

## Chapter 3 – VITAMIN D: PRODUCTION, METABOLISM AND MECHANISMS OF ACTION

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### ABSTRACT

Rickets became a public health problem with the movement of the population from the farms to the cities during the Industrial Revolution. Various foods such as cod liver oil and irradiation of other foods including plants were found to prevent or cure this disease, leading eventually to the discovery of the active principle—vitamin D. Vitamin D comes in two forms ( $D_2$  and  $D_3$ ) which differ chemically in their side chains. These structural differences alter their binding to the carrier protein vitamin D binding protein (DBP) and their metabolism, but in general the biologic activity of their active metabolites is comparable. For complete coverage of this and related areas in Endocrinology, visit our free web-books, [www.endotext.org](http://www.endotext.org) and [www.thyroidmanager.org](http://www.thyroidmanager.org).

### INTRODUCTION

Vitamin  $D_3$  is produced in the skin from 7-dehydrocholesterol by UV irradiation, which breaks the B ring to form pre- $D_3$ . Pre- $D_3$  isomerizes to  $D_3$  or with continued UV irradiation to tachysterol and lumisterol.  $D_3$  is preferentially removed from the skin, bound to DBP. The liver and other tissues metabolize vitamin D, whether from the skin or oral ingestion, to 25OHD, the principal circulating form of vitamin D. Several enzymes have 25-hydroxylase activity, but CYP2R1 is the most important. 25OHD is then further metabolized to  $1,25(OH)_2D$  principally in the kidney, by the enzyme CYP27B1, although other tissues including various epithelial cells, cells of the immune system, and the parathyroid gland contain this enzymatic activity.  $1,25(OH)_2D$  is the principal hormonal form of vitamin D, responsible for most of its biologic actions. The production of  $1,25(OH)_2D$  in the kidney is tightly controlled, being stimulated by parathyroid hormone, and inhibited by calcium, phosphate and FGF23. Extrarenal production of  $1,25(OH)_2D$  as in keratinocytes and macrophages is under different control, being stimulated primarily by cytokines such as tumor necrosis factor alfa ( $TNF\alpha$ ) and interferon gamma ( $IFN\gamma$ ).  $1,25(OH)_2D$  reduces  $1,25(OH)_2D$  levels in cells primarily by stimulating its catabolism through the induction of CYP24A1, the 24-hydroxylase. 25OHD and  $1,25(OH)_2D$  are hydroxylated in the 24 position by this enzyme to form  $24,25(OH)_2D$  and  $1,24,25(OH)_3D$ , respectively. This 24-hydroxylation is generally the first step in the catabolism of these active metabolites to the final end product of calcitroic acid, although  $24,25(OH)_2D$  and  $1,24,25(OH)_3D$  have their own biologic activities. CYP24A1 also has 23-hydroxylase activity that leads to a different end product. Different species differ in their ratio of 23-hydroxylase/24-hydroxylase activity in their CYP24A1 enzyme, but in humans the 24-hydroxylase activity predominates. CYP24A1 is induced by  $1,25(OH)_2D$ , which serves as an important feedback mechanism to avoid vitamin D toxicity. In macrophages,

CYP24A1 is either missing or defective, so in situations such as granulomatous diseases like sarcoidosis in which macrophage production of  $1,25(\text{OH})_2\text{D}$  is increased, hypercalcemia and hypercalciuria due to elevated  $1,25(\text{OH})_2\text{D}$  can occur.

The vitamin D metabolites are transported in blood bound to DBP and albumin. Very little circulates as the free form. The liver produces DBP and albumin, production that is decreased in liver disease, and these proteins may be lost in protein losing enteropathies or the nephrotic syndrome. Thus individuals with liver, intestinal or renal diseases which result in low levels of these transport proteins may have low total levels of the vitamin D metabolites without necessarily being vitamin D deficient as their free concentrations may be normal.

The receptor for  $1,25(\text{OH})_2\text{D}$  (VDR) is a transcription factor regulating the expression of genes which mediate its biologic activity. VDR is a member of a rather large family of nuclear hormone receptors which includes the receptors for glucocorticoids, mineralocorticoids, sex hormones, thyroid hormone, and vitamin A metabolites or retinoids. The VDR is widely distributed, and is not restricted to those tissues considered the classic target tissues of vitamin D. The VDR upon binding to  $1,25(\text{OH})_2\text{D}$  heterodimerizes with other nuclear hormone receptors, in particular the family of retinoid X receptors. This complex then binds to special DNA sequences called vitamin D response elements (VDRE) generally within the genes it regulates. There are thousands of the VDREs in hundreds of genes, and the profile of active VDREs (and regulated genes) varies from cell to cell. A variety of additional proteins called coregulators complex with the VDR to activate (coactivators) or inhibit (corepressors) VDR transcriptional activity. The coactivators may form a bridge from the VDR binding to the VDRE to the proteins such as RNA polymerase II at the transcription start site or help unravel the chromatin at the site of the gene via recruitment of histone acetyl transferases (HAT) and demethylases (DME), allowing transcription to proceed. The vitamin D receptor Interacting Protein complex (DRIP, also known as Mediator) is an example of the first type of coactivator complex, the Steroid Receptor Coactivator family (SRC 1-3) are examples of the latter type of coactivator. Different genes differ in their regulation by these coactivators. In addition to coactivators there are a number of corepressors. One recently discovered corepressor of VDR action in the skin is called hairless, in that its loss or mutation, like that of the VDR, leads to altered hair follicle cycling resulting in baldness. Corepressors typically work by recruiting histone deacetylases (HDAC) or methyl transferases (MT) to the gene which reverses the actions of HAT and DME, leading to a reduction in access to the gene by the transcription machinery. Like the coactivators, the corepressors can be specific for different genes, and in either case different cells differentially express these coregulators, providing some specificity for the actions of  $1,25(\text{OH})_2\text{D}$  and VDR.

In addition to regulating gene expression,  $1,25(\text{OH})_2\text{D}$  has a number of non genomic actions including the ability to stimulate calcium transport across the plasma membrane. The mechanisms mediating these non genomic actions and their physiologic significance remain unclear. Similarly, it is not clear that all actions of the VDR require the ligand  $1,25(\text{OH})_2\text{D}$ . The best example of this is the hair loss in animals and subjects with VDR mutations but not in animals and subjects with mutations in CYP27B1, the enzyme producing  $1,25(\text{OH})_2\text{D}$ . As mentioned, the VDR is widely distributed, and the actions of  $1,25(\text{OH})_2\text{D}$  are quite varied. The classic target tissues—bone, gut, and kidney—are involved with calcium homeostasis. The mechanisms by which  $1,25(\text{OH})_2\text{D}$  regulates transcellular calcium transport are best understood in the intestine. Here  $1,25(\text{OH})_2\text{D}$  stimulates calcium entry across the brush border membrane into the cell,

transport of calcium through the cell, and removal of calcium from the cell at the basolateral membrane. Calcium entry at the brush border membrane occurs down a steep electrochemical gradient. It is controlled in large measure by a specific calcium channel called TRPV6. Transport of calcium through the cell is regulated by a class of calcium binding proteins called calbindins. Much of the transport occurs within vesicles that form in the terminal web. Removal of calcium from the cell at the basolateral membrane requires energy and is mediated by the ATP requiring calcium pump or CaATPase.  $1,25(\text{OH})_2\text{D}$  induces TRPV6, the calbindins, and the CaATPase, but not all aspects of transcellular calcium transport are a function of new protein synthesis. Animals null for calbindin 9k (the major calbindin in mammalian intestine) have little impairment of intestinal calcium transport. Animals null for TRPV6, on the other hand, have a reduction in intestinal calcium transport, but the deficit is not profound. Thus it is likely that compensatory mechanisms for intestinal calcium transport exist that have yet to be discovered. Similar mechanisms mediate  $1,25(\text{OH})_2\text{D}$  regulated calcium reabsorption in the distal tubule of the kidney. The proteins involved are homologous but not identical. The situation in bone, however, is less clear. VDR are found in osteoblasts, the bone forming cells.  $1,25(\text{OH})_2\text{D}$  promotes the differentiation of osteoblasts and regulates the production of proteins such as collagen, alkaline phosphatase, and osteocalcin thought to be important in bone formation.  $1,25(\text{OH})_2\text{D}$  also induces RANKL, a membrane bound protein in osteoblasts that enables osteoblasts to stimulate the formation and activity of osteoclasts. Thus  $1,25(\text{OH})_2\text{D}$  regulates both bone formation and bone resorption. Some evidence suggests that the major effect of  $1,25(\text{OH})_2\text{D}$  on bone is to provide adequate levels of calcium and phosphate from the intestine. The rickets of patients with a mutated VDR or of mice in which the VDR has been deleted can be prevented/corrected by normalizing serum calcium and phosphate levels by dietary means. On the other hand normal bone formation is not restored, and with time the VDR null mice become osteoporotic despite the high calcium/phosphate diet. Whether subjects with VDR mutations also develop osteoporosis prematurely has not been reported.

The non classic actions of  $1,25(\text{OH})_2\text{D}$  include regulation of cellular proliferation and differentiation, regulation of hormone secretion, and regulation of immune function. The ability of  $1,25(\text{OH})_2\text{D}$  to inhibit proliferation and stimulate differentiation has led to the development of a number of analogs in the hopes of treating hyperproliferative disorders such as psoriasis and cancer without raising serum calcium. Psoriasis is now successfully treated with several vitamin D analogs. Observational studies are promising with respect to adequate vitamin D nutrition and cancer prevention, but definitive randomized controlled trials are lacking.  $1,25(\text{OH})_2\text{D}$  inhibits parathyroid hormone secretion and stimulates insulin secretion. A number of analogs and  $1,25(\text{OH})_2\text{D}$  itself are currently available for use in the treatment of secondary hyperparathyroidism accompanying renal failure. Epidemiologic evidence indicates that vitamin D deficiency is associated with increased risk of both type 1 and type 2 diabetes mellitus, but prospective clinical trials to demonstrate a role for vitamin D or  $1,25(\text{OH})_2\text{D}$  have not provided consistent results. The ability of  $1,25(\text{OH})_2\text{D}$  to regulate immune function is likely part of its efficacy in the treatment of psoriasis. A number of other autoimmune diseases have been found in animal studies to respond favorably to vitamin D and  $1,25(\text{OH})_2\text{D}$  or its analogs, and epidemiologic evidence linking vitamin D deficiency to increased incidence of these diseases has been reported. However, as yet no large prospective trials demonstrating efficacy have been reported.

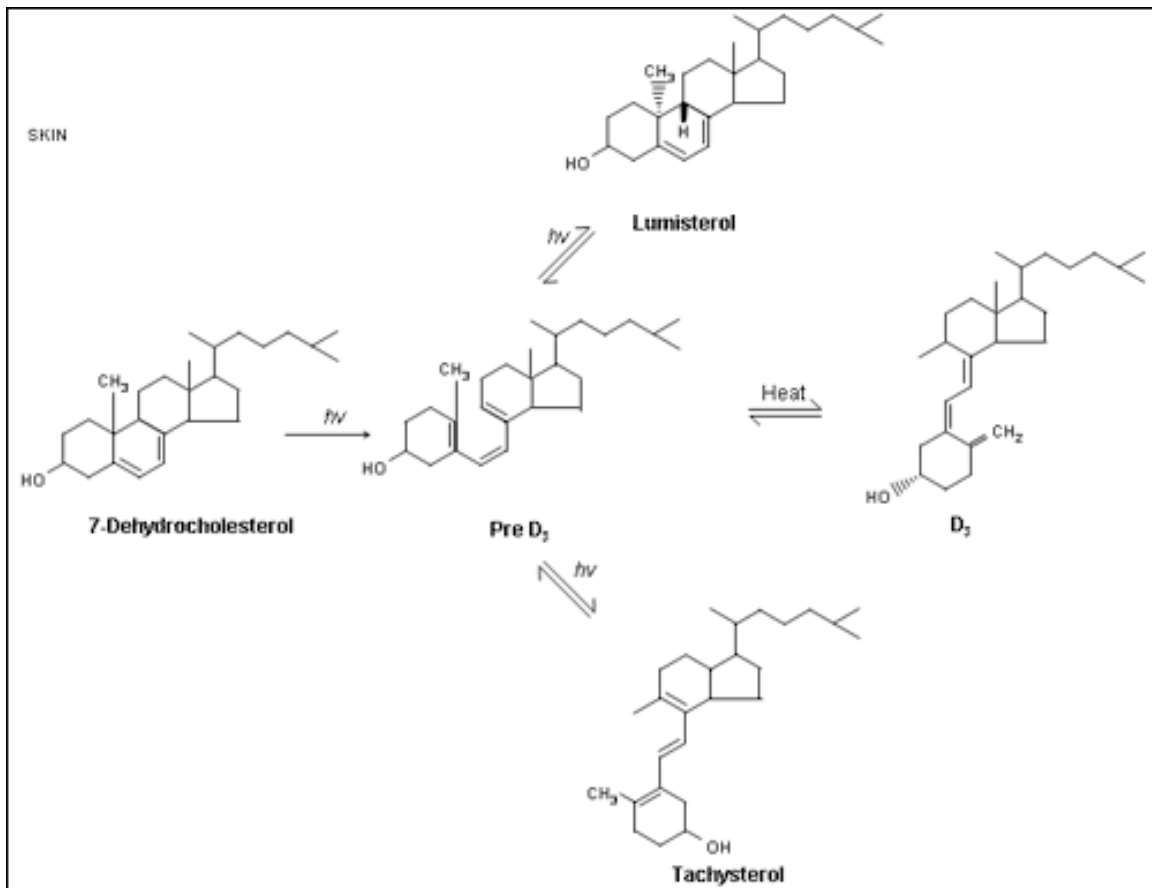
**Take home points:**

1. Vitamin D production in the skin under the influence of sunlight (UVB) is maximized at levels of sunlight exposure that do not burn the skin.
2. Further metabolism of vitamin D to its major circulating form (25(OH)D) and hormonal form (1,25(OH)<sub>2</sub>D) takes place in the liver and kidney, respectively, but also in other tissues where the 1,25(OH)<sub>2</sub>D produced serves a paracrine/autocrine function: examples include the skin, cells of the immune system, intestinal epithelium, prostate, and breast
3. Parathyroid hormone, FGF23, calcium and phosphate are the major regulators of the renal 1-hydroxylase; regulation of the extra renal 1-hydroxylase differs from that in the kidney and involves cytokines.
4. The major enzyme that catabolizes 25(OH)D and 1,25(OH)<sub>2</sub>D is the 24-hydroxylase; like the 1-hydroxylase it is tightly controlled in the kidney in a manner opposite to that of the 1-hydroxylase, but like the 1-hydroxylase it is widespread in other tissues where its regulation is different from that of the kidney.
5. Vitamin D and its metabolites are carried in the blood bound to vitamin D binding protein (DBP) and albumin--for most tissues it is the free (ie. unbound) metabolite that enters the cell; however, DBP bound metabolites can enter some cells such as the kidney and parathyroid gland through a megalin/cubilin mechanism.
6. Most but not all actions of 1,25(OH)<sub>2</sub>D are mediated by the vitamin D receptor (VDR). VDR is a transcription factor that partners with other transcription factors such as retinoid X receptor that when bound to 1,25(OH)<sub>2</sub>D regulates gene transcription either positively or negatively depending on other cofactors to which it binds.
7. The VDR is found in most cells, not just those involved with bone and mineral homeostasis (ie. bone, gut, kidney) resulting in wide spread actions of 1,25(OH)<sub>2</sub>D on most physiologic and pathologic processes.
8. Animal studies indicate that vitamin D has beneficial effects on various cancers, blood pressure, heart disease, immunologic disorders, but these non skeletal effects have been difficult to prove in humans.
9. Analogs of 1,25(OH)<sub>2</sub>D are being developed to achieve specificity for non skeletal

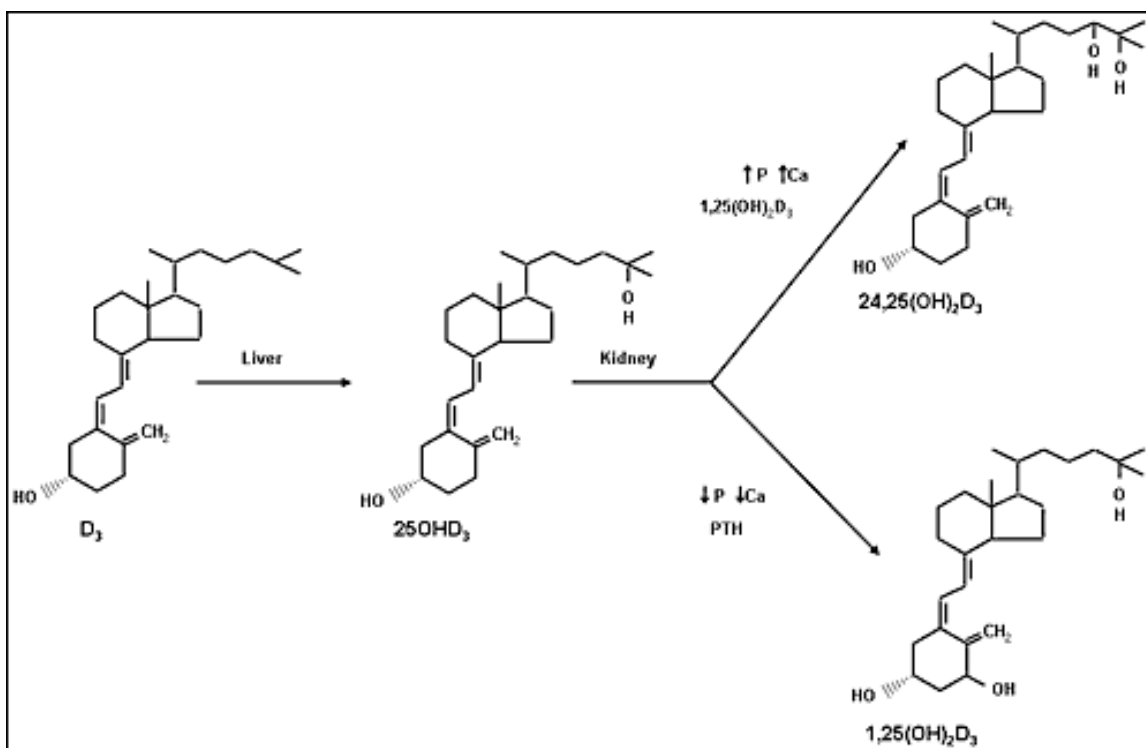
**DISCOVERY**

The first clear description of rickets was by Whistler (1) in 1645. However, it was not until the Industrial Revolution with the mass movement of the population from the farms to the smoke filled cities that rickets became a public health problem, most notably in England where sunlight intensity was already marginal for much of the year. Mellanby (2) in Great Britain and McCollum (3) in the United States developed animal models for rickets and showed that rickets could be cured with cod liver oil. McCollum heated the cod liver oil to destroy its vitamin A content and found that it still had antirachitic properties; he named the antirachitic factor vitamin D. Steenbock and Black (4) then demonstrated that UV irradiation of food, in particular non saponifiable lipids, could treat rickets. Meanwhile, clinical investigations revealed that rickets could be prevented or cured in children with sunlight or artificial UV exposure (5,6) suggesting that what subsequently became known as vitamin D could be produced by irradiation of precursors *in vivo*. Ultimately, Askew et al. (7) isolated and determined the structure of vitamin D<sub>2</sub> (ergocalciferol) from irradiated plant sterols (ergosterol), and Windaus et al. (8) determined the structures and pathway by which 7-dehydrocholesterol (7-DHC) in the skin is converted to vitamin D<sub>3</sub> (cholecalciferol). The name vitamin D<sub>1</sub> refers to what proved to be an error of an earlier identification, and is not used. The structures and pathways of production of vitamin D<sub>2</sub> and D<sub>3</sub> are shown in figure 1. The structures of vitamins D<sub>2</sub> and D<sub>3</sub> differ in the side chain where D<sub>2</sub> contains a double bond (C<sub>22-23</sub>)

and an additional methyl group attached to C<sub>24</sub>. In this chapter the designation of D will refer to both D<sub>3</sub> and D<sub>2</sub>.



of vitamin D<sub>3</sub> from 7-dehydrocholesterol in the epidermis. Sunlight (the ultraviolet B component) breaks the B ring of the cholesterol steroid, and a thermal induced rearrangement to form D<sub>3</sub>. Continued irradiation of pre- D<sub>3</sub> leads to the reversible formation of lumisterol<sub>3</sub> and tachysterol<sub>3</sub>.



