# Identification of the Sterol- and Actin-Binding Domains of Plasma Vitamin D Binding Protein (Gc-Globulin)†

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ABSTRACT: The mammalian plasma vitamin D binding protein (DBP), or Gc-globulin, is recognized to have at least two functional properties: sterol binding and G-actin sequestration. Affinity labeling of the sterol binding site with the radioactive electrophilic ligand, 3β-(bromoacetoxy)-25-hydroxycholecalciferol, followed by limited proteolysis, permitted the isolation and identification of three overlapping peptides in the amino terminus of the molecule. When G-actin affinity chromatography was applied to other proteolytic fragments, two fragments from the carboxy terminus of the molecule were isolated and identified. Another, large, tryptic fragment displayed both sterol- and actin-binding properties. The amino-terminal assignment of the sterol-binding domain was confirmed by demonstrating sterol-specific binding by an in vitro transcribed and translated product of a mutated rat DBP cDNA encoding a protein truncated in its carboxy terminus. The sterol-binding domain was localized to the region between the first-amino-terminal disulfide bond, and the actin-binding domain was found between residues 350 and 403. A high degree of sequence conservation in these regions was found among human, rat, and mouse DBP's. These functional domain assignments confirm the apparent independence of these two binding activities and help to explain the observed triprotein complex of DBP–actin–DNase I and the competition between DBP and profilin for G-actin binding. Our findings should facilitate more precise delineation of the binding domains by site-directed mutagenesis experiments.

A major globulin in the blood of all mammalian and avian species examined to date, the vitamin D binding protein (DBP)[1] or group-specific component (Gc-globulin), displays features and properties that establish it to be a multifunctional, monomeric constituent of plasma (Cooke & Haddad, 1989). It is also recognized as the group-specific component (Gc-globulin) of human plasma (Daiger et al., 1987). As the major carrier for vitamin D and its metabolites, the protein's concentration in plasma far exceeds that of its sterol ligands, suggesting other roles than transporter and reservoir for vitamin D sterols (Cooke & Haddad, 1989). DBP has been shown to bind to monomeric actin (Van Baelen et al., 1980) with high affinity ($K_d$ = 1 nM) (McLeod et al., 1989), and DBP–G-actin complexes have been demonstrated in plasma from humans and animals experiencing tissue injuries (Lee et al., 1985, 1987). As an actin monomer sequestrant, DBP complements plasma gelsolin's severing activity on F-actin, and these two proteins are now thought to constitute a powerful actin scavenger system in plasma (Haddad et al., 1990).

A variety of other functional activities have been suggested for DBP. The protein has been associated with the immunoglobulin receptor on lymphocytes (Petrini et al., 1983, 1985) and the macrophage Fc receptor activation that occurs in mixed immunocyte incubations (Yamamoto et al., 1991). DBP binds to C5a and C5a-des-Arg, possibly working as a cochemotaxin for neutrophils (Kew & Webster, 1988). The protein has also been observed on the surface of monocytes (McLeod et al., 1986; Guoth et al., 1990) and cytotrophoblasts (Nestler et al., 1987), but not on erythrocytes or platelets. Cell-surface DBP is thought to be acquired from external sources since endogenous DBP synthesis has only been observed in hepatocytes. However, low levels of DBP mRNA have been detected in several rat tissues (McLeod & Cooke, 1989). Since a $Gc^0$ homozygote is not recognized, in spite of extensive searches, complete deletion of DBP may be a lethal event. It seems likely, therefore, that one or more functions encoded by the DBP gene are necessary to life.

We have studied the two best-established properties of DBP with the goal of identifying structure–function relationships. Sterol- and G-actin-binding domains were sought by limited enzymatic proteolysis of human DBP (hDBP), fragment isolation, and amino acid sequencing. To confirm these findings, we also studied the binding properties of products prepared by in vitro transcription and translation of full-length and 3′ truncated rat DBP (rDBP) cDNA's. We report here findings that establish functional domains within the DBP molecule and compare the regions to those in the other members of the albumin/α-fetoprotein/DBP multigene family.

