 220nm to 330nm. The concentration

in $\mu\text{g}/\mu\text{l}$ was determined by dividing the absorbance at 260nm by 0.02 times the dilution factor, if any.

Eukaryotic Cell Transfection Assay

African green monkey kidney Cos-1 cells (*Cercopithecus aethiops*) (American Type Culture Collection, Manassas, VA) were grown to 60%-80% confluency in Dulbecco's modification of Eagle's medium (Cellgro) supplemented with 10% newborn calf serum (Biowhittaker) in a Forma Scientific 37900 incubator at 37°C in a 92% humidified atmosphere of 95% air and 5% CO₂ at least 36 hours prior to transfection. Transfection of DNA into Cos-1 cells was done using the DEAE-Dextran assisted transfection (McCutchan and Pagano, 1968). The following steps were performed using aseptic techniques. DMEM media supplemented with 10% NCS was removed from six tissue culture treated dishes (100mm size, Costar brand) containing cells at 60%-80% confluency, using vacuum aspiration. The cells were washed with 5mL of PBS (preheated to 37°C) and allowed to sit for 15 minutes. After 15 minutes, the PBS was removed and 4mL of diluted DEAE-dextran (10-fold in sterile PBS) was added to each dish. After exactly 9 minutes, the DEAE-dextran solution was removed. The cells were washed two times with 5mL of PBS. DNA solution (5.3 μg pcDNA-VDR wild-type or mutant combined with 0.6mL PBS) was added to each dish. The dishes were then incubated at 37°C for 24 minutes with gentle rocking every 4 minutes. After this incubation period, 6mL of medium containing chloroquine (80 μM) was added to each dish. The dishes were incubated at 37°C for 3-4 hours. The media was then replaced

with new media, containing no chloroquine, and the dishes were allowed to incubate for 2-3 days at 37°C.

Saturation Ligand Binding Assay

Seventy-two hours post transfection, the cells were harvested with trypsin-EDTA and lysed in 3ml of ice-cold TED buffer by aspirating five times with a 20-gauge needle syringe. The cell lysate was incubated with increasing concentrations of $1\alpha,25-(\text{OH})_2$ -[26,27-methyl- ^3H]- D_3 (0.125- 5.0nM) (Amersham Pharmaceuticals: 158-186 Ci/mmol specific activity) for 4 hours at 4°C in the presence or absence of a 100-fold molar excess of non-radioactive $1,25-(\text{OH})_2\text{D}_3$ (Makin et al., 1989). Bound and free ligands were separated with 200 μL 50% hydroxyapatite (BIORAD) in TED buffer (Wecksler and Norman, 1979). The hydroxyapatite was then washed 3 times with TED-buffer supplemented with 0.5% triton solution (Triton X-100) by centrifugation at 2245 x g for 1 minute. The final pellet was washed with 1mL 200- proof ethanol to extract bound ligand. The ethanol was decanted into scintillation vials. Ecoscint scintillation fluid (3mL) (National Diagnostics) was added to each vial. Bound ligand was quantified using a Beckman LS 6500 scintillation counter.

VDR Cotransfection Assay

African green monkey kidney Cos-1 cells were grown in 24-well plates to approximately 70% confluency with DMEM media supplemented with 10% newborn calf serum in a 37°C incubator of 92% humidity of 95% air and 5% CO_2 . Circular

plasmid DNA-VDR (7µg) wild-type or mutant was used together with 35µg of pcVDRE-SEAP reporter plasmid in 12mL of PBS for each 24-well plate. Transfection was performed as described above. Twenty-four hours post-transfection, increasing concentrations of 1,25(OH)₂D₃ (0.1nM to 10µM in ethanol vehicle) diluted 1/100 in MEM media without phenol red was added to each well. After 2 days, 30µl of media was collected from each well for the secreted alkaline phosphatase assay.

Secreted Alkaline Phosphatase Assay

The activity of secreted alkaline phosphatase was measured using the Phospha-Light™ System (Applied Biosystems). Seventy-two hours post-transfection, 30µl of cell culture medium from each well was transferred into a 96-well plate. The plate containing the samples was incubated at 65°C for 30 min and then placed on ice to cool. CSPD® chemiluminescent substrate (1.25mM) was prepared by diluting CSPD 1:20 with Phospha-Light™ Reaction Buffer Diluent and allowed to equilibrate to room temperature. A sufficient amount of Phospha-Light™ Assay Buffer required for the entire experiment was allowed to equilibrate to room temperature as well. Dilution Buffer (20µL of 1X) was added to each sample. Then, 50µl of Assay Buffer was added and the plate was allowed to incubate at room temperature for 5 min. Finally, 50µl of diluted CSPD substrate was added to each well and incubated for 20 min. Light output for each well was measured using a chemiluminometer (Luminoskan, Ascent Labsystems) at 10 minute intervals, at a default PMT voltage of 835nm wavelength. All experiments were done in triplicate. A dilution series of an alkaline phosphatase standard

provided in the kit (Applied Biosystems) confirmed that the detection method for the alkaline phosphatase activity worked.

RESULTS

Construction and Verification of Mutated Receptors

In this project, a comparison analysis was performed on wild-type VDR and mutated VDR. Prior to executing such analysis, the mutant receptors were generated by introducing specific mutations in the cDNA encoding the VDR. The mutations for the three plasmids were detected by using the chain terminator method of DNA sequencing. One plasmid contained a mutation at the 305 position of the vitamin D receptor, changing the CAC code for histidine to the GCC code for alanine, while the other contained a mutation at the 397 position changing the CAC code for histidine to the TTC code for phenylalanine, and the third mutant contained both the 305 and 397 changes (see Appendix B for complete sequence of the three mutants). Figure 8 illustrates the confirmation of the mutation at the 305 position of the vitamin D receptor. The mutation at the 397 position was also confirmed (data not shown).

Figure 8. An autoradiograph of a sequencing gel. Picture depicts the histidine to alanine mutation at the 305 position of the cDNA encoding the vitamin D receptor. A plasmid pcDNA-305 was purified from a bacterial culture using the Plasmid Maxi Prep (QIAGEN) and sequenced via the chain-terminator method. Sequence is read from bottom to top. The GCC codon on the right of autoradiograph depicts desired mutation at the 305 position.