**Figure 2.** The metabolism of vitamin D. The liver converts vitamin D to 25OHD. The kidney converts 25OHD to 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D. Other tissues contain these enzymes, but the liver is the main source for 25-hydroxylation, and the kidney is the main source for 1 $\alpha$ -hydroxylation. Control of metabolism of vitamin D to its active metabolite, 1,25(OH)<sub>2</sub>D, is exerted primarily at the renal level where calcium, phosphorus, parathyroid hormone, FGF23, and 1,25(OH)<sub>2</sub>D regulate the levels of 1,25(OH)<sub>2</sub>D produced.

## METABOLISM

Vitamin D<sub>3</sub> produced in the epidermis must be further metabolized to be active. The first step, 25-hydroxylation, takes place primarily in the liver, although other tissues have this enzymatic activity as well. As will be discussed below, there are several 25-hydroxylases. 25OHD is the major circulating form of vitamin D. However, in order for vitamin D metabolites to achieve maximum biologic activity they must be further hydroxylated in the 1 $\alpha$  position by the enzyme CYP27B1; 1,25(OH)<sub>2</sub>D is the most potent metabolite of vitamin D and accounts for most of its biologic actions. The 1 $\alpha$  hydroxylation occurs primarily in the kidney, although as for the 25-hydroxylase, other tissues have this enzyme. Vitamin D and its metabolites, 25OHD and 1,25(OH)<sub>2</sub>D, can also be hydroxylated in the 24 position. In the absence of 25-hydroxylation this may serve to activate the metabolite or analog as 1,25(OH)<sub>2</sub>D and 1,24(OH)<sub>2</sub>D have similar biologic potency. However, 24-hydroxylation of metabolites with an existing 25OH group reduces their activity and leads to further catabolism. The details of these reactions are described below.

## Cutaneous Production of Vitamin D<sub>3</sub>.

Although irradiation of 7-DHC was known to produce pre-D<sub>3</sub> (which subsequently undergoes a temperature rearrangement of the triene structure to form D<sub>3</sub>), lumisterol, and tachysterol (figure 1), the physiologic regulation of this pathway was not well understood until the studies of Holick and his colleagues (9-11). They demonstrated that the formation of pre-D<sub>3</sub> under the influence of solar or UV irradiation (maximal effective wavelength between 290-310) is relatively rapid and reaches a maximum within hours. UV irradiation further converts pre-D<sub>3</sub> to lumisterol and tachysterol. Both the degree of epidermal pigmentation and the intensity of exposure correlate with the time required to achieve this maximal concentration of pre-D<sub>3</sub>, but do not alter the maximal level achieved. Although pre-D<sub>3</sub> levels reach a maximum level, the biologically inactive lumisterol continues to accumulate with continued UV exposure. Tachysterol is also formed, but like pre-D<sub>3</sub>, does not accumulate with extended UV exposure. The formation of lumisterol is reversible and can be converted back to pre-D<sub>3</sub> as pre-D<sub>3</sub> levels fall. At 0°C, no D<sub>3</sub> is formed; however, at 37°C pre-D<sub>3</sub> is slowly converted to D<sub>3</sub>. Thus, short exposure to sunlight would be expected to lead to a prolonged production of D<sub>3</sub> in the exposed skin because of the slow thermal conversion of pre-D<sub>3</sub> to D<sub>3</sub> and the conversion of lumisterol to pre-D<sub>3</sub>. Prolonged exposure to sunlight would not produce toxic amounts of D<sub>3</sub> because of the photoconversion of pre-D<sub>3</sub> to lumisterol and tachysterol as well as the photoconversion of D<sub>3</sub> itself to suprasterols I and II and 5,6 transvitamin D<sub>3</sub> (12).

Melanin in the epidermis, by absorbing UV irradiation, can reduce the effectiveness of sunlight in producing D<sub>3</sub> in the skin. This may be one important reason for the lower 25OHD levels (a well documented surrogate measure for vitamin D levels in the body) in Blacks and Hispanics living in temperate latitudes (13). Sunlight exposure increases melanin production, and so provides another mechanism by which excess D<sub>3</sub> production can be prevented. The intensity of UV irradiation is also important for effective D<sub>3</sub> production. The seasonal variation of 25OHD levels can be quite pronounced with higher levels during the summer months and lower levels during the winter. The extent of this seasonal variation depends on the latitude, and thus the intensity of the sunlight striking the exposed skin. In Edmonton, Canada (52°N) very little D<sub>3</sub> is produced in exposed skin from mid-October to mid-April; Boston (42°N) has a somewhat longer period for effective D<sub>3</sub> production; whereas in Los Angeles (34°N) and San Juan (18°N) the skin is able to produce D<sub>3</sub> all year long (14). These findings apply to sea level. At higher elevations there is less atmospheric absorption of UVB, so that skiers can make vitamin D even in winter on sunny days. Peak D<sub>3</sub> production occurs around noon, with a larger portion of the day being capable of producing D<sub>3</sub> in the skin during the summer than other times of the year. Clothing (15) and sunscreens (16) effectively prevent D<sub>3</sub> production in the covered areas. This is one likely explanation for the observation that the Bedouins in the Middle East, who totally cover their bodies with clothing, are more prone to develop rickets and osteomalacia than the Israeli Jews with comparable sunlight exposure.

## Hepatic production of 25OHD.

The next step in the bioactivation of D<sub>2</sub> and D<sub>3</sub>, hydroxylation to 25OHD, takes place primarily in the liver although a number of other tissues express this enzymatic activity. 25OHD is the major circulating form of vitamin D and provides a clinically useful marker for vitamin D status. DeLuca and colleagues were the first to identify 25OHD and demonstrate its production in the liver over 30 years ago, but ambiguity remains as to



the actual enzyme(s) responsible for this activity. 25-hydroxylase activity has been found in both the liver mitochondria and endoplasmic reticulum, and the enzymatic activities appear to differ indicating different proteins. At this point most attention has been paid to the mitochondrial CYP27A1 and the microsomal CYP2R1. However, in mouse knockout studies and in humans with mutations in these enzymes, only CYP2R1 loss is associated with decreased 25OHD levels (17,18). However, deletion or mutation of CYP2R1 does not totally eliminate 25OHD production. These are mixed function oxidases, but differ in apparent  $K_m$ s and substrate specificities.

The mitochondrial 25-hydroxylase is now well accepted as CYP27A1, an enzyme first identified as catalyzing a critical step in the bile acid synthesis pathway. This is a high capacity, low affinity enzyme consistent with the observation that 25-hydroxylation is not generally rate limiting in vitamin D metabolism. Although initial studies suggested that the vitamin D<sub>3</sub>-25-hydroxylase and cholestane triol 27-hydroxylase activities in liver mitochondria were due to distinct enzymes with differential regulation, the cloning of CYP27A1 and the demonstration that it contained both activities has put this issue to rest (19-21). CYP27A1 is widely distributed throughout different tissues with highest levels in liver and muscle, but also in kidney, intestine, lung, skin, and bone (19-22). Mutations in CYP27A1 lead to cerebrotendinous xanthomatosis (23,24), and are associated with abnormal vitamin D and/or calcium metabolism in some but not all of these patients (24-26). However, mice in which CYP27A1 is deleted actually have elevated 25OHD levels along with the disruption in bile acid synthesis (27). CYP27A1 can hydroxylate vitamin D and related compounds at the 24, 25, and 27 positions. However, D<sub>2</sub> appears to be preferentially 24-hydroxylated, whereas D<sub>3</sub> is preferentially 25-hydroxylated (28). The 1 $\alpha$ OH derivatives of D are more rapidly hydroxylated than the parent compounds(30). These differences between D<sub>2</sub> and D<sub>3</sub> and their 1 $\alpha$ OH derivatives may explain the differences in biologic activity between D<sub>2</sub> and D<sub>3</sub> or between 1 $\alpha$ OHD<sub>2</sub> and 1 $\alpha$ OHD<sub>3</sub>.

The major microsomal 25-hydroxylase is CYP2R1, although other enzymes have been shown in vitro studies to have 25-hydroxylase activity. This enzyme like that of CYP27A1 is widely distributed, although it is most abundantly expressed in liver, skin and testes (29). Unlike CYP27A1, CYP2R1 25-hydroxylates D<sub>2</sub> and D<sub>3</sub> equally (29). Several Nigerian families have been shown to have CYP2R1 mutations in family members with rickets (18,30). These subjects respond to D therapy but suboptimally (18,30). Mice lacking CYP2R1 have reduced 25OHD levels, unlike mice lacking CYP27A1, but even the combined deletion of CYP2R1 and CYP27A1 does not reduce these levels more than about 70% (17). Thus neither CYP27A1 nor CYP2R1 by themselves account for all 25-hydroxylase activity in the body, suggesting a role of other yet to be described 25-hydroxylases.

Studies of the regulation of 25-hydroxylation have not been completely consistent, most likely because of the initial failure to appreciate that at least two enzymatic activities were involved and because of species differences. In general, 25-hydroxylation in the liver is little affected by vitamin D status. However, CYP27A1 expression in the intestine (31) and kidney (32) is reduced by 1,25(OH)<sub>2</sub>D. Not surprisingly bile acids decrease CYP27A1 expression (33) as does insulin (34) through an unknown mechanism. Dexamethasone, on the other hand, increases CYP27A1 expression (35). The regulation of CYP2R1 has been less well studied. Whether any of these manipulations alters 25-hydroxylase activity in the liver remains unknown. Estrogen given to male rats increases 25-hydroxylase activity, whereas testosterone given to female rats has the



opposite effect (36). However, evidence for such sex steroid hormone regulation of 25-hydroxylase activity in humans is lacking.

## Renal production of 1,25(OH)<sub>2</sub>D.

1,25(OH)<sub>2</sub>D is the most potent metabolite of vitamin D, and mediates most of its hormonal actions. 1,25(OH)<sub>2</sub>D is produced from 25OHD by the enzyme 25OHD-1 $\alpha$  hydroxylase (CYP27B1). The cloning of CYP27B1 by four independent groups (37-40) ended a long effort to determine the structure of this critical enzyme in vitamin D metabolism. Mutations in this gene are responsible for the rare autosomal disease of pseudovitamin D deficiency rickets (37,39,41,42). An animal model in which the gene is knocked out by homologous recombination reproduces the clinical features of this disease including retarded growth, rickets, hypocalcemia, hyperparathyroidism, and undetectable 1,25(OH)<sub>2</sub>D (43). Unlike VDR null mice and VDR mutations in humans, alopecia is not part of this phenotype.

CYP27B1 is a mitochondrial mixed function oxidase with significant homology to other mitochondrial steroid hydroxylases including CYP27A1 (39%), CYP24A1 (30%), CYP11A1 (32%), and CYP11 $\beta$  (33%) (37). However, within the heme-binding domain the homology is much greater with 73% and 65% sequence identity with CYP27A1 and CYP24A1 (37). These mitochondrial P450 enzymes are located in the inner membrane of the mitochondrion, and serve as the terminal acceptor for electrons transferred from NADPH through ferredoxin reductase and ferredoxin. Expression of CYP27B1 is highest in epidermal keratinocytes (37), cells that previously had been shown to contain high levels of this enzymatic activity (44). However, the kidney also expresses this enzyme in the renal tubules as do the brain, placenta, testes, intestine, lung, breast, macrophages, lymphocytes, parathyroid gland, osteoblasts and chondrocytes (37,45-48). That said, the kidney is generally considered the major source of circulating levels of 1,25(OH)<sub>2</sub>D, with the extrarenal CYP27B1 activities providing for local needs under normal circumstances. However, extrarenal sources can lead to increased 1,25(OH)<sub>2</sub>D and calcium levels in some pathologic conditions to be discussed subsequently.

The principal regulators of CYP27B1 activity in the kidney are parathyroid hormone (PTH), FGF23, calcium, phosphate, and 1,25(OH)<sub>2</sub>D. Extrarenal production tends to be stimulated by cytokines such as IFN- $\gamma$  and TNF- $\alpha$  more effectively than PTH (49) and may be less inhibited by calcium, phosphate, and 1,25(OH)<sub>2</sub>D depending on the tissue. Administration of PTH in vivo (50) or in vitro (51,52) stimulates renal production of 1,25(OH)<sub>2</sub>D. This action of PTH can be mimicked by cAMP (50,52) and forskolin (53,54) indicating that at least part of the effect of PTH is mediated via its activation of adenylate cyclase. However, PTH activation of protein kinase C (PKC) also appears to be involved in that concentrations of PTH sufficient to stimulate PKC activation and 1,25(OH)<sub>2</sub>D production are below that required to increase cAMP levels (55). Furthermore, synthetic fragments of PTH lacking the ability to activate adenylate cyclase but which stimulate PKC activity were found to increase 1,25(OH)<sub>2</sub>D production (56). Direct activation of PKC with phorbol esters results in increased 1,25(OH)<sub>2</sub>D production. Although the promoter of CYP27B1 contains several AP-1 (PKC activated) and cAMP response elements, it is not yet clear how PTH regulates CYP27B1 gene expression (57). However, several mechanisms have been proposed. In one study the nuclear receptor 4A2 acting through a C/EBP consensus element appears to be involved (58). Another mechanism involves VDIR that is proposed to bind to a negative VDRE in the CYP27B1 promoter. When PKA is activated by PTH VDIR is phosphorylated and recruits the p300

complex with HAT activity, inducing gene transcription (59). Calcium modulates the ability of PTH to increase  $1,25(\text{OH})_2\text{D}$  production. Calcium by itself can decrease CYP27B1 activity (60,61) and block the stimulation by PTH (62). Given in vivo, calcium can exert its effect in part by reducing PTH secretion, but this does not explain its direct actions in vitro or its effects in parathyroidectomized or PTH infused animals. Phosphate deprivation can stimulate CYP27B1 activity in vivo (63,64) and in vitro (65). The in vivo actions of phosphate deprivation can be blocked by hypophysectomy (66,67) and partially restored by growth hormone (GH) (67,68) and insulin-like growth factor (IGF-I) (69). However, like PTH, the exact mechanism by which GH and/or IGF-I mediates the effects of phosphate on CYP27B1 expression remains unclear. More recently FGF23 has been shown to inhibit CYP27B1 activity in vivo and in vitro (70). FGF23 has been implicated as at least one of the factors responsible for impaired phosphate reabsorption and  $1,25(\text{OH})_2\text{D}$  production in conditions such as X-linked and autosomal dominant hypophosphatemic rickets and oncogenic osteomalacia (71,72). FGF23 acts through FGF receptors 1 and 3 in conjunction with the coreceptor Klotho, but the mechanism by which FGF23 regulates CYP27B1 remains obscure. High phosphate stimulates FGF23 production from bone, and this is likely the major mechanism by which phosphate leads to decreased CYP27B1 activity (73).  $1,25(\text{OH})_2\text{D}$  administration leads to reduction in CYP27B1 activity. It was initially thought that this feedback inhibition was mediated at the level of gene expression. However, no vitamin D response element has been identified in the promoter of the  $1\alpha$ -hydroxylase gene (57). In keratinocytes,  $1,25(\text{OH})_2\text{D}$  has little or no effect on CYP27B1 mRNA and protein levels when given in vitro. When 24-hydroxylase activity is blocked,  $1,25(\text{OH})_2\text{D}$  administration fails to reduce the levels of  $1,25(\text{OH})_2\text{D}$  produced (74,75). Thus the apparent feedback regulation of CYP27B1 activity by  $1,25(\text{OH})_2\text{D}$  appears to be due to its stimulation of CYP24A1 and subsequent catabolism, not to a direct effect on CYP27B1 expression or activity. Moreover,  $1,25(\text{OH})_2\text{D}$  stimulates FGF23 production and inhibits PTH production. Both actions will decrease, indirectly, the ability of  $1,25(\text{OH})_2\text{D}$  to inhibit its own production (73). However, in one renal cell line a chromatin remodeling complex (WINAC) has been described that mediates the ability of the vitamin D receptor to regulate CYP27B1 gene expression in a non classic manner enabling  $1,25(\text{OH})_2\text{D}$  suppression of this gene (59) (76). In this mechanism  $1,25(\text{OH})_2\text{D}$  binds to VDR in turn stimulating VDR binding to VDR recruiting an HDAC corepressor complex as well as methyl transferases that block CYP27B1 transcription. Thus, renal and extrarenal regulation of CYP27B1 by  $1,25(\text{OH})_2\text{D}$  may differ.

