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VITAMIN D RECEPTORS: NATURE AND FUNCTION

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PERSPECTIVES AND SUMMARY

The discovery of receptors for vitamins, hormones, neurotransmitters, and other biological modifiers has contributed greatly to our understanding of the molecular aspects of nutrition, physiology, and medicine. Notable examples

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are the receptors for LDL-cholesterol, estrogen, insulin, acetylcholine, and the opiates. In many cases, the characterization of receptors has yielded information about the nature and quantitation of the endogenous ligand, its biochemical mechanism of action, and the pathophysiology of its related disease states. Such is the case for the role of vitamin D in controlling calcium and bone metabolism. The vitamin D receptor was first revealed in 1969 as a chromosomal protein in intestinal mucosa nuclei that specifically bound the most active metabolite of the parent vitamin (80). This metabolite was later identified (90) as 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the hormonal ligand that occupies the vitamin D receptor in vivo. The vitamin D story parallels other scenarios of the interface between nutrition and endocrine systems. For example, iodine is a crucial nutritional factor in thyroid hormone biosynthesis, and tetraiodothyronine is metabolized to its more active metabolite, triiodothyronine, which localizes in target cell nuclei via a receptor similar to that for $1,25(OH)_2D_3$ (20). The receptor for $1,25(OH)_2D_3$ has been employed in radioreceptor assays for circulating $1,25(OH)_2D_3$ in animals and humans (14, 50), which gave new insight into both the physiology and pathology of the vitamin D–endocrine system (78). Recently, genetic defects have been discovered in the $1,25(OH)_2D_3$ receptor that are manifested by a type of vitamin D resistance that clinically resembles classical rickets (117).

Biochemical studies of the $1,25(OH)_2D_3$ receptor indicate that the mode of action of the vitamin D sterol is similar to that of steroid and thyroid hormones, with the active metabolite complexing with a selective high-affinity binding protein that concentrates the hormone in the nucleus. The occupied receptor is then postulated to regulate gene expression and induce proteins that alter the functions of target cells. The $1,25(OH)_2D_3$ receptor is a DNA-binding protein (157) with biochemical properties remarkably similar to those of steroid and thyroid hormone receptors. $1,25(OH)_2D_3$ receptor proteins have molecular weights between 50,000 and 60,000, they bind $1,25(OH)_2D_3$ with very high affinity ($K_d = 10^{-11}$M) and selectivity over other vitamin D metabolites, and they possess essential sulfhydryl groups in both their hormone- and DNA-binding domains (75). Recent work suggests that the $1,25(OH)_2D_3$ receptor is itself modulated, both quantitatively in that it is up-regulated by vitamin D metabolites (35) and qualitatively by phosphorylation in the presence of $1,25(OH)_2D_3$ (160). Monoclonal antibodies generated against the receptor (39, 155, 159) have facilitated unequivocal identification of the monomeric protein through immunoblotting, and permitted the analysis of its biosynthesis in cultured cells or via translation of its mRNA in vitro. Using antibody screening of expression vector cDNA libraries, it may soon be possible to clone the vitamin D receptor gene, as has just been accomplished for estrogen (194) and glucocorticoid receptors (91). At this point, there is little doubt that the receptor mediates the actions of $1,25(OH)_2D_3$ at the level of DNA and the goal of the

future is to elucidate the chemical and physical details of this biochemical event.

Finally, using the $1,25(OH)_2D_3$ receptor as a marker for vitamin D target organs, surprising new sites for vitamin D function have been postulated, including a number of endocrine glands, skin, breast, brain, thymus, and bone marrow. Therefore, vitamin D apparently has a myriad of biological actions beyond those involved in traditional control of bone and mineral metabolism at the intestine, kidney, and bone. It is probable that $1,25(OH)_2D_3$, like the vitamin A metabolite retinoic acid, plays a basic role in cell differentiation—especially in the hematopoietic system (124). There are also indications that, by analogy with vitamin A, vitamin D may act as an anticancer agent in certain situations (28). Thus, what was originally considered an antirachitic vitamin is now recognized as the precursor to a powerful sterol hormone capable not only of effecting calcium and skeletal homeostasis, but also of fundamental actions on cell proliferation and differentiation. The current challenge is to characterize the detailed molecular mechanism of action of $1,25(OH)_2D_3$ in its spectrum of target cells and to integrate these individual functions into a picture of vitamin D–mediated cell development as well as nutritional and physiological adaptation.

BACKGROUND AND SIGNIFICANCE

Vitamin D Metabolism and Biologic Functions

The focus of the present chapter is the receptor protein for the vitamin D hormone, but it is necessary to review first the significant advances in our comprehension of vitamin D metabolism and actions that have occurred in the last 20 years. This material is outlined only briefly because it has been the subject of several extensive reviews (42, 78, 86, 142).

As summarized in Figure 1, the functional metabolism of vitamin D_3, the sunlight vitamin, consists of an initial 25-hydroxylation primarily in liver, followed by 1-hydroxylation of 25-hydroxyvitamin D_3 [$25(OH)D_3$] in the kidney. The $1,25(OH)_2D_3$ product is likely the sole biologically significant metabolite of the vitamin that acts in target cells (13, 71, 73, 81). Its main functions are the stimulation of intestinal calcium and phosphate absorption, mediation of bone remodeling, and conservation of minerals at the kidney. $1,25(OH)_2D_3$ also has biologic effects not directly related to mineral transport or mineralization, including affecting sterol metabolism in skin (52), stimulating macrophage differentiation (124), modifying T-lymphocyte activity (193), and influencing the secretion of a number of peptide hormones [e.g. parathyroid hormone (43), prolactin (200), and insulin (102)]. Predominantly in kidney, but probably to some degree in all target cells, $1,25(OH)_2D_3$ induces a 24-hydroxylase (24-OHase) enzyme that initiates a catabolic cascade for the

side-chain oxidation, cleavage, and ultimate metabolic elimination of the $1,25(OH)_2D_3$ hormone (Figure 1) as well as its $25(OH)D_3$ precursor (21, 100, 130, 141). Considering that the renal production of $1,25(OH)_2D_3$ is strictly controlled by the calcium and phosphorus needs of the organism (Figure 1; see also 78), it is clear that the levels and actions of the $1,25(OH)_2D_3$ hormone are dynamically regulated. For instance, during nutritional calcium deprivation, $1,25(OH)_2D_3$ biosynthesis is enhanced by parathyroid hormone (PTH) and in part by low blood calcium itself (94). Intestinal calcium absorption is then accelerated to correct the hypocalcemia and spare skeletal mineral, while at the same time $1,25(OH)_2D_3$ initiates its own biodegradation via the 24-OHase cascade. In this fashion the metered production of $1,25(OH)_2D_3$ and its biochemical actions delicately coordinate the physiological adaptation of the organism to varying situations of mineral nutrition.

Receptor Discovery and Evidence that it Mediates Vitamin D Action

The first insight that a biologically active vitamin D metabolite functions analogously to steroid hormones was obtained in 1968 when Haussler et al (79) demonstrated that this metabolite was localized in target tissue nuclear chromatin in a saturable and specific manner after vitamin D administration in vivo. It was next reported by Haussler & Norman (80) in 1969 that the vitamin D metabolite could be extracted from chromatin by KCl, with 50% extraction occurring at 0.2-M KCl. Significantly, the metabolite remained bound to a receptor-like protein in the KCl extract as demonstrated by ammonium sulfate precipitation and CsCl ultracentrifugation. In fact, gel filtration suggested a M_r of 50,000–70,000 for the protease-sensitive metabolite receptor (80). These data constitute the discovery of the vitamin D receptor and, interestingly, predate the structural identification of the active vitamin D metabolite as $1,25(OH)_2D_3$ (90).

Tsai & Norman (192) next showed that $1,25(OH)_2D_3$ association with chromatin in reconstituted systems was facilitated by a soluble factor. This factor was conclusively shown to be receptor-like by Brumbaugh & Haussler (15–17), who reported the following three critical characteristics of the macromolecule: (*a*) binds vitamin D analogs in a rank order corresponding to their biologic potencies (15), (*b*) sediments at 3.0–3.5S in high salt-sucrose gradients, and (*c*) displays saturable high-affinity binding in vitro (17). These studies in 1973 and 1974 comprised the first pharmacological and biochemical identification of the receptor in vitro. An important property of the receptor was revealed in 1979 when Pike & Haussler (157) observed that it is a DNA-binding protein and the molecule was first purified utilizing DNA cellulose chromatography. Denaturing electrophoresis demonstrated that the purified chick intestinal $1,25(OH)_2D_3$ receptor consisted of several protein species of 50,000–65,000 daltons (157).