Binding Assays

The wild-type and mutant receptors were compared based on their affinity of binding $1,25(\text{OH})_2\text{D}_3$. At least three binding assay trials were done for each mutant. The raw data, shown in disintegrations per minute (dpm), and a graph for each experiment is shown in Appendixes C and D, respectively. For each experiment, three dpm values

were obtained for total binding and two dpm values were obtained for nonspecific binding. Total binding refers to tubes that contained only the radioactive [^3H]-1,25(OH) $_2$ D $_3$, while nonspecific binding refers to the tubes containing both radioactive [^3H]-1,25(OH) $_2$ D $_3$ and 100-fold molar excess of non-radioactive 1,25(OH) $_2$ D $_3$. The average of the total binding dpm values minus the average of the nonspecific dpm values was used to determine specific binding. Dpm values were converted to units of femtomoles, which represent the quantity of receptor bound to 1,25(OH) $_2$ D $_3$, by using the following general equation:

$$\text{DPM counts} / (2.22 \times 10^{12} \text{ DPM/ Ci}) = \text{Ci of } [^3\text{H}]\text{-1,25(OH)}_2\text{D}_3$$

Ci [^3H]-1,25(OH) $_2$ D $_3$ is then divided by the specific activity of [^3H]-1,25(OH) $_2$ D $_3$, expressed in Ci/mmol, to give mmols of [^3H]-1,25(OH) $_2$ D $_3$.

The femtomoles of receptor bound were graphed as a function of [^3H]-1,25(OH) $_2$ D $_3$ concentrations using nonlinear regression (GraphPad Prism software, version 3.2). The one-site binding dissociation constant (K_D) and Bmax value were calculated. The K_D value, expressed in units of moles/liter, refers to the concentration of ligand which occupies half of the receptors at equilibrium. The equilibrium equation for the dissociation of the vitamin D receptor and hormone is shown below.

$$[\text{VDR}] \cdot [1,25\text{D}_3] \cdot k_{\text{on}} = [\text{VDR}-1,25\text{D}_3] \cdot k_{\text{off}}$$

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[\text{VDR}] [1,25\text{D}_3]}{[\text{VDR}-1,25\text{D}_3]}$$

The Bmax value represents the total number of receptors present. The K_D values obtained in each experiment for each receptor were averaged and are summarized in Table 3.

Table 3. Binding Assay Results

VDR construct	Saturation Analysis K_D (nM)
Wild-type	0.67 ± 0.18 (n=3)*
H305A	1.49 ± 0.66 (n=6)
H397F	1.03 ± 0.06 (n=3)
H305A/H397F	1.92 ± 0.74 (n=5)

*n is the number of replicates

A saturation binding curve representing one experiment for each receptor with their respective K_D values is shown in Figure 9.

Figure 9. Saturation binding curves for wild-type VDR and mutants. Each curve represents one binding experiment. Wild-type or mutant VDR was transfected into Cos-1 cells using DEAE-dextran. After 48-72 hours, the transfected cells were harvest with trypsin and lysed using a syringe. Increasing concentrations of 1,25-dihydro[26,27-methyl- ^3H]-vitamin D_3 was added in the presence or absence of 100nM excess of unlabeled $1,25(\text{OH})_2\text{D}_3$. Receptor-ligand complex was separated from unbound ligand using hydroxyapatite. Specific binding data was determined based on the average of non-specific binding subtracted from the average of total binding.

Transcriptional Activation Assays

The wild-type and mutant receptors were also compared by determining the concentration of $1,25(\text{OH})_2\text{D}_3$ required for each to induce a half-maximal response of secreted alkaline phosphatase. When $1,25(\text{OH})_2\text{D}_3$ is added to the transfected cells 24

hours post-transfection, the hormone-receptor complex undergoes a conformational change that attracts RXR (see Mode of Action). The complex, RXR-VDR- $1,25(\text{OH})_2\text{D}_3$, recognizes the VDRE sequence on the second plasmid containing the cDNA for secreted alkaline phosphatase and binds to it, thus inducing the expression of the secreted alkaline phosphatase. Since the secreted alkaline phosphatase lacks the membrane anchoring domain, it is efficiently secreted from the cells into the culture medium. Levels of secreted alkaline phosphatase in the culture medium are directly proportional to changes in intracellular concentrations of secreted alkaline phosphatase mRNA and represent transcriptional activity.

The dosage of $1,25(\text{OH})_2\text{D}_3$ required to induce production of secreted alkaline phosphatase was determined by measuring the light output of a chemiluminescent substrate. When CSPD® substrate is dephosphorylated by secreted alkaline phosphatase, it produces an unstable dioxetane anion which decomposes and emits light. The light output was measured and the data, in relative light units, for all experiments is shown in Appendix E. Trans-activation assays were done at least two times for each receptor.

Relative light units (RLU) were graphed as a function of the log of $1,25(\text{OH})_2\text{D}_3$ concentrations using nonlinear regression (GraphPad Prism software, version 3.2). From the graphs, an effective concentration (EC_{50}) value was obtained. The EC_{50} , expressed in moles/liter, is the concentration of $1,25(\text{OH})_2\text{D}_3$ needed to induce a half-maximal response. The EC_{50} values obtained from each experiment for each receptor were averaged and are summarized in Table 4.

Table 4. Transcriptional Activation Results.

VDR Construct	Transactivation Assay EC50 (nM)
Wild-type	2.77 ± 0.74 (n=2)
H305A	215 ± 160 (n=4)
H397F	139 ± 88 (n=3)
H305A/H397F	362 ± 330 (n=4)

A graph for each experiment is found in Appendix F. A dose-response curve representing one experiment for each receptor is shown in Figure 10.

Figure 10. Transcriptional activation curves for wild-type VDR and mutants. Each curve represents one trans-activation experiment. VDR wild-type or mutant plasmid cDNA was cotransfected into Cos-1 cells with a plasmid containing an alkaline phosphatase gene and VDRE

using DEAE-dextran. After 24 hours, cells were treated with increasing doses of $1,25(\text{OH})_2\text{D}_3$. Forty-eight hours after transfection, culture medium was collected and alkaline phosphatase activity was determined by chemiluminescence.

Statistical Analysis

T-tests were performed for both binding assays and transcriptional activation assays at a 95% confidence interval ($P < 0.05$) using nonparametric Dunnett's Multiple Comparison Test. The mutant receptor values were compared to the wild-type values in both cases. In saturation binding analysis, the H305A mutant and the H305A/H397F mutant received a P-value of less than 0.001, indicating that the K_D values obtained for each are significantly different compared to the wild-type. The H397F mutant was not significantly different than the wild-type (Table 5).

