MINIREVIEW

Molecular Recognition in Vitamin D–Binding Protein (44020)

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Abstract. **Vitamin D–binding protein (DBP) or group-specific component (Gc) is a relatively abundant serum protein with multiple functions, the majority of which are initiated by the highly specific recognition and binding of a ligand by this protein. During the past decade and a half, several structure-functional studies have been carried out to shed light on the physiological significance of the multiple functions of DBP. Results of these studies are discussed.** [P.S.E.B.M. 1996, Vol 212]

In a biological system, the molecular recognition process, which usually involves specific recognition and binding of a small molecule (ligand) by a relatively large macromolecule (usually a protein, receptor), is a phenomenon of immense importance. The majority of the physiological processes are now known to be either strongly influenced or driven by ligand-receptor interactions. Manifestation of this molecular recognition process is also highly prominent in the vitamin D endocrine system.

Vitamin D_3 (vitamin D), a seco-steroid, is biosynthesized in the skin by the interaction of the ultraviolet (UV) light from the sun with 7-dehydrocholesterol which is plentiful in the epidermis. The product of this photolytic reaction is predominantly a ring-opened compound called previtamin D_3, which isomerizes slowly by the body temperature to an equilibrium mixture containing primarily vitamin D_3. After entering circulation, cutaneously synthesized vitamin D_3 is specifically bound by a serum protein (vitamin D–binding protein [DBP], also called group-specific component [Gc]) which transports it to various target organs where sequential regio- and stereospecific hydroxylations take place, to produce 25-hydroxyvitamin D_3 (25-OH-D_3, in the liver) and 1α,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$, in the kidney). From the kidney, $1,25[OH]_2D_3$ is transported (by DBP) to its target tissues, including intestine, where it is responsible for intestinal calcium absorption, and bone, where it stimulates bone calcium mobilization (Fig. 1). In addition, $1,25[OH]_2D_3$ is also involved in normal growth and maturation of cells (1, 2). These biologic manifestations of $1,25[OH]_2D_3$ are mediated by its high-affinity binding to a nuclear receptor/transcriptional factor called the $1,25[OH]_2D_3$ receptor (vitamin D receptor [VDR]).

DBP (Gc), a member of the albumin gene family, is a genetically polymorphic glycoprotein which is present fairly abundantly in the α_2-globulin fraction of serum (3). The two major phenotypes are Gc1 and Gc2, which differ from each other by four amino acids (152, 311, 416, and 420) as well as attached polysaccharide structures (4, 5). VDR, on the other hand, is DNA-binding protein and a typical member of the steroid hormone receptor superfamily (6).

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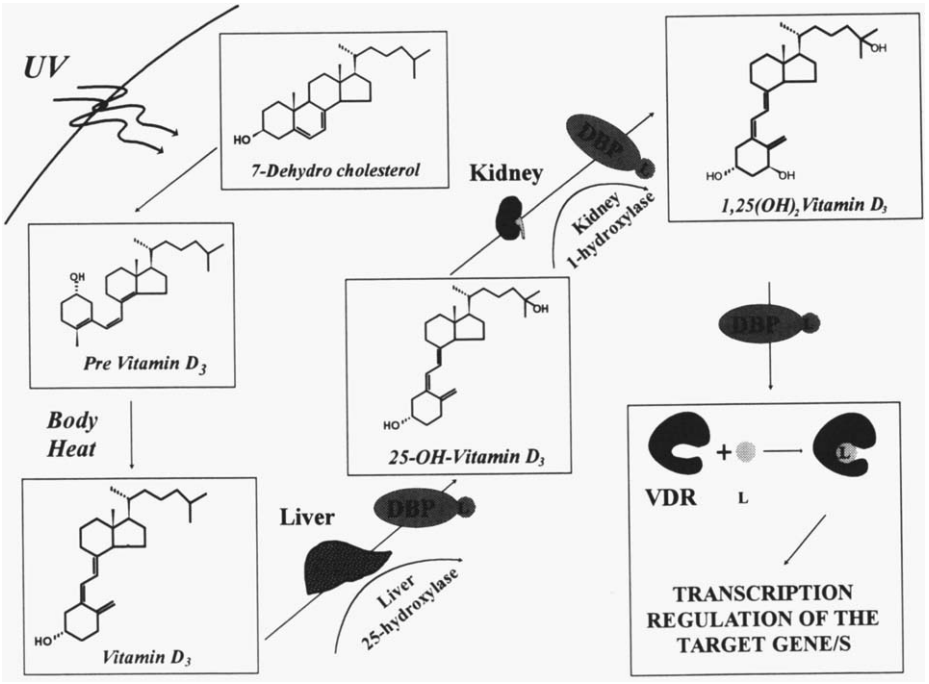

Figure 1. Biosynthesis and modes of action of vitamin D_3 and its biologically important metabolites.