## **Renal production of $24,25(\text{OH})_2\text{D}$ .**

The kidney is also the major producer of a second important metabolite of 25OHD, namely  $24,25(\text{OH})_2\text{D}$ , and the enzyme responsible is 25OHD-24 hydroxylase (CYP24A1) [75]. CYP24A1 and CYP27B1 are homologous enzymes that coexist in the mitochondria of tissues where both are found, such as the kidney tubule. However, they are located on different chromosomes (chromosome 20q13 and chromosome 12q14 for CYP24A1 and CYP27B1, respectively, in humans). They share the same ferredoxin and ferredoxin reductase components. While CYP27B1 activates the parent molecule, 25OHD, CYP24A1 initiates a series of catabolic steps that lead to its inactivation. However, in some tissues  $24,25(\text{OH})_2\text{D}$  has been shown to have biologic effects different from  $1,25(\text{OH})_2\text{D}$  as will be described subsequently. CYP24A1 24-hydroxylates both 25OHD and  $1,25(\text{OH})_2\text{D}$ . The 24-hydroxylation is then followed by oxidation of 24OH to a 24-keto group, 23-hydroxylation, cleavage between C23-24, and the eventual production of calcitroic acid, a metabolite with no biologic activity. CYP24A1 also has 23-

hydroxylase activity, initiating steps that lead to 23/26 lactone formation. Different species have CYP24A1s that differ in their preference for the 24-hydroxylation vs 23-hydroxylation pathway. Analogs with differences in their side chain are also likely to differ in the pathway utilized. CYP24A1 catalyzes all the steps in this catabolic pathway (77) (78). Although CYP24A1 is highly expressed in the kidney tubule, its tissue distribution is quite broad. In general, CYP24A1 can be found wherever the VDR is found. The affinity for 1,25(OH)<sub>2</sub>D is higher than that for 25OHD, making this enzyme an efficient means for eliminating 1,25(OH)<sub>2</sub>D. Thus, CYP24A1 is likely to play the important role of protecting the body against excess 1,25(OH)<sub>2</sub>D. Indeed, inactivating mutations in CYP24A1 have been found to underlie the disease idiopathic infantile hypercalcemia (79), manifesting as the name suggests with elevated serum calcium and 1,25(OH)<sub>2</sub>D levels. These individuals may present for the first time as adults, often in the context of increased 1,25(OH)<sub>2</sub>D production as in pregnancy (80). An animal model in which CYP24A1 has been knocked out likewise showed very high levels of 1,25(OH)<sub>2</sub>D when treated with vitamin D and impaired mineralization of intramembranous bone (81). The skeletal abnormalities could be corrected by crossing this mouse to one lacking the VDR suggesting that excess 1,25(OH)<sub>2</sub>D (which acts through the VDR) rather than deficient 24,25(OH)<sub>2</sub>D (which does not) is to blame (81).

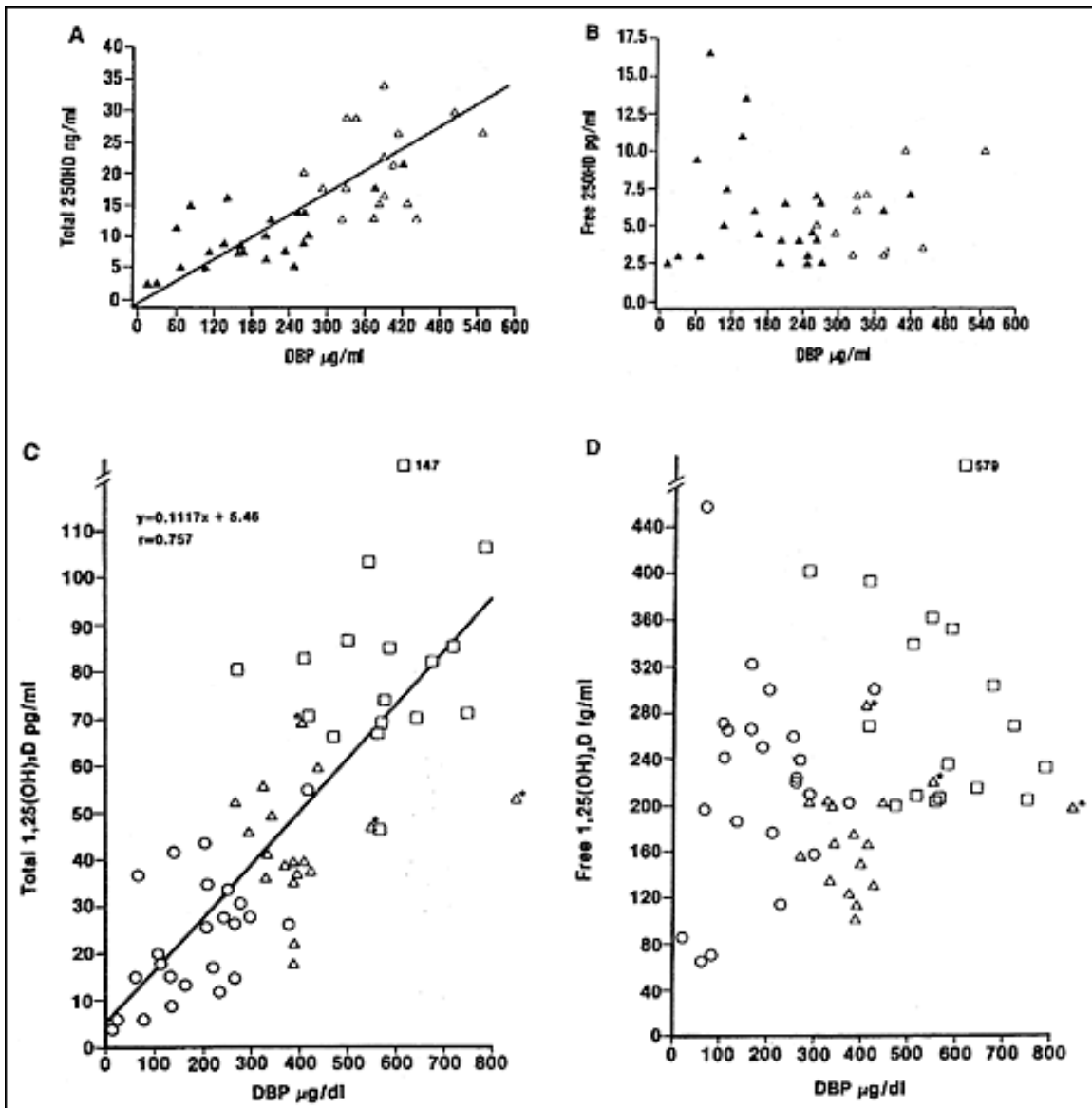
The regulation of CYP24A1 in the kidney is almost the mirror image of that of CYP27B1. PTH and 1,25(OH)<sub>2</sub>D are the dominant regulators, but calcium, phosphate, insulin, FGF23, IGF-I, GH, and sex steroids may also play a role. 1,25(OH)<sub>2</sub>D induces CYP24A1. The promoter of CYP24A1 has two vitamin D response elements (VDREs) critical for this induction (82-84). Protein kinase C activation as by phorbol esters enhances this induction by 1,25(OH)<sub>2</sub>D (85). An AP-1 site is found adjacent to the proximal VDRE, but mutation of this site does not appear to block phorbol ester enhancement of CYP24A1 induction by 1,25(OH)<sub>2</sub>D (86). PTH, on the other hand, inhibits the expression of CYP24A1 in the kidney (87). This action can be reproduced with cAMP (88) and forskolin (53) indicating the role of PTH activated adenylate cyclase (89). PTH has no effect on intestinal CYP24A1, most likely because the intestine does not have PTH receptors. Surprisingly, however, PTH is synergistic with 1,25(OH)<sub>2</sub>D in stimulating CYP24A1 expression and activity in bone cells which do have PTH receptors, again through a cAMP mediated mechanism (90). This synergism is further potentiated by the addition of insulin (91). The region(s) of the CYP24A1 promoter mediating these disparate actions of PTH has not been identified so the mechanisms responsible remain unclear. FGF23 also induces CYP24A1 expression (92). Surprisingly this requires the VDR (92), since FGF23 also inhibits 1,25(OH)<sub>2</sub>D production and so would be expected to reduce CYP24A1 via a 1,25(OH)<sub>2</sub>D/VDR mechanism. Restriction in dietary phosphate reduces CYP24A1 expression consistent with a decrease in FGF23, but also in a manner blocked by hypophysectomy (93). GH and IGF-I can reduce CYP24A1 expression in hypophysectomized animals, suggesting that the phosphate effect on CYP24A1 like its opposing effect on CYP27B1, is mediated by GH and IGF-I (93) as well as FGF23. Thus, although different regulators tend to have opposite effects on CYP24A1 and CYP27B1, the molecular mechanisms by which the regulation occurs also differ for each enzyme.

## TRANSPORT IN BLOOD

The vitamin D metabolites are transported in blood bound primarily to vitamin D binding protein (DBP) (85-88%) and albumin (12-15%) (94-96). DBP concentrations are normally 4-8μM, well above the concentrations of the vitamin D metabolites, such that DBP is only

about 2% saturated. DBP has high affinity for the vitamin D metabolites ( $K_a=5 \times 10^8 \text{M}^{-1}$  for 25OHD and  $24,25(\text{OH})_2\text{D}$ ,  $4 \times 10^7 \text{M}^{-1}$  for  $1,25(\text{OH})_2\text{D}$  and vitamin D), such that under normal circumstances only approximately 0.03% 25OHD and  $24,25(\text{OH})_2\text{D}$  and 0.4%  $1,25(\text{OH})_2\text{D}$  are free (95-97). Conditions such as liver disease and nephrotic syndrome resulting in reduced DBP and albumin levels will lead to a reduction in total 25OHD and  $1,25(\text{OH})_2\text{D}$  levels without necessarily affecting the free concentrations (98) (figure 3). Similarly DBP levels are reduced during acute illness, potentially obscuring the interpretation of total 25OHD levels (99). Earlier studies with a monoclonal antibody to measure DBP levels suggested a decreased level in African Americans consistent with their lower total 25OHD levels, but these results were not confirmed using polyvalent antibody based assays (100). Vitamin D intoxication can increase the degree of saturation sufficiently to increase the free concentrations of  $1,25(\text{OH})_2\text{D}$  and so cause hypercalcemia without necessarily raising the total concentrations (101).

The vitamin D metabolites bound to DBP are in general not available to most cells. Thus the free or unbound concentration is that which is critical for cellular uptake. Therefore, there is currently a debate as to whether the free concentration of 25OHD, for example, is a better indicator of vitamin D nutritional status than total 25OHD, given that DBP levels, and hence total 25OHD levels, can be influenced by liver disease, nephrotic syndrome, pregnancy, and inflammatory states (102,103). However, certain tissues such as the kidney, placenta, and parathyroid gland express the megalin/cubilin complex which is able to transport vitamin D metabolites bound to DBP into the cell. This is critical for preventing renal losses of the vitamin metabolites (104) and may be important for vitamin D metabolite transport into the fetus and regulation of PTH secretion.



**Figure 3.** Correlation of total 25OHD (A) and 1,25(OH)<sub>2</sub>D (C) levels to DBP; lack of correlation of free 25OHD (B) and 1,25(OH)<sub>2</sub>D (D) levels to DBP. Data from normal subjects (open triangles), subjects with liver disease (closed triangles, open circles), subjects on oral contraceptives (open triangles\*), and pregnant women (open squares) are included. These data demonstrate the dependence of total 25OHD and 1,25(OH)<sub>2</sub>D concentrations on DBP levels which are reduced by liver disease. However, the free concentrations of 25OHD and 1,25(OH)<sub>2</sub>D are normal in most patients with liver disease. Reprinted with permission from the American Society for Clinical Investigation.

DBP was originally known as group specific component (Gc-globulin) before its properties as a vitamin D transport protein became known. It has three common polymorphisms which are useful in population genetics. These alleles have somewhat different affinities for the vitamin D metabolites (105), but which do not appear to alter its function. DBP is a 58kDa protein with 458 amino acids that is homologous to albumin and  $\alpha$ -fetoprotein ( $\alpha$ FP) (40% homology at the nucleotide level, 23% at the amino acid level) (106). These three genes cluster on chromosome 4q11-13 (107). DBP like albumin and  $\alpha$ FP is made primarily but not exclusively in the liver-other sites include the



kidney, testes, and fat. DBP like other steroid hormone binding proteins is increased by oral (not transdermal) estrogens and pregnancy (95). In vitro, glucocorticoids and cytokines such as EGF, IL-6 and TGF- $\beta$  have been shown to increase (glucocorticoids, EGF, IL-6) or decrease (TGF- $\beta$ ) DBP production (108).

Although transport of the vitamin D metabolites may be the major function for DBP, it has other properties. DBP has high affinity for actin, and may serve as a scavenger for actin released into the blood during cell death (109). DBP has also been shown to activate macrophages (110) and osteoclasts (111). However, in a mouse rendered deficient in DBP by homologous recombination (knock out) no obvious abnormality was observed except for increased turnover in vitamin D and increased susceptibility to osteomalacia on a vitamin D deficient diet (112). Evidence for osteopetrosis (indicating failure of osteoclast function) was not found.

## **MECHANISM OF ACTION**

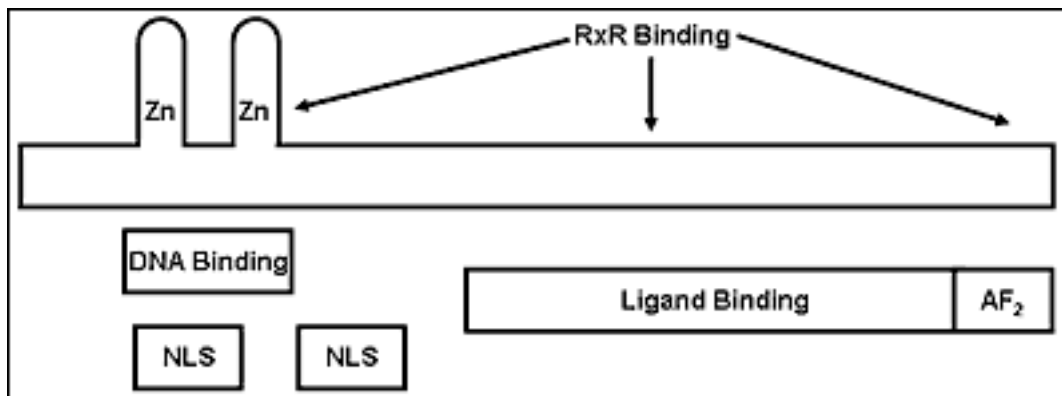
The hormonal form of vitamin D, 1,25(OH)<sub>2</sub>D, is the ligand for a transcription factor, the vitamin D receptor (VDR). Most if not all effects of 1,25(OH)<sub>2</sub>D are mediated by VDR acting primarily by regulating the expression of genes whose promoters contain specific DNA sequences known as vitamin D response elements (VDREs). There are thousands of VDREs throughout the genome, often thousands of base pairs away from the coding portion of the gene regulated. However, some actions of 1,25(OH)<sub>2</sub>D are more immediate, and may be mediated by a membrane bound vitamin D receptor that has been less well characterized than the nuclear VDR or by the VDR acting outside of the nucleus. On the other hand some actions of VDR do not require its ligand 1,25(OH)<sub>2</sub>D. Our understanding of the mechanism by which VDR regulates gene expression has increased enormously over the past few years.

## **VDR and Transcriptional Regulation.**

The VDR was discovered in 1969 (113) (although only as a binding protein for an as yet unknown vitamin D metabolite subsequently identified as 1,25(OH)<sub>2</sub>D), and was eventually cloned and sequenced in 1987 (114,115). Inactivating mutations in the VDR result in hereditary vitamin D resistant rickets (HVDRR) (116). Animal models in which the VDR has been knocked out (117) (118) have the full phenotype of severe vitamin D deficiency indicating that the VDR is the major mediator of vitamin D action. The one major difference is the alopecia seen in HVDRR and VDR knockout animals, a feature not associated with vitamin D deficiency, suggesting that the VDR may have functions independent of 1,25(OH)<sub>2</sub>D at least in hair follicle cycling. The VDR is a member of a large family of proteins (over 150 members) that includes the receptors for the steroid hormones, thyroid hormone, vitamin A family of metabolites (retinoids), and a variety of cholesterol metabolites, bile acids, isoprenoids, fatty acids and eicosanoids. A large number of family members have no known ligands, and are called orphan receptors. VDR is widely, although not universally, distributed throughout the different tissues of the body (119). Many of these tissues were not originally considered target tissues for 1,25(OH)<sub>2</sub>D. The discovery of VDR in these tissues along with the demonstration that 1,25(OH)<sub>2</sub>D altered function of these tissues has markedly increased our appreciation of the protean effects of 1,25(OH)<sub>2</sub>D.

The VDR is a molecule of approximately 50-60kDa depending on species. The basic structure is shown in figure 4. The VDR is unusual in that it has a very short N-terminal

domain before the DNA binding domain when compared to other nuclear hormone receptors. The human VDR has two potential start sites. A common polymorphism (Fok 1) alters the first ATG start site to ACG. Individuals with this polymorphism begin translation three codons downstream such that in these individuals the VDR is three amino acids shorter (424 aas vs 427 aas). This polymorphism has been correlated with reduced bone density suggesting it is of functional importance (120). The most conserved domain in VDR from different species and among the nuclear hormone receptors in general is the DNA binding domain. This domain is comprised of two zinc fingers. The name derives from the cysteines within this stretch of amino acids that form tetrahedral complexes with zinc in a manner which creates a loop or finger of amino acids with the zinc complex at its base. The proximal (N-terminal) zinc finger confers specificity for DNA binding to the VDREs while the second zinc finger and the region following provide at least one of the sites for heterodimerization of the VDR to the retinoid X receptor (RXR). The second half of the molecule is the ligand binding domain, the region responsible for binding 1,25(OH)<sub>2</sub>D, but which also contains regions necessary for heterodimerization to RXR. At the C-terminal end is the major activation domain, AF-2, which is critical for the binding to coactivators such as those in the steroid receptor coactivator (SRC) and vitamin D receptor interacting protein (DRIP) or Mediator families (121). In mutation studies of the homologous thyroid receptor corepressors were found to bind in overlapping regions with coactivators in helices 3 and 5, a region blocked by helix 12 (the terminal portion of the AF2 domain) in the presence of ligand (122). Deletion of helix 12 promoted corepressor binding while preventing that of coactivators (122).