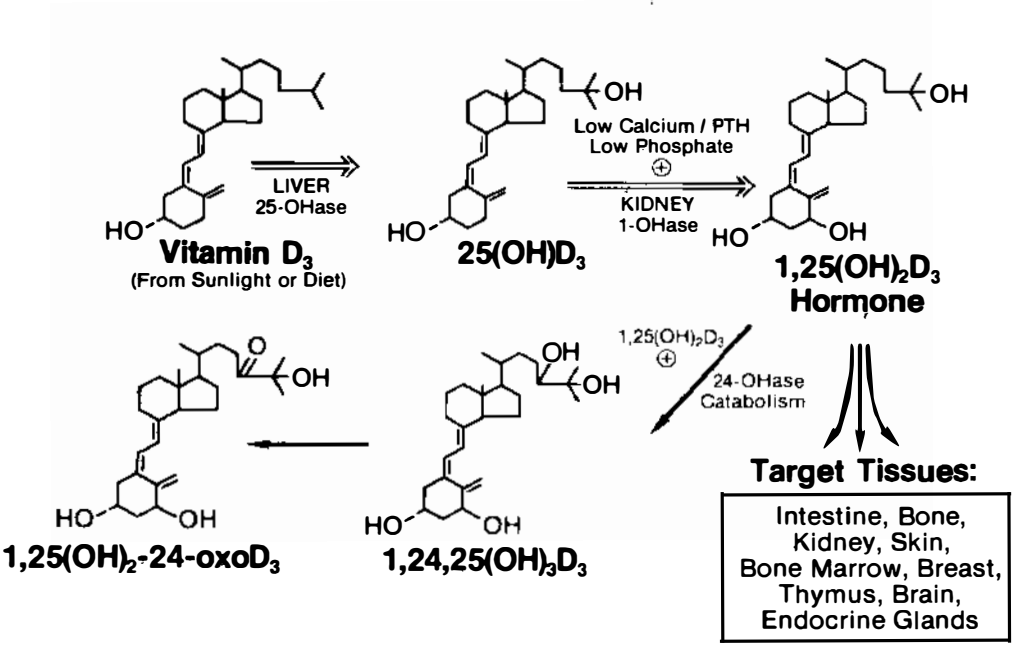

Figure 1 The vitamin D endocrine system. The scheme for 24-OHase-initiated side-chain oxidation of 1,25(OH)$_2D_3$ also occurs with 25(OH)D_3 as a starting substrate, although for simplicity this pathway is not shown. 1,25(OH)$_2$-24-oxoD_3 is eventually converted via side-chain cleavage to calcitroic acid (1α-hydroxy-23-carboxytetranorvitamin D), the probable excreted form of the hormone. Vitamin D_2 taken as a supplement or from diet is metabolized similarly to vitamin D_3.

There are now many lines of evidence indicating that the receptor protein mediates most, if not all, of the biologic functions of vitamin D. One of the more convincing of these is the strong positive correlation between binding potency of a series of vitamin D metabolites and analogs and their biological activity. This correlation holds when the analogs are assayed in vivo (70, 162), and in isolated systems such as calcium mobilization from bone in tissue culture (181). It is important to recognize that the relatively high association constant (10^{11} M^{-1}) with which the intracellular receptor protein binds $1,25(OH)_2D_3$ compared to other metabolites like $25(OH)D_3$ is reversed in the case of the serum vitamin D–binding protein (DBP) (71, 81). Thus DBP binds $25(OH)D_3$ and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] more avidly than the $1,25(OH)_2D_3$ hormone, which allows the intracellular receptor selectively to concentrate and retain $1,25(OH)_2D_3$ in vitamin D target cells (126).

The fact that the receptor was originally discovered in the major vitamin D target organ, namely intestinal mucosa, and was subsequently revealed in other sites of mineral translocation or regulation, i.e. parathyroid gland (19, 85), bone (110), and kidney (22, 29, 33), adds credence to its proposed role in vitamin D action. These tissues persist as locations with the highest concentrations of $1,25(OH)_2D_3$ receptor, but many new target tissues and cell types for $1,25(OH)_2D_3$ have been identified by biochemical detection of receptor and via autoradiographic localization of tritiated $1,25(OH)_2D_3$ in vivo (82, 184). Many of these new targets are mineral transport sites (shell gland, chorioallantoic membrane, etc), endocrine organs (pancreas, pituitary, ovary, testis, etc), and reproductive tissues (breast, placenta, uterus, etc). The skin, where the photobiosynthesis of vitamin D occurs, is also a location for the $1,25(OH)_2D$ receptor (54, 178).

These exciting findings do not negate the concept that the receptor is a marker for the calcium homeostatic actions of vitamin D, but instead bring to light the theory that vitamin D functions in a vast array of target cells, more akin to vitamin A and steroid hormones like the glucocorticoids. The physiologic significance of $1,25(OH)_2D_3$ action in its newly identified target cells has not been fully evaluated, but it is apparent that disorders of calcium absorption and bone mineralization dominate the clinical picture in simple vitamin D–deficient rickets or osteomalacia. In type II vitamin D–dependent rickets, which is a hereditary hypocalcemic syndrome characterized by resistance to $1,25(OH)_2D_3$, the receptor protein in fibroblasts from the affected patients is defective (49, 53, 117, 154). Despite the fact that these patients have excessive circulating levels of $1,25(OH)_2D_3$, they still display hypocalcemia and severe rickets. Since their genetic defect is presumably a point mutation destroying $1,25(OH)_2D_3$ receptor function, it is clear that this disease constitutes the most compelling evidence that the $1,25(OH)_2D_3$ receptor is obligatory and mediates the action of vitamin D to prevent rickets.

Occurrence and Subcellular Distribution of the Receptor

As outlined above, it is now apparent that the distribution of the $1,25(OH)_2D_3$ receptor is broad, including a vast array of putative target cell types. In some cases these new target tissues were identified by classical ligand-binding experiments (19, 22, 29, 33, 42, 54, 82, 85, 110, 142, 156, 178).

However, two new developments in $1,25(OH)_2D_3$ binding studies have been the use of autoradiographic localization of $1,25(OH)_2D[^3H]D_3$ in vivo (183), and of cultured cell lines in vitro. Autoradiographic localization of the hormone has not only confirmed that $1,25(OH)_2D_3$ is concentrated in nuclei of target tissues like intestine, parathyroid, bone, and kidney, but has verified (and in some cases predicted) new target sites for the hormone. More importantly, autoradiographic techniques allow for the identification of individual cell types that localize and probably respond to the hormone. In this fashion, Stumpf and associates (183) demonstrated the following unique target cells for $1,25(OH)_2D_3$: neurons in brain and spinal cord, anterior pituitary thyrotrophs, gastric endocrine cells, pancreatic beta cells, bone marrow reticular cells, chondroblasts, and osteoblasts. Cultured cells also offer systems where homogeneous populations of cells can be probed for the $1,25(OH)_2D_3$ receptor. The receptor has been detected in human breast cancer cells (51), rat osteogenic sarcoma cells (44, 127), rat somatomammotrophic pituitary cells (77), human melanoma cells (32), human promyelocytic leukemia cells (124), human lymphoblasts (163), and embryonic mouse fibroblasts (158). These established cell lines are excellent systems for investigating the $1,25(OH)_2D_3$ receptor and its functions and are currently under intensive investigation in many laboratories. One caveat to using these model systems is that they are transformed cell lines that may have altered receptor dynamics because of their uncontrolled proliferation and relative state of dedifferentiation. However, studies to date seem to indicate that they are valid models that reflect the biochemistry of vitamin D action in their respective normal counterparts.

Table 1 presents a compilation of the known target locations for the $1,25(OH)_2D_3$ receptor, combining biochemical, autoradiographic and established cell line results. It should be noted that $1,25(OH)_2D_3$ receptors have also been detected in amphibians (82), as well as in specialized avian and fish tissues. Unique avian vitamin D receptor locations include the shell gland (37) and chorioallantoic membrane (38), while those in the fish are the gill, corpuscles of Stannius, and, surprisingly, the liver (128). Clearly, more tissues contain the $1,25(OH)_2D_3$ receptor than do not, although a clear pattern is emerging to indicate that vitamin D functions in select cell types, predomonantly to mediate mineral, endocrine, and reproductive actions. It is also apparent that, like other steroid hormones, $1,25(OH)_2D_3$ is potentially a neuromodulator and immune regulator. What must be determined is whether there is some common denominator in the action of $1,25(OH)_2D_3$ in all cells, such as

intracellular calcium fluctuation, or whether $1,25(OH)_2D_3$ is a basic regulator of cell maturation. It is conceivable that vitamin D affects all cells during a certain period of their differentiation. Thus in fully differentiated intestinal epithelial cells, $1,25(OH)_2D_3$ is a potent inducer of calcium transport and this action correlates with the appearance and escalation of the receptor during embryonic and neonatal development in the chick (176) and rat (68), respectively. In contrast, adult avian and mammalian liver are not considered targets for vitamin D, but recent evidence from our laboratory (K. Yamaoka, S. L. Marion, J. W. Pike, and M. R. Haussler, unpublished information) suggests that early chick embryo liver possesses the $1,25(OH)_2D_3$ receptor and loses it during embryonic development. Do $1,25(OH)_2D_3$ and its receptor play some role in initial liver cell differentiation, or are such embryonic receptors vestigal? Answering this question is critical to evaluating the biological relevance of $1,25(OH)_2D_3$ receptor presence in embryonic and cancer cells as well as the significance of the wide receptor distribution detailed in Table 1. Perhaps there is a threshold number (e.g. 500 copies/cell) of receptors required to initiate a biological response, making quantitation of receptors as important as their qualitative presence in a cell.