Multiple Ligand Binding by DBP

As described above, vitamin D and all its major metabolites are known to bind strongly to DBP, but the strongest binding is reserved for 25-OH-D_3 (K_D = 10^{-10}–10^{-12} *M*) (3, 7). Binding efficiencies, however, decrease significantly for vitamin D_3 and 1,25[OH]$_2D_3$, although these molecules differ from 25-OH-D_3 by subtraction or addition of a hydroxyl group (to 25-OH-D_3).

Another interesting phenomenon about DBP involves its presence in the serum in a large excess over that necessary for vitamin D sterol transport. Studies have shown, that at any given time only 1%–2% of its sterol-binding site is utilized (8, 9). This phenomenon is further augmented by the fact that DBP plays host to a wide variety of other ligands. DBP, in conjunction with plasma gelsolin, plays a major role in scavenging monomers of actin (K_a = 10^{-8} *M*) (10, 11). DBP also binds lipids and fatty acids with high affinity (12–15) and enhances complement activation on neutrophil chemotaxis by binding to complement C5a and C5a des Arg (16). In addition, DBP has been found to be associated with a variety of cell types including B lymphocytes (17), subpopulations of T lymphocytes (18), and cytotrophoblasts of placental yolk sac (19).

Primary structures of human DBP (hDBP) and rat DBP (rDBP) have been deduced recently from the cDNA sequences as well as by direct sequencing (of the human Gc2 isomorph only) (4, 5, 20). These data have revealed that, structurally, DBP is highly homologous with albumin (ALB) and α-fetoprotein (AFP), the other members of this globulin gene family (3). Comparison of the amino acid sequences of these proteins reveal that all of them have high cysteine contents, and positions of these cysteine residues are highly conserved among these proteins. In the case of DBP, all the cysteine residues are present as disulfides. As a result, there are fourteen (14) disulfide bonds in DBP, giving it a triple-domained modular structure. The question is, How are these structural features of DBP related to its varied functions?

Vitamin D Sterol Binding by DBP. During the past several years several laboratories, including ours, have delved into structurally defining the vitamin D sterol–binding pocket in DBP by photoaffinity and affinity labeling techniques. For several decades, these methods have been popular among biochemists, enzymologists, and endocrinologists to determine organelle-distribution and polymorphic structure of proteins, as well as to define structurally the ligand-binding pockets of proteins (21).

In general, these methods involve chemical synthesis of substrate analog/analogs which contain either a chemically reactive group (which can form a covalent bond with an amino acid residue at the ligand-binding pocket—affinity labeling), or a functional group which can be activated by photolysis of the protein-ligand complex (thereby forming a covalent bond at the ligand-binding pocket—photoaffinity labeling) (Fig. 2). After the chemical synthesis, the analogs are tested for their ability to bind specifically to the target

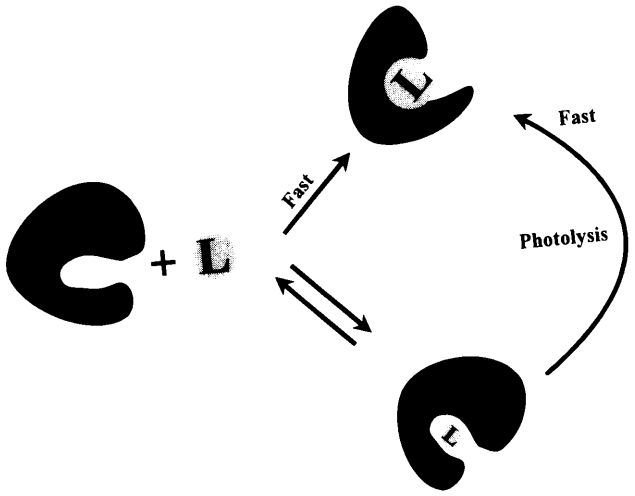

Figure 2. Events representing the processes of affinity and photoaffinity labeling of macromolecules.

protein, and suitable analogs are chosen. This process is followed by radiochemical synthesis of the selected analogs, and covalent labeling of the target protein. Over the years, several proteins have been labeled by this multistep process, and in some cases the labeled protein has been cleaved with site-specific enzymes and/or chemical reagents, and the "ligand-binding domain" has been identified and "mapped."