In the transcriptional activation experiments, all of the mutants were significantly different from the wild-type, although, there were no significant differences amongst the mutant receptors themselves. This is probably a result of their large standard deviations (Table 5).

Table 5. Summary of Results. * $p < 0.001$

VDR Construct	Saturation Binding K_D (nM)	Transactivation Assay EC_{50} (nM)
Wildtype	0.67 ± 0.18 (n=3)	2.77 ± 0.74 (n=2)
H305A	$1.49 \pm 0.66^*$ (n=6)	$215 \pm 160^*$ (n=4)
H397F	1.03 ± 0.06 (n=3)	$139 \pm 88^*$ (n=3)
H305A/H397F	$1.92 \pm 0.74^*$ (n=5)	$362 \pm 330^*$ (n=4)

DISCUSSION

In the present study, the importance of the histidine residues at the 305 position and at the 397 position were evaluated by comparison studies. Three mutant receptors were constructed: H305A, H397F, H305A/H397F. The mutant receptors were compared with the wild-type receptor based on their ability to bind $1,25(\text{OH})_2\text{D}_3$ and based on the dosage concentration of $1,25(\text{OH})_2\text{D}_3$ required for each to induce a half-maximal response of a reporter gene, secreted alkaline phosphatase. In the crystal structure (Rochel et al., 2000), the residues interact with the 25-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$, and there is a naturally occurring mutation at the 305 position replacing the histidine with a glutamine residue (Q) that causes vitamin D resistance rickets, type II (Malloy et al., 1997).

Saturation Binding Analysis

The literature K_D value for wild-type VDR bound to $1,25(\text{OH})_2\text{D}_3$ falls between $1\text{--}50 \times 10^{-10}$ M (Wecksler and Norman, 1980; Wecksler et al., 1980a; Wecksler et al., 1980b). The K_D value for wild-type VDR obtained in this study was 0.67nM and lies within the literature value range. The two single mutants, H305A and H397F obtained average K_D values of 1.49nM and 1.03nM, respectively. The double mutant, H305A/H397F, had an average K_D value of 1.92nM (Table 3). The binding studies revealed that the mutations at H305A and H397F did not result in large differences in affinity for $1,25(\text{OH})_2\text{D}_3$, although the K_D of the H305A mutant was statistically different than the wild-type receptor. To further support this data, the naturally occurring mutant,

H305Q, decreased binding to $1,25(\text{OH})_2\text{D}_3$ by approximately 8-fold less than the normal receptor (Malloy et al., 1997). Perhaps, the difference for observing a two-fold decrease in binding in the present study versus the eight-fold decrease from Malloy's study is due to the difference in size of the residues glutamine and alanine. The binding affinity of mutant H305A to $1,25(\text{OH})_2\text{D}_3$ was concurrently studied by the group in Riverside and the K_D value they found for the H305A mutant was 1.3nM (Bula et al., 2003). Although only one experiment value was obtained for this mutant, it supports the data in the present study.

In the present study, it is surprising to discover that the mutant at the 397 position was not significantly different than the wild-type receptor since the histidine-397 residue is shown to hydrogen bond with the 25-hydroxyl group of $1,25(\text{OH})_2\text{D}_3$ (Rochel et al., 2000). The Riverside group found a K_D value of 1.1nM for the H397F mutant, a value slightly higher than the value obtained in the present study ($K_D=1.03\text{nM}$) (Bula et al., 2003); however, it is not obvious if their value was significantly different than wild-type since they obtained only one K_D value for this mutant. In contrast with the binding data obtained in this study, Collins and Norman found that the H397F mutant decreased binding by five-fold compared with the wild-type receptor's affinity for $1,25(\text{OH})_2\text{D}_3$ (Collins and Norman, 2000). In addition, Yamamoto and co-workers had previously mutated the histidine residue at 397 to an alanine residue and found that the ability of the mutated receptor to bind to the natural ligand was completely abolished (Yamamoto et al., 2000). Their data indicated that H397 was essential for ligand binding. Perhaps the alanine residue may have disrupted folding of the receptor when bound to the ligand.

The data shown in this study, however, shows that the affinity of $1,25(\text{OH})_2\text{D}_3$ for H397 is not significantly different than its affinity for the wild-type. Perhaps, no difference in binding was determined because the histidine was replaced with a similar in size phenylalanine residue as opposed to a less bulky residue such as alanine, although hydrogen bonding was abolished.

In the present study, the data suggests that the removal of both histidines, as in the double mutant, decreases VDR binding to $1,25(\text{OH})_2\text{D}_3$ (Table 5). The removal of both histidines significantly alters binding compared to the removal of only one histidine. To further support the data obtained in the present study, Bula and coworkers found that for the double mutant, H305A/H397F, the K_D value was 1.7nM (Bula et al., 2003); a value similar to that found in the present study.

Ligand binding measures the affinity of the receptor to the ligand, but binding is not enough to permit the detection of any conformational changes in the receptor that may lead to trans-activation. Therefore, a second experiment was done to further characterize the receptor-ligand interaction.

Transcriptional Activation Analysis

The ability of the mutant receptors to activate transcription was evaluated in receptor-deficient Cos-1 cells. The EC_{50} for the three mutant VDRs are 215nM, 139nM, 362nM, for H305A, H397F, and H305A/H397F, respectively. The EC_{50} for wild-type VDR in this study was found to be 2.77nM, which lies within the standard deviation of a effective dosage concentration (ED_{50}) value previously obtained by Liu et al. of $3.0 \pm$