In terms of the subcellular distribution of the vitamin D receptor, nuclear localization is supported indirectly by the striking nuclear occurrence of tritiated $1,25(OH)_2D_3$ via autoradiography (101, 182–184) and by original biochemical detection of the receptor protein in nuclear chromatin after administration of labeled vitamin D in vivo (79, 80). Subsequent biochemical fractionation of target tissues labeled with $1,25(OH)_2D[^3H]D_3$ in vitro, and studies in reconstituted systems (16–18), verified the predominant nuclear location of the occupied vitamin D receptor. Evidence was also gathered to suggest that the unoccupied receptor was primarily a cytosolic molecule, with the $1,25(OH)_2D_3$ receptor complex migrating to the nucleus upon hormone binding (17, 18). Thus, the $1,25(OH)_2D_3$ receptor system seemed to fit perfectly with the prevailing steroid hormone receptor dogma of a two-step process of hormone binding in cytoplasm and subsequent localization in the nucleus (78, 98).

Although nuclear localization of occupied $1,25(OH)_2D_3$ receptor remains undisputed, in vitamin D and several other steroid hormone systems the subcellular distribution of the unoccupied receptor is now less well defined. Initial data showing predominantly cytosolic receptors were obtained in experiments using homogenizing buffers of intermediate (17) or high (110) ionic strength. Walters and associates (197–199) have used low ionic strength buffers to reexamine the assumption that the unoccupied vitamin D receptor is cytosolic. They observed that as much as 90% of the unoccupied $1,25(OH)_2D_3$ receptor is associated with purified nuclei or chromatin under low ionic strength fractionation conditions. Thus, according to Walters et al (199), the nature of the $1,25(OH)_2D_3$ receptor may approach that of the thyroid hormone receptor,

Table 1 Relevant tissues and cells that possess the $1,25(OH)_2D_3$ receptor[a]

Category	Tissues	Specific cell types[b]	Established cell lines	Reference
Calcium control	Intestine	Absorptive epithelial	Intestine 407 (human)	39, 80, 152
	Bone/Cartilage	Osteoblast	ROS17/2.8 osteosarcoma (rat)	27, 110, 127
		Chondroblast	—	185
	Kidney	Distal epithelial	LLC-PK_1 (pig)	21, 22, 29, 3
Endocrine	Parathyroid	Chief	—	19, 85
	Pancreas	β-cell	—	29, 150, 156
	Pituitary	Somatomammotroph	GH_3 tumor (rat)	76, 77, 156
	Ovary	?	Chinese hamster ovary	45
	Testis	Sertoli/seminiferous tubule	—	115, 136, 19
	Thyroid	C-cell	—	59
Reproductive	Breast	Epithelial	MCF-7, T47D carcinoma (human)	51, 58, 168,
	Placenta	?	—	156
	Uterus	?	—	195
Other	Skin	Epidermal	3T6 fibroblast (mouse) G361 melanoma (human)	32, 49, 54, 158, 178
	Muscle	Myoblast	G-8 myoblast (mouse)	12, 180
	Parotid	Acinar	—	146
	Brain	Certain neurons/hippocampus	—	31, 182
	Thymus	Reticular/T lymphocytes	HSB-2 lymphoblast (human)	75, 163, 16
	Bone marrow	Monocyte	HL-60 leukemia (human) U937 monoblast (human)	124, 138, 16

[a]Receptor-positive tissues generally contain a concentration of ≥10 fmol/mg protein or approximately 500 copies per
[b]Conclusions reached from autoradiography (183, 184) and biochemical studies in homogeneous populations of cul cells.

which is an intrinsic nonhistone chromosomal protein (20). A general model has been proposed (199) to explain the $1,25(OH)_2D_3$ receptor in relation to steroid and thyroid hormone receptors. This model is based upon the concept that variations in the affinity of receptors for nuclear components account for apparent differences in subcellular distribution upon biochemical fractionation of the tissue. Unoccupied receptors are thought to have affinities for homologous nuclei in the following order: steroid hormones $<1,25(OH)_2D_3<$thyroid hormones. These relative affinities for nuclear binding, plus the ionic strength and volume (free water content) of homogenizing buffers, explain the marked differences seen in the distribution of unoccupied

hormone receptors in vitro. Recently, independent methodologies of immunocytochemistry and enucleation have been applied to determining the subcellular location of the unoccupied estrogen receptor (106, 202), with the surprising result that it is primarily a nuclear macromolecule. Thus, even this weakly nucleophilic macromolecule may reside principally in the nucleus in situ. Preliminary findings employing immunocytochemistry of the $1,25(OH)_2D_3$ receptor (11, 31) also indicate nuclear localization of the unoccupied receptor in human breast cancer tissue, mouse osteoblasts and kidney, and rat hippocampus. The present working hypothesis is that the $1,25(OH)_2D_3$ receptor, like other steroid receptors, is a loosely associated chromosomal protein, with affinity for nuclear components increasing upon hormone binding (96, 149, 158, 199). It is probable that a fraction of unoccupied receptors for $1,25(OH)_2D_3$ exists in the cytoplasm—in equilibrium with the majority of unoccupied receptors on chromosomes. In any event, it does not seem to be critically important whether the receptor receives the $1,25(OH)_2D_3$ hormone in the cytoplasm or while it is attached in the nucleus. What is significant is that the occupied receptor apparently is capable of translocating within the chromosome and recognizing upstream regulatory regions of vitamin D–controlled genes, with a resulting alteration in transcription.

In summary, based upon its tissue, cellular, and subcellular distribution, its pharmacologic profile for binding vitamin D congeners, and the clinical consequences of genetic defects in the molecule, it is reasonable to conclude that this protein is the biochemical mediator of $1,25(OH)_2D_3$ action. Although extranuclear (nongenomic) actions of $1,25(OH)_2D_3$ have not been ruled out (166), the properties of the vitamin D receptor convincingly place it in the family of genomic regulators with the thyroid and steroid receptors, but distinguish it from the smaller cellular vitamin A–binding proteins, which are not tightly associated with DNA or nuclei (83, 186).

BIOCHEMICAL PROPERTIES AND STRUCTURE

Avian Receptors

The most extensively studied $1,25(OH)_2D_3$ receptor is that from chick intestinal mucosa (15–17, 109, 149, 157, 176, 201). The chick intestinal receptor is biochemically indistinguishable from that found in other avian tissues like parathyroid gland, bone, pancreas, and ovary. There is general agreement that the receptor is a macromolecule sedimenting at 3.0–3.7S and is present even in this richest known site at $\leq 0.001\%$ of the soluble protein. It is an acidic protein, with a pI of 6.2, has a Stokes radius of 36 Å, and possesses distinct domains for $1,25(OH)_2D_3$ hormone binding ($K_d = 10^{-10}$–10^{-11} M) and for association with DNA (5, 133). Both the $1,25(OH)_2D_3$-binding region (36) and the DNA-binding domain (148) contain essential sulfhydryl groups. The receptor has

been purified by multiple chromatographic steps including DNA-cellulose, gel filtration, and DEAE-cellulose to yield a protein of 63,000 daltons (179) or 64,000 daltons (159) via gel electrophoresis under denaturing conditions. This approximate molecular weight for the monomeric receptor is in reasonable agreement with recent immunoblot identification of the receptor as a 60,000-dalton species by Pike (152) in our laboratory. However, this molecular weight is not consistent with data suggesting that a form of larger size (95,000–110,000 daltons) exists in the presence of protease inhibitors (9, 96, 103) or dithiothreitol (67). Although the final resolution of this conflict will require the cloning and sequencing of the receptor gene, the studies with protease inhibitors and dithiothreitol involve crude preparations and probably reflect either the measurement of nonreceptor proteins or their complexing with the monomeric receptor. The more definitive denaturing electrophoresis of purified receptor and immunoblotting techniques are no doubt identifying the authentic receptor as a 60,000-dalton protein. Further characterization of the 60,000-M_r chick intestinal receptor through amino acid sequencing has not been completed, but much additional information about the receptor has been obtained using impure or partially purified preparations.