During the past few years, our laboratory and that of several others have synthesized several affinity and photoaffinity analogs of 25-OH-D_3 to probe the vitamin D sterol–binding domain of DBP. Names and structures of these analogs, and their corresponding radiolabeled (^3H or ^{14}C) versions are shown in Figure 3.

It was observed that all these analogs (unlabeled) displaced ^3H-25-OH-D_3, bound specifically to DBP in a dose-dependent manner, although with varying efficiencies. For example, the 3-epoxy derivative, 25-hydroxyvitamin D_3-3β-(1,2-epoxypropyl)ether (II), displayed DBP-binding efficiency and specificity very similar to those of 25-OH-D_3, the naturally occurring ligand. The other analogs, however, displayed 20–50 times less binding affinity (compared with 25-OH-D_3) towards DBP. In general, DBP was found to be quite tolerant towards modification at the 3 position of the 25-OH-D_3 molecule, as well as incorporation of groups of different nature and sizes in the tether attached to the 3-hydroxyl group of 25-OH-D_3 molecule.

Incubation of DBP (in serum or in pure form) with radiolabeled versions of the affinity analogs I and II (I* and II*, ^{14}C as well as [^3H]-labeled analogs; Fig. 3) produced covalently labeled DBP (affinity labeling). On the other hand, incubation of serum or pure DBP with III–V*, followed by photolysis, produced covalently labeled DBP (photoaffinity labeling). In each of the above-mentioned cases, the extent of covalent labeling was drastically reduced when the incubation was carried out in the presence of a large excess of 25-OH-D_3. These results demonstrated that these sub-