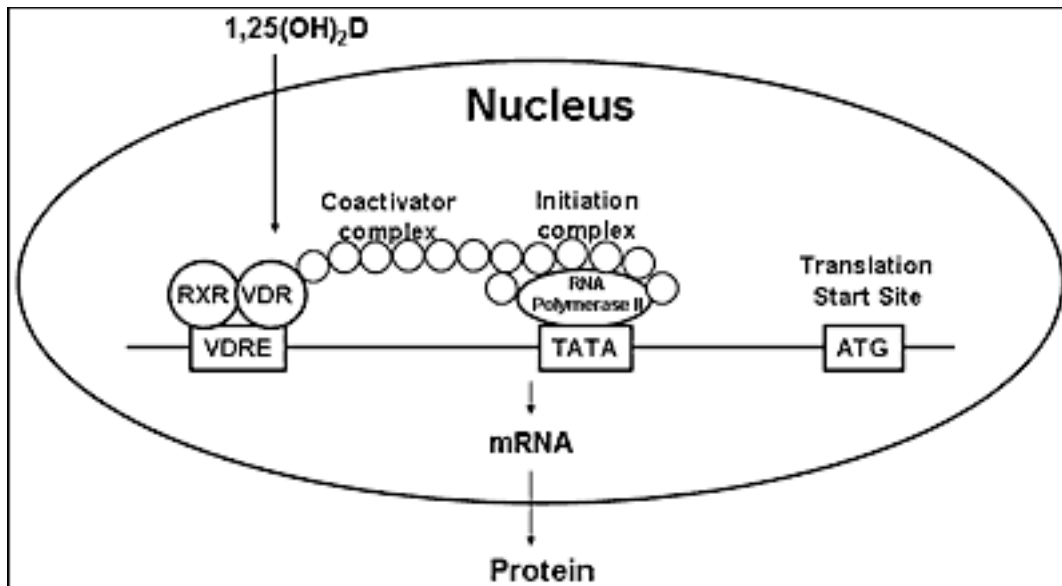


**Figure 4.** Model of the vitamin D receptor (VDR). The N terminal region is short relative to other steroid hormone receptors. This region is followed by two zinc fingers which constitute the principal DNA binding domain. Nuclear localization signals (NLS) are found within and just C-terminal to the DNA binding domain. The ligand binding domain makes up the bulk of the C-terminal half of the molecule, with the AF2 domain comprising the most C-terminal region. The AF2 domain is largely responsible for binding to co-activators such as the SRC family and DRIP (Mediator) in the presence of ligand. Regions on the second zinc finger and within the ligand binding domain facilitate heterodimerization with RXR. Corepressor binding is less well characterized but appears to overlap that of coactivators in helices 3 and 5, a region blocked by helix 12 in the presence of ligand.

The ligand binding domain (LBD) for VDR has been crystallized and its structure solved (123). More recently the structure of the VDR/RXR heterodimer has been analyzed by high resolution cryo electron microscopy (124). These studies show that the VDR has a



high degree of structural homology to other nuclear hormone receptors. It is comprised of 12 helices joined primarily by beta sheets. The  $1,25(\text{OH})_2\text{D}$  is buried deep in the ligand binding pocket and covered by helix 12 (the terminal portion of the AF-2 domain). Assuming analogy with the unliganded LBD of RXR $\alpha$  and the ligand bound LBD of RAR $\gamma$  (125), the binding of  $1,25(\text{OH})_2\text{D}$  to the VDR triggers a substantial movement of helix 12 from an open position to a closed position, covering the ligand binding pocket and putting helix 12 in position with critical residues from helices 3, 4, and 5 to bind coactivators. Coactivator complexes bridge the gap from the VDRE to the transcription machinery at the transcription start site (figure 5) (126).



**Figure 5.**  $1,25(\text{OH})_2\text{D}$ -initiated gene transcription.  $1,25(\text{OH})_2\text{D}$  enters the target cell and binds to its receptor, VDR. The VDR then heterodimerizes with the retinoid X receptor (RXR). This increases the affinity of the VDR/RXR complex for the vitamin D response element (VDRE), a specific sequence of nucleotides in the promoter region of the vitamin D responsive gene. Binding of the VDR/RXR complex to the VDRE attracts a complex of proteins termed coactivators to the VDR/RXR complex. The DRIP (Mediator) coactivator complex spans the gap between the VDRE and RNA polymerase II and other proteins in the initiation complex centered at or around the TATA box (or other transcription regulatory elements). SRC coactivators recruit histone acetyl transferases (HAT) to the gene promoting the opening up of its structure to enable the transcription machinery to work. Transcription of the gene is initiated to produce the corresponding mRNA, which leaves the nucleus to be translated to the corresponding protein.

Nuclear hormone receptors including the VDR are further regulated by protein complexes that can be activators or repressors (127). The role of corepressors in VDR function has been demonstrated (128) but is less well studied than the role of coactivators. One such corepressor, hairless, is found in the skin and may regulate  $1,25(\text{OH})_2\text{D}$  mediated epidermal proliferation and differentiation as well as ligand independent VDR regulation of hair follicle cycling (129-131). The coactivators, which are essential for VDR function, form two distinct complexes, the interaction of which remains unclear (121). The SRC family has three members, SRC 1-3, all of which can bind to the VDR in the presence of ligand ( $1,25(\text{OH})_2\text{D}$ ) (132). These coactivators recruit additional coactivators such as CBP/p300 and p/CAF that have histone acetyl

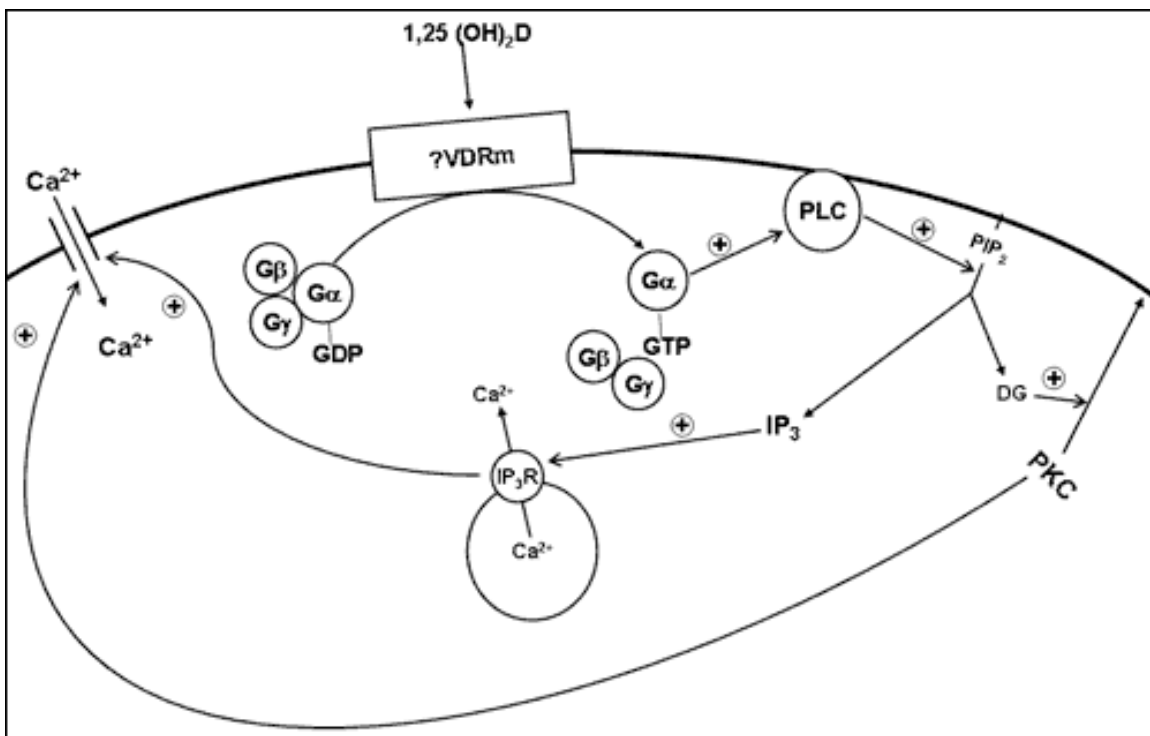
transferase activity (HAT), an enzyme that by acetylation of lysines within specific histones appears to help unravel the chromatin allowing the transcriptional machinery to do its job. The domain in these molecules critical for binding to the VDR and other nuclear hormone receptors is called the NR box, and has as its central motif LxxLL where L stands for leucine and x for any amino acid. Each SRC family member contains three well conserved NR boxes in the region critical for nuclear hormone receptor binding. The DRIP (Mediator) complex is comprised of 15 or so proteins several of which contain LxxLL motifs (133). However, DRIP205 (Mediator 1) is the protein critical for binding the complex to VDR. It contains 2 NR boxes. Different NR boxes in these coactivators show specificity for different nuclear hormone receptors (134). Unlike the SRC complex, the DRIP complex does not have HAT activity (121). Rather the DRIP complex spans the gene from the VDRE to the transcription start site linking directly with RNA polymerase II and its associated transcription factors. DRIP and SRC appear to compete for binding to the VDR. In keratinocytes DRIP binds preferentially to the VDR in undifferentiated cells, whereas SRC 2 and 3 bind in the more differentiated cells in which DRIP levels have declined (135). Thus in these cells DRIP appears to regulate the early stages of  $1,25(\text{OH})_2\text{D}$  induced differentiation, whereas SRC may be more important in the later stages, although overlap in gene specificity is also observed (136,137) (138). Recently, SMAD 3, a transcription factor in the TGF- $\beta$  pathway, has been found to complex with the SRC family members and the VDR, enhancing the coactivation process (139). Phosphorylation of the VDR may also control VDR function (140). Furthermore, VDR has been shown to suppress  $\beta$ -catenin transcriptional activity (141), whereas  $\beta$ -catenin enhances that of VDR (142). Thus, control of VDR activity may involve crosstalk between signaling pathways originating in receptors at the plasma membrane as well as within the nucleus.

VDR acts in concert with other nuclear hormone receptors, in particular RXR (143). Unlike VDR, there are three forms of RXR-- $\alpha$ ,  $\beta$ ,  $\gamma$ --and all three are capable of binding to VDR with no obvious differences in terms of functional effect. RXR and VDR form heterodimers that optimize their affinity for the vitamin D response elements (VDREs) in the genes being regulated. RXR appears to be responsible for keeping VDR in the nucleus in the absence of ligand (144). VDR may also partner with other receptors including the thyroid receptor (TR) and the retinoic acid receptor (RAR) (145,146), but these are the exceptions, whereas RXR is the rule. The VDR/RXR heterodimers bind to VDREs, which typically are comprised of two half sites each with six nucleotides separated by three nucleotides of nonspecific type; this type of VDRE is known as a DR3 (direct repeats with three nucleotide spacing). RXR binds to the upstream half site, while VDR binds to the downstream site (147). However, a wide range of VDRE configurations have been found at nearly any location within a gene (5', 3', introns) (148). Moreover, different tissues differ as to which VDREs actively bind VDR (149).  $1,25(\text{OH})_2\text{D}$  is required for high affinity binding and activation, but the RXR ligand, 9-cis retinoic acid, may either inhibit (150) or activate (151)  $1,25(\text{OH})_2\text{D}$  stimulation of gene transcription. A DR6 has been identified in the phospholipase C- $\gamma$ 1 gene that recognizes VDR/RAR heterodimers (145), and a DR4 has been found in the mouse calbindin 28k gene (152). Inverted palindromes with 7 to 12 bases between half sites have also been found (142). Furthermore, the half sites of the various known VDREs show remarkable degeneracy (table 1). The G in the second position of each site appears to be the only nearly invariant nucleotide.  $1,25(\text{OH})_2\text{D}$  can also inhibit gene transcription through its VDR. This may occur by direct binding of the VDR to negative VDREs that in the PTH and PTHrP genes are remarkably similar in sequence to positive VDREs of other genes (153,154). However, inhibition may also be indirect. For example,  $1,25(\text{OH})_2\text{D}$  inhibits IL-2 production by blocking the NFATp/AP-1 complex of transcription factors from

activating this gene (155) through a mechanism not yet clear. Similarly, as previously mentioned 1,25(OH)<sub>2</sub>D inhibits CYP27B1 in at least one renal cell line by an indirect mechanism involving VDR binding to VDRE (59,76). Thus a variety of factors including the flanking sequences of the genes around the VDREs and tissue specific factors play a large role in dictating the ability of 1,25(OH)<sub>2</sub>D to regulate gene expression.

## Non Genomic Actions.

A variety of hormones that serve as ligands for nuclear hormone receptors also exert biologic effects that do not appear to require gene regulation and may work through membrane receptors rather than their cognate nuclear hormone receptors. Examples include estrogen (156), progesterone (157), testosterone (158), corticosteroids (159), and thyroid hormone (160). 1,25(OH)<sub>2</sub>D has also been shown to have rapid effects on selected cells that are not likely to involve gene regulation and that appear to be mediated by a different, probably membrane receptor. A model for such effects is shown in figure 6. Similar to other steroid hormones, 1,25(OH)<sub>2</sub>D has been shown to regulate calcium and chloride channel activity, protein kinase C activation and distribution, and phospholipase C activity in a number of cells including osteoblasts (161), liver (162), muscle (163), and intestine (164,165). These rapid effects of 1,25(OH)<sub>2</sub>D have been most extensively studied in the intestine. Norman's laboratory coined the term transcalcachia to describe the rapid onset of calcium flux across the intestine of a vitamin D replete chick perfused with 1,25(OH)<sub>2</sub>D (166). This increased flux could not be blocked with actinomycin D pretreatment (167), but was blocked by voltage gated L type channel inhibitors (168) and protein kinase C inhibitors (169). These animals had to be vitamin D replete and contain the VDR, indicating that the basic machinery for calcium transport was intact. On the other hand L type channel activators such as BAY K-8644 (170) and protein kinase C activators such as phorbol esters (168) could activate transcalcachia similar to 1,25(OH)<sub>2</sub>D.



**Figure 6.** Model for the non genomic actions of  $1,25(\text{OH})_2\text{D}$ .  $1,25(\text{OH})_2\text{D}$  binds to a putative membrane receptor. This leads to activation of a G protein (GTP displacement of GDP and dissociation of the  $\beta$  and  $\gamma$  subunits from the now active  $\alpha$  subunit).  $\text{G}\alpha$  -GTP activates phospholipase C (PLC) ( $\beta$  or  $\gamma$ ) to hydrolyze phosphatidyl inositol bis phosphate ( $\text{PIP}_2$ ) to inositol tris phosphate ( $\text{IP}_3$ ) and diacyl glycerol (DG).  $\text{IP}_3$  releases calcium from intracellular stores via the  $\text{IP}_3$  receptor in the endoplasmic reticulum; DG activates protein kinase C (PKC). Both calcium and PKC may regulate the influx of calcium across the plasma membrane through various calcium channels including L-type calcium channels.

A putative membrane receptor for  $1,25(\text{OH})_2\text{D}$  ( $1,25(\text{OH})_2\text{D}$  membrane associated rapid response steroid binding protein ( $1,25\text{D}$ -MARRSBP) also known as ERp57) has been purified from the intestine (171) and subsequently cloned and sequenced (172). Its size is approximately 66kDa. Antibodies have been made against this putative receptor (173). These antibodies block the ability of  $1,25(\text{OH})_2\text{D}$  to stimulate calcium uptake by isolated chick intestinal cells (174) and to stimulate protein kinase C activity in resting zone chondrocytes while inhibiting proliferation of both resting zone and proliferating zone chondrocytes (173). Analog studies also support the existence of a separate membrane receptor for  $1,25(\text{OH})_2\text{D}$ . Because of the breaking of the B ring during vitamin  $\text{D}_3$  production from 7-dehydrocholesterol, the A ring can assume a conformation similar to the parent cholesterol molecule (6-s-cis) (shown as previtamin  $\text{D}_3$  in figure 1) or the more commonly depicted 6-s-trans form in which the A ring rotates away from the rest of the molecule (shown as vitamin  $\text{D}_3$  in figure 1). Analogs of  $1,25(\text{OH})_2\text{D}$  can be produced which favor the 6-s-cis conformation or the 6-s-trans conformation.  $1,25(\text{OH})_2\text{d5}$ -previtamin  $\text{D}_3$  is one such analog locked into the 6-s-cis conformation. This analog has only weak activity with respect to VDR binding or transcriptional activation but is fully effective in terms of stimulating transcalcitachia and calcium uptake by osteosarcoma cells when compared to  $1,25(\text{OH})_2\text{D}$  (175). 6-s-trans analogs are not effective. However, some of these rapid actions of  $1,25(\text{OH})_2\text{D}$  are not found in cells from VDR null mice suggesting that the VDR may be required for the expression and/or function of the membrane receptor or be the membrane receptor (176). In other cells both  $1,25\text{D}$ -MARRSBP and VDR appear to be required for these rapid effects of  $1,25(\text{OH})_2\text{D}$  (177).