The receptor is unstable and both hormone- and DNA-binding capacity decay in a time- and temperature-dependent fashion. Dithiothreitol (5 mM) preserves hormone binding to some degree but the DNA-binding function is quite labile and readily destroyed by endogenous proteolysis (57, 135). Recent data from our lab (E. A. Allegretto, K. Yamaoka, J. W. Pike, M. R. Haussler, unpublished information) reveal that an endogenous protease cleaves the 60,000-M_r receptor into a fragment of M_r 45,000 that binds hormone but not DNA. It is likely that this fragment is the "cytosolic" form of the receptor that has been reported not to bind DNA (139), which indicates that this species is a proteolytic product and not a novel form of unactivated receptor. As with other steroid receptors, molybdate appears to stabilize the $1,25(OH)_2D_3$ receptor by interacting with its DNA-binding region (57, 140; K. Yamaoka, J. W. Pike, and M. R. Haussler, unpublished information).

Although most of the investigations have been carried out on monomeric chick intestinal receptor, there is evidence for aggregation and formation of multimeric configurations. In the case of well-characterized prokaryotic regulators of transcription, there is precedent for dimeric and tetrameric proteins constituting the active species (175). Accordingly, Wilhelm & Norman (203) have observed positive cooperativity in the binding of $1,25(OH)_2D_3$ to its intestinal receptor protein in concentrated preparations. One possibility they propose to explain their data is that the receptor can form a homodimer, with the process of dimerization affecting the K_d for the second $1,25(OH)_2D_3$ bound. It is established that aggregation of the receptor to faster sedimenting forms in low-salt sucrose gradients requires an intact DNA-binding domain (57). Thus,

it is reasonable to assume that, in its native form in association with DNA in situ, the receptor could exist as a homo- or heteropolymer.

Much additional information is now available about the DNA or polynucleotide binding characteristics of the chick intestinal $1,25(OH)_2D_3$ receptor. The receptor elutes from DNA-cellulose with a higher salt concentration (0.22-M KCl) when occupied with $1,25(OH)_2D_3$ than when unoccupied (0.16-M KCl) (96, 158). The intercalating agent, ethidium bromide, is a potent blocker of receptor-DNA association (164) and the receptor prefers double-stranded over single-stranded DNA. These data indicate that receptor-DNA interaction is not solely electrostatic, but involves hydrophobic interactions with the major and minor grooves of the DNA double helix (164). The receptor also interacts with RNA (56), but elutes at a lower KCl concentration than from DNA, which suggests that it may bind less avidly to RNA. Some base sequence selectivity is observed when polydeoxyribonucleotides are used instead of natural DNA, with adenine:thymine (AT) preference occurring when DNA interactions are assessed by competition assay (164). The polynucleotide binding domain of the receptor appears to be the site of interaction with triazinyl dye-ligands (132) and, like DNA-cellulose, resins such as blue dextran Sepharose are powerful tools in purifying the receptor (131). Clearly, specific amino acid sequence information will be required to characterize fully the DNA-binding domain and ultimately to identify its interactions with the regulatory regions of vitamin D–modulated genes. Another challenging question will be to determine how $1,25(OH)_2D_3$ binding alters the conformation or activity of the DNA-binding domain to render it functional in controlling gene expression. Finally, the hormone-binding region of the receptor must be characterized, perhaps with the availability of photoaffinity probes such as recently reported by Ray et al (167).

Mammalian Receptors

In addition to characterizing the rat intestinal mucosa receptor for $1,25(OH)_2D_3$ (111), biochemical studies of mammalian receptors have included some unique tissue locations such as mouse colon (87) and rat yolk sac (41). Recently, mammalian vitamin D receptors have been intensely investigated in primary cultured cells and continuous cell lines (See Table 1). Mammalian receptors are remarkably similar to their avian counterparts, possessing corresponding dissociation constants for $1,25(OH)_2D_3$ binding of 10^{-11}–10^{-10} M, and virtually identical specificity in that other prominent circulating vitamin D metabolites like $25(OH)D_3$ and $24,25(OH)_2D_3$ bind less than 4% as effectively as the hormone. The mammalian $1,25(OH)_2D_3$ receptor has DNA-binding properties indistinguishable from the avian receptor, which reveals that both the hormone- and DNA-binding domains are conserved in these two species. Sucrose gradient centrifugation experiments do suggest that the mammalian receptor

(3.2–3.3S) is slightly smaller than the avian receptor, which is considered to be a 3.7S macromolecule (18, 109, 201), although sedimentation coefficients as low as 3.1–3.3S have been reported for the avian vitamin D receptor (19, 155). More definitive immunoblot data recently revealed that mammalian receptors are significantly smaller than the 60,000-dalton avian form. Employing immunoblotting of cultured cells, Pike (152) in our laboratory observed the following molecular weights for mammalian $1,25(OH)_2D_3$ receptors: mouse fibroblasts (3T6)=55,000; rat osteosarcoma (ROS 17/2.8)=54,000; porcine kidney (LLC-PK_1)=54,000; human leukemia (HL-60)=52,000. Similarly, Dame et al (39) identified the porcine intestinal receptor as a 55,000-dalton protein. Thus, mammalian $1,25(OH)_2D_3$ receptor monomers range in M_r from 52,000 to 55,000 daltons, but are otherwise strikingly similar in biochemical properties to the 60,000-dalton avian protein.

Evolution of the Receptor

The $1,25(OH)_2D_3$ receptor has been detected in vitamin D target cells in mammals (human, bovine, porcine, murine, and rat), birds, and amphibians, where it is prominent in such sites as skin, gut, and kidney (82). Fish also contain the $1,25(OH)_2D_3$ receptor in tissues like intestine, liver, pituitary, gills, and corpuscles of Stannius (47, 128, 159). These observations suggest that the vitamin D receptor is an evolutionarily ancient molecule. Biochemical properties such as $1,25(OH)_2D_3$-binding affinity and specificity, as well as DNA-binding characteristics, are indistinguishable for the receptor in all tissues from fish to humans. This indicates that the molecule is highly conserved and that similar mechanisms constitute the action of $1,25(OH)_2D_3$ in each of its target cells. The development of monoclonal antibodies to chick (155, 159) and porcine (39) receptors has strengthened this conclusion. Most, but not all, antibodies generated against the avian receptor cross-react with mammalian and fish vitamin D receptors (159), while virtually all antibodies to the porcine receptor react with other mammalian as well as avian receptors (40). Clearly the antigenic determinants in the receptor from various species are homologous and these regions of the molecule have undergone only minimal biochemical evolution. Because many of the epitopes are near or in the DNA-binding domain (40, 151), the conservation of this region and its potential functional significance are again highlighted. As with other hormone-receptor systems, the vitamin D receptor and its endogenous ligand have probably evolved new biological functions. Primitive but more fundamental roles of this system in cell proliferation and maturation may have preceded its more clinically significant involvement in stimulating mineral absorption and bone remodeling. Elucidating and cataloging the tissue-specific and specialized functions of the vitamin D receptor through evolution is obviously a significant problem for the future. Equally exciting will be the comparison of the amino acid sequence of the

vitamin D receptor with other hormone receptors and proteins that modify DNA transcription to disclose any homology and perhaps point to a common ancestral gene or genes.

PHYSIOLOGICAL AND CLINICAL APPLICATIONS

Radioreceptor Assay of 1,25-Dihydroxyvitamin D

The renal production and blood level of $1,25(OH)_2D$ [i.e. $1,25(OH)_2D_3$ + $1,25(OH)_2D_2$] are affected by a number of physiologic and nutritional agents that influence mineral metabolism (78; see Figure 1). In addition to this physiological regulation, circulating $1,25(OH)_2D$ concentrations are altered in a number of diseases of bone and mineral metabolism, notably renal osteodystrophy, parathyroid disorders, and various types of heritable rickets (69, 78). Because of the importance of $1,25(OH)_2D$ in the pathophysiology of calcium metabolism, considerable effort has been directed toward development of assay methods for monitoring the hormone in biological fluids. The receptor has been the basis of most assay methodology because of its high affinity and specific binding of $1,25(OH)_2D$.

Brumbaugh et al (14) created the first competitive radioassay for $1,25-(OH)_2D$ using the chick intestinal cytosol-chromatin receptor system and filtration to separate bound and free hormone. The procedure included a $1,25(OH)_2D$ purification scheme with Celite partition chromatography as the final step. Eisman et al (50) reported a conceptually similar method utilizing polyethylene glycol precipitation of soluble receptor to assess bound hormone and HPLC isolation of the sterol prior to assay. In the ensuing years modifications and improvements have been made in the radioreceptor assay of $1,25(OH)_2D$ in many laboratories (10, 48, 64, 93, 122, 134). Rapid extraction/purification procedures for the hormone have been devised and charcoal adsorption has been adopted as the method of choice for separating bound and free sterol. With the increased specific activity of $1,25(OH)_2D[^3H]D_3$ to ≥ 150 Ci/mmol and improved binding conditions to yield apparent dissociation constants approaching 10^{-11} M for the association of $1,25(OH)_2D_3$ with receptor, radioassays sensitive to 0.5–1 pg of $1,25(OH)_2D$ have been devised. The $1,25(OH)_2D_3$ receptor from chick intestine and more recently from calf thymus (170) are utilized in this clinically important assay. A variation on the radioreceptor assay, known as the cytoreceptor assay, has also been reported (125). Using intact rat osteosarcoma cells as a source of receptor and incubating in the presence of serum DBP to sequester interfering sterols like 25(OH)D and $24,25(OH)_2D$, Manolagas et al (125) were able to assay serum $1,25(OH)_2D$ with only minimal extraction and purification. Although the cytoreceptor assay is esthetically pleasing in that the receptor is employed for competition binding

in its natural location within the cell, and although it is a rapid technique for clinical estimation of $1,25(OH)_2D$, it may not be as rigorous or accurate as radioreceptor assays involving HPLC purification and lipid equalization strategies (48). Regardless of the procedure chosen, it is evident that radioreceptor assay of $1,25(OH)_2D$ in animals and humans facilitated a quantum jump in our understanding of the pathophysiology of the vitamin D endocrine system (69, 78).