1.2nM (Liu et al., 1997) and matches the value obtained by Swann and coworkers ($EC_{50}=2nM$) (Swann et al., 2002). The three mutant receptors required approximately two to three orders of magnitude more $1,25(OH)_2D_3$ to induce the same response as that required for the wild-type receptor. The data represented in this study is supported by Choi and coworkers, who found that H397A abolished transcriptional activity and H305A decreased transcriptional activity by 25% (Choi et al., 2001). Likewise, Yamamoto and coworkers determined that H397A completely abolished transcriptional activity using Cos-7 cells (Yamamoto et al., 2000). Collins and Norman found that the H397F mutant was able to activate gene transcription at slightly higher doses than the wild-type VDR required (Collins and Norman, 2000). In addition, Bula and coworkers found that H397F required approximately 7-fold more of $1,25(OH)_2D_3$ to induce a transcriptional response (Bula et al., 2003). The discrepancies found between the Yamamoto group and the Choi group versus the Bula group and Collins and Norman's work is probably due to the residues that replace the histidine. The Bula group as well as Collins and Norman maintained the size of the histidine residue with the phenylalanine residue at the 397 position, while the other two groups mutated a bulky residue histidine to a less bulky alanine residue possibly changing the conformation of the ligand binding pocket and altering protein folding. It is possible that altering protein folding can reveal or cover co-activator sites so as to up-regulate or inhibit transcriptional activity, respectively. In connection with the H305A mutant in this study, which required an approximate 80-fold higher dose of $1,25(OH)_2D_3$, Bula and coworkers found that the H305A mutant significantly required an approximate 18-fold greater dose of

1,25(OH)₂D₃ than that required for the wild-type receptor (Bula et al., 2003). Bula and coworkers also mutated the histidine residue at the 305 position to a phenylalanine residue and found that H305F required an EC₅₀ of 3.2nM in order to induce the same response (Bula et al., 2003). According to their data, this value was not significantly different than the value required for the wild-type receptor (Bula et al., 2003), thereby illustrating that the size of the replaced residues does matter. In addition, the naturally occurring mutant H305Q required a 5-10 fold higher concentration of 1,25(OH)₂D₃ to achieve the same effect observed in the cells transfected with the normal gene (Malloy et al., 1997). Malloy and coworkers described that this mutation is unique in that it marks the first time that a patient with HVDRR has shown clinical improvement when treated with high doses of 1,25(OH)₂D₃ (Malloy et al., 1997). On the other hand, Gardezi and coworkers found that the naturally occurring mutant H305Q required an 80-fold higher concentration of 1,25(OH)₂D₃ to induce a transcriptional response (Gardezi et al., 2001). Their data correlates with the data found in this study.

As for the double mutant, the value obtained in the present study was supported by the value obtained by the Riverside group (31±10nM) in that both mutants required a larger dose of 1,25(OH)₂D₃ to induce a transcriptional response. Additionally, the Riverside group synthesized a second double mutant replacing both histidine residues with phenylalanine residues, H305F/H397F, and determined that the H305F/H397F mutant required a dosage concentration of 1.6±0.13nM (Bula et al., 2003) to induce the same response. Their data corresponds to a 19-fold difference in the requirement for

1,25(OH)₂D₃ between the two double mutants further validating the theory regarding the importance of conserving the size of the replaced residues.

The data in this study suggests that both H305 and H397 directly affect trans-activation. Mutation of these histidine residues results in a decrease of transcriptional activation, and transcriptional reduction may be circumvented by administering higher doses of 1,25(OH)₂D₃. However, in a clinical setting, therapeutic doses of 1,25(OH)₂D₃ are not ideal due to the hypercalcemic properties of the natural hormone (Bouillon et al., 1995). Therefore, analogs of 1,25(OH)₂D₃ that possess reduced calcemic side effects, yet maintain the anti-proliferative and pro-differentiative effects of the natural ligand have been developed and studied.

Gardezi and coworkers studied an epi analog of the natural hormone, 20-epi-1,25(OH)₂D₃ (Gardezi et al., 2001), in patients with HVDRR. Their work was of relevance to the present study because one of their selected three HVDRR mutants was the histidine 305 to glutamine (H305Q) mutant, in which they studied the binding affinity and the transcriptional activity between the analog and both the wild-type and mutant receptor. They found that 20-epi-1,25(OH)₂D₃ had the same binding affinity for both the wild-type receptor and the mutant receptor and that the potency of 20-epi-1,25(OH)₂D₃ to induce transcription through the H305Q mutant was decreased only 4-fold compared with the wild-type VDR (Gardezi et al., 2001).

Although no analogs were used in the present study, the work done by Gardezi and coworkers provides insight to the H305A mutant and its contact point at the 25-hydroxyl group of the natural hormone. The Gardezi group found that although histidine

305 is in contact with the 25-hydroxyl group in the x-ray crystal structure, it is not essential for the high affinity binding of $1,25(\text{OH})_2\text{D}_3$ (Gardezi et al., 2001). They proposed that in the H305Q mutant, the natural hormone may use an alternative contact point that fails to stabilize a functional conformation of the protein, whereas the analog does so more successfully.

In the present study, the natural hormone bound with the H305A mutant may have disrupted the conformation of the protein in such a way that resulted in lower binding affinity and decreased trans-activation. Likewise, the natural hormone bound to the H397F mutant failed to stabilize the conformation to a lesser extent than that of the H305A mutant because binding affinity for the H397F was insignificant, but enough to alter trans-activation function. Replacing the residues in both mutants may have comprised the functional interface of the proteins resulting in decreased transcription. To determine any conformational changes in the mutant receptors, further experiments will need to be performed.

The experiments done in the present study are important because they focus on the mechanism of action of $1,25(\text{OH})_2\text{D}_3$ with respect to VDR binding and activation. Through these experiments, the understanding of the interaction between $1,25(\text{OH})_2\text{D}_3$ and amino acid contact points of VDR were further enhanced. Such steps are critical towards the development of potential analogs for therapeutic use in treating osteoporosis, cancer, autoimmune diseases and psoriasis. Over a thousand analogs have been developed including those with increased cell differentiating activity and increased intestinal calcium absorption and bone calcium mobilization: $1,25-(\text{OH})_2-20\text{-epi-D}_3$,

1,25(OH)₂-20-epi-24-homo-D₃, 1,25(OH)₂-20-epi-24a,26a,27a-trihomo-D₃, 20-epi-22-oxa-24-homo-1,25(OH)₂D₃, 1,25(OH)₂-20-epi-22-oxa-24a, 26a, 27a-trihomo-D₃ (Bouillon et al., 1995). One analog, 1,24(S)-(OH)₂-22-ene-26,27-cyclopropyl-D₃ (Leo Pharmaceuticals) is already in clinical use for the local treatment of psoriasis (Binderup and Kragballe, 1992).