## EXPERIMENTAL PROCEDURES

### *Materials*

Pure hDBP was isolated as previously described (Haddad et al, 1984) or purchased (Calbiochem). Monomeric rabbit skeletal muscle actin was prepared as previously reported (Spudich & Watt, 1971; McLeod, et al., 1989). Radioinert 25-hydroxycholecalciferol (25-$OHD_3$) and 3β-(bromoacetoxy)-25-hydroxycholecalciferol were kindly provided by Dr. Milan Uskokovic of Hoffman-LaRoche (Nutley, NJ). 25-OH[$^3$H]-

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[1] Abbreviations: DBP, plasma vitamin D binding protein; 25-$OHD_3$ or calcifidiol-25, hydroxycholecalciferol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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26,27]$D_3$ (sp act. 19.9 Ci/mmol) and bromo[1-$^{14}$C]acetic acid (sp act. 51 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, IL. Silica gel thin-layer chromatography sheets were obtained from Gelman, Ann Arbor, MI. Staphyloccal V8 protease (Miles Labs), carboxypeptidase Y (Pierce, Rockford, IL), and trypsin and radioactive and radioinert polyacrylamide gel electrophoresis (PAGE) molecular weight markers (Sigma) were purchased. En$^3$Hance was acquired from New England Nuclear (Boston, MA). Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA) and Bethesda Research Laboratories (Gaithersburg, MD). [$\alpha$-$^{32}$P]dNTP's (3000 Ci/mmol) and [$^{35}$S]methionine were obtained from Amersham Corp.

*Methods*

*Synthesis of 3-β-(Bromoacetoxy)calcifidiol Ester.* A modification of techniques (Haddad et al., 1981; Warren et al., 1975) was used. Dichloromethane was dried over calcium hydride pellets, and 25-hydroxy[$^3$H]cholecalciferol was mixed with radioinert 25-$OHD_3$ to provide a final specific activity of 6 μCi/μmol of sterol. Monobromoacetic acid (boiling point 202–204 °C) or bromo[1-$^{14}$C]acetic acid (Amersham; sp act. 51 mCi/mmol) was added in different preparations of the ester, the latter used with radioinert 25-$OHD_3$.

To a stirred solution of 6 μmol (2.4 mg) of sterol in 0.2 mL of $CH_2Cl_2$ at 0–4 °C were sequentially added 6 μmol of bromoacetic acid and 8 μmol of dicyclohexylcarbodiimide in 0.05 mL of the same solvent. After 5 min, 6.5 μmol of dry pyridine was added, and the mixture was stirred at 0–4 °C for 1 h, and gradually to 23 °C over the next 2–3 h. Aliquots of the reaction mixture were analyzed by thin-layer chromatography (TLC) on silica gel layers in chloroform/acetone (19:1 v/v). 3β-(Bromoacetoxy)-25-$OHD_3$ (relative front = 0.5) and 25-$OHD_3$ (relative front = 0.33), respectively, were located under an ultraviolet lamp. The reaction neared completion in 2–4 h, and the reaction mixture was dried under a stream of nitrogen. Two 2-mL aliquots of acetone were added and dried similarly to remove traces of dichloromethane. The residue was mixed with 3 mL of acetone/absolute ethanol (1:1 v/v) and filtered through gauze in a 5-cm glass column. Solid residue (dicyclohexylurea) was extracted again in the same solvent. The combined eluates were pooled, dried under nitrogen, and dissolved in the TLC solvent for the preparative isolation of the ester. The ultraviolet light-absorbing ester band was cut from the silica layer, added to absolute ethanol/ether (2:1 v/v), and mixed. The eluate was filtered through gauze, nitrogen-dried, and solubilized in absolute ethanol. Aliquots were used for UV spectroscopy and for scintillation spectroscopy. A molar extinction coefficient of 18 000 was used for absorbance at 264 nm in absolute ethanol, and the specific activity of the ester was calculated, indicating a yield of 50–60% of the ester product. The product transformed to 25-$OHD_3$ during saponification in 0.1 N KOH in ethanol overnight, as judged by TLC.