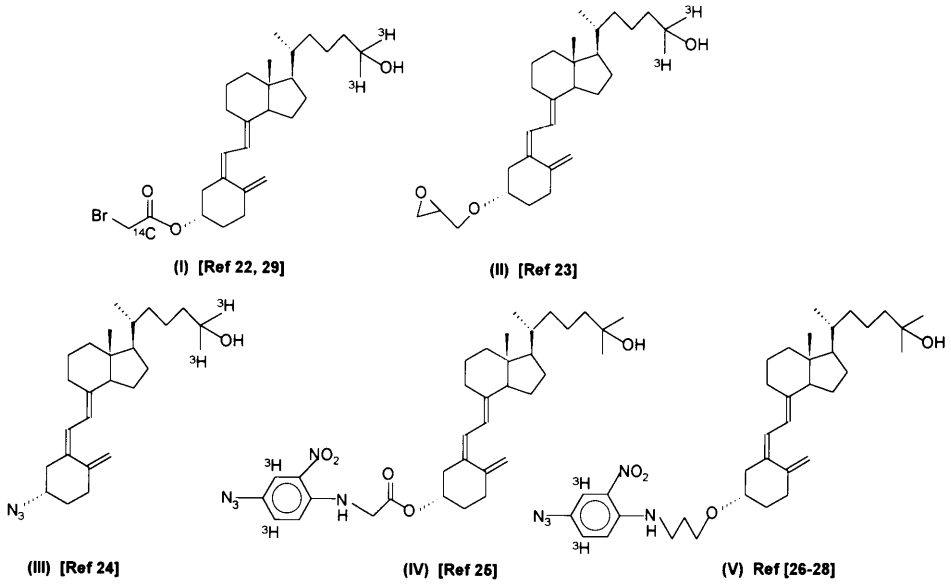

Figure 3. Chemical structures of various affinity and photoaffinity analogs of 25-hydroxyvitamin D_3 and their radiolabeled counterparts. Positions of the radioisotopes (^3H or ^{14}C) are marked directly on the structures. (I) 25-Hydroxyvitamin D_3-3β-bromoacetate; (I*) 25-hydroxy[26(27)-methyl-^3H]vitamin D_3-3β-bromoacetate or 25-hydroxyvitamin D_3-3β-bromo[1-^{14}C]acetate; (II) 25-hydroxyvitamin D_3-3β-(1,2-epoxypropyl)ether; (II*) 25-hydroxy[26(27)-methyl-^3H]vitamin D_3-3β-(1,2-epoxypropyl)ether; (III) 25-hydroxy-3-deoxy-3β-azido-vitamin D_3; (III*) 25-hydroxy[26(27)-methyl-^3H]-3-deoxy-3β-azido-vitamin D_3; (IV) 25-hydroxyvitamin D_3-3β-[N-(4-amido-2-nitrophenyl) glycinate]; (IV*) 25-hydroxy[26(27)-methyl-^3H]vitamin D_3-3β-[N-(4-azido-2-nitrophenyl)glycinate]; (V) 25-hydroxyvitamin D_3-3β-[N-(4-azido-2-nitrophenyl)amino]propylether; (V*) 25-hydroxy[26(27)-methyl-^3H]vitamin D_3-3β-[N-(4-azido-2-nitrophenyl)amino]propylether. Corresponding references are denoted in parenthesis.

strate-analogs covalently modified the binding-site in DBP which is specific for 25-OH-D_3 and other metabolites of vitamin D.

It should be noted that, although all the above-mentioned analogs covalently modified DBP, efficiency and specificity of labeling varied significantly among these compounds. For example, the photoaffinity analog IV*, which contains a nitrophenyl azido photoactive probe attached *via* a short chain to the 3β position of 25-OH-D_3, almost exclusively labeled DBP in human serum (25). In contrast, analog III*, containing a photoactive 3β-azido group in the place of the 3β-hydroxyl, labeled several proteins, including DBP, in human serum (24). Reason for this discrepancy is unclear. It could, however, be related to the nonpolar nature of the azido group (as in III), which could have changed the polarity of the parent 25-OH-D_3 molecule much more drastically than all other analogs listed in Figure 3. As a result nonspecific (random) labeling by analog III* was significantly enhanced.

In addition to covalently labeling the vitamin D sterol–binding site in DBP, a significant amount of data is currently available regarding the location of the 25-OH-D_3–binding site in hDBP. For example, a sample of hDBP, photoaffinity labeled with V*, was digested with BNPS-skatole, a chemical reagent that specifically cleaves peptide bonds next to a tryptophan (Trp). Due to the unique positioning of Trp-145, the only Trp in hDBP (4, 5), such treatment produced only two fragments. Importantly, only the smaller fragment (1–145) carried all the radioactivity as evidenced by SDS-PAGE/fluorography (27). This result demonstrated that the photoaffinity analog V* covalently modified the N terminus of hDBP. Furthermore, cyanogen bromide-cleavage of this photoaffinity-labeled sample (of hDBP) also produced a N-terminal peptide (1–107) bearing almost exclusively all the radioactive marker (27). These results strongly suggested that the putative 25-OH-D_3–binding domain in hDBP is located in the N terminus of the protein.

Recently, Haddad *et al.* and our laboratory developed 25-hydroxyvitamin D_3-3-bromoacetate (I) as an affinity labeling analog of 25-OH-D_3 (22, 29). Synthesis of this analog, and its tritium (^{3}H)– and radiocarbon (^{14}C)–labeled versions, was much simpler than that of all other analogs listed in Figure 3. This reagent was used to focus further in the 25-OH-D_3–binding pocket of hDBP. For example, Haddad *et al.* have identified a N-terminal decapeptide from the tryptic digestion of hDBP-labeled with I* (^{14}C), although the amino acids that are modified by this reagent (contact points) have not been identified (22). Currently, our laboratory is involved in determining the topography of the 25-OH-D_3–binding pocket of DBP utilizing this analog of high specific activity and other affinity analogs in which the molecular probes are attached to C-6 and C-19 positions of 25-OH-D_3 (*vide infra*).