Hereditary Vitamin D–Resistant Syndromes

In addition to nutritional rickets and osteomalacia, there are several inherited rachitic syndromes in which patients are resistant to physiologic doses of vitamin D and require massive supplements of vitamin D and/or minerals. Familial hypophosphatemic rickets, also known as vitamin D–resistant rickets, is characterized by a primary phosphate leak at the kidney that is also associated with a blunted adaptive increase in circulating $1,25(OH)_2D$ in response to the hypophosphatemia (120). Patients respond well to therapy with oral phosphate plus $1,25(OH)_2D_3$, which means that $1,25(OH)_2D_3$ responsiveness is intact (119), a conclusion supported by the presence of normal receptors and biochemical response in skin fibroblasts (4). There are two types of vitamin D–dependent rickets or hereditary hypocalcemic vitamin D–resistant rickets: (*a*) type I disease caused by a lack of adequate $1,25(OH)_2D_3$ production at the kidney, presumably because of a genetic error in the 1-OHase; and (*b*) type II disease resulting from tissue resistance to the $1,25(OH)_2D_3$ hormone (72).

Vitamin D–dependent rickets, type II (VDDR II), is a rare, heritable syndrome characterized clinically by hypocalcemia, secondary hyperparathyroidism, and rickets, all of which persist despite high circulating levels of $1,25(OH)_2D_3$. Patients also often display alopecia (53, 117). This constellation of features results from peripheral target organ resistance to $1,25(OH)_2D_3$ that is analogous to clinical disorders of resistance to glucocorticoids and androgens. Cultured skin fibroblasts from patients with VDDR II have been used to evaluate the underlying defects associated with this disorder. These studies showed that this disease is heterogeneous and arises primarily from defects in either the receptor's interaction with $1,25(OH)_2D_3$ or its site of action in the nucleus (53, 117). One particularly abundant phenotype identified in fibroblasts of a number of kindreds appears to be a receptor-deficient variant, as deduced by the absence of hormone-binding activity. There is a possibility that the syndrome is caused by an inherited deletion of the gene for the $1,25(OH)_2D_3$ receptor; we have recently evaluated this by using a radioligand immunoassay (46) to examine these fibroblasts for $1,25(OH)_2D_3$ receptors. Our results indicated the presence of normal amounts of material in these cells cross-reacting with the receptor antibody (154). This suggests that tissue resistance associated with phenotypes in which hormone binding is lacking may be caused

by mutations in the hormone-binding domain of the receptor protein and is rarely the result of genetic deletion.

Two recent studies report on novel subtypes of VDDR II in which mutations selectively influence the DNA-binding domain of the vitamin D receptor, with a reduction in affinity for DNA and a presumed compromise in receptor biopotency (89, 118). These findings of cellular resistance to $1,25(OH)_2D_3$ being associated with altered functional domains in the receptor provide dramatic confirmation of the biochemical results that point to the significance of the hormone- and DNA-binding regions of the receptor (153). The generality of the receptor defect in VDDR II fibroblasts is highlighted by reports that defective $1,25(OH)_2D_3$ receptors also occur in keratinocytes (30), mononuclear cells (107), and cultured bone cells (116). Moreover, $1,25(OH)_2D_3$ responsiveness in terms of vitamin D-24-OHase induction was shown to be absent or blunted in cells from patients with defective receptors, which proves a lack of functional bioresponsiveness to $1,25(OH)_2D_3$ (26, 30, 53, 61, 107, 116). In one case, normal receptors were associated with impaired stimulation of 24-OHase(65), indicative of a variety of VDDR II with postreceptor resistance to $1,25(OH)_2D_3$. As stated above, nature's experiment of VDDR II and its subtypes strengthens the conclusion that the $1,25(OH)_2D_3$ receptor is an obligate mediator of vitamin D action in bone and mineral homeostasis. When the normal receptor gene is cloned, it should be possible to transfect it into genetically resistant cells and restore responsiveness to $1,25(OH)_2D_3$.

Further study of vitamin D resistance will be facilitated by the use of certain cell culture and animal models. Kelly et al (105) have discovered that a monkey kidney cell line ($LLC\text{-}MK_2$) contains receptors with an abnormally low affinity for $1,25(OH)_2D_3$ that is reflected by the need for pharmacologic doses of the hormone to induce the 24-OHase enzyme. An identical situation is seen in some patients with VDDR II (61), where excessive doses of $1,25(OH)_2D_3$ cause a calcemic response in patients and elicit 24-OHase induction in cultured fibroblasts. Finally, as is the case with certain other steroid hormones, New World monkeys appear resistant to $1,25(OH)_2D_3$ (3, 187). These primates have evolved with high circulating hormone levels and complementary low receptor levels, resulting in a situation of "adaptive resistance" to steroid hormones, including $1,25(OH)_2D_3$.

NEW INSIGHTS

Monoclonal Antibodies

In order to characterize the vitamin D receptor fully, it was necessary to create immunochemical reagents. The low abundance of the receptor protein, its lability, and the resulting difficulties encountered in purifying it made this a formidable task. Pike et al (155, 159) extensively purified the receptor from 10

kg of chick intestinal mucosa, and were able to generate monoclonal antibodies to this highly enriched avian receptor. These antibodies displaced the native hormone-receptor complex in a sucrose gradient (155, 159) and, most importantly, were found to interact with both avian and mammalian $1,25(OH)_2D_3$ receptors with very high affinity ($K_d=10^{-11}$ M) (151). The latter property enabled our laboratory to develop immunoblot methodology capable of identifying the molecular mass of receptor proteins (152). Immunoblots of chick intestine high-salt extracts revealed a major immunoreactive protein in intestine at 60,000 daltons and a minor band at 58,000 daltons. We therefore concluded that the avian $1,25(OH)_2D_3$ receptor consists of two monomeric forms, a major species of 60,000 daltons and a minor form of 58,000 daltons. It is not known if both immunoreactive species in the avian intestine bind the $1,25(OH)_2D_3$ hormone and it is conceivable that the minor form arises through proteolytic degradation of the major receptor. Recent experiments involving the in vitro translation of chick intestinal mRNA similarly reveal a 60,000/58,000 dalton doublet of immunoprecipitable receptor (D. J. Mangelsdorf, J. W. Pike, and M. R. Haussler, unpublished information). Thus, these two forms of the receptor could result from alternative mRNA splicing or differing transcription start/termination sites, although proteolysis cannot be ruled out during the in vitro translation and subsequent immunoprecipitation procedures.

Recently, Dame et al (39) developed monoclonal antibodies to the highly enriched porcine intestinal receptor for $1,25(OH)_2D_3$. These workers employed a strategy similar to that of Pike et al (155, 159) to obtain monoclonal anti-receptor antibodies, most of which complexed with native receptors of mammalian as well as avian origin (40). As was detailed above, mammalian $1,25(OH)_2D_3$ receptors were identified by immunoblotting with monoclonal antibodies as single monomeric proteins of M_r 52,000–55,000 (39, 152). In vitro translation of the respective mRNAs of mammalian $1,25(OH)_2D_3$ receptors followed by immunoprecipitation with monoclonal antibody has confirmed the molecular masses of the various mammalian receptors (D. J. Mangelsdorf, J. W. Pike, and M. R. Haussler, unpublished information). The $1,25(OH)_2D_3$ receptor monomer is therefore similar in size to the thyroid (20) and estrogen (194) receptors, which have molecular weights of 57,000 and 65,000 daltons, respectively. Also, as discussed above, like all thyroid and steroid hormone-binding proteins, the $1,25(OH)_2D_3$ receptor is a DNA-binding protein that now appears to be predominantly associated with the nucleus, even in the unoccupied state. However, in spite of the functional resemblance of the various receptors, and the fact that they likely are endowed with analogous structural motifs, each is unique in terms of the hormonal ligand. Antibodies to the vitamin D receptor do not cross-react with other receptors, such as those for estrogen and glucocorticoids (159). Hence if there is a superfamily of thyroid and steroid receptors that regulate gene expression, distinct immunologic epitopes are present in the individual proteins.