CONCLUSION

In the present study, the interactions of the 25-hydroxyl group of 1,25(OH)₂D₃ and two histidine residues in the VDR were analyzed. Mutations of VDR were successfully constructed using site-directed mutagenesis in order to determine the importance of the histidine residues. The binding affinities of the wild-type VDR and the VDR mutants were compared. The single mutants H305A and H397F had a small decrease in binding affinity, whereas the double mutant H305A/H397F decreased binding affinity by approximately three times the amount of the wild-type K_D value. Also, transcriptional activation assays were utilized to determine the amount of the hormone necessary to induce an enzymatic response. All mutants required an effective dosage concentration several orders of magnitude greater of 1,25(OH)₂D₃ than that required for wild-type. The transcriptional activity of the H305A single mutant and H305A/H397F double mutant was in parallel with their binding affinity for 1,25(OH)₂D₃. The data obtained for the mutants showed that replacing the histidine residues had a greater effect in gene transcription than on binding.

APPENDIXES

Appendix A. Amino Acid Sequence of the Human Vitamin D Receptor

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1  MEAMAASTSL PDPGDFDRNV PRICGVCGR ATGFHFNAMT CEGCKGFFRR SMKRKALFTC
61  PFNGDCRITK DNRRHCQACR LKRCVDIGMM KEFILTDEEV QRKREMILKR KEEEALKDSL
121 RPKLSEEQQR IIAILLDAH KTYDPTYSD CQFRPPVRVN DGGGSHPSRP NSRHTPSFSG
181 DSSSSCSDHC ITSSDMMDS SFSNLDLSEE DSDDPSVTLE LSQLSMLPHL ADLVSYSIQK
241 VIGFAKMIPG FRDLTSEDQI VLLKSSAIEV IMLRSNESFT MDDMSWTCGN QDYKYRVSDV
301 TKAGHSLELI EPLIKFQVGL KKLNLHEEEH VLLMAICIVS PDRPGVQDAA LIEAIQDRLS
361 NTLQTYIRCR HPPPGSHLLY AKMIQKLADL RSLNEEHSKQ YRCLSFQPEC SMKLTPLVLE
421 VFGNEIS

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one letter symbol for the amino acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic Acid	P	Proline
E	Glutamic Acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

Appendix B. Nucleotide Sequence for the Human Vitamin D Receptor

(From the NCBI website; accession # NM_000376)

Wild-type

61 acctcacaga agagcacccc tgggctccac ttacctgccc cctgctcctt cagggatgga
121 ggcaatggcg gccagcactt ccttgctga cctggagac ttgacgga acgtgccccg
181 gatctgtggg gtgtgtggag accgagccac tggctttcac ttcaatgcta tgacctgtga
241 aggctgcaaa ggcttcttca ggcaagcat gaagcggaag gcactattca cctgcccctt
301 caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccgggtcaa
361 acgctgtgtg gacatcggca tgatgaagga gttcattctg acagatgagg aagtgcagag
421 gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
481 caagctgtct gaggagcagc agcgcatcat tgccatactg ctggacgccc accataagac
541 ctacgacccc acctactccg acttctgcca gttccggcct ccagttcgtg tgaatgatgg
601 tggaggggagc catccttcca ggcccaactc cagacacact ccagcttct ctggggactc
661 ctctctctcc tgctcagatc actgtatcac ctcttcagac atgatggact cgtccagctt
721 ctccaatctg gatctgagtg aagaagattc agatgaccct tctgtgaccc tagagctgtc
781 ccagctctcc atgctgcccc acctggctga cctggtcagt tacagcatcc aaaaggctcat
841 tggctttgct aagatgatac caggattcag agacctcacc tctgaggacc agatcgtact
901 gctgaagtca agtgccattg aggtcatcat gttgcgctcc aatgagtcct tcaccatgga
961 cgacatgtcc tggacctgtg gcaaccaaga ctacaagtac cgcgtcagtg acgtgaccaa
1021 agccggacac agcctggagc tgattgagcc cctcatcaag ttccagggtg gactgaagaa
1081 gctgaacttg catgaggagg agcatgtcct gctcatggcc atctgcatcg tctcccaga
1141 tcgtcctggg gtgcaggagc ccgcgctgat tgaggccatc caggaccgcc tgtccaacac
1201 actgcagacg tacatccgct gccgccaccc gccccgggc agccacctgc tctatgccaa
1261 gatgatccag aagctagccg acctgcgcag cctcaatgag gagcactcca agcagtaccg
1321 ctgcctctcc ttccagcctg agtgcagcat gaagctaacg ccccttggtg tcgaagtgtt
1381 tggcaatgag atctcctgac taggacagcc tgtgcggtgc ctgggtgggg ctgctctctc

* bold nucleotides designate start and stop codons

H305A

61 acctcacaga agagcacccc tgggctccac ttacctgccc cctgctcctt caggg**atgga**
121 ggcaatggcg gccagcactt ccttgccctga ccctggagac ttgaccgga acgtgccccg
181 gatctgtggg gtgtgtggag accgagccac tggctttcac ttcaatgcta tgacctgtga
241 aggctgcaaa ggcttcttca ggcgaagcat gaagcggaag gcactattca cctgccccctt
301 caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa
361 acgctgtgtg gacatcggca tgatgaagga gttcattctg acagatgagg aagtgcagag
421 gaagcgggag atgatcctga agcgggaagga ggaggaggcc ttgaaggaca gtctgcggcc
481 caagctgtct gaggagcagc agcgcacatc tgccatactg ctggacgccc accataagac
541 ctacgacccc acctactccg acttctgcca gttccggcct ccagttcgtg tgaatgatgg
601 tggaggggag catccttcca ggcccaactc cagacacact ccagcttct ctggggactc
661 ctctctctcc tgctcagatc actgtatcac ctcttcagac atgatggact cgtccagctt
721 ctccaatctg gatctgagtg aagaagattc agatgaccct tctgtgacce tagagctgtc
781 ccagctctcc atgctgcccc acctggctga cctggtcagt tacagcatcc aaaaggtcat
841 tggctttgct aagatgatac caggattcag agacctcacc tctgaggacc agatcgtact
901 gctgaagtca agtgccattg aggtcatcat gttgcgctcc aatgagtcct tcaccatgga
961 cgacatgtcc tggacctgtg gcaaccaaga ctacaagtac cgcgtcagtg acgtgaccaa
1021 agccggagcc agcctggagc tgattgagcc cctcatcaag ttccaggtgg gactgaagaa
1081 gctgaacttg catgaggagg agcatgtcct gctcatggcc atctgcatcg tctccccaga
1141 tcgtctctgg gtgcaggacg ccgcgctgat tgaggccatc caggaccgcc tgtccaacac
1201 actgcagacg tacatccgct gccgccaccc gccccgggc agccacctgc tctatgccaa
1261 gatgatccag aagctagccg acctgcgcag cctcaatgag gagcactcca agcagtaccg
1321 ctgcctctcc ttccagctg agtgcagcat gaagctaacy ccccttgtgc tcgaagtgtt
1381 tggcaatgag atctcct**gac** taggacagcc tgtgcggtgc ctgggtgggg ctgctctccc