*Labeling of hDBP with 3β-(Bromoacetoxy)-25-$OHD_3$.* In order to label the sterol-binding site in hDBP, we carried out saturation analyses with radioinert ester, radioactive and radioinert 25-$OHD_3$, and pure hDBP, using dextran-coated charcoal to remove unbound sterol (Haddad & Chyu, 1971). Sterols were added in ethanol (<10% of final volume) to hDBP in 0.01 M Tris, pH 8.0. The ester was 10–12-fold less potent than 25-$OHD_3$ in displacing 25-OH[$^3$H]$D_3$ from hDBP, in equilibrium incubations, at 23 °C over 2 h. However, when equimolar amounts of the ester and hDBP were incubated for 2 h prior to the addition of 25-OH[$^3$H]$D_3$, >95% of the tritiated sterol was available to the adsorbent, indicating that the ester had competed for, and covalently bound to the specific binding site. Under identical conditions, 10 nmol of radiolabeled ester was preincubated with 10.5 nmol of hDBP in 0.01 M Tris, pH 8.0, for 2 h at 23 °C. In parallel, 10% of the above ingredients were mixed in the presence of a 100-fold molar excess of radioinert 25-$OHD_3$, 17β-estradiol, or testosterone. Aliquots of the supernatant from the charcoal step were heated at 70 °C in 1% SDS for 1 h prior to analysis in SDS–PAGE under reducing conditions. The $^3$H- and $^{14}$C-labeled esters were shown to be covalently bound to hDBP. The hDBP labeled with radioactive 3β-(bromoacetoxy)-25-OH[$^3$H]$D_3$ was used in the proteolytic cleavage analyses.

*Analyses of Proteolytic Fragments of Sterol-Labeled hDBP.* Pure hDBP was incubated with varying amounts of trypsin for increasing periods of time and aliquots were boiled for 5 min in 2% SDS prior to SDS–PAGE under reducing conditions. With suitable conditions, the radioactive ester-labeled hDBP was partially digested with the enzyme and the products were separated in highly cross-linked polyacrylamide gels (Huang & Matthews, 1990; Schagger & von Jagow, 1987). In the 20-cm separating gels using Tricine buffer, a 5–15 mA constant current was used over 25–30 h. Gels were analyzed for discrete peptides by Coomassie blue staining and their radioactivity content was determined by scintillation spectrometry of paired lanes. For sequencing, identified peptides were electroblotted onto Immobilon membranes in a Hoeffer system at 800 mA for 1 h at 40 °C. For peptides correlating with radioactivity in paired gel lanes, their stained membrane bands were isolated with a razor blade and were analyzed for amino-terminal sequence in an Applied Biosystems 473A protein sequencer. The results obtained were compared with the known sequence of hDBP (Cooke & David, 1985). Sequences for peptide fragments were then used to predict the likely enzyme cleavage site at the carboxy terminus of the identified peptide (Cooke & David, 1985).

*Strategies for Identification of G-Actin-Binding Regions in hDBP.* We determined whether proteolytic fragments of hDBP could be identified by their binding affinity for G-actin immobilized on a gel column (Haddad et al., 1984, 1985). Pilot experiments with trypsin and *Staphylococcus aureus* V8 protease digests of hDBP indicated that the latter enzyme was suitable. The V8 protease (50 μg) was incubated with 500 μg of hDBP in 250 μL of 0.01 M sodium phosphate buffer, pH 7.8, for 40 h at 37 °C. The hDBP digest was then chilled in an ice bath and applied to a column packed with G-actin–Affigel-15 (5 mg of protein/mL of packed gel) equilibrated in 0.15 PBS, pH 7.4, at 4 °C. The column was rinsed sequentially with the PBS and 0.5 M NaCl/buffered to pH 7.4 with sodium phosphate (0.01 M), until the effluent stream displayed no absorbancy at 280 nm. Adherent peptides were eluted in 0.01 M Tris, pH 7.4, containing 5% SDS. The peptides in the final eluates were separated in 20 cm, 15% polyacrylamide gels using a Tris/Tricine cathode buffer under denaturing and reducing conditions. Other aliquots were applied to the same PAGE system using 0.002% thioglycolic acid in the running buffer, and peptides from the gel were electroblotted onto a Pro-Blott membrane (Applied Biosystems, Foster City, CA) in 10 mM CAPS, pH 11, with 10% methanol at 800 mA for 1 h at 4 °C. The gel and membrane were stained in 0.1% Coomassie blue and destained in aqueous 40% methanol/10% acetic acid until the background was clear.

Discrete bands were cut from the dried membrane and stored in dry vials until amino acid sequence analyses were performed.