In summary, monoclonal antibodies have facilitated unequivocal identification of $1,25(OH)_2D_3$ receptors from various species through immunoblot analysis and immunoprecipitation of internally labeled products of in vitro mRNA translation. The ability to assess and possibly enrich receptor mRNA with the aid of in vitro translation, combined with antibody screening of cDNA libraries through expression vector technology, should ultimately lead to the cloning of the $1,25(OH)_2D_3$ receptor gene. In the meantime, monoclonal antibody immunoprecipitation is proving useful in investigating another exciting area—receptor regulation and covalent modification.

Quantitative and Qualitative Modification of the Receptor

Cultured cells have provided excellent new model systems for studying the biochemical action of $1,25(OH)_2D_3$, especially as it relates to the receptor molecule and its possible modulation. Chen et al (27) first reported the existence of the $1,25(OH)_2D_3$ receptor in a cultured cell, namely primary mouse calvarial cells. Subsequently, Chen & Feldman (24) reported that the $1,25(OH)_2D_3$ receptor is regulated in culture in that levels correlate positively with the rate of cell division in primary bone cells. In LLC-PK_1 kidney cells, medium change caused a transient increase in $1,25(OH)_2D_3$ receptor followed by a decline to 40% of control values by 18 h (88). Glucocorticoids have been found to slow cell division in mouse cells and correspondingly reduce $1,25(OH)_2D_3$ receptor number (23). Conversely, in rat osteoblast-like cells, glucocorticoids stimulate cell division and enhance $1,25(OH)_2D_3$ receptor concentration. The significance of these results is unclear, but species dependence of the regulation of the receptor was recently highlighted by the demonstration that glucocorticoids administered to dogs in vivo double the intestinal $1,25(OH)_2D_3$ receptor level (108). The vitamin A metabolite retinoic acid has also been observed to modulate $1,25(OH)_2D_3$ receptors in cultured cells. Retinoic acid stimulates $1,25(OH)_2D_3$ receptors in rat osteosarcoma cells (147) and in mouse osteoblast-like cells (25), but it reduces receptor levels in primary rat osteoblast-like cells (25). These results emphasize the complexity of receptor control, with opposite effects of retinoic acid on receptor number in transformed vs primary (normal) rat bone cells, and species dependence as was observed in the case of glucocorticoid effects on the receptor.

Compounds such as glucocorticoids and retinoic acid are known to change the rate of cell proliferation and, in some instances, differentiation of cultured cells. Human promyelocytic leukemia (HL-60) cells are a good model system for studying $1,25(OH)_2D_3$ receptors during differentiation. These cells differentiate to macrophage-like cells when treated with the tumor promotor TPA and with $1,25(OH)_2D_3$; they also contain significant quantities of the $1,25(OH)_2D_3$ receptor (up to 4000 copies per cell) (124). We have observed that differentiation of HL-60 cells to macrophages elicits an 80% reduction in $1,25(OH)_2D_3$ receptor number (M. R. Haussler, D. J. Mangelsdorf, C. A.

Donaldson, S. L. Marion, and J. W. Pike, unpublished information). It is also known that the receptor level increases dramatically in normal human T lymphocytes when they are activated (163). In our hands, the receptor copy number per T cell rises from 200 to 2000 when the cells are activated for 72 h with concanavalin A (75). Thus it is evident that the concentration of the $1,25(OH)_2D_3$ receptor is critically dependent on the state of differentiation and/or activation of certain vitamin D target cells, probably to restrict or amplify the action of the hormone at various stages in the physiology of the cell.

The $1,25(OH)_2D_3$ hormone also appears to alter the level of its receptor in certain cultured cell lines. Sher et al (177) reported that in T47D breast cancer cells $1,25(OH)_2D_3$ induces processing of the receptor to a nonbinding form. Conversely, Costa et al (35) observed that $1,25(OH)_2D_3$ causes a significant up-regulation of $1,25(OH)_2D_3$ receptor binding activity in cultured kidney cells as well as other cell types. In our own studies involving immunoblot detection of the receptor in $1,25(OH)_2D_3$-treated 3T6 mouse fibroblasts (J. W. Pike, N. M. Sleator, and M. R. Haussler, unpublished information) we found an enhancement in receptor levels as early as 4 h and as high as 10-fold within 48–72 h. Our data in 3T6 cells and those of Costa et al (35) may be explained by invoking the principle that $1,25(OH)_2D_3$ influences receptor levels via an alteration of the proliferation and differentiation state of the cultured cells. However, in view of the rapidity of this event, it is more likely that the hormone is capable of altering the turnover rate of the receptor, either by enhancing its biosynthesis or by decreasing its degradation, or both. The mechanism whereby $1,25(OH)_2D_3$ apparently up-regulates the level of its receptor in mouse 3T6 and other cells and the possible relevance of this finding to the biology of the receptor in mammalian systems in vivo are currently under study.

Recently, a striking qualitative modification in the receptor was observed in our laboratory (160) when mouse fibroblast (3T6) cells were exposed to the $1,25(OH)_2D_3$ hormone. When a high-salt cytosol preparation from untreated 3T6 cells was immunoblotted, the receptor appeared as a band at 54,000 daltons. In contrast, after treatment of intact 3T6 cells with $1,25(OH)_2D_3$ in culture for 90 min, the receptor extracted from the nucleus exhibited a small but significant reduction in electrophoretic mobility, with the upshifted form of the 3T6 cell $1,25(OH)_2D_3$ receptor displaying an approximate molecular weight of 55,000. These data indicate that the occupied receptor isolated from the nuclear fraction had undergone covalent modification that caused it to migrate more slowly upon denaturing gel electrophoresis (160).

The hormone-dependent alteration in the receptor was further characterized by labeling the protein internally with [^{35}S]methionine in 3T6 cells. Metabolic labeling, immunoprecipitation, and denaturing electrophoresis confirmed the hormone-dependent upshift in electrophoretic mobility (160) that was seen via immunoblot analysis. An intense doublet appeared at 55,000 daltons representing the residual unmodified receptor and its upshifted derivative in the 3T6 total

cell lysate (160). When cells were instead incubated with [^{32}P]orthophosphate in the presence or absence of 1,25(OH)$_2$D$_3$, no ^{32}P was detected in the receptor without addition of 1,25(OH)$_2$D$_3$ to the cells, but significant and selective phosphorylation of the upshifted band occurred when 3T6 cells were treated for 4 h with 1,25(OH)$_2$D$_3$. Thus, the anomalous migration of the 1,25(OH)$_2$D$_3$ receptor was due to phosphorylation (160). Preliminary data indicate that phosphorylation in the presence of 1,25(OH)$_2$D$_3$ under these conditions (i.e. no phosphatase inhibitors) occurs on a serine residue(s) (J. W. Pike, N. M. Sleator, M. R. Haussler, N. Weigel, unpublished information). Results of in vitro translation of 3T6 cell mRNA followed by immunoprecipitation of biosynthesized receptors (D. J. Mangelsdorf, J. W. Pike, M. R. Haussler, unpublished information) are consistent with the upper receptor band being a posttranslationally modified form; only the lower nonphosphorylated receptor band at 54,000 daltons appears during in vitro translation.

A similar phosphorylation of the nuclear progesterone receptor in uterine slices was recently reported by Logeat et al (121). Therefore, the 1,25(OH)$_2$D$_3$ receptor and probably other steroid receptors become phosphorylated upon hormone binding in culture and perhaps in vivo. The interaction of the 1,25(OH)$_2$D$_3$ hormone with the receptor probably alters the conformation of the protein, rendering it a good substrate for a nuclear protein kinase. Because phosphorylation is an early, hormone-dependent event coinciding with nuclear localization, it is likely of functional importance. The phosphorylated receptor could be the form of the receptor that binds most avidly to upstream regulatory regions of vitamin D–controlled genes. In addition to functional activation, phosphorylation may act as a signal for receptor processing to inactive forms. Regardless of the role of 1,25(OH)$_2$D$_3$ receptor phosphorylation, this qualitative modification of the receptor is probably a key biochemical event in the mechanism of action of 1,25(OH)$_2$D$_3$.