We have also probed the vitamin D sterol-binding domain of hDBP by studies involving specific chemical modifications of amino acids in the protein. These modifications were coupled with 25-OH-D_3 binding and internal fluorescence assays. For example, treatment of hDBP with N-bromosuccinimide (NBS, specific modifier of Trp) reduced the internal fluorescence of ^{3}H–25-OH-D_3 binding by the protein in a dose-dependent manner (30). However, the loss of DBP fluorescence and ^{3}H–25-OH-D_3 binding were almost completely protected by pre-incubation of hDBP samples with an excess of 25-OH-D_3. These results strongly emphasized the importance of Trp (single residue at position 145) to 25-OH-D_3 binding. In a similar fashion, histidine residues (His, total six) of hDBP were targeted by specific modification with diethylpyrocarbonate (DEPC). It was observed that DEPC-treatment of hDBP reduced ^{3}H–25-OH-D_3 binding in a dose-dependent manner; and this loss in binding was almost completely protected by pre-incubation with 25-OH-D_3. Furthermore, UV absorbance studies of DBP samples, with or without DEPC, and with or without 25-OH-D_3 and DEPC, demonstrated that one His is important to 25-OH-D_3 binding. Collectively, these results strongly suggested that Trp-145 and one His residue (out of a total of six) are integrally involved in 25-OH-D_3 binding.

According to the primary structure of DBP, there are two His residues that are present in the 1–145 region (domain I) of hDBP, and one is implicated in 25-OH-D_3 binding by the above-mentioned "chemical mutation" studies. Furthermore, position of Trp 145 is unique for DBP, because there is no Trp in domains I of ALB and AFP, the other members of DBP-gene family. In addition, DBP contains two additional cysteines at positions 13 and 59, providing an extra disulfide linkage, and an additional fold in the first internal domain of the molecule (4, 5). Our photoaffinity/affinity and chemical modification studies strongly suggest that 25-OH-D_3 binding indeed may occur entirely through this sequence. This notion also leads one to think that DBP may have a molecular structure in which functionally distinct domains are located in structurally distinct regions.

Actin and Fatty Acid Binding by DBP

Numerous studies have been carried out to show that DBP plays an important role in depolymerizing actin polymers in circulation. During injury and cell lysis, DBP sequesters monomers of actin (G-actin) with very high affinity ($K_a = 10^{-8}$ *M*), thus preventing the harmful effects of polymeric actin (F-actin) in clogging the arteries (10–12). This beneficial effect of DBP

is known to be assisted by gelsolin, another plasma protein (31). It has also been observed that this sequestration process is further aided by the rapid clearance of DBP-actin complex (31).

During the past few years, actin-binding by DBP has been subjected to several structure-functional studies. For example, binding of G-actin by DBP has been shown to change the conformation of the latter, which can be monitored by the change in the fluorescence of 2-*p*-toluidinylnapthalene-6-sulfonate, an external hydrophobic probe (32). It has also been shown that DBP displays high-affinity binding towards a 33.5-kDa tryptic fragment of G-actin. Furthermore, presence of DBP prevented further degradation of actin (33). Collectively, these results indicated that only a fraction of G-actin, instead of the whole protein, is required for DBP-binding.

In a recent study, Haddad *et al.* have affinity chromatographed a tryptic digest of DBP on a G-actin–immobilized affinity column, which specifically bound the "actin-binding domain" of DBP (22). Structural analysis of this peptide showed that the actin-binding activity is concentrated in the domain III of DBP. Houmeida *et al.*, on the other hand, have demonstrated that the DBP-actin interaction site is located in the carboxy-terminal sequence of actin (34). It is noteworthy that only fragments of the whole proteins (DBP and G-actin) are required for the interaction between these proteins.

It is well documented that, structurally, DBP is highly homologous with ALB and AFP, yet DBP differs from these proteins in being a specific binder of vitamin D metabolites, as well as a specific scavenger of G-actin. In addition, DBP differs from ALB and AFP in possessing relatively weak binding affinities for saturated and unsaturated fatty acids ($K_a = 10^{-5}$–10^{-6} *M*) (3). This discrepancy could be related to a shorter third domain (C terminus) of DBP compared with that of ALB. ALB contains several high- and low-affinity fatty acid–binding sites (35). In comparison, DBP is known to possess a single fatty-acid–binding site (14). In addition, the fatty acid-binding sites in ALB are primarily located in domain III of the protein, although some are present in domains 1 and 2 also. In the case of DBP, such a situation will probably lead to overlapping of the binding domains for various ligands.