In separate studies, we carried out native 10.5% PAGE shift assays of hDBP digests preincubated with or without G-actin in depolymerizing or G-actin buffer. The G-actin–DBP complex is retarded in this system relative to free DBP, and we identified band shifts as a result of actin binding by peptides from partial proteolytic digests of hDBP. When a 50-kDa peptide from a trypsin digest was eluted from the native gel slice for size estimation by reducing SDS–PAGE, it was found also to exhibit specific binding of 25-$OHD_3$. Therefore, the blotted peptide was analyzed for amino acid sequence at its amino terminus. Since its carboxy-terminal cleavage site was judged to be critical, we eluted the native PAGE-purified 50-kDa fragment for treatment with carboxypeptidase Y in order to determine the carboxy-terminal portion of hDBP that was unnecessary for actin binding. Timed aliquots from a 50-kDa fragment–enzyme (50:1) incubation (3, 6, and 9 h) were mixed with 10 volumes of cold acetone to precipitate the protein. The acetone supernatants were dried under a stream of $N_2$ and subjected to amino acid analyses.

*In Vitro Transcription and Translation of Full-Length and Truncated Rat DBP.* A previously described mutant rDBP cDNA fragment was utilized as template for transcription and translation of a truncated DBP. This clone, isolated from a rat kidney cDNA library, contains a deletion at codon 264 (GAA to GA–), presumed to be due to a reverse transcriptase error. This deletion results in a frameshift and subsequent termination at codon 267, predicting translation of a 31.5-kDa protein (McLeod & Cooke, 1989). The 5′ *Eco*RI subclone of this cDNA (prkDBP875) that encompasses the mutation was subcloned into the pGEM3 transcription vector (Strategene), and its orientation in the vector was determined by restriction digestions. The plasmid was linearized with *Xba*I for use as a transcription template.

To generate a template for synthesis of full-length rDBP mRNA encoding the entire protein, the 5′ *Eco*RI subclone of liver DBP cDNA (Cooke, 1986), prlDBP875, was ligated to the 3′ *Eco*RI subclone, prlDBP760, at their common *Eco*RI site (codon 277). A clone containing these fragments was selected, and their orientation was confirmed by DNA sequence analysis (Sanger et al., 1977). However, RNA generated from this full-length clone (prlDBP1635) encoded a protein of about 20 kDa, not the expected 58 kDa. It was suspected that a mutation had occurred during the cloning, but this was not further investigated. Instead, a second full-length clone was generated by ligating a gel-purified *Nde*I–*Xba*I fragment from kidney DBP cDNA subclone (prkDBP875), which included the 5′-nontranslated region, and the initiation codon through codon 210, to the *Nde*I–*Xba*I fragment from liver DBP cDNA subclone (prlDBP1635) that included codon 210 through the 3′-nontranslated region and the pGEM3 vector. The final construction, prklDBP1635, was therefore a hybrid between the 5′ end of the kidney clone, which was known to translate beyond the 20-kDa block, and the 3′ end of the liver clone. Rat kidney and liver DBP cDNAs are identical except for polymorphisms at codons 116, 159, and 194 and in the 3′ nontranslated region (McLeod & Cooke, 1989). This plasmid was linearized with *Pvu*II for use as a transcription template.

The linearized cDNA templates were transcribed in the presence of T7 RNA polymerase (rkDBP875) or SP6 RNA polymerase (rklDBP1635) at 40 °C for 1 h. The transcription products were subsequently purified on a G-50 Sephadex spin column (Boehringer Mannheim, Indianapolis, IN) that was prewashed with sterile water. The transciption reaction was analyzed on an 8 M urea, 6% polyacrylamide gel to confirm that the transcripts were intact as judged from their size. A total of 1–5 μL of the transcription reaction was translated in vitro in a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine at 30 °C for 40 min as previously described (Liebhaber et al., 1984). Translation products were analyzed on 10.5% polyacrylamide–SDS gels that were treated with Resolution Enhancer (EM Corp, Chestnut Hill, MA), dried, and exposed to X-ray film (Kodak AR-5) for various times at 70 °C.

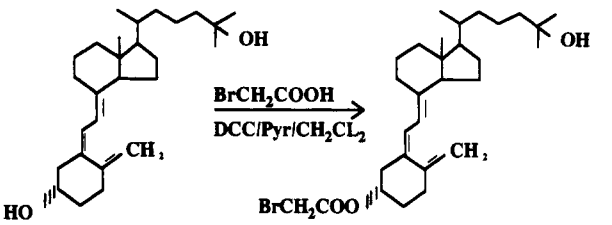


FIGURE 1: Synthesis of 3-(bromoacetoxy)calcifidiol, using calcifidiol, bromoacetic acid, dicyclohexylcarbodiimide, and pyridine in dichloromethane.