BIOLOGIC ROLES AS A MEDIATOR OF VITAMIN D FUNCTION

Calcium and Mineral Homeostasis

In the case of stimulating intestinal calcium absorption, there is compelling evidence for the involvement of the 1,25(OH)$_2$D$_3$ receptor. In the developing neonatal rat, the receptor appears at a time coincident with 1,25(OH)$_2$D$_3$-elicited (active) calcium absorption (68). Moreover, exchange assays have been devised for quantitating both occupied and unoccupied 1,25(OH)$_2$D receptors (97, 129, 165), exploiting the selective action of L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) on unoccupied receptors (97) or mersalyl to dissociate bound hormone (129, 165). Hunziker et al (95) have evaluated the dynamic equilibrium between in vivo occupied and unoccupied receptors and correlated this with differing vitamin D status and other physi-

ologic conditions in the chick. These parameters were related also to a biologic response, the concentration of intestinal vitamin D_3–dependent calcium-binding protein (CaBP). Intestinal receptor occupancy correlated directly with serum $1,25(OH)_2D$, which indicates that the quantity of occupied receptor is determined by a simple equilibrium between serum $1,25(OH)_2D$ and unoccupied receptors. Under normal physiologic conditions, only 10–20% of the total receptor is occupied by ligand in both chicks (46, 95) and rats (129). Finally, under all experimental situations in chicks, there was a positive correlation between occupied receptor and CaBP levels (95). These data clearly support the participation of the occupied receptor in the calcium translocation response to $1,25(OH)_2D_3$.

Regulation of Vitamin D Metabolism

In addition to its role in renal mineral conservation, a direct consequence of $1,25(OH)_2D_3$ action in the kidney is decreased production of $1,25(OH)_2D_3$ and, concomitantly, enhanced production of 24-hydroxylated vitamin D_3 metabolites (84, 143, 189, 191). This feedback regulatory action is known to result from the reciprocal action of $1,25(OH)_2D_3$ on the relative specific activities of the renal 1-OHase and 24-OHase enzymes. Substantial evidence has been accumulated to suggest that the expression/induction of the 24-OHase enzyme activity represents a classic steroid hormone action of $1,25(OH)_2D_3$. The presence of functional receptors for $1,25(OH)_2D_3$ has been demonstrated in a number of cultured cell systems and there is a good correlation between the presence of these receptors and $1,25(OH)_2D_3$-responsive 24-OHase activity.

In the established pig kidney line, LLC-PK_1, $1,25(OH)_2D_3$ receptors are present (21, 34) and have been correlated with the ability of $1,25(OH)_2D_3$ to induce the $25(OH)D_3$-24-OHase enzyme. The dose dependency of 24-OHase enhancement by $1,25(OH)_2D_3$ treatment in culture resembles the binding kinetics for the hormone to cytosol preparations from LLC-PK_1 cells. The time course of $1,25(OH)_2D$ action (2-h lag; 8-h maximal 24-OHase), plus the fact that actinomycin D blocks this effect of $1,25(OH)_2D_3$, indicates that 24-OHase increase is an induction event (21). Moreover, the occurrence of the $1,25(OH)_2D_3$ receptor in these cells and its recognized property of nuclear localization implicate the receptor as the mediator of the genomic effects of the hormone on 24-OHase enzyme levels. Impressive extensions of the data in kidney cells have been accomplished in the case of bone cells, circulating mononuclear cells, and fibroblasts. Osteoblast $1,25(OH)_2D_3$ receptors are significantly altered by treatment with retinoic acid and there is a positive correlation between receptor number in a particular circumstance and the magnitude of $1,25(OH)_2D_3$'s ability to induce the 24-OHase enzyme (25). Utilizing circulating mononuclear cells (107) or fibroblasts derived from patients with vitamin D–dependent rickets type II (26, 53, 61, 89), Feldman's and Liberman's groups observed a striking association between biochemically

competent $1,25(OH)_2D_3$ receptors and the induction of 24-OHase by the hormone. This association has even been extended to New World monkey fibroblasts (3), which display resistance to vitamin D caused by reduced $1,25(OH)_2D_3$ receptor number. Taken together, these findings strongly suggest that the $1,25(OH)_2D_3$ receptor mediates 24-OHase induction. But in all cell culture experiments reported thus far, there is only a correlation between the $1,25(OH)_2D_3$ receptor and the bioresponse.

Control of the Immune System, Bone Remodeling, and Mineralization

Because nonclassical sites for the $1,25(OH)_2D_3$ receptor include cells derived from bone marrow and thymus, $1,25(OH)_2D_3$ may be an immunomodulator like the glucocorticoids and other steroids. The most intensively investigated model system within the cell-mediated immune system is the differentiation of HL-60 leukemia cells. This leukemic line differentiates into macrophage-like cells when treated with $1,25(OH)_2D_3$ in culture (7, 124, 188). Our laboratory has shown that saturation of the HL-60 nuclear receptor by $1,25(OH)_2[^3H]D_3$ in complete culture medium correlates with the kinetics of $1,25(OH)_2D_3$-induced FMLP (chemotaxin) receptors (124), which indicates that this expression of the differentiated phenotype can be mediated by the $1,25(OH)_2D_3$ receptor protein. However, suppression of proliferation and differentiation of HL-60 cells into macrophages requires at least 72 h, even though the saturation of nuclear receptors with $1,25(OH)_2D_3$ can be achieved as early as 4 h in suspended cells. One rapid action of $1,25(OH)_2D_3$ in HL-60 cells that has been observed by Reitsma et al (171) is the attenuation of c-*myc* oncogene mRNA levels. While it is tempting to speculate that the $1,25(OH)_2D_3$ receptor complex regulates c-*myc* transcription via direct binding in the c-*myc* gene regulatory region, it is plausible that this regulation is secondary and that the hormone-receptor complex controls the transcription of a key gene(s) whose product(s) in turn controls c-*myc* mRNA levels. Since $1,25(OH)_2D_3$ regulates CaBP in traditional target cells, it is equally possible that intracellular calcium fluctuations modulated by CaBP represent a second messenger in $1,25(OH)_2D_3$-induced differentiation of HL-60 cells. It should also be noted that a number of other compounds that differentiate HL-60 cells, such as retinoic acid and a phorbol ester tumor promoter (TPA), elicit suppressions in c-*myc* mRNA levels similar to that produced by $1,25(OH)_2D_3$ (66). Thus, this effect on c-*myc* expression may reflect either a declining proliferation rate or possibly the general triggering of differentiation.

As noted above, there is an 80% reduction in the concentration of $1,25(OH)_2D_3$ receptors that accompanies the process of macrophage differentiation. This may relate to the proposed role of monocytes and macrophages as osteoclast precursors (144). $1,25(OH)_2D_3$ increases osteoclast number in vivo (190) and is proposed to mediate bone resorption, in part by increasing the

differentiation of osteoclast progenitors, as illustrated in Figure 2. Osteoclasts do not possess $1,25(OH)_2D_3$ receptors (137) and therefore their number but not their activity is directly affected by $1,25(OH)_2D_3$. The observation that $1,25(OH)_2D_3$ receptor concentrations are dramatically reduced upon differentiation of HL-60 cells to macrophages could reflect the first stage in the attenuation of receptor expression that is presumably complete upon fusion of macrophages to multinucleate osteoclasts.

As illustrated in Figure 2, it is important to note that $1,25(OH)_2D_3$ indirectly augments the final stages of osteoclast differentiation through a newly recognized operation on T lymphocytes. T cells contain $1,25(OH)_2D_3$ receptors (163) and both $1,25(OH)_2D_3$ receptor number and lymphokine production are modulated by $1,25(OH)_2D_3$; at least one of the regulated lymphokines has been shown to elicit macrophage fusion to giant multinucleate osteoclast-like cells (2). A second T-cell-derived lymphokine acts synergistically with $1,25(OH)_2D_3$ in causing U937 monoblastic cells to elaborate IL-1 (6), which is itself a strong bone resorbing agent (63). Finally, $1,25(OH)_2D_3$ could cause bone resorption by binding to its well-known receptors in osteoblasts (27, 127) and bringing about the release of osteoblast-derived resorption factors, as depicted in Figure 2. $1,25(OH)_2D_3$-stimulated osteoblasts also presumably remineralize bone during the remodeling cycle.