* purple codons translate to desired alanine mutation

H397F

61 acctcacaga agagcacccc tgggctccac ttacctgccc cctgctcctt cagggatgga
121 ggcaatggcg gccagcactt cctgacctga ccctggagac tttgaccgga acgtgccccg
181 gatctgtggg gtgtgtggag accgagccac tggctttcac ttcaatgcta tgacctgtga
241 aggctgcaaa ggcttcttca ggcgaaagcat gaagcggaag gcactattca cctgccccct
301 caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa
361 acgctgtgtg gacatcgga tgatgaagga gttcattctg acagatgagg aagtgcagag
421 gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
481 caagctgtct gaggagcagc agcgcatcat tgccatactg ctggacgccc accataagac
541 ctacgacccc acctactccg acttctgcca gttccggcct ccagttcgtg tgaatgatgg
601 tggagggagc catccttcca ggcccaactc cagacacact ccagcttct ctggggactc
661 ctctcctcc tgetcagatc actgtatcac ctcttcagac atgatggact cgtccagctt
721 ctccaatctg gatctgagtg aagaagattc agatgaccct tctgtgacct tagagctgtc
781 ccagctctcc atgctgcccc acctggctga cctggctcagt tacagcatcc aaaaggtcat
841 tggctttgct aagatgatac caggattcag agacctcacc tctgaggacc agatcgtact
901 gctgaagtca agtgccattg aggtcatcat gttgcgctcc aatgagtcct tcaccatgga
961 cgacatgtcc tggacctgtg gcaaccaaga ctacaagtac cgcgtcagtg acgtgaccaa
1021 agccggacac agcctggagc tgattgagcc cctcatcaag ttccagggtg gactgaagaa
1081 gctgaacttg catgaggagg agcatgtcct gctcatggcc atctgcatcg tctccccaga
1141 tcgtcctggg gtgcaggacg ccgcgctgat tgaggccatc caggaccgcc tgtccaacac
1201 actgcagacg tacatccgct gccgccaccc gccccgggc agccacctgc tctatgccaa
1261 gatgatccag aagctagccg acctgcgcag cctcaatgag gagttctcca agcagtaccg
1321 ctgcctctcc ttccagcctg agtgcagcat gaagctaacg ccccttgtgc tcgaagtgtt
1381 tggcaatgag atctcctgac taggacagcc tgtgcggtgc ctgggtgggg ctgctcctcc

* purple codons translate to desired phenylalanine mutation

H305A/H397F

61 acctcacaga agagcacccc tgggctccac ttacctgccc cctgctcctt cagggatgga
121 ggcaatggcg gccagcactt cctgacctga cctgggagac ttgaccgga acgtgccccg
181 gatctgtggg gtgtgtggag accgagccac tggctttcac ttcaatgcta tgacctgtga
241 aggctgcaaa ggctttctca ggcgagcat gaagcggaag gcactattca cctgccccct
301 caacggggac tgccgcatca ccaaggacaa ccgacgccac tgccaggcct gccggctcaa
361 acgctgtgtg gacatcggca tgatgaagga gttcattctg acagatgagg aagtgcagag
421 gaagcgggag atgatcctga agcggaagga ggaggaggcc ttgaaggaca gtctgcggcc
481 caagctgtct gaggagcagc agcgcatcat tgccatactg ctggacgccc accataagac
541 ctacgacccc acctactccg acttctgcca gttccggcct ccagttcgtg tgaatgatgg
601 tggagggagc catccttcca ggcccaactc cagacacact cccagcttct ctggggactc
661 ctctctctcc tgctcagatc actgtatcac ctcttcagac atgatggact cgtccagctt
721 ctccaatctg gatctgagtg aagaagattc agatgaccct tctgtgaccc tagagctgtc
781 ccagctctcc atgctgcccc acctggctga cctgggtcagt tacagcatcc aaaaggatcat
841 tggctttgct aagatgatac caggattcag agacctcacc tctgaggacc agatcgtact
901 gctgaagtca agtgccattg aggtcatcat gttgcgctcc aatgagtctt tcaccatgga
961 cgacatgtcc tggacctgtg gcaaccaaga ctacaagtac cgcgtcagtg acgtgaccaa
1021 agccggagoc agcctggagc tgattgagcc cctcatcaag ttccagggtg gactgaagaa
1081 gctgaacttg catgaggagg agcatgtctt gctcatggcc atctgcatcg tctccccaga
1141 tcttcttggg gtgcaggacg ccgcgtgat tgaggccatc caggaccgcc tgtccaacac
1201 actgcagacg tacatccgct gccgccaccc gccccgggc agccacctgc tctatgccaa
1261 gatgatccag aagctagccg acctgcgcag cctcaatgag gagttctcca agcagtaccg
1321 ctgcctctcc ttccagcctg agtgcagcat gaagctaacg ccccttgtgc tgaagtgtt
1381 tggcaatgag atctcctgac taggacagcc tgtgcggtgc ctgggtgggg ctgctcctcc

Appendix C. Binding Assay – Raw Data

Disintegrations per Minute of Total, Nonspecific, and Specific Binding

nanoMolar concentrations of 1,25(OH)₂D₃

Wild-type

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.1	237.51	189.29	260.48	229.093	39.55	54.25	46.9	182.19
0.2	220.9	231.42	210.26	220.860	37.98	57.17	47.575	173.29
0.5	628.74	678.96	722.78	676.827	120.52	90.65	105.585	571.24
1	657.83	578.61	893.39	709.943	133.5	125.43	129.465	580.48
2	726.79	908.74	1257.48	964.337	48.01	28.51	38.26	926.08
4	1313.88	1070.98	2066.29	1483.717	547.07	425.36	486.215	997.50