Translation products of the full-length and prematurely terminated rDBP mRNAs were evaluated by SDS–PAGE and autoradiography. Neither product entered native polyacrylamide gels, presumably due to complexing with unidentified macromolecules in the reticulocyte lysates. To evaluate sterol binding, each product was incubated for 1 h at 4 °C with 25-OH[$^3$H]$D_3$ in the absence or presence of a 100-fold excess of radioinert 25-$OHD_3$ or 17β-estradiol. Radioligand binding was assessed in supernatants after adsorbent removal of unbound ligand (Haddad & Chyu, 1971).

*Protein Structure Prediction and Hydrophobicity.* For relevant peptides, secondary structure was predicted from the tables of Chou and Fasman (1978) and the Robson–Garnier method (Garnier et al., 1978). The distribution of hydrophobic residues in peptides involved in sterol binding was also examined (Kyte & Doolittle, 1982; Eisenberg et al., 1982).

## RESULTS

The 3β-(bromoacetoxy) ester of 25-$OHD_3$ (3-(bromoacetoxy)calcifidiol) (Figure 1) was synthesized as described in Methods and then tested to determine its ability to serve as an affinity ligand for hDBP. Following the synthesis, only the monoacetylated product was observed under the conditions employed. This was presumed to be due to steric hindrance of the 25-hydroxyl group by the neighboring 26 and 27 carbon atoms. The ability of 3β-(bromoacetoxy)calcifidiol to competitively displace 25-OH[$^3$H]$D_3$ from its binding site on hDBP has been studied in equilibrium binding competition with 25-$OHD_3$. 3β-(Bromoacetoxy)calcifidiol was 10–12-fold less potent that 25-$OHD_3$ in this study (Haddad et al, 1981). However, preincubation of hDBP with an equimolar concentration of the 3β-(bromoacetoxy)calcifidiol prevented more than 95% of the specific binding of 25-OH[$^3$H]$D_3$, indicating that this electrophilic ligand was associating with the hDBP sterol-binding site.

Radioactive 3-(bromoacetoxy)calcifidiols were synthesized and studied under similar binding conditions. The radioactive compounds bound covalently to hDBP as indicated by their mobility in SDS–polyacrylamide gels under denaturing conditions (Figure 2: panel A, lanes 1 and 2; panel B). A single