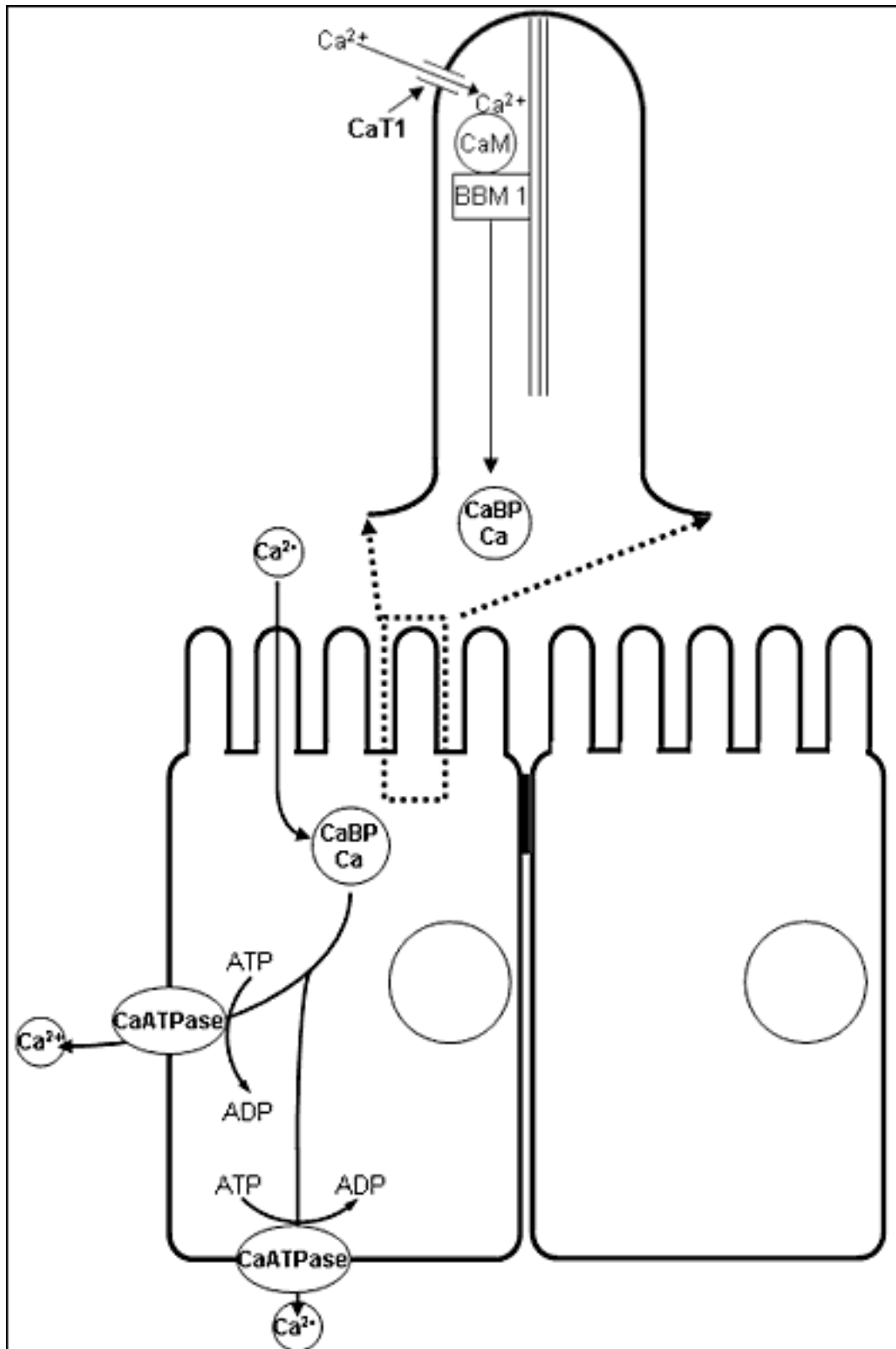
The model (figure 6) emerging from these studies is that  $1,25(\text{OH})_2\text{D}$  interacts with a membrane receptor to activate phospholipase C possibly through a G protein coupled process. Phospholipase C then hydrolyzes phosphatidyl inositol bis phosphate ( $\text{PIP}_2$ ) in the membrane releasing inositol tris phosphate ( $\text{IP}_3$ ) and diacyl glycerol (DG). These second messengers may then activate both the intracellular release of calcium from intracellular stores via the  $\text{IP}_3$  receptor and protein kinase C, either one or both of which could stimulate calcium channel activity leading to a further rise in intracellular calcium levels. In the intestine and kidney the increased flux of calcium across the brush border membrane is then transported out of the cell at the basolateral membrane, completing transcellular transport. In other cells the increased calcium would need to be removed by other mechanisms after the signal conveyed by the rise in calcium is no longer required. Much work remains to prove this model including the physiologic requirement for a unique membrane receptor.

## TARGET TISSUE RESPONSES: CALCIUM REGULATING ORGANS

### Intestinal calcium absorption

Intestinal calcium absorption, in particular the active component of transcellular calcium absorption, is one of the oldest and best known actions of vitamin D having been first

described in vitro by Schachter and Rosen (178) in 1959 and in vivo by Wasserman et al. (179) in 1961. Absorption of calcium from the luminal contents of the intestine involves both transcellular and paracellular pathways. The transcellular pathway dominates in the duodenum, and this is the pathway primarily regulated by 1,25 dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) (180) (reviews in (181)). Figure 7 shows a model of our current understanding of how this process is regulated by  $1,25(\text{OH})_2\text{D}$ . Calcium entry across the brush border membrane (BBM) occurs down a steep electrical-chemical gradient and requires no input of energy. Removal of calcium at the basolateral membrane must work against this gradient, and energy is required. This is achieved by the CaATPase (PMCA1b), an enzyme induced by  $1,25(\text{OH})_2\text{D}$  in the intestine. Calcium movement through the cell occurs with minimal elevation of the intracellular free calcium concentration (182) by packaging the calcium in calbindin containing vesicles (183-185) that form in the terminal web following  $1,25(\text{OH})_2\text{D}$  administration.



**Figure 7.** Model of intestinal calcium transport. Calcium enters the microvillus of the intestinal epithelial cell through TRPV6 (previously known as CaT1) calcium channel. Within the microvillus calcium is bound to calmodulin (CaM) which is itself bound to brush border myosin I (BBMI). BBMI may facilitate the movement of the calcium/CaM complex into the terminal web where the calcium is picked up by calbindin (CaBP) and transported



through the cytoplasm in endocytic vesicles. At the basolateral membrane the calcium is pumped out of the cell by the Ca-ATPase (PMCAb).  $1,25(\text{OH})_2\text{D}$  enhances intestinal calcium transport by inducing TRPV6, CaBP, and PMCAb as well as increasing the amount of CaM bound to BBMI in the brush border.

$1,25(\text{OH})_2\text{D}$  regulates transcellular calcium transport using a combination of genomic and nongenomic actions. The first step, calcium entry across the BBM, is accompanied by changes in the lipid composition of the membrane including an increase in linoleic and arachidonic acid (186,187) and an increase in the phosphatidylcholine:phosphatidylethanolamine ratio (188). These changes are associated with increased membrane fluidity (187), which we have shown results in increased calcium flux (189). The changes in lipid composition occur within hours after  $1,25(\text{OH})_2\text{D}$  administration and are not blocked by pretreatment with cycloheximide (188). In addition an epithelial specific calcium channel, TRPV6, is expressed in the intestinal epithelium (190). This channel has a high degree of homology to TRPV5, a channel originally identified in the kidney (191,192). The tissue distributions of these channels are overlapping and can be found in other tissues, but TRPV6 appears to be the main form in the intestine (193,194). TRPV6 mRNA levels in the intestine of vitamin D deficient mice are markedly increased by  $1,25(\text{OH})_2\text{D}$ , although similar changes are not found in the kidney (195). Mice null for TRPV6 have decreased intestinal calcium transport (196).

Calcium entering the brush border must then be moved into and through the cytoplasm without disrupting the function of the cell. Electron microscopic observations indicate that in the vitamin D deficient animal, calcium accumulates along the inner surface of the plasma membrane of the microvilli (197,198). Following vitamin D or  $1,25(\text{OH})_2\text{D}$  administration calcium leaves the microvilli and subsequently can be found in mitochondria and vesicles within the terminal web (183,184,197,198). The vesicles appear to shuttle the calcium to the lateral membrane where it is pumped out of the cell by the basolateral CaATPase, PMCA1b. These morphologic observations have been confirmed by direct measurements of calcium using xray microanalysis that demonstrate equivalent amounts of calcium within the microvilli of D deficient and  $1,25(\text{OH})_2\text{D}$  treated animals but much higher amounts of calcium in the mitochondria and vesicles of the  $1,25(\text{OH})_2\text{D}$  treated animals (184,199). Such data suggest that  $1,25(\text{OH})_2\text{D}$  controls calcium entry into the cell primarily by regulating its removal from the microvillus and accumulation by subcellular organelles in the terminal web, although flux through calcium channels in the membrane such as TRPV6 also plays a major role.

The ability of  $1,25(\text{OH})_2\text{D}$  to stimulate calcium entry into and transport from the microvillus does not require new protein synthesis (183,188,200). Cycloheximide does not block the ability of  $1,25(\text{OH})_2\text{D}$  to increase the capacity of brush border membrane vesicles (BBMV) to accumulate calcium, although it does block the increase in alkaline phosphatase in the same BBMV [193]. Likewise, cycloheximide does not block the increase in mitochondrial calcium following  $1,25(\text{OH})_2\text{D}$  administration, although it blocks the rise in calbindin and prevents the normal vesicular transport of calcium through the cytosol (183,201). Thus, nongenomic actions underlie at least some of these first steps in  $1,25(\text{OH})_2\text{D}$  stimulated intestinal calcium transport within the microvillus, although the changes take hours, not minutes, to observe. The exact role for these nongenomic effects on calcium influx relative to the role of TRPV6 remains to be elucidated.



Calmodulin is the major calcium binding protein in the microvillus (202). Its concentration in the microvillus is increased by  $1,25(\text{OH})_2\text{D}$ ; no new calmodulin synthesis is required or observed after  $1,25(\text{OH})_2\text{D}$  administration (203). Calmodulin is likely to play a major role in calcium transport within the microvillus, and inhibitors of calmodulin block  $1,25(\text{OH})_2\text{D}$  stimulated calcium uptake by BBMV (204). Within the microvillus calmodulin is bound to a 110kD protein, myosin 1A (myo1A) (previously referred to as brush border myosin 1).  $1,25(\text{OH})_2\text{D}$  increases the binding of calmodulin to myo1A in brush border membrane preparations (203), although binding of calmodulin to the myo1A attached to the actin core following detergent extraction of the membrane appears to be reduced (205). The calmodulin/myo1A complex appears late in the development of the brush border, and is found in highest concentration in the same cells of the villus which have the highest capacity for calcium transport (206). Myo1A is located primarily in the microvillus of the mature intestinal epithelial cell, although small amounts have been detected associated with vesicles in the terminal web (207). Thus, the calmodulin/myo1A complex may be responsible for moving calcium out of the microvillus. Its exact role in calcium transport is unclear in that mice null for myo1A do not show reduced intestinal calcium transport (Bikle and Munson, unpublished observations). Calbindin is the dominant calcium binding protein in the cytoplasm (202,208), where it appears to play the major role in calcium transport from the terminal web to the basolateral membrane (181). The increase in calbindin levels in the cytosol following  $1,25(\text{OH})_2\text{D}$  administration is blocked by protein synthesis inhibitors (200). Indeed, calbindin was the first protein discovered to be induced by vitamin D (208). Glenney and Glenney (202) observed that calbindin has a higher affinity for calcium than does calmodulin. The differential distribution of calmodulin and calbindin between microvillus and cytosol combined with the differences in affinity for calcium led Glenney and Glenney (202) to propose that in the course of calcium transport calcium flowed from calmodulin in the microvillus to calbindin in the cytosol with minimal change in the free calcium concentration in either location. However, the role of calbindin in intestinal calcium transport does not appear to be critical in that mice null for calbindin9k grow normally, have normal intestinal calcium transport, and their serum calcium levels and bone mineral content are equivalent to wildtype mice regardless of the calcium content of the diet (209). The CaATPase (PMCA1b) at the basolateral membrane and the sodium/calcium exchanger (NCX) are responsible for removing calcium from the cell against the same steep electrochemical gradient as favored calcium entry at the brush border membrane (210). Related proteins are found in the renal distal tubule. As its name implies, the extrusion of calcium from the cell by the calcium pump requires ATP. This pump is a member of the PMCA family, and in the intestine the isoform PMCA1b is the major isoform found. This pump is induced by  $1,25(\text{OH})_2\text{D}$  (211). Calmodulin activates the pump, but calbindin may do likewise (212). Whether deletion of PMCA1b affects calcium transport has not been evaluated, although the deletion of protein 4.1R, which regulated PMCA1b expression in the intestine, results in decreased intestinal calcium transport (213). The role of NCX is not considered to be as important as PMCA1b for intestinal calcium transport (214).

Although less studied, intestinal phosphate transport is also under the control of vitamin D. This was first demonstrated by Harrison and Harrison (215) in 1961. Active phosphate transport is greatest in the jejunum, in contrast to active calcium transport that is greatest in the duodenum. Cycloheximide blocks  $1,25(\text{OH})_2\text{D}$  stimulated phosphate transport (216), indicating that protein synthesis is involved. Phosphate transport at both the brush border and basolateral membranes requires sodium. A sodium-phosphate transporter in the small intestine ( $\text{NaP}_i\text{-IIb}$ ), homologous to the type IIa sodium phosphate transporter in kidney, has been cloned and sequenced (217). Expression of  $\text{NaP}_i\text{-IIb}$  is increased by  $1,25(\text{OH})_2\text{D}$  (218). Transport of phosphate

through the cytosol from one membrane to the other is poorly understood. However, cytochalasin B, a disrupter of microfilaments, has been shown to disrupt this process (219) suggesting that as for calcium, intracellular phosphate transport occurs in vesicles.

## **Actions of Vit D on Bone.**

Nutritional vitamin D deficiency, altered vitamin D responsiveness such as vitamin D receptor mutations (hereditary vitamin D resistant rickets), and deficient production of  $1,25(\text{OH})_2\text{D}$  such as mutations in the CYP27B1 gene (pseudo vitamin D deficiency) all have rickets as their main phenotype. This would suggest that vitamin D, and in particular  $1,25(\text{OH})_2\text{D}$ , is of critical importance to bone. Furthermore, VDR are found in bone cells (220,221), and vitamin D metabolites have been shown to regulate many processes in bone. However, the rickets resulting from vitamin D deficiency or VDR mutations (or knockouts) can be corrected by supplying adequate amounts of calcium and phosphate either by infusions or orally [214-217]. Moreover, deletion of VDR from bone cells does not result in rickets (222). This would suggest either that vitamin D metabolites do not directly impact bone, or that substantial redundancy has been built into the system. However, arguing for a physiologically non redundant direct action of vitamin D on bone is the development of osteoporosis and decreased bone formation in these VDR or CYP27B1 null mice not corrected by the high calcium/phosphate diet (223). A further complicating factor in determining the role of vitamin D metabolites in bone is the multitude of effects these metabolites have on systemic calcium homeostatic mechanisms which themselves impact on bone. The lack of vitamin D results in hypocalcemia and hypophosphatemia that as implied above is sufficient to cause rickets. Moreover, part of the skeletal phenotype in vitamin D deficiency is also due to the hyperparathyroidism that develops in the vitamin D deficient state as PTH has its own actions on bone and cartilage. Furthermore, within bone the vitamin D metabolites can alter the expression and/or secretion of a large number of skeletally derived factors including insulin like growth factor-1 (IGF-I) (224), its receptor (225), and binding proteins (226,227), transforming growth factor  $\beta$  (TGF $\beta$ ) (228), vascular endothelial growth factor (VEGF) (229), interleukin-6 (IL-6) (230), IL-4 (231), and endothelin receptors (232) all of which can exert effects on bone of their own as well as modulate the actions of the vitamin D metabolites on bone. Understanding the impact of vitamin D metabolites on bone is additionally complicated by species differences, differences in responsiveness of bone and cartilage cells according to their states of differentiation, and differences in responsiveness in terms of the vitamin D metabolite being examined. Thus the study of vitamin D on bone has had a complex history, and uncertainty remains as to how critical the direct actions of the vitamin D metabolites on bone are for bone formation and resorption.

Bone develops intramembranously (eg. skull) or from cartilage (endochondral bone formation, eg. long bones with growth plates). Intramembranous bone formation occurs when osteoprogenitor cells proliferate and produce osteoid, a type I collagen rich matrix. The osteoprogenitor cells differentiate into osteoblasts which then deposit calcium phosphate crystals into the matrix to produce woven bone. This bone is remodeled into mature lamellar bone. Endochondral bone formation is initiated by the differentiation of mesenchymal stem cells into chondroblasts that produce the proteoglycan rich type II collagen matrix. These cells continue to differentiate into hypertrophic chondrocytes that shift from making type II collagen to producing type X collagen. These cells also initiate the degradation and calcification of the matrix by secreting matrix vesicles filled with degradative enzymes such as metalloproteinases and phospholipases, alkaline

phosphatase (thought to be critical for the mineralization process), and calcium phosphate crystals. Vascular invasion and osteoclastic resorption are stimulated by the production of VEGF and other chemotactic factors from the degraded matrix. The hypertrophic chondrocytes also begin to produce markers of osteoblasts such as osteocalcin, osteopontin, and type I collagen resulting in the initial deposition of osteoid. Moreover, at least some of these chondrocytes further differentiate (or trans differentiate) into osteoblasts (233). Terminal differentiation of the hypertrophic chondrocytes and the subsequent calcification of the matrix are markedly impaired in vitamin D deficiency leading to the flaring of the ends of the long bones and the rachitic rosary along the costochondral junctions of the ribs, classic features of rickets. Although supply of adequate amounts of calcium and phosphate may correct most of these defects in terminal differentiation and calcification, the vitamin D metabolites,  $1,25(\text{OH})_2\text{D}$  and  $24,25(\text{OH})_2\text{D}$ , have been shown to exert distinct roles in the process of endochondral bone formation.