H305A (Trial 1)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	117.76	214.71	184.92	172.463	58.43	46.58	52.505	119.96
0.25	126.93	196.12	223.61	182.220	81.93	127.86	104.895	77.33
0.49	286.28	231.55	299.73	272.520	126.45	78	102.225	170.30
1.23	1011.5	548.86	911.17	823.843	138.39	231.57	184.98	638.86
2.46	759.56	757.89	822.98	780.143	547.02	328.18	437.6	342.54
4.91	1214.43	1170.74	1108.96	1164.710	334.76	453.99	394.375	770.34

H305A (Trial 2)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0.000	0	0	0	0.00
0.12	189.92	176.05	165.49	177.153	90.1	60.51	75.305	101.85
0.25	141.66	403.22	334.51	293.130	295.4	122.59	208.995	84.14
0.49	565.07	574.51	543.31	560.963	157.43	237.05	197.24	363.72
1.23	1128.8	974.4	913.82	1005.673	236.82	436.5	336.66	669.01
2.46	1397.1	1464.46	1827.99	1563.183	363.48	628.35	495.915	1067.27
4.91	1869.91	1966.89	2371.25	2069.350	1303.26	882.98	1093.12	976.23

H305A (Trial 3)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	102.13	65.18	66.44	77.917	42.93	30.22	36.575	41.34
0.24	131.29	144.13	88.29	121.237	67.2	84.79	75.995	45.24
0.58	291.64	296.38	303.08	297.033	147.14	250.95	199.045	97.99
1.2	398.52	362.44	370.47	377.143	196.88	260.58	228.73	148.41
2.3	601.93	614.33	568.64	594.967	447.1	397.13	422.115	172.85
4.6	1165.19	1158.23	828.63	1050.683	970.79	519.49	745.14	305.54

H305A (Trial 4)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	129.97	103.74	117.58	117.097	36.61	45.6	41.105	75.99
0.24	142.5	225.57	106.04	158.037	46.33	69.56	57.945	100.09
0.48	117.26	181.99	174.62	157.957	57.56	58.05	57.805	100.15
1.19	383.97	464.96	506.84	451.923	173.77	154.19	163.98	287.94
2.38	711.54	684.52	673.41	689.823	224.13	293.59	258.86	430.96
4.77	1048.27	1019.29	998.02	1021.860	631.93	423.8	527.865	494.00

H305A (Trial 5)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	187.39	234.95	208.73	210.357	46.28	31.23	38.755	171.60
0.24	319.42	319.72	295.15	311.430	48.21	48.11	48.16	263.27
0.47	477.28	583.06	602.34	554.227	67.96	64.34	66.15	488.08
1.18	947.51	967.83	996.95	970.763	171.93	152	161.965	808.80
2.36	826.73	976.63	1209.44	1004.267	260.4	235.37	247.885	756.38
4.73	1463.42	1445.17	2332.2	1746.930	486.77	568.79	527.78	1219.15

H305A (Trial 6)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.25	226.41	308.98	271.29	268.893	35.53	60.5	48.015	220.88
0.5	368.86	288.11	424.48	360.483	57.04	60.93	58.985	301.50
1	579	637.68	646.38	621.020	196.7	107.5	152.1	468.92
4	942.38	1045.09	1191.43	1059.633	302.57	336.45	319.51	740.12

H397F (Trial 1)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	178.81	129.46	100.24	136.170	56.57	41.26	48.915	87.26
0.25	279.11	207.95	228.63	238.563	73.01	52.67	62.84	175.72
0.49	384.32	394.64	397.87	392.277	71.46	85.66	78.56	313.72
1.23	707.31	709.25	545.43	653.997	182.45	161.71	172.08	481.92
2.46	1105.35	986.1	988.63	1026.693	222.54	216.34	219.44	807.25
4.91	1199.12	1183.45	1275.81	1219.460	465.42	473.94	469.68	749.78

H397F (Trial 2)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	152.36	123.36	139.26	138.327	21.01	49.25	35.13	103.20
0.24	181.11	201.76	227.57	203.480	56.39	40.46	48.425	155.06
0.47	441.86	460.55	520.1	474.170	53.63	78.35	65.99	408.18
2.36	917.08	1036.56	1210.23	1054.623	242.31	209.03	225.67	828.95
4.73	1256.98	1393.23	1503.87	1384.693	414.34	450.9	432.62	952.07

H397F (Trial 3)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	137.94	164.96	163.15	155.350	36.26	29.11	32.685	122.67
0.24	278.37	273.5	230.68	260.850	51.67	46.37	49.02	211.83
0.47	544.06	511.68	497.74	517.827	63.81	63.09	63.45	454.38
1.18	876.18	841.99	882.33	866.833	131.35	167.28	149.315	717.52
2.36	977.77	1040.87	1054.29	1024.310	213.66	218.89	216.275	808.04
4.73	1384.99	1599.73	1331.98	1438.900	404.63	336.65	370.64	1068.26

H305A/H397F (Trial 1)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.12	211.55	165.18	189.58	188.770	165.78	50.06	107.92	80.85
0.25	360.24	375.93	335.53	357.233	66.81	71.69	69.25	287.98
0.49	558.35	564.88	500.43	541.220	77.37	89.78	83.575	457.65
1.23	942.18	950.97	1103.19	998.780	191.12	171.27	181.195	817.59
2.46	1621.75	1289.18	1278.84	1396.590	463.16	296.72	379.94	1016.65
4.91	1791.3	1771.61	2296.54	1953.150	597.77	383.49	490.63	1462.52

H305A/H397F (Trial 2)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0.000	0	0	0	0.00
0.12	211.056	214.75	160.2	195.335	117.42	104.16	110.79	84.55
0.24	302.59	354.2	198.84	285.210	182.48	158.56	170.52	114.69
0.48	531.62	420.43	415.22	455.757	183.09	146.42	164.755	291.00
1.19	578.03	682.88	644.94	635.283	286.02	297.29	291.655	343.63
2.38	985.24	1424.2	966.39	1125.277	367.92	442.87	405.395	719.88
4.77	1526.79	1644.77	1357.12	1509.560	586.9	611.77	599.335	910.23

H305A/H397F (Trial 3)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0.000	0	0	0	0.00
0.12	100.71	65.04	116.72	94.157	96.82	64.38	80.6	13.56
0.24	281.48	226.46	298.82	268.920	79.87	95.45	87.66	181.26
0.48	314.12	471.4	478.84	421.453	233.51	334.2	283.855	137.60
1.19	898.77	1341.92	800.26	1013.650	257.64	267.86	262.75	750.90
2.38	1454.82	1397.44	1795.03	1549.097	701.78	681.11	691.445	857.65
4.77	1849.36	3060.95	2420.58	2443.630	1052.12	1366.55	1209.335	1234.30