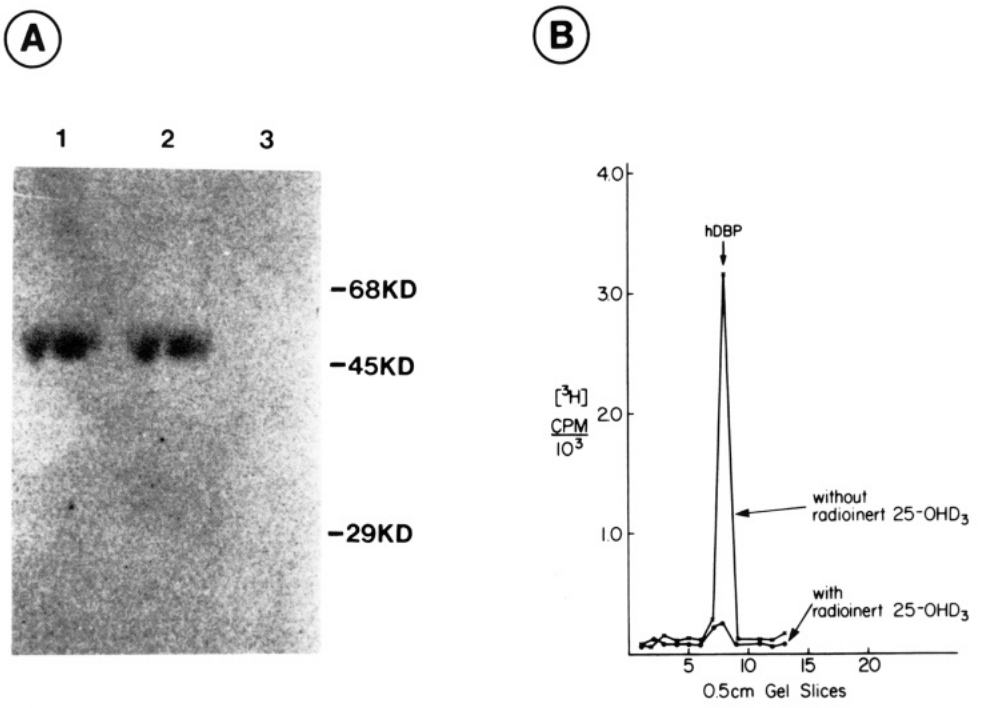


FIGURE 2: Specific labeling of the sterol-binding site of hDBP with radiolabeled 3-(bromoacetoxy)calcifidiol. (A) Autoradiogram of SDS–polyacrylamide gel: lanes 1 and 2 represent incubations of hDBP with 3-(bromo[$^{14}$C]acetoxy)calcifidiol; lane 3 shows the same incubation mixture in the presence of a 100-fold molar excess of radioinert calcifidiol. (B) Radioactivity contained in gel slices from an SDS–polyacrylamide gel following electrophoresis of incubations of hDBP with 3-(bromoacetoxy)-[$^3$H]calcifidiol in the absence or presence of a 100-fold excess of radioinert calcifidiol. The arrow indicates the position of stained, intact hDBP in a companion lane.

radiolabeled band was observed migrating at 58 kDa, the expected mobility of hDBP. In the presence of a 100-fold molar excess of 25-OHD$_3$, 3$\beta$-(bromoacetoxy)-$^3$H]calcifidiol and 3-bromo[$^{14}$C]acetoxycalcifidiol were prevented from binding to hDBP (panel A, lane 3 and panel B). Other steroid hormones such as estradiol and testosterone at a 100-fold excess were unable to displace these radiolabeled esters from hDBP under these conditions. On the basis of the specificity of the competitive binding data, we concluded that the radiolabeled 3-(bromoacetoxy)calcifidiol esters were covalently bound near the native sterol-binding site of hDBP.

Limited proteolysis of the covalent hDBP–radioactive ester complex with trypsin yielded three peptides that exhibited radioactive content, as assessed by staining and radioactive content of parallel lane gel slices (Figure 3 and Table I). Additional, parallel, gel lanes were electroblotted onto Pro-Blott membranes, and the bands corresponding to [$^3$H] content were cut out and analyzed for sequence. Repeat analyses of this digest in 20-cm, 15% polyacrylamide gels confirmed the labeled ester's association with these peptides. Amino acid sequencing from the amino termini of the fragments revealed two peptides cleaved at Lys-35 and one at Arg-5. All three peptides are located in the amino-terminal region of the hDBP molecule. These regions, as identified by sequencing and peptide size, are indicated in Table I.

The partial digestion of hDBP with V8 protease, followed by actin affinity column chromatography was found to yield two prominent fragments (29 and 13 kDa) that displayed high-affinity actin binding (Figure 4, lane 1). Following transfer of Pro-Blott membranes from 20-cm, 15% polyacrylamide gels run under denaturing and reducing conditions, both peptides were sequenced (Table I, section B). Both peptides were derived from the carboxy terminus of hDBP. The 29-kDa peptide began at Glu-212 and the 13-kDa peptide began at Glu-350.

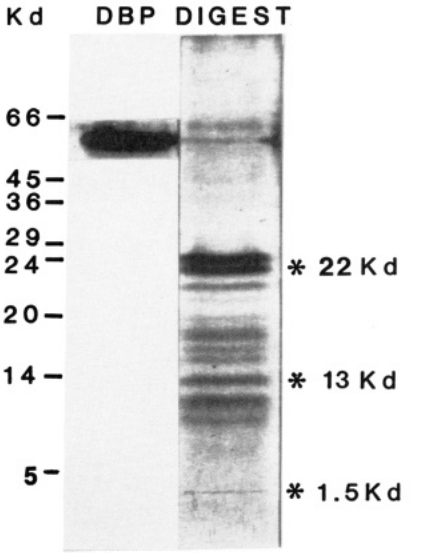


FIGURE 3: Coomassie blue stain of SDS–PAGE of intact and a partial trypsin digest of hDBP after its covalent linkage to 3-(bromoacetoxy)-[$^3$H]calcifidiol. Positions of size markers are shown on the left. Asterisks indicate the positions of peptides in neighboring gel slices that contained radioactivity.