Recently, we have extended our ligand-binding site probing studies of DBP to fatty acids due to our general interest in studying the structure-function relationship of DBP. In this effort we have synthesized several affinity labeling analogs of palmitic acid and their radiolabeled counterparts (unpublished results). We observed that, while *p*-nitrophenyl ester of ^{14}C-palmitic acid specifically and covalently labeled bovine serum albumin, the same reagent failed to label in hDBP (unpublished results). Recently, Reed has employed a conjugate of Woodward K reagent and ^{14}C-palmitic acid to label the fatty acid–binding site in bovine serum albumin (36). In the case of hDBP, this reagent covalently and specifically labeled the palmitic acid–binding site in the protein (manuscript in preparation).

It is noteworthy that our results clearly indicated that the chemical nature of the primary fatty acid-binding domain in DBP differs from that of bovine serum ALB. For example, bovine serum ALB could tolerate a hydrophobic (*p*-nitrophenyl ester) as well as a hydrophillic (Woodward K reagent) head group at the carboxyl end of palmitic acid. However, DBP could only accommodate a polar and Zwitterionic carboxyl head group. In any event, this affinity analog and others will be used in the future to locate and characterize structurally the fatty acid–binding site in DBP.

DBP is a multidomained protein, and the results discussed above clearly indicate that each domain is largely reserved for a specific ligand binding. The question is whether the binding of a ligand to a specific site and in a specific domain can influence the binding of another in a different domain, and can bring about any possible change in its function. Such interaction among domains ("cross-talk") has been invoked to explain the strong competition between 25-OH-D_3 and arachidonic acid in terms of their binding to DBP (14). In comparison, palmitic acid did not compete with 25-OH-D_3 at all. This striking difference has been explained by possible changes in the conformation of the protein upon arachidonic acid binding. However, no direct proof of such an event has been forwarded. It will be interesting to probe the interdependence among other endogenous ligands of DBP (i.e., actin/vitamin D sterols) and actin/fatty acids.

Future Research

Development of New Generation Analogs of 25-OH-D_3 to Identify Additional Contact Points within the 25-OH-D_3–Binding Pocket of DBP. It is noteworthy that, in all the analogs shown in Figure 3 (except III), the 3-hydroxyl group of 25-OH-D_3 is modified with a short leash (tether), the end of which contains the affinity/photoaffinity probes. We reason that covalent modification of an amino acid/amino acids within the 25-OH-D_3–binding pocket will depend on the proximity of affinity/photoaffinity probes (attached to 25-OH-D_3) to the amino acid/amino acids (contact points) within this binding pocket. Hence, it is necessary that DBP be labeled with future generation affinity/photoaffinity analogs where the probes are attached to the parent molecule (25-OH-D_3) in positions

other than 3. These analogs could potentially identify "other" contact points within the 25-OH-D_3–binding pocket of DBP. Recently, we have developed synthetic schemes to introduce a tether, with a chemically reactive end-group, at the 6 and 19 positions of vitamin D_3. Currently, we are in the process of applying similar synthetic strategy to obtain C-6 and C-19 derivatized affinity/photoaffinity analogs of 25-OH-D_3. These analogs will enable us to identify various contact points within the 3-dimensional architecture of the vitamin D sterol–binding domain in DBP.

Development of DBP Mutants and Independent Domains. Advent of molecular biology has offered us a powerful tool to complement the chemical/biochemical method (described above) with "point mutation" studies. For example, when the "contact points" within the binding pocket for vitamin D sterols in DBP are known (by affinity/photoaffinity and "chemical mutation" studies), these amino acids could be changed to amino acids of varying polarity/charge by site-directed mutagenesis. Competitive binding assays of these mutant DBP proteins with 25-OH-D_3 and other metabolites of vitamin D_3 will determine the importance of these "contact points" towards vitamin D sterol binding.

Recently, we developed a bacterial system to express full-length human DBP as a partner in glutathione S-transferase-DBP fusion protein. The recombinant hDBP, obtained by the thrombin-cleavage of the fusion protein, was functional as judged by its specific binding ability for 25-OH-D_3 (37). In the future, this expression system could be used for expressing point mutants as well as independent domains of DBP. We have recently expressed a truncated (277–456) version of DBP (domain III) and observed that this independent domain (of DBP) had no binding for 25-OH-D_3 (unpublished results). This result supported our earlier observation that vitamin D sterol binding may be restricted to the N terminus (domain I) of DBP. Development of other domains and parts of DBP as well as mutants will be extremely helpful in determining the interdependence among various endogenous ligands as well as determining the extent of "cross talk" among various domains, and ultimately in assigning functions to various structural motifs in DBP.