The VDR makes its first appearance in the fetal rat at day 13 of gestation in the condensing mesenchyme of the vertebral column then subsequently in osteoblasts and the proliferating and hypertrophic chondrocytes by day 17 (234). However, fetal development is quite normal in vitamin D deficient rats (235) and VDR knockout mice (118) suggesting that vitamin D and the VDR are not critical for skeletal formation. Rickets develops postnatally, becoming most manifest after weaning. The impairment of endochondral bone formation observed in vitamin D deficiency is associated with decreased alkaline phosphatase activity of the hypertrophic chondrocytes (236), alterations in the lipid composition of the matrix (237) perhaps secondary to reduced phospholipase activity (238), and altered proteoglycan degradation (239) due to changes in metalloproteinase activity (239,240). Both  $1,25(\text{OH})_2\text{D}$  and  $24,25(\text{OH})_2\text{D}$  appear to be required for optimal endochondral bone formation (241). However, in the CYP24A1 knockout mouse, that fails to produce any 24-hydroxylated metabolites of vitamin D, the skeletal lesion is defective mineralization of intramembranous (not endochondral) bone. Furthermore, the skeletal abnormality appears to be due to high circulating  $1,25(\text{OH})_2\text{D}$  levels in that crossing this mouse with one lacking the VDR corrects the problem (81). Whether this reflects species differences between mice and other species (most studies demonstrating the role of  $24,25(\text{OH})_2\text{D}$  in bone and cartilage have used rats and chicks) remains unknown. Chondrocytes from the resting zone of the growth plate of rats tend to be more responsive to  $24,25(\text{OH})_2\text{D}$  than  $1,25(\text{OH})_2\text{D}$ , whereas the reverse is true for chondrocytes from the growth zone with respect to stimulation of alkaline phosphatase activity (242), regulation of phospholipase A2 (stimulation by  $1,25(\text{OH})_2\text{D}$ , inhibition by  $24,25(\text{OH})_2\text{D}$ ) (243), changes in membrane fluidity (increased by  $1,25(\text{OH})_2\text{D}$ , decreased by  $24,25(\text{OH})_2\text{D}$ ) (244), and stimulation of protein kinase C activity (245). These actions of  $1,25(\text{OH})_2\text{D}$  and  $24,25(\text{OH})_2\text{D}$  do not require the VDR and are non genomic in that they take place with isolated matrix vesicles and membrane preparations from these cells (242). As discussed earlier membrane receptors for these vitamin D metabolites have been found in chondrocytes that may mediate these non genomic actions (246). Osteoblasts also differ in their response to  $1,25(\text{OH})_2\text{D}$  depending on their degree of maturation (247). In the latter stages of differentiation, rat osteoblasts respond to  $1,25(\text{OH})_2\text{D}$  with an increase in osteocalcin production (248), but do not respond to  $1,25(\text{OH})_2\text{D}$  in the early stages. Mice, however, differ from rats in that  $1,25(\text{OH})_2\text{D}$  inhibits osteocalcin expression (248). Similarly, the effects of  $1,25(\text{OH})_2\text{D}$  on alkaline phosphatase (249) and type I collagen (250) are inhibitory in the early stages of osteoblast differentiation but stimulatory in the latter stages (247). Osteopontin is better stimulated by  $1,25(\text{OH})_2\text{D}$  in the early stages than the late stages of differentiation (247,251). Osteocalcin and osteopontin in human and rat cells have well described

VDREs in their promoters (252-254) (the mouse does not) (255). However, alkaline phosphatase and the COL1A1 and COL1A2 genes producing type I collagen do not have clearly defined VDREs, so it remains unclear how these genes are regulated by  $1,25(\text{OH})_2\text{D}$ . These maturation dependent effects of  $1,25(\text{OH})_2\text{D}$  on bone cell function may explain the surprising ability of excess  $1,25(\text{OH})_2\text{D}$  to block mineralization leading to hyperosteoridosis (256,257) as such doses may prevent the normal maturation of osteoblasts.

In addition to its role in promoting bone formation,  $1,25(\text{OH})_2\text{D}$  also promotes bone resorption by increasing the number and activity of osteoclasts (258). Whether mature osteoclasts contain the VDR and are regulated directly by  $1,25(\text{OH})_2\text{D}$  remains controversial (259,260), but the VDR in osteoclast precursors is not required for osteoclastogenesis. Rather, the stimulation of osteoclastogenesis by  $1,25(\text{OH})_2\text{D}$  is mediated by osteoblasts. Rodan and Martin (261) originally proposed the hypothesis that osteoblasts were required for osteoclastogenesis, and the mechanism has now been elucidated (262). Osteoblasts produce a membrane associated protein known as RANKL (receptor activator of nuclear factor (NF)- $\kappa\text{B}$  ligand) that activates RANK on osteoclasts and their hematopoietic precursors. This cell to cell contact in combination with m-CSF also produced by osteoblasts stimulates the differentiation of precursors to osteoclasts, and promotes their activity.  $1,25(\text{OH})_2\text{D}$  regulates this process by inducing RANKL (263) as does PTH, PGE2, and IL-11, all of which stimulate osteoclastogenesis.  $1,25(\text{OH})_2\text{D}$  requires the VDR in osteoblasts for this purpose, although the other hormones and cytokines do not. Osteoblasts from VDR knockout mice fail to support  $1,25(\text{OH})_2\text{D}$  induced osteoclastogenesis, whereas osteoclast precursors from VDR knockout mice can be induced by  $1,25(\text{OH})_2\text{D}$  to form osteoclasts in the presence of osteoblasts from wildtype animals (264).

## Kidney.

The regulation of calcium and phosphate transport by vitamin D metabolites in the kidney has received less study than that in the intestine, but the two tissues have similar although not identical mechanisms. Eight grams of calcium are filtered by the glomerulus each day, and 98% of that is reabsorbed. Most is reabsorbed in the proximal tubule. This is a paracellular, sodium dependent process with little or no regulation by PTH and  $1,25(\text{OH})_2\text{D}$ . Approximately 20% of calcium is reabsorbed in the thick ascending limb of the loop of Henle, 10-15% in the distal tubule, and 5% in the collecting duct (265). Regulation by vitamin D takes place in the distal tubule where calcium moves against an electrochemical gradient (presumably transcellular) in a sodium independent fashion (266). Phosphate, on the other hand, is approximately 80% reabsorbed in the proximal tubule, and this process is regulated by PTH (267). In parathyroidectomized (PTX) animals Puschett et al. (268-270) demonstrated acute effects of  $25\text{OHD}$  and  $1,25(\text{OH})_2\text{D}$  on calcium and phosphate reabsorption. Subsequent studies indicated that PTH could enhance or was required for the stimulation of calcium and phosphate reabsorption by vitamin D metabolites (271,272).

The molecules critical for calcium reabsorption in the distal tubule appear to be the **VDR, calbindin, TRPV5, and the BLM calcium pump (PMCA1b as in the intestine),** a situation similar to the mechanism for calcium transport in the intestine. However, the calbindin in the kidney in most species is 28kDa, whereas the 9kDa form is found in the intestine in most species. The kidney has mostly TRPV5, whereas the intestine is primarily TRPV6. The calcium pump is the same isoform in both tissues (PMCA1b) although other forms

of PMCA are also present. Calmodulin and a brush border myosin I like protein are also found in the kidney brush border, but their role in renal calcium transport has not been explored. VDR, calbindin, TRPV5, and PMCA1b colocalize in the distal tubule, but not all distal tubules contain this collection of proteins (191,192,273,274) suggesting that not all distal tubules are involved in calcium transport.  $1,25(\text{OH})_2\text{D}$  upregulates the VDR (216), an action opposed by PTH (219). Calbindin is also induced by  $1,25(\text{OH})_2\text{D}$  in the kidney(275,276). The activity of the calcium pump is increased by  $1,25(\text{OH})_2\text{D}$  (277), but it is not clear that the protein itself is induced. The increased activity may be due to the induction of calbindin that increases its activity. The effect of  $1,25(\text{OH})_2\text{D}$  on TRPV5 expression is stimulatory (195).

Phosphate reabsorption in the proximal tubule is mediated at the brush border by sodium dependent phosphate transporters (NaPi-2a and NaPi-2c) that rely on the basolateral membrane Na,K ATPase to maintain the sodium gradient that drives the transport process (278). It is not clear whether  $1,25(\text{OH})_2\text{D}$  regulates the expression or activities of these transporters as it does in the intestine, although PTH clearly does. Like PTH, FGF23 blocks phosphate reabsorption, presumably by blocking NaPi-2a activity. Unlike PTH, FGF23 also blocks the renal production of  $1,25(\text{OH})_2\text{D}$ , as discussed earlier. The link between phosphate reabsorption and  $1,25(\text{OH})_2\text{D}$  production remains unclear.

## **TARGET TISSUE RESPONSES: NON CALCIUM TRANSPORTING TISSUES**

In addition to the its effects on tissues directly responsible for calcium homeostasis,  $1,25(\text{OH})_2\text{D}$  regulates the function of a wide number of other tissues. These all contain the VDR. Regulation of differentiation and proliferation is one common theme; regulation of hormone secretion is another; regulation of immune function is the third. In most cases  $1,25(\text{OH})_2\text{D}$  acts in conjunction with calcium. Selected examples follow.

### **Regulation of Hormone Secretion:**

#### **Parathyroid gland (PTH secretion):**

As previously mentioned, PTH stimulates the production of  $1,25(\text{OH})_2\text{D}$ . In turn  $1,25(\text{OH})_2\text{D}$  inhibits the production of PTH (279,280). The regulation occurs at the transcriptional level. Within the promoter of the PTH gene is a region that binds the VDR and mediates the suppression of the PTH promoter by  $1,25(\text{OH})_2\text{D}$  (153,275,281-285). However, there is substantial controversy about whether this site is a single half site (281) or a more classic DR3 (274), whether one VDRE is involved or two (282), whether only VDR binds (281,285), whether VDR/RXR heterodimers bind (153,282), or whether VDR partners with a different protein (283). Some of the differences may reflect different species, but the nature of PTH gene suppression by  $1,25(\text{OH})_2\text{D}$  remains incompletely understood. Calcium alters the ability of  $1,25(\text{OH})_2\text{D}$  to regulate PTH gene expression. Calcium is a potent inhibitor of PTH production and secretion, acting through the calcium sensing receptor (CaSR) on the plasma membrane of the parathyroid cell.  $1,25(\text{OH})_2\text{D}$  induces the CaSR in the parathyroid gland making it more sensitive to calcium (286). Animals placed on a low calcium diet have an increase in PTH and  $1,25(\text{OH})_2\text{D}$  levels indicating that the low calcium overrides the inhibition by  $1,25(\text{OH})_2\text{D}$  on PTH secretion (287,288). One possible explanation involves the protein calreticulin that binds to nuclear hormone receptors including VDR at KXGFFKR sequences, and inhibits their activity (289,290). Low dietary calcium has been shown to increase calreticulin levels in the parathyroid gland (291). The ability of  $1,25(\text{OH})_2\text{D}$  to inhibit PTH production and secretion has been exploited clinically in that  $1,25(\text{OH})_2\text{D}$  and several of its analogs are



used to prevent and/or treat secondary hyperparathyroidism associated with renal failure. The parathyroid gland also expresses CYP27B1 and so can produce its own  $1,25(\text{OH})_2\text{D}$  that may act in an autocrine or paracrine fashion to regulate PTH production (292). As noted earlier, the parathyroid gland is one of several tissues expressing the megalin/cubilin complex potentially enabling it to take up  $25\text{OHD}$  and other D metabolites still bound to DBP.

### **Pancreatic beta cells (insulin secretion):**

$1,25(\text{OH})_2\text{D}$  stimulates insulin secretion, although the mechanism is not well defined (293,294). VDR, CYP27B1 and calbindin- $\text{D}_{28\text{k}}$  are found in pancreatic beta cells (295-297), and studies using calbindin- $\text{D}_{28\text{k}}$  null mice have suggested that calbindin- $\text{D}_{28\text{k}}$ , by regulating intracellular calcium, can modulate depolarization-stimulated insulin release (298). Furthermore, calbindin- $\text{D}_{28\text{k}}$ , by buffering calcium, can protect against cytokine mediated destruction of beta cells (299). A number of mostly case control and observational studies have suggested that vitamin D deficiency contributes to increased risk for type 2 diabetes mellitus (300), although results of randomized controlled trials with vitamin D have not been conclusive (301).

### **Fibroblast growth factor (FGF23):**

FGF23 is produced primarily by bone, and in particular by osteoblasts and osteocytes.  $1,25(\text{OH})_2\text{D}_3$  stimulates this process, but the mechanism is not clear (302). Inasmuch as FGF23 inhibits  $1,25(\text{OH})_2\text{D}$  production by the kidney, this feedback loop like that for PTH secretion maintains a balance in the levels of these important hormones. Mutations in the Phosphate regulating gene with Homologies to Endopeptidases on the X chromosome (PHEX) or FGF23 itself (which prevent its proteolysis) or conditions such as McCune-Albright disease and tumor induced osteomalacia in which FGF23 is overexpressed in the involved tissue lead to hypophosphatemia and inappropriately low  $1,25(\text{OH})_2\text{D}$  accompanied by osteomalacia. The role of PHEX, which was originally thought to cleave FGF23, in regulating FGF23 levels is not clear. In contrast mutations in UDP-N-acetyl- $\alpha$ -D galactosamine:polypeptide N-acetylgalactosaminyltransferase (GALNT3), which glycosylates FGF23, or in FGF23 which blocks this glycosylation result in inhibited FGF23 secretion leading to hyperphosphatemia, increased  $1,25(\text{OH})_2\text{D}$ , and tumoral calcinosis (303).

## **Regulation of proliferation and differentiation.**

### **Cancer:**

$1,25(\text{OH})_2\text{D}$  has been evaluated for its potential anticancer activity in animal and cell studies for nearly 40 years (304). The list of malignant cells that express VDR is now quite extensive, and the list of those same cells that express CYP27B1 is growing. The accepted basis for the promise of  $1,25(\text{OH})_2\text{D}$  in the prevention and treatment of malignancy includes its antiproliferative, prodifferentiating effects on most cell types. The list of mechanisms proposed for these actions is extensive, and to some extent cell specific (305). Among these mechanisms  $1,25(\text{OH})_2\text{D}$  has been shown to stimulate the expression of cell cycle inhibitors p21 and p27 (306) and the expression of the cell adhesion molecule E-cadherin (141), while inhibiting the transcriptional activity of  $\beta$ -

catenin (141,307,308). In keratinocytes,  $1,25(\text{OH})_2\text{D}$  has been shown to promote the repair of DNA damage induced by ultraviolet radiation (UVR) (309) (310), reduce apoptosis while increasing survival after UVR (311), and increase p53 (312). Epidemiologic evidence supporting the importance of adequate vitamin D nutrition (including sunlight exposure) for the prevention of a number of cancers (313-317) is extensive. Although numerous types of cancers show reduction (318), most attention has been paid to cancers of the breast, colon, and prostate. I (319) recently reviewed a number of meta-analyses of epidemiologic studies evaluating the association of vitamin D intake and/or 25OHD levels and the risk of developing these cancers. The data supporting a reduction in risk for developing colorectal cancer and breast cancer in premenopausal females with higher vitamin D intake or higher serum 25OHD levels were considerably stronger than that for the prevention of prostate cancer. Prospective randomized controlled trial data are limited. In a prospective 4 yr trial with 1100iu vitamin D and 1400-1500 mg calcium originally designed to look at osteoporosis the authors showed a 77% reduction in cancers after excluding the initial year of study (320), including a reduction in both breast and colon cancers. In this study, vitamin D supplementation raised the 25OHD levels from a mean of 28.8ng/ml to 38.4ng/ml with no changes in the placebo or calcium only arms of the study. However, this was a relatively small study in which cancer prevention was not the primary outcome variable. Trials of  $1,25(\text{OH})_2\text{D}$  and its analogs for the treatment of cancer have been disappointing. In a small study involving 7 subjects with prostate cancer treated with doses of  $1,25(\text{OH})_2\text{D}$  up to  $2.5\mu\text{g}$  for 6-15 months, 6/7 showed a decrease in the rise of prostate specific antigen (PSA), a marker of tumor progression (321), and one patient showed a decline. However, hypercalciuria was common and limiting. A preliminary report of a larger study involving 250 patients with prostate cancer using  $45\mu\text{g}$   $1,25(\text{OH})_2\text{D}$  weekly in combination with docetaxel demonstrated a non significant decline in PSA, although survival was significantly improved (HR 0.67) (322). A larger followup study did not show increased survival (323). The incidence of either hypercalcemia or hypercalciuria was not reported. Most likely until an analog of  $1,25(\text{OH})_2\text{D}$  is developed which is both efficacious and truly non hypercalcemic, treatment of cancer with vitamin D metabolites will remain problematic.

## Skin.

Epidermal keratinocytes are the only cells in the body with the entire vitamin D metabolic pathway. As described earlier, production of vitamin  $\text{D}_3$  from 7-dehydrocholesterol takes place in the epidermis. However, the epidermis also contains CYP27A1 (324), the mitochondrial enzyme that 25-hydroxylates vitamin D, and CYP27B1 (37,44), the enzyme that produces  $1,25(\text{OH})_2\text{D}$  from 25OHD. The CYP27B1 in keratinocytes is differently regulated than CYP27B1 in renal cells. Although PTH stimulates CYP27B1 activity in the keratinocyte, the mechanism appears to be independent of cAMP (325). Cytokines such as tumor necrosis factor- $\alpha$  and interferon- $\gamma$  stimulate CYP27B1 activity (326,327).  $1,25(\text{OH})_2\text{D}$  does not exert a direct effect on CYP27B1 expression in keratinocytes, but regulates  $1,25(\text{OH})_2\text{D}$  levels by inducing CYP24A1 thus initiating the catabolism of  $1,25(\text{OH})_2\text{D}$  (75). CYP27B1 is expressed primarily in the basal cells of the epidermis (47); as the cells differentiate the mRNA and protein levels of CYP27B1 and its activity decline (328).

$1,25(\text{OH})_2\text{D}$  regulates keratinocyte differentiation in part by modulating the ability of calcium to do likewise (329). Therefore, it is important to understand the actions of



calcium on this cell prior to examining the influence of  $1,25(\text{OH})_2\text{D}$  (330-335) (336). If keratinocytes are grown at calcium concentrations below 0.07mM, they continue to proliferate but either fail or are slow to develop intercellular contacts, stratify little if at all, and fail or are slow to form cornified envelopes. Acutely increasing the extracellular calcium concentration ( $\text{Cao}$ ) above 0.1mM (calcium switch) leads to the rapid redistribution of desmoplakin, cadherins, integrins, catenins, plakoglobin, vinculin, and actinin from the cytosol to the membrane where they participate in the formation of intercellular contacts. Calcium also stimulates the redistribution to the membrane of protein kinase  $\text{Ca}$  ( $\text{PKCa}$ ) (337,338) and the tyrosine-phosphorylated p62 associated protein of ras GAP (339,340) where they further the calcium signaling process. These early events are accompanied by a rearrangement of actin filaments from a perinuclear to a radial pattern which if disrupted blocks the redistribution of these proteins and blocks the differentiation process. Within hours of the calcium switch keratinocytes switch from making the basal keratins K5 and K14 and begin making keratins K1 and K10 (335) followed, subsequently, by increased levels of profilaggrin (the precursor of filaggrin, an intermediate filament associated protein), involucrin and loricrin (precursors for the cornified envelope) (341,342). Loricrin, involucrin and other proteins (343) are cross linked into the insoluble cornified envelope by the calcium sensitive, membrane bound form of transglutaminase (344,345), which like involucrin and loricrin increases within 24 hours after the calcium switch (346). Within 1-2 days of the calcium switch cornified envelope formation is apparent (334,347), paralleling transglutaminase activation (348). The induction of these proteins represents a genomic action (likely indirect) of calcium as indicated by a calcium induced increase in mRNA levels and transcription rates (335,342,348,349). The relevance of calcium induced differentiation *in vitro* to the *in vivo* situation is indicated by the steep gradient of calcium within the epidermis, with the highest levels in the uppermost (most differentiated) nucleated layers (350). Current evidence for the importance of calcium in epidermal function is that barrier disruption, which results in increased proliferation, is associated with loss of the calcium gradient, whereas increasing the calcium concentration in the epidermis with sonophoresis stimulates lamellar body secretion (351-355).