Aliquots of hDBP were partially cleaved with trypsin and then incubated with or without G-actin in depolymerizing buffer. These samples were analyzed on a native 10.5% polyacrylamide gel for alterations in their mobility. One major tryptic fragment that migrated slower than intact DBP (Figure 5A, lane 4) was retarded in its migration when preincubated with G-actin (Figure 5A, lane 3). This peptide was eluted from the native gel and sized by SDS–PAGE and blotting (Figure 5B, lane 3) and was found to migrate at 50 kDa. The eluted 50-kDa fragment was subjected to competitive binding studies and found to bind 25-OH[$^3$H]D$_3$ specifically. The peptide was partially sequenced and found to have an amino-

Table I: Proteolytic Fragments of hDBP

| amino-terminal amino acid sequence[a] | region (amino acid residues)[b] | fragment size[c] (kDa) |
|---|---|---|
| A. Sterol-Binding Regions (Trypsin) | | |
| 1. Lys-Phe-Pro-Ser-Gly-Thr | 35–260 | 22 |
| 2. Arg-Asp-Tyr-Glu-Lys-Asn | 6–137 | 13 |
| 3. Lys-Phe-Pro-Ser-Gly-Thr | 35–49 | 1.5 |
| B. Actin-Binding Regions (V8 Protease) | | |
| 1. Glu-Lys-Lys-Ser-Arg-Leu-Ser-Asn-Leu-Ile-Lys | 212–458 | 29 |
| 2. Glu-Pro-Thr-Leu-Lys-Ser-Leu-Gly-Glu-Cys-Cys | 350–458 | 13 |
| C. Region with Both Functions (Trypsin) | | |
| 1. Arg-Gly-Arg-Asp-Tyr-Glu-Lys-Asn-Lys-Val-Cys | 3–403[d] | 50 |

[a] Consensus amino acid sequence found in the peptides indicated. Includes dibasic amino acid (trypsin) or Glu (V8 protease) cleavage residue. [b] Estimated amino acid residue span of the peptide, as judged from amino acid sequence, peptide size, and cleavage sites. [c] As estimated in SDS–PAGE along with molecular markers. [d] As determined by carboxypeptidase Y digestion and amino acid analyses.

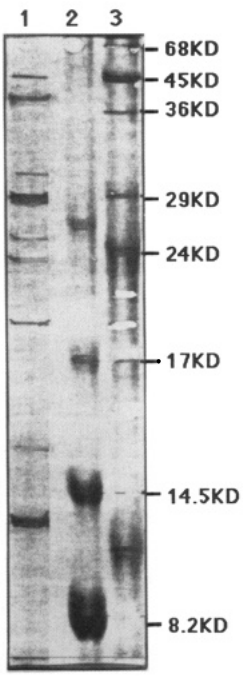


FIGURE 4: Stained SDS–PAGE blot from a partial V8 protease digestion of hDBP that had bound to the G-actin–agarose affinity column (lane 1). Lanes 2 and 3 show the positions of molecular weight markers on the stained blot. The prominent peptides, at 29 and 13 kDa, in lane 1 of the blot were isolated and sequenced. Due to a photographic mishap, an enhanced (lane 1) xerox copy of lane 1 is shown.

terminal cleavage site at Arg-3 (Table I, section C). Analysis of amino acid residues liberated during a timed carboxypeptidase Y digestion revealed Lys, Tyr, and Glu. This series of residues were uniquely found adjacent to Lys-403.

The region assignment of the sterol-binding site of DBP to the amino-terminal half of the molecule was confirmed by studies of the 25-OHD$_3$ binding properties of the translation products of in vitro transcribed full-length rDBP mRNA and rDBP mRNA prematurely terminating at codon 267. The [$^{35}$S]Met-labeled, full-length 52-kDa rDBP and the 32-kDa mutant protein products of in vitro transcription and translation are shown in Figure 6 (lanes 3 and 2, respectively). Identical products, without $^{35}$S labeling, were demonstrated to specifically bind 25-OH[$^3$H]D$_3$ by saturation analyses (Table II).