Direct effects of $1,25(OH)_2D_3$ on osteoblast-like cells include enhanced vitamin K–dependent bone γ-carboxyglutamic acid (Gla) containing protein (BGP) (161) and alkaline phosphatase (123) biosynthesis, and suppressed collagen formation at the mRNA level (173). While the exact functions of these modulated proteins have not been characterized, attenuation of collagen

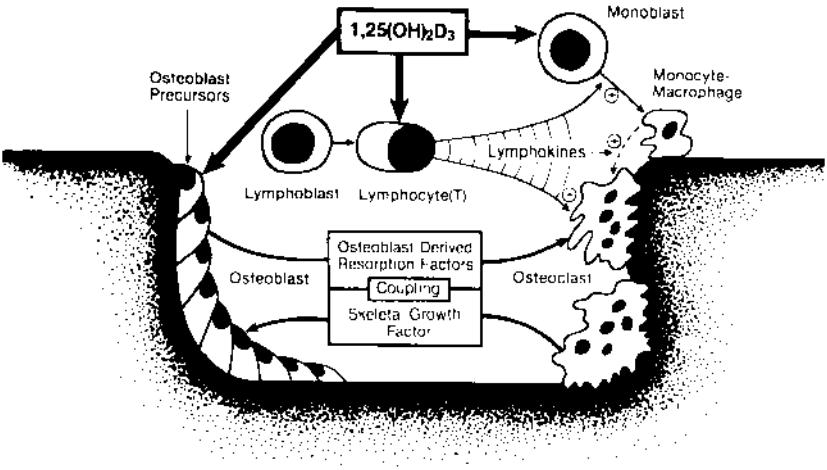

Figure 2 Proposed function of $1,25(OH)_2D_3$ and its receptor in bone remodeling and immunomodulation.

biosynthesis and secretion could be the "final event" in $1,25(OH)_2D_3$-induced mineralization of existing collagen. Similarly, $1,25(OH)_2D_3$ has potent actions on blood mononuclear cells. The hormone suppresses immunoglobin production by activated human peripheral blood mononuclear cells, probably by inhibiting proliferation of antibody-producing B cells or helper T cells (113, 114). $1,25(OH)_2D_3$ blocks phytohemagglutinin- and antigen-induced lymphocyte blast transformation, apparently via attenuation of IL-2 production (8, 113, 172, 193). All of this new information could have clinical relevance to osteoporosis, since T-cell subsets are abnormal in this disorder (60) and the regulation by $1,25(OH)_2D_3$ and sex hormones of the immune system has been invoked to explain the etiology of postmenopausal osteoporosis (74). Therefore, it is clear that, along with its mediating receptor, $1,25(OH)_2D_3$ accomplishes a complex yet elegant regulation of the bone remodeling cells. $1,25(OH)_2D_3$-responsive cells of the immune system appear to play a central role in the function of $1,25(OH)_2D_3$ on bone resorption, but this may be only one facet of a more general action of the hormone as a novel immunoregulator.

Cell Proliferation, Differentiation, and Anticancer Actions

$1,25(OH)_2D_3$ has been found to inhibit proliferation in a number of cancer cell lines that possess the vitamin D receptor (32, 44, 55, 75, 124). In some cases, inhibition of cell growth is coupled to morphological changes (44, 55) and differentiation of the cells (1, 7, 24). Our laboratory has found that the $1,25(OH)_2D_3$-elicited inhibition of colony formation by tumorigenic cells in soft agar is directly correlated to vitamin D receptor number (75). Clonal lines with low $1,25(OH)_2D_3$ receptor numbers do not respond to $1,25(OH)_2D_3$ in terms of growth regulation and morphological differentiation (44, 124). These results from cultured cells suggest that $1,25(OH)_2D_3$ inhibits tumor cell growth and may even be capable of suppressing the malignant phenotype by initiating normal differentiation. Honma et al (92) extended these findings to the in vivo situation by showing that $1,25(OH)_2D_3$ prolongs the survival time of *nude* mice inoculated with M1 leukemia cells. Also, 1α-hydroxyvitamin D_3 [an efficient precursor of $1,25(OH)_2D_3$] suppresses pulmonary metastases of Lewis lung carcinoma (174).

The above observations would seem to be consistent with preliminary data from a prospective study showing that colorectal cancer risk is inversely correlated with dietary vitamin D and calcium (62). Additionally, two groups (2, 205) have demonstrated that $1,25(OH)_2D_3$ can inhibit phorbol ester–dependent chemical carcinogenesis in mouse skin. However, until more in vivo trials are performed, these data must be viewed with caution. For example, as with vitamin A compounds, in certain circumstances $1,25(OH)_2D_3$ can act as a tumor enhancer (99, 112, 204; K. Yamaoka, S. L. Marion, A. Gallegos, and M. R. Haussler, unpublished information). Thus, it is premature to conclude that $1,25(OH)_2D_3$ is a natural anticancer agent. What is evident is that, in

combination with its receptor, $1,25(OH)_2D_3$ is a potent regulator of cell growth and maturation. Whether this bioeffect can be exploited in the prevention or treatment of certain malignancies is open to question.

INTEGRATED MODEL FOR THE ACTION OF THE VITAMIN D RECEPTOR

In the integrated model of $1,25(OH)_2D_3$ receptor biochemistry and biology pictured in Figure 3, a fraction of the unoccupied receptor is hypothesized to be present in the cytoplasm, but the equilibrium probably favors the nuclear compartment, where the receptor exists as a loosely associated chromosomal protein. When complexed with the $1,25(OH)_2D_3$ hormone, the receptor is phosphorylated—possibly at multiple sites. The phosphorylated receptor is proposed to be the form that locates upstream regulatory regions of vitamin D–modulated genes in order to control transcription. These upstream activating sequences are analogous to those found for other steroid hormone receptors (104). Altered DNA transcription then results in enhanced or repressed levels of various mRNAs. The altered mRNA concentrations depicted in Figure 3 are hypothetical [i.e. extrapolated from $1,25(OH)_2D_3$-elicited protein changes] except for CaBP, which is increased (145), and c-*myc* and collagen, which are decreased (171, 173). The concept that the $1,25(OH)_2D_3$ receptor complex

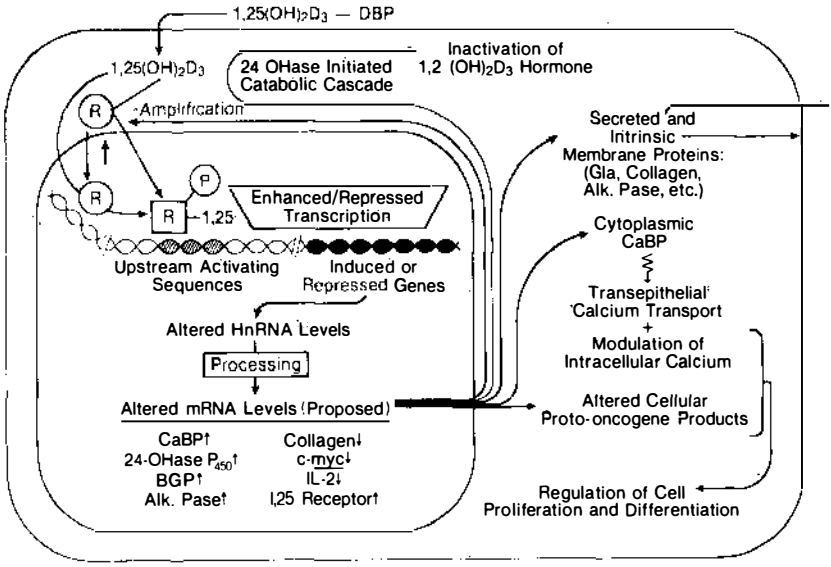

Figure 3 Model for receptor-mediated actions of $1,25(OH)_2D_3$ at the molecular level. A hypothetical composite target cell for vitamin D is depicted. An R in a circle represents the unoccupied receptor, while an R in a square designates the occupied and phosphorylated receptor. BGP means bone Gla containing protein, CaBP is calcium-binding protein, and 24-OHase is 24-hydroxylase.

binds to regulatory/promotor sequences for each of the putative induced mRNAs is only one possible mechanism. Regardless of the exact sequence of events, there is little doubt that control of mRNA levels is the key manner in which $1,25(OH)_2D_3$ ultimately orchestrates the concentration of its bioactive proteins.

As summarized in Figure 3, there are a number of $1,25(OH)_2D_3$-induced proteins that occur in various target cells. Individual cell types are presumably preprogrammed to modulate certain of the induced proteins, which then effect the appropriate altered cell function. 3T6 fibroblasts would be an example of a cell type in which receptor up-regulation or amplification occurs. Other target cells, such as those of the kidney, may primarily inactivate $1,25(OH)_2D_3$ via the induced 24-OHase initiated cascade and still carry out transepithelial transport of minerals. Bone cells apparently respond to $1,25(OH)_2D_3$ by modulating an array of proteins (e.g. BGP, collagen, alkaline phosphatase, etc) required for the complex process of bone mineralization and remodeling. CaBP undoubtedly functions as a part of intestinal calcium transport, but could also modulate intracellular calcium in other targets. Along with control of oncogene products, intracellular calcium may act to regulate cell proliferation and differentiation. This is particularly true in certain hematopoietic and transformed cells that appear to respond to $1,25(OH)_2D_3$ by differentiation and suppression of the embryonic or malignant phenotype.

CONCLUSION

Clearly, the $1,25(OH)_2D_3$ receptor is an integral part of the biochemical action of vitamin D. Perhaps the receptor protein is the molecular key that will enable us to unlock the mechanism through which vitamin D controls gene expression and ultimately influences cell function. The receptor is therefore important to basic science, but it also has significant ramifications for nutrition and clinical medicine. Analysis of the receptor and its use in radioreceptor assay have dramatically increased our understanding of several disorders of bone and mineral metabolism. Perhaps most exciting, potential new actions of vitamin D in the neuroendocrine and immune systems have been revealed. As a nutritional principle, vitamin D has assumed a pivotal position as the precursor of a biological modifier of major significance.

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