H305A/H397F (Trial 4)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0	0	0	0	0
0.25	262.84	260.43	271.76	265.010	61.19	45.72	53.455	211.56
0.5	383.96	471.08	394.16	416.400	81.58	63.16	72.37	344.03
1.0	638.78	735.84	721.24	698.620	165.73	154.39	160.06	538.56
2.0	891.07	848.56	896.24	878.623	191.68	229.48	210.58	668.04
4.0	1285.96	1235.99	1274.22	1265.390	332.19	346.67	339.43	925.96

H305A/H397F (Trial 5)

nM	Total	Total	Total	Avg Total	Nonspec.	Nonspec.	Avg Nons	Specific
0	0	0	0	0.000	0	0	0	0.00
0.125	115.85	115.79	143.53	125.057	38.03	35.02	36.525	88.53
0.25	212.02	196.75	212.95	207.240	53.9	47.98	50.94	156.30
0.5	320.16	329.37	283.3	310.943	84.85	64.87	74.86	236.08
1.25	740.04	673.37	741.24	718.217	156.12	142.84	149.48	568.74
2.5	956.65	1022.35	975.75	984.917	274.09	273.39	273.74	711.18
5.0	1233.49	1189.29	1270.4	1231.060	469.26	425.38	447.32	783.74

Appendix D. Binding Assay- Curves

Wildtype

H305A (Trial 1)

H305A (Trial 2)

H305A (Trial 3)

H305A (Trial 4)

H305A (Trial 5)

H305A (Trial 6)

H397F (Trial 1)

H397F (Trial 2)

H397F (Trial 3)

H305A/H397F (Trial 1)

H305A/H397F (Trial 2)

H305A/H397F (Trial 3)

H305A/H397F (Trial 4)

H305A/H397F (Trial 5)

Appendix E. Transcriptional Activation Assay- Raw Data

Data is expressed in Relative Light Units (RLU)

Molar concentrations of 1,25(OH)₂D₃

Wildtype

(Trial 1)

Log(M)	RLU	RLU	RLU
-10	67.13	76.68	90.21
-9	170	126.4	153.8
-8	366.2	212.7	269.8
-7	352	281.8	374.2
-6	294.5	390.5	352.4
-5	296.3	360	335.6

(Trial 2)

Log(M)	RLU	RLU	RLU
-10	31.8433	27.57	30.61
-9	107.4833	74.45	44.7
-8	196.2	124.2	232.1
-7	219.7	218.4	182.4
-6	268.2333	285.1	204

H305A

(Trial 1)

Log(M)	RLU	RLU	RLU
-10	57.85	49.02	49.75
-9	48.85	55.075	56.82
-8	82.98	73.24	99.035
-7	92.21	109.1	113.8
-6	125.2	134	94.07
-5	139.1	137.1	121.9

(Trial 2)

Log(M)	RLU	RLU	RLU
-10	45.39	31.5	31.17
-9	60.5	39.8	36.7
-8	52.25	71.44	35.46
-7	123.8	132.6	65.9
-6	191.4	161.3	188
-5	252.85	136.2	223.1

Log(M)	RLU	RLU	RLU
-10	15.94	15.07	15.6
-9	13.96	13.4	14.52
-8	13.82	13.77	13.92
-7	14.48	14.46	15.67
-6	17.9	15.51	20.29
-5	19.165	19.65	18.66

(Trial 3)

Log(M)	RLU	RLU	RLU
-10	17.87	18.67	16.29
-9	14.84	15.33	21.16
-8	24.55	23.07	39.73
-7	32.05	79.71	59.38
-6	85.88	95.87	91.78
-5	100.2	99.97	100.27

(Trial 4)

H397F

(Trial 1)

Log(M)	RLU	RLU	RLU
-10	34	41.75	32.22
-9	55.04	37.83	44.49
-8	65.96	69.81	49.01
-7	82.91	107.3	86.91
-6	126.7	134.7	138.6
-5	154.1	147.9	148.96

Log(M)	RLU	RLU	RLU
-10	18.32	18.41	21.02
-9	17.83	16.81	19.78
-8	20.62	22.74	21
-7	43.21	45.37	44.8
-6	49.21	52.8	51.6
-5	64.37	63.2	64.8

(Trial 3)

(Trial 2)

Log(M)	RLU	RLU	RLU
-10	12.13	11.55	13.56
-9	13.66	14.37	13.65
-8	13.88	13.97	13.79
-7	15.09	15.68	14.9
-6	19.965	18.9	18.9
-5	18.92	22.631	19.2

H305A/H397F

(Trial 1)

Log(M)	RLU	RLU	RLU
-10	22.67	20.55	20.2325
-9	19.52	20.3	20.095
-8	23.62	35.97	25.05
-7	48.94	71.92	57.967
-6	100.81	86.18	93.3
-5	163.7	67.97	97.56

Log(M)	RLU	RLU	RLU
-10	59.83	81.9	64.82
-9	57.19	61.84	64.37
-8	76	91.773	71.3
-7	132.1	133.5	159.6
-6	222	208.8	140
-5	171	205.1	186.9

(Trial 3)

(Trial 2)

Log(M)	RLU	RLU	RLU
-10	11.99	12.23	13.83
-9	13.34	14.37	14.3
-8	15.36	13.91	14.51
-7	17.19	20.16	16.88
-6	30.989	31.69	31.694

Log(M)	RLU	RLU	RLU
-10	46.18	36.48	38.15
-9	39.83	41.63	41.81
-8	40.14	59.04	37.36
-7	72.19	42.87	56.09
-6	181.515	142.5	124.7
-5	243	179.7	228.05

(Trial 4)

Appendix F. Transcriptional Activation Assay- Curves

Wildtype (Trial 1)

Wildtype (Trial 2)

H305A (Trial 1)

H305A (Trial 2)

H305A (Trial 3)

H305A (Trial 4)

H397F (Trial 1)

H397F (Trial 2)

H397F (Trial 3)

H305A/H397F (Trial 1)

H305A/H397F (Trial 2)

H305A/H397F (Trial 3)

H305A/H397F (Trial 4)

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