Conversion of DBP to DBP-Macrophage Activating Factor. It was recently discovered that DBP converted *in vivo* to a very potent macrophage activating factor (DBP-MAF) (38–40). This conversion involves stepwise deglycosylation of glycosylated DBP molecules (natural DBP is only 0%–5% glycosylated) by membraneous β-galactosidase and sialidase of inflammation-primed B and T cells. According to this mechanism the core N-acetylgalactosamine (galNAc), which is retained by the protein after deglycosylation, is essential for the observed biological properties of DBP-MAF.

In addition to simulating macrophages to induce phagocytosis of target antigens, DBP-MAF has recently been found to be a potent regulator of osteoclasts (41, 42). Schneider *et al.* have demonstrated that infusions of *ex vivo*–generated DBP-MAF (picogram quantities) to osteopetrotic rats resulted in a significant decrease in bone mass (43). Similar results were also obtained in normal rats which were used as controls, suggesting that the observed bone resorption property of DBP-MAF is mediated by the activation of osteoclasts. Based on these results, it has been suggested that DBP-MAF is a major contributor to localized tissue destruction in several osteolytic diseases (43).

Recently, we have carried out preliminary studies in which DBP-MAFs, obtained from natural DBP and recombinant full-length DBP, were subjected to macrophage superoxide production and bone resorption assays. We observed that, while the natural DBP-MAF significantly enhanced macrophage and osteoclast activities over the control, recombinant DBP-MAF had no effect on these activities (unpublished results). Since the bacterially expressed DBP did not contain any sugar, these results strongly supported the earlier hypothesis that the core sugar moiety (galNAc), which remains attached to DBP after deglycosylation, is essential for DBP-MAF activities.

It is well-established that the core galNAc moiety, described above, is present in the third domain of DBP (44). Since this region of the protein is implicated in the actin and fatty acid bindings, one wonders whether DBP-MAF activities are influenced by these endogenous ligands. Similar question can be raised about vitamin D sterol binding, which takes place in the domain I. Further research is required to answer these questions, which may be coupled with the potential therapeutic applications of DBP-MAF (43).

Possible Relationship between DBP and VDR. DBP and VDR belong to two entirely different classes of proteins. DBP is a typical member of the globulin family and is structurally similar to ALB and AFP. VDR, on the other hand, is a DNA-binding nuclear transcriptional factor and is similar to other classical steroid hormone receptors. These two proteins, however, share a common function: both of them bind metabolites of vitamin D with high efficiency and specificity. Thus, it is not completely unlikely that these two proteins may have had a common progenitor/primordial gene which has undergone profound mutations during evolution. If there is any truth to this statement, some structural motifs could still be traced in these proteins, particularly in the vitamin D sterol–binding domains. During the past few years our labo-

ratory and that of others have carried out affinity/photoaffinity labeling studies to label the $1,25[OH]_2D_3$–binding site in VDR (45–51). These studies could potentially lead to the identification and mapping of the $1,25[OH]_2D_3$–binding domain of VDR, just as in the case of DBP described earlier. Successful completion of these studies will provide important information about the possible link between genes producing these disparate proteins.

Conclusion

Physiological implications of the varied functions of DBP are unclear till today. For example, in 1987 Eales *et al.* reported that Gc1f isomorph of Gc (DBP) could be used as a potential marker for susceptibility to human immunodeficiency virus (HIV) and subsequent progression towards acquired immunodeficiency syndrome (AIDS) (52). This work, however, could not be confirmed by other researchers; and the claim has since been refuted (53–55). In our estimation, any putative biological role of DBP, related to any of its multiple functions, must depend on the initial binding of a ligand by DBP. This, in turn, should depend on the structure of the ligand as well as the 3-dimensional architecture of the binding pocket reserved for this particular ligand. Thus, information about the structural elements in the ligand-binding pocket/pockets will be absolutely essential for the proper understanding of the multiple ligand-binding by DBP and its putative physiological implications.

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