The keratinocyte senses calcium via a seven transmembrane domain, G protein coupled receptor (CaSR) (356) originally cloned from the parathyroid cell by Brown et al (357,358). Knocking out the CaSR blocks calcium induced differentiation *in vitro* (359,360) and *in vivo* (361). However, keratinocytes also produce an alternatively spliced variant of the CaSR as they differentiate (362). This variant CaSR lacks exon 5 and so would be missing residues 461-537 in the extracellular domain. A mouse model in which the full length CaSR has been knocked out continues to produce the alternatively spliced form of CaSR, but its epidermis contains lower levels of the terminal differentiation markers loricrin and profilaggrin, and keratinocytes from these mice fail to respond normally to calcium (362) consistent with the results when the full length calcium receptor was deleted *in vitro* (359,360). We have produced a conditional knockout of the CaSR allowing us to delete CaSR in the tissue of choice using cell specific cre recombinases that avoids the problem with the original global knockout (363). When the CaSR is deleted specifically in the keratinocyte, this mouse has a reduction in epidermal differentiation and barrier repair (361), but unlike the global knockout does not have abnormalities in overall calcium homeostasis, and rather than showing an increased calcium gradient in the epidermis has a blunted one. The conditional knockout mouse also lacks the alternatively spliced CaSR.

Inositol 1,4,5 tris phosphate ( $\text{IP}_3$ ) and diacylglycerol levels increase within seconds to minutes after the calcium switch implicating activation of the phospholipase C (PLC)

pathway (364,365). Similar to intracellular calcium levels (Cai), the levels of inositol phosphates (IPs) remain elevated for hours after the calcium switch. The prolonged increase in IPs after the calcium switch may contribute to the plateau phase of Cai elevation and a prolonged elevation of diacylglycerol (DG) that would stimulate the protein kinase C (PKC) pathway. This prolonged increase in IPs appears to be due to calcium induction and activation of PLC (145,365,366), especially PLC- $\gamma$ 1. Activation of PLC- $\gamma$ 1 by calcium involves a chain of events involving src kinase activation of phosphatidylinositol 3 kinase and phosphatidylinositol 4 phosphate 5 kinase 1 $\alpha$  within the context of a membrane complex with E-cadherin leading to the formation of phosphatidylinositol trisphosphate in the membrane which activates PLC- $\gamma$ 1 via its PH domain (367). Phosphorylation of PLC- $\gamma$ 1 is not part of its activation by calcium unlike its activation by EGF (368). Knocking out PLC- $\gamma$ 1 blocks the ability of calcium to increase Cai and to induce involucrin and transglutaminase (366). Thus, like CaSR, PLC- $\gamma$ 1 is critical for the ability of calcium to regulate keratinocyte differentiation.

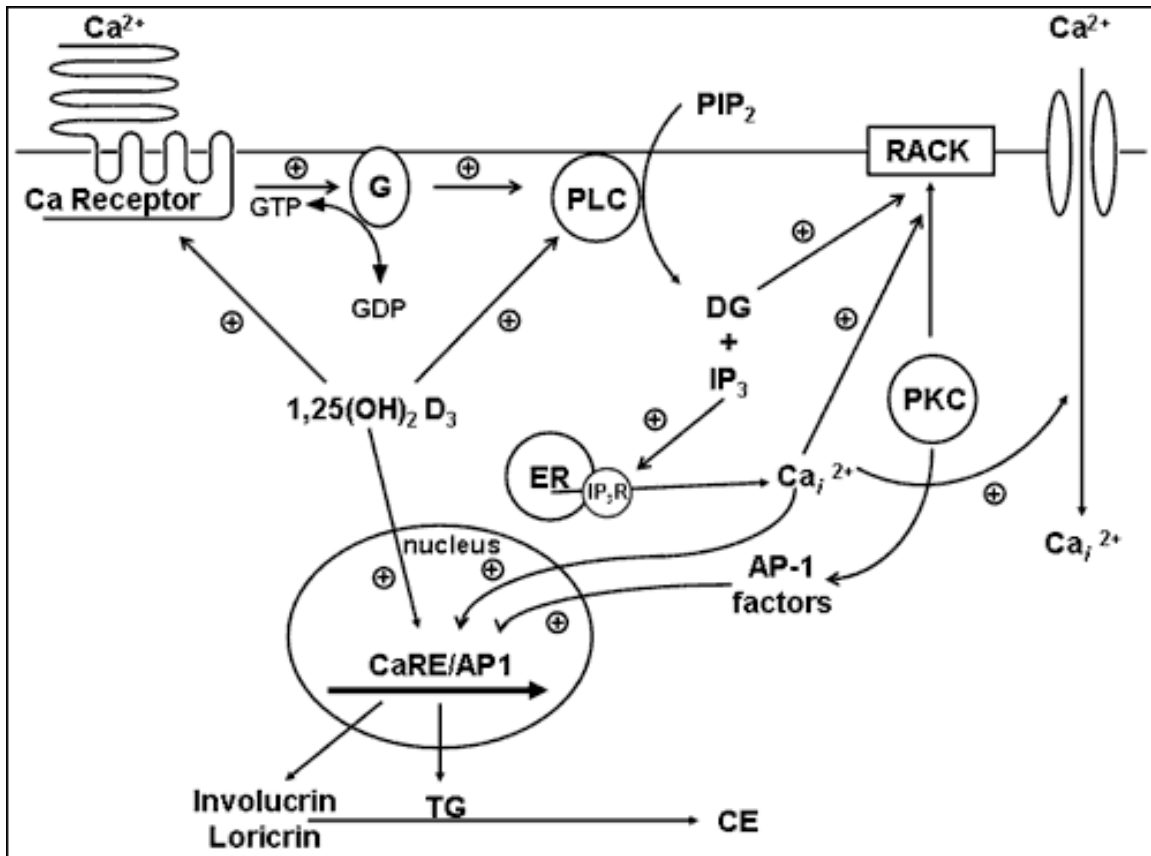
Phorbol esters, which bind to and activate PKC, are well known tumor promoters in skin. However, the initial effects of phorbol esters in vitro are to promote differentiation in cells grown in low calcium (337,369,370), effects which are potentiated by calcium (362). Phorbol esters stimulate PKC, and PKC inhibitors block the ability of both calcium and phorbol esters to promote differentiation (370). Phorbol esters as well as calcium stimulate the expression of both keratin 1 and involucrin gene constructs each of which contains an AP-1 site within the calcium response element (CaRE) of the promoter for these genes (371,372). If the AP-1 site within the CaRE is mutated, neither calcium nor phorbol esters are effective (371,372). These CaREs also contain VDREs (DR3), which at least in the involucrin gene has been shown to mediate 1,25(OH) $_2$ D regulation of this gene (373). Phorbol esters do not reproduce all the actions of calcium on the keratinocyte, and vice versa, but cross talk between their signaling pathways is clearly present.

The observation that 1,25(OH) $_2$ D induces keratinocyte differentiation was first made by Hosomi et al. (374) and provided a rationale for the previous and unexpected finding of 1,25(OH) $_2$ D receptors in the epidermis (375). 1,25(OH) $_2$ D increases the mRNA and protein levels for involucrin and transglutaminase, and promotes CE formation at subnanomolar concentrations in preconfluent keratinocytes (349,376-378). Calcium affects the ability of 1,25(OH) $_2$ D to stimulate keratinocyte differentiation, and vice versa. Calcium in the absence of 1,25(OH) $_2$ D and 1,25(OH) $_2$ D at low (0.03mM) calcium raise the mRNA levels for involucrin and transglutaminase in a dose dependent fashion by stimulating gene expression. The stimulation of mRNA levels by calcium and 1,25(OH) $_2$ D is synergistic at early time points; however, longer periods of incubation lead to a paradoxical fall in the mRNA levels for these proteins. This is due to the fact that although transcription is increased by calcium and 1,25(OH) $_2$ D, stability of the mRNA is reduced in cells incubated with calcium and 1,25(OH) $_2$ D.

The transcriptional regulation by 1,25(OH) $_2$ D is both direct and indirect. Several genes contain VDREs (eg. involucrin), but VDREs have not been found in all genes that are regulated by 1,25(OH) $_2$ D. Inhibition of PKC activity or mutation of the AP-1 site in the CaRE of the involucrin gene also blocks the ability of 1,25(OH) $_2$ D to regulate expression of involucrin (373). The ability of 1,25(OH) $_2$ D to increase intracellular calcium (Cai) (298) accounts for at least part of the ability of 1,25(OH) $_2$ D to induce differentiation. A rapid (presumably nongenomic) effect of 1,25(OH) $_2$ D on Cai has been described (379), although this response is controversial (377). Our studies indicate that the ability of

1,25(OH)<sub>2</sub>D to increase Cai requires time and gene transcription. 1,25(OH)<sub>2</sub>D increases CaSR mRNA levels and prevents their fall in cells grown in 0.03mM calcium (380). This results in an enhanced Cai response to extracellular calcium (Cao). 1,25(OH)<sub>2</sub>D also induces the family of PLCs (381). PLC-γ1 contains a VDRE in its promoter (145), which unlike the usual VDRE is a DR6 which binds VDR/RAR rather than VDR/RXR. Knocking out PLC-γ1 blocks 1,25(OH)<sub>2</sub>D induced differentiation (382) as well as calcium induced differentiation mentioned earlier. The other PLCs have not been studied as extensively, but are likely to show similar means of regulation by 1,25(OH)<sub>2</sub>D.

Our current working model for the mechanisms by which calcium and 1,25(OH)<sub>2</sub>D regulate keratinocyte differentiation is shown in figure 8. The keratinocyte expresses a CaSR that by coupling to and activating PLC controls the production of two important second messengers, IP<sub>3</sub> and DG. PLC-β is likely to be activated acutely by CaSR via a G protein coupled mechanism, whereas PLC-γ1 is activated acutely by calcium stimulated non receptor tyrosine kinases and subsequently by PIP3 in the membrane. Both PLCs are induced by calcium and 1,25(OH)<sub>2</sub>D. IP<sub>3</sub> stimulates the release of calcium from intracellular stores thus raising Cai. The initial release of calcium from these stores activates the Stim1/Orai1 channel in the membrane (383) that may stimulate proliferation of the basal keratinocytes and initiate their movement out of the basal layer. The increase in Cai and DG stimulates the activation of critical PKCs and their translocation to membrane receptors (RACK). PKC-α appears to be the most critical PKC for the subsequent events triggered by calcium in the keratinocyte, although PKCδ has also been implicated. Activated PKC leads to the induction and activation of AP-1 transcription factors which regulate the transcription of a number of genes including keratin 1, transglutaminase, involucrin, loricrin, and profilaggrin required for the differentiation process. Activation of the CaSR also activates the RhoA kinase leading to activation of src kinases which by phosphorylating various catenins leads to the formation of the Ecadherin/catenin complex in the membrane (384). This complex recruits both PI3K and PIP5K1α required to maintain the PIP2 and PIP3 levels in the membrane (336). PIP3 activates PLC-γ, that is in turn activates the TRPC channels in the membrane to enable the prolonged increase in Cai required for differentiation (385). 1,25(OH)<sub>2</sub>D, which is produced by the keratinocyte in a highly regulated fashion, modulates calcium regulated differentiation at several steps. First, 1,25(OH)<sub>2</sub>D increases CaSR expression, thus making the cell more responsive to calcium. Secondly, 1,25(OH)<sub>2</sub>D induces all the PLCs again increasing the responsiveness of the cell to calcium. Finally, 1,25(OH)<sub>2</sub>D has a direct effect on the transcription of the genes such as involucrin. The net result is that both calcium and 1,25(OH)<sub>2</sub>D promote keratinocyte differentiation through interactive mechanisms.



**Figure 8.** A model of  $1,25(\text{OH})_2\text{D}$  and calcium regulated keratinocyte differentiation. The G-protein coupled calcium receptor (CaSR) when activated by extracellular calcium activates  $G_\alpha$  as described in the legend to figure 6.  $G_\alpha$  stimulates PLC mediated hydrolysis of  $\text{PIP}_2$  to  $\text{IP}_3$  and  $\text{DG}$ .  $\text{IP}_3$  releases  $\text{Ca}_i$  from intracellular stores, and  $\text{DG}$  activates PKC. Depletion of intracellular calcium stores leads to influx of calcium across store operated calcium channels. PKC stimulation leads to activation of AP-1 transcription factors which along with calcium and  $1,25(\text{OH})_2\text{D}$  activated transcription factors stimulate the expression of genes essential for the differentiation process.  $1,25(\text{OH})_2\text{D}$  regulates this process by inducing CaSR and PLC as well as genes essential for cornified envelope formation such as involucrin and transglutaminase.

The VDR is also critical for hair follicle (HF) cycling. Unlike epidermal differentiation, hair follicle cycling is not dependent on  $1,25(\text{OH})_2\text{D}$ . Alopecia is a well described characteristic of mice and humans lacking VDR (117,118,386) due to failure to regenerate the cycling lower portion of the HF after the initial developmental cycle is completed. Deletion of CYP27B1 (387) and CaSR (361) do not result in alopecia. Cianferotti et al. (388) attributed the loss of HF cycling in VDR null mice to a gradual loss of the proliferative potential in the stem cells of the HF bulge region. However, this conclusion has been challenged by Palmer et al. (389), who attributed the failure of HF cycling in the VDR null mouse in part to a failure of the progeny of these stem cells to migrate out of the bulge rather than their loss of proliferative potential suggesting a loss of activation. The role of VDR in the stem cells that regulate both HF cycling and epidermal regeneration is also important in the skin wound healing process. When the skin is wounded the progeny of stem cells from all regions of the HF and interfollicular epidermis (IFE) contribute at least initially (390,391), although the stem cells in the IFE

make the most lasting contribution. Tian et al. (392) observed that topical  $1,25(\text{OH})_2\text{D}$  enhanced wound healing, suggesting that unlike HF cycling, the wound repair required this VDR ligand. Luderer et al. (393) observed that in the global VDRKO, there was a reduction in  $\text{TGF}\beta$  signaling in the dermis, and subsequently demonstrated that the VDR in macrophages but not in keratinocytes was responsible for macrophage recruitment during the inflammatory phase of cutaneous wound healing (394). Our studies have focused on the VDR in epidermal keratinocytes. We have observed that re-epithelialization by the keratinocytes over the wound is impaired when the deletion of VDR from keratinocytes is accompanied by either a low calcium diet or a deletion of the CaSR (395). Thus like the role of calcium and CaSR in vitamin D regulated keratinocyte differentiation so a similar synergism is seen in wound healing. These results are consistent with the loss of E-cadherin/catenin complex formation in the VDRKO keratinocyte, a complex that maintains stem cells in their niches (396), regulates when stem cell division is symmetric (to maintain stem cell numbers) or asymmetric (initiating differentiation) (397), and is essential for the ability of keratinocytes to migrate as a sheet to re-epithelialize the wound (398). As noted previously calcium and the CaSR along with  $1,25(\text{OH})_2\text{D}$  and VDR are required for E-cadherin/catenin complex formation during the differentiation process and so are involved in enabling its role in wound healing (399).

## **Immune system.**

The potential role for vitamin D and its active metabolite  $1,25(\text{OH})_2\text{D}_3$  in modulating the immune response has long been recognized since the discovery of vitamin D receptors (VDR) in macrophages, dendritic cells (DC) and activated T and B lymphocytes, the ability of macrophages and DC as well as activated T and B cells to express CYP27B1, and the ability of  $1,25(\text{OH})_2\text{D}_3$  to regulate the proliferation and function of these cells. While these are the key cells mediating the adaptive immune response,  $1,25(\text{OH})_2\text{D}$ , VDR, and CYP27B1 are also expressed in a large number of epithelial cells which along with the aforementioned members of the adaptive immune response contribute to host defense by their innate immune response. The totality of the immune response involves both types of responses in complex interactions involving numerous cytokines. The regulation of these different responses and their interactions by  $1,25(\text{OH})_2\text{D}_3$  is nuanced. In general  $1,25(\text{OH})_2\text{D}_3$  enhances the innate immune response primarily via its ability to stimulate cathelicidin, an antimicrobial peptide important in defense against invading organisms, whereas it inhibits the adaptive immune response primarily by inhibiting the maturation of dendritic cells (DC) important for antigen presentation, reducing T cell proliferation, and shifting the balance of T cell differentiation from the Th1 and Th17 pathways to Th2 and Treg pathways. Inflammatory autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis involve Th17 activation, a cell that expresses RANKL, and so can drive osteoclastogenesis leading to bone loss.

## **Adaptive immune response:**

The adaptive immune response is initiated by cells specialized in antigen presentation, DC and macrophages in particular, activating the cells responsible for subsequent antigen recognition, T and B lymphocytes. These cells are capable of a wide repertoire of responses that ultimately determine the nature and duration of the immune response. Activation of T and B cells occurs after a priming period in tissues of the body, eg. lymph nodes, distant from the site of the initial exposure to the antigenic substance, and is marked by proliferation of the activated T and B cells accompanied by post translational modifications of immunoglobulin production that enable the cellular response to adapt

specifically to the antigen presented. Importantly, the type of T cell activated, CD4 or CD8, or within the helper T cell class Th1, Th2, Th17, Treg, and subtle variations of those, is dependent on the context of the antigen presented by which cell and in what environment. Systemic factors such as vitamin D influence this process. Vitamin D in general exerts an inhibitory action on the adaptive immune system.  $1,25(\text{OH})_2\text{D}_3$  decreases the maturation of DC as marked by inhibited expression of the costimulatory molecules HLA-DR, CD40, CD80, and CD86, decreasing their ability to present antigen and so activate T cells (400). Furthermore, by suppressing IL-12 production, important for Th1 development, and IL-23 and IL-6 production important for Th17 development and function,  $1,25(\text{OH})_2\text{D}_3$  inhibits the development of Th1 cells capable of producing  $\text{IFN-}\gamma$  and IL-2, and Th17 cells producing IL-17(401). These actions prevent further antigen presentation to and recruitment of T lymphocytes (role of  $\text{IFN-}\gamma$ ), and T lymphocyte proliferation (role of IL-2). Suppression of IL-12 increases the development of Th2 cells leading to increased IL-4, IL-5, and IL-13 production, which further suppresses Th1 development shifting the balance to a Th2 cell phenotype. Treatment of DCs with  $1,25(\text{OH})_2\text{D}_3$  can also induce CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells (Treg) cells (402) as shown by increased FoxP3 expression, critical for Treg development. These cells produce IL-10, which suppresses the development of the other Th subclasses. Treg are critical for the induction of immune tolerance (403). In addition,  $1,25(\text{OH})_2\text{D}_3$  alters the homing of properties of T cells for example by inducing expression of CCR10, the receptor for CCL27, a keratinocyte specific cytokine, while suppressing that of CCR9, a gut homing receptor (404). The actions of  $1,25(\text{OH})_2\text{D}_3$  on B cells have received less attention, but recent studies have demonstrated a reduction in proliferation, maturation to plasma cells and immunoglobulin production (405).

$1,25(\text{OH})_2\text{D}_3$  has both direct and indirect effects on regulation of a number of cytokines involved with the immune response (review in (406)). TNF has a VDRE in its promoter to which the VDR/RXR complex binds.  $1,25(\text{OH})_2\text{D}_3$  both blocks the activation of  $\text{NF}\kappa\text{B}$  via an increase in  $\text{I}\kappa\text{B}\alpha$  expression and impedes its binding to its response elements in the genes such as IL-8 and IL-12 that it regulates.  $1,25(\text{OH})_2\text{D}_3$  has also been shown to bring an inhibitor complex containing histone deacetylase 3 (HDAC3) to the promoter of rel B, one of the members of the  $\text{NF}\kappa\text{B}$  family, thus suppressing gene expression. Thus, TNF/ $\text{NF}\kappa\text{B}$  activity is markedly impaired by  $1,25(\text{OH})_2\text{D}_3$  at multiple levels. In VDR null fibroblasts,  $\text{NF}\kappa\text{B}$  activity is enhanced. Furthermore,  $1,25(\text{OH})_2\text{D}_3$  suppresses  $\text{IFN}\gamma$ , and a negative VDRE has been found in the  $\text{IFN}\gamma$  promoter. GM-CSF is regulated by VDR monomers binding to a repressive complex in the promoter of this gene, competing with nuclear factor of T cells 1(NFAT1) for binding to the promoter.

The ability of  $1,25(\text{OH})_2\text{D}_3$  to suppress the adaptive immune system appears to be beneficial for a number of conditions in which the immune system is directed at self—ie. autoimmunity (review in (407)). In a number of experimental models including inflammatory arthritis, psoriasis, autoimmune diabetes (eg. NOD mice), systemic lupus erythematosus (SLE), experimental allergic encephalitis (EAE) (a model for multiple sclerosis), inflammatory bowel disease (IBD), prostatitis, and thyroiditis VDR agonist administration has prevented and/or treated the disease process. As will be discussed later, a number of these conditions are associated with bone loss either directly (eg. inflammatory arthritis) or indirectly presumably via increased serum levels of inflammatory cytokines. These actions of  $1,25(\text{OH})_2\text{D}_3$  were originally ascribed to inhibition of Th1 function, but Th17 cells have recently been shown to play important roles in a number of these conditions including psoriasis [321], experimental colitis(401), and rheumatoid arthritis (408), conditions that respond to  $1,25(\text{OH})_2\text{D}_3$  and its analogs.

Although few prospective, randomized, placebo controlled trials in humans have been performed, epidemiologic and case control studies indicate that a number of these diseases in humans are favorably impacted by adequate vitamin D levels. For example, the incidence of multiple sclerosis correlates inversely with 25OHD levels and vitamin D intake, and early studies suggested benefit in the treatment of patients with rheumatoid arthritis and multiple sclerosis with VDR agonists (406,407). Similarly, IBD is associated with low vitamin D levels (409). Children who are vitamin D deficient have a higher risk of developing type 1 diabetes mellitus, and supplementation with vitamin D during early childhood reduces the risk of developing type 1 diabetes (review in (400)). In VDR null mice myelopoeisis and the composition of lymphoid organs are normal, although a number of abnormalities in the immune response have been found. Some of the abnormalities in macrophage function and T cell proliferation in response to anti-CD3 stimulation in these animals could be reversed by placing the animals on a high calcium diet to normalize serum calcium (410), indicating the important role of calcium in vitamin D regulated immune function as in skeletal development and maintenance, hormone regulation, and keratinocyte differentiation. Other studies have noted an increased number of mature DCs in the lymph nodes of VDR null mice, which would be expected to promote the adaptive immune response (411). Somewhat surprisingly, RANKL also increases the number and retention of DCs in lymph nodes (412) suggesting that at least this mechanism is not mediated via the RANKL/RANK system in VDR null mice, which I will discuss at length subsequently. In contrast to these inhibitory actions of  $1,25(\text{OH})_2\text{D}_3$ , Th2 function as indicated by increased IgE stimulated histamine from mast cells is increased in VDR null mice (413). The IL-10 null mouse model of IBD shows an accelerated disease profile when bred with the VDR null mouse with increased expression of Th1 cytokines (414). Surprisingly, despite a reduction in natural killer T cells and Treg cells and a decreased number of mature DCs, VDR null mice bred with NOD mice do not show accelerated development of diabetes (415). Part of the difference in tissue response in VDR null mice may relate to differences in the ability of  $1,25(\text{OH})_2\text{D}_3$  to alter the homing of T cells to the different tissues (404). In allergic airway disease (asthma) Th2 cells, not Th1 cells, dominate the inflammatory response.  $1,25(\text{OH})_2\text{D}_3$  administration to normal mice protected these mice from experimentally induced asthma in one study, blocking eosinophil infiltration, IL-4 production, and limiting histologic evidence of inflammation (416). However, a study with VDR null mice using a comparable method of inducing asthma showed that lack of VDR also protected the mice from an inflammatory response in their lungs (417). In an extension of this study the investigators showed that wildtype (WT) splenocytes were only minimally successful at restoring experimental airway inflammation to VDR null mice, whereas splenocytes from these mice were able to transfer experimental airway inflammation to the unprimed WT host (418). Thus the impact of vitamin D signaling on adaptive immunity depends on the specifics of the immune response being evaluated.

Inhibition of the adaptive immune response may also have benefit in transplantation procedures (419). In experimental allograft models of the aorta, bone, bone marrow, heart, kidney, liver, pancreatic islets, skin, and small bowel VDR agonists have shown benefit generally in combination with other immunosuppressive agents such as cyclosporine, tacrolimus, sirolimus, and glucocorticoids (419). Much of the effect could be attributed to a reduction in infiltration of Th1 cells, macrophages and DC into the grafted tissue associated with a reduction in chemokines such as CXCL10, CXCL9, CCL2, and CCL5. CXCL10, the ligand for CXCR3, may be of particular importance for acute rejection in a number of tissues, whereas CXCL9 as well as CXCL10 (both CXCR3 ligands) may be more important for chronic rejection at least in the heart and kidney, respectively. Although there are no prospective trials of the use of VDR agonists



in transplant patients, several retrospective studies in patients with renal transplants treated with  $1,25(\text{OH})_2\text{D}_3$  have suggested benefit with respect to prolonged graft survival and reduced numbers of acute rejection episodes.

Suppression of the adaptive immune system may not be without a price. Several publications have demonstrated that for some infections including *Leishmania major* (420) and toxoplasmosis (421),  $1,25(\text{OH})_2\text{D}_3$  promotes the infection (421), while the mouse null for VDR is protected (420). This may be due at least in part to loss of  $\text{IFN}\gamma$  stimulation of ROS and NO production required for macrophage antimicrobial activity (420). Furthermore, atopic dermatitis, a disease associated with increased Th2 activity (422), and allergic airway disease, likewise associated with increased Th2 activity, (416-418), may be aggravated by  $1,25(\text{OH})_2\text{D}_3$  and less severe in animals null for VDR.

### **The innate immune response:**

The innate immune response involves the activation of toll-like receptors (TLRs) in polymorphonuclear cells (PMNs), monocytes and macrophages as well as in a number of epithelial cells including those of the epidermis, gingiva, intestine, vagina, bladder and lungs (review in (423)). There are 10 functional TLRs in human cells (of 11 known mammalian TLRs). TLRs are an extended family of host noncatalytic transmembrane pathogen-recognition receptors that interact with specific membrane patterns (PAMP) shed by infectious agents that trigger the innate immune response in the host. A number of these TLRs signal through adapter molecules such as myeloid differentiation factor-88 (MyD88) and the TIR-domain containing adapter inducing  $\text{IFN}\beta$  (TRIF). MyD88 signaling includes translocation of NF $\kappa$ B to the nucleus, leading to the production and secretion of a number of inflammatory cytokines. TRIF signaling leads to the activation of interferon regulatory factor-3 (IRF-3) and the induction of type 1 interferons such as  $\text{IFN}\beta$ . MyD88 mediates signaling from TLRs 2, 4, 5, 7 and 9, whereas TRIF mediates signaling from TLR 3 and 4. TLR1/2, TLR4, TLR5, TLR2/6 respond to bacterial ligands, whereas, TLR3, TLR7, and TLR 8 respond to viral ligands. The TLR response to fungi is less well defined. CD14 serves as a coreceptor for a number of these TLRs. Activation of TLRs leads to the induction of antimicrobial peptides (AMPs) and reactive oxygen species, which kill the organism. Among these AMPs is cathelicidin. Cathelicidin plays a number of roles in the innate immune response. The precursor protein, hCAP18, must be cleaved to its major peptide LL-37 to be active. In addition to its antimicrobial properties, LL-37 can stimulate the release of cytokines such as IL-6 and IL-10 through G protein coupled receptors, and IL-18 through ERK/P38 pathways, stimulate the EGF receptor leading to activation of STAT1 and 3, induce the chemotaxis of neutrophils, monocytes, macrophages, and T cells into the skin, and promote keratinocyte proliferation and migration(424). The expression of this antimicrobial peptide is induced by  $1,25(\text{OH})_2\text{D}_3$  in both myeloid and epithelial cells(425,426). In addition,  $1,25(\text{OH})_2\text{D}_3$  induces the coreceptor CD14 in keratinocytes(427). Stimulation of TLR2 by an antimicrobial peptide in macrophages (428) or stimulation of TLR2 in keratinocytes by wounding the epidermis (427) results in increased expression of CYP27B1, which in the presence of adequate substrate (25OHD) stimulates the expression of cathelicidin. Lack of substrate (25OHD) or lack of CYP27B1 blunts the ability of these cells to respond to a challenge with respect to cathelicidin and/or CD14 production (426-428). In diseases such as atopic dermatitis, the production of cathelicidin and other antimicrobial peptides (AMPs) is reduced, predisposing these patients to microbial superinfections (429). Th2 cytokines such as IL-4 and 13 suppress the induction of AMPs(430). Since  $1,25(\text{OH})_2\text{D}_3$

stimulates the differentiation of Th2 cells, in this disease  $1,25(\text{OH})_2\text{D}_3$  administration may be harmful. An important role of these AMPs besides their antimicrobial properties is to help link the innate and adaptive immune response.

Although many cells are capable of the innate immune response including bone cells, most studies have focused on the macrophage and the keratinocyte. Vitamin D regulation of the innate immune response in these two cell types is comparable, but differences exist.

**Macrophages.** The importance of adequate vitamin D nutrition for resistance to infection has long been appreciated but poorly understood. This has been especially true for tuberculosis. Indeed, prior to the development of specific drugs for the treatment of tuberculosis, getting out of the city into fresh air and sunlight was the treatment of choice. In a recent survey of patients with tuberculosis in London (431) 56% had undetectable 25OHD levels, and an additional 20% had detectable levels but below 9 ng/ml (22 nM). In 1986 Rook et al. (432) demonstrated that  $1,25(\text{OH})_2\text{D}_3$  could inhibit the growth of *Mycobacterium tuberculosis*. The mechanism for this remained unclear until the publication by Liu et al. (428) of their results in macrophages. They observed that activation of the Toll-like receptor TLR2/1 by a lipoprotein extracted from *M. tuberculosis* reduced the viability of intracellular *M. tuberculosis* in human monocytes and macrophages concomitant with increased expression of the VDR and of CYP27B1 in these cells. Killing of *M. tuberculosis* occurred only when the serum in which the cells were cultured contained adequate levels of 25OHD, the substrate for CYP27B1. This provided clear evidence for the importance of vitamin D nutrition (as manifested by adequate serum levels of 25OHD) in preventing and treating this disease, and demonstrated the critical role for endogenous production of  $1,25(\text{OH})_2\text{D}_3$  by the macrophage to enable its antimycobacterial capacity. Activation of TLR2/1 or directly treating these cells with  $1,25(\text{OH})_2\text{D}_3$  induced the antimicrobial peptide cathelicidin, which is toxic for *M. tuberculosis*. If induction of cathelicidin is blocked as with siRNA, the ability of  $1,25(\text{OH})_2\text{D}_3$  to enhance the killing of *M. tuberculosis* is prevented (433). Furthermore,  $1,25(\text{OH})_2\text{D}_3$  also induces the production of reactive oxygen species which if blocked likewise prevents the antimycobacterial activity of  $1,25(\text{OH})_2\text{D}_3$  treated macrophages (434). The murine cathelicidin gene lacks a known VDR response element in its promoter, and so might not be expected to be induced by  $1,25(\text{OH})_2\text{D}_3$  in mouse cells, yet  $1,25(\text{OH})_2\text{D}_3$  stimulates antimycobacterial activity in murine macrophages. Murine macrophages, unlike human macrophages, utilize inducible nitric oxide synthase (iNOS) for their TLR and  $1,25(\text{OH})_2\text{D}_3$  mediated killing of *M. tuberculosis* (434,435). Clinical trials attempting to treat tuberculosis patients with high levels of vitamin D have shown mixed results (436), (437)

**Keratinocytes.** Cathelicidin and CD14 expression in epidermal keratinocytes is induced by  $1,25(\text{OH})_2\text{D}_3$  (424,427). In these cells butyrate, which by itself has little effect, potentiates the ability of  $1,25(\text{OH})_2\text{D}_3$  to induce cathelicidin (438). Keratinocytes treated with  $1,25(\text{OH})_2\text{D}_3$  are substantially more effective in killing *Staphylococcus aureus* than are untreated keratinocytes. Wounding the epidermis induces the expression of TLR2 and that of its co-receptor CD14 and cathelicidin (427). This does not occur in mice lacking CYP27B1 (427). Unlike macrophages,  $1,25(\text{OH})_2\text{D}_3$  stimulates TLR2 expression in keratinocytes as well as in the epidermis when applied topically (427) providing a feed forward loop to amplify the innate immune response. Wounding also increases the expression of CYP27B1. This may occur as a result of increased levels of cytokines such as  $\text{TNF-}\alpha$  and  $\text{IFN-}\gamma$ , both of which we have shown stimulate  $1,25(\text{OH})_2\text{D}_3$  production, as well as by  $\text{TGF-}\beta$  and the TLR2 ligand Malp-2 (427). When the levels of VDR or one of its principal coactivators, SRC3, are reduced using siRNA technology, the

ability of  $1,25(\text{OH})_2\text{D}_3$  to induce cathelicidin and CD14 expression in human keratinocytes is markedly blunted (438).

### **Other tissues.**

The VDR is widespread (119,439) (reviews). In some of these tissues the functional significance of the VDR and/or the effect of  $1,25(\text{OH})_2\text{D}$  are unclear. Since several of the functions regulated by  $1,25(\text{OH})_2\text{D}$  in some of these tissues may have clinical relevance, this section will focus on a select number of these tissues.

### **Heart.**

A reduction in contractility has been observed in vitamin D deficient animals (440). This may be due to lack of vitamin D or the accompanying hypocalcemia and hypophosphatemia. However, *in vitro*  $1,25(\text{OH})_2\text{D}$  stimulates calcium uptake by cardiac muscle cells (441,442). In addition  $1,25(\text{OH})_2\text{D}$  inhibits the expression of atrial natriuretic factor, one of the few genes with a negative VDRE in its promoter (443). Deletion of the VDR specifically in cardiac muscle leads to hypertrophy and fibrosis (444). Low circulating levels of  $25\text{OHD}$  are associated with increased risk of myocardial infarction in men [436].

### **Skeletal muscle.**

Proximal muscle weakness is a hallmark of vitamin D deficiency, and reduced high energy substrates (ATP, creatinine phosphate) have been observed in that condition (445). Myoblasts contain VDR, although the expression of VDR in mature muscle cells is controversial. Muscle weakness may reflect the lower levels of calcium and phosphate rather than a reduction in  $1,25(\text{OH})_2\text{D}$ . However, evidence for a direct role of  $1,25(\text{OH})_2\text{D}$  and VDR in muscle function is increasing (446). Moreover,  $1,25(\text{OH})_2\text{D}$  may have actions on muscle that do not require the VDR, at least the genomic functions of VDR. The Boland laboratory (447) has demonstrated acute effects of  $1,25(\text{OH})_2\text{D}$  on calcium uptake, PLC,  $\text{PLA}_2$ , PLD, PKC, and adenylate cyclase activities, all of which may alter muscle function.

### **Pituitary.**

VDR have been found primarily in thyrotropes *in vivo* and in GH and prolactin secreting cell lines *in vitro* (448,449).  $1,25(\text{OH})_2\text{D}$  increases TRH stimulated TSH secretion by a mechanism involving increased  $\text{Ca}^{2+}$  and  $\text{IP}_3$  production (450,451), suggesting that induction of PLC by  $1,25(\text{OH})_2\text{D}$  may be involved.

### **Breast**

The breast contains VDR (452), and vitamin D plays a role in normal breast development (453). Moreover, breast cancer cells also contain VDR (454), and  $1,25(\text{OH})_2\text{D}$  and its analogs reduce their proliferation *in vivo* and *in vitro* (455,456). This has obvious clinical implications for the treatment of breast cancer.

## **Liver.**

Low levels of VDR have been found in the liver (457). Hepatic regeneration is impaired in vitamin D deficient animals, even when the serum calcium is normalized by a high calcium diet (458), suggesting a role for 1,25(OH)<sub>2</sub>D in hepatic cell growth.

## **Lung.**

VDR have been found in type II epithelial pneumocytes (459). 1,25(OH)<sub>2</sub>D stimulates their maturation including increased phospholipid production and surfactant release [437]. These results are consistent with the abnormal alveolar development observed in pups born to vitamin D deficient mothers (460). In addition 1,25(OH)<sub>2</sub>D stimulates the innate immune response in bronchial epithelial cells and may provide protection in patients with cystic fibrosis with recurrent lung infections (461).

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