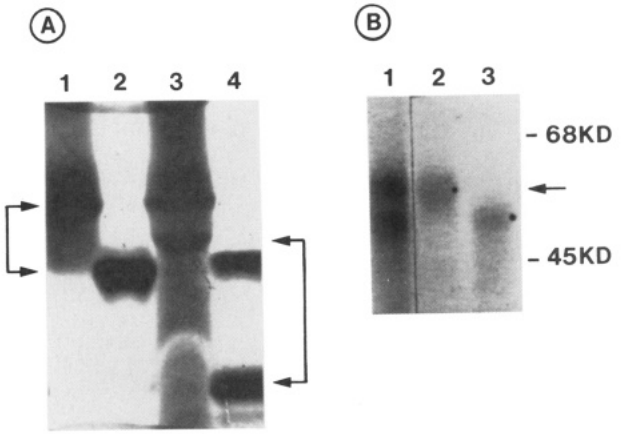


FIGURE 5: (A) Stained, native, 10.5% polyacrylamide gel of intact hDBP and a limited trypsin digest of hDBP after incubation without or with G-actin. Lane 2: intact hDBP. Lane 1: intact hDBP after preincubation with G-actin. Lane 4: partial tryptic digest of hDBP. Lane 3: the digest after preincubation with G-actin. Left bracket indicates positions of actin–hDBP (top) and free hDBP (bottom). The right bracket indicates the positions of actin–hDBP fragment (top) and free hDBP fragment (bottom). Oligomers of actin can be seen closer to the gel origin (top) in lanes 1 and 3. (B) SDS–polyacrylamide gel blot of intact hDBP and the major peptide below it in lane 4 of (A), following their elution from native PAGE gel segments. Lane 1: intact hDBP (top) and the major peptide. Lane 2: intact hDBP. Lane 3: the peptide fragment of hDBP, indicating a size of approximately 50 kDa. Elution of preparative amounts of the hDBP fragment from native polyacrylamide gel segments allowed analyses of its sterol-binding property and its C-terminus residues (carboxypeptidase Y). The fragment in the SDS–PAGE blot was used for N-terminus amino acid sequencing.

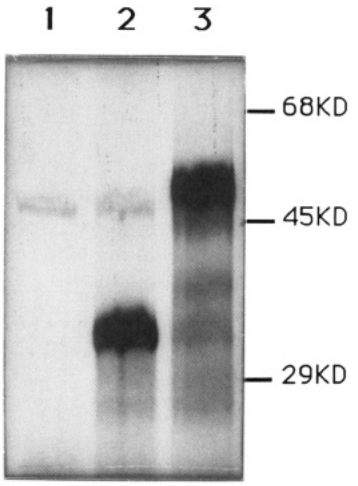


FIGURE 6: Autoradiogram of SDS–PAGE gels following in vitro translation of full-length rat DBP mRNA and carboxy-terminal-truncated rat DBP and mRNA. Lane 1: reticulocyte lysate in the absence of added mRNA. Lane 2: [$^{35}$S]methionine signals from incubation of the truncated rDBP mRNA in the translation system. Lane 3: the full-length rDBP synthesized in the lysate incubated with intact rDBP mRNA.

A graphic presentation of the peptides identified to be related to sterol binding, or to G-actin binding, or both, is shown in Figure 7. From the three peptides covalently bound by the radiolabeled sterol ester, the sterol binding site is seen to be in the amino terminus of the molecule. Since the size estimation of smaller peptides is not precise in the lower molecular weight range (Huang & Matthews, 1990), our best estimates of the sterol-binding region are residues 35–49, or 35–71, the latter reflecting the next tryptic site at Arg-71. On the other hand, overlapping peptides, which describe the actin-binding domain, are located in the carboxy terminus of the protein between residues 350 and 403.

Table II: Sterol-Binding Activities of In Vitro Transcribed and Translated Full-Length and Carboxy-Terminal-Shortened, Mutant cDNA's of Rat DBP[a]

| product | Binding[b] (dpm) 25-OH[$^3$H]$D_3$ alone | add estradiol | add 25-$OHD_3$ |
|---|---|---|---|
| reticulocyte lysate without added mRNA | 380 | 392 | 394 |
| reticulocyte lysate with full-length rDBP mRNA | 1422 | 1442 | 368 |
| reticulocyte lysate with mutant rDBP mRNA | 1378 | 1396 | 438 |

[a] Radioinert products were synthesized in the reticulolysate system in parallel with the same mRNA's used with [$^{35}$S]methionine in order to validate the products translated (Figure 5). Products were incubated with 0.16 pmol of 25-OH[$^3$H]$D_3$ in the absence or presence of 30 pmol of radioinert 17$\beta$-estradiol or 25-$OHD_3$. After coated charcoal removal of unbound sterol, supernatants were assayed for $^3$H in a scintillation spectrometer. In these experiments, [$^3$H]sterol binding was 15–20% of the total 25-OH[$^3$H]$D_3$ used. [b] Means of duplicate analyses in one experiment. Results of another experiment were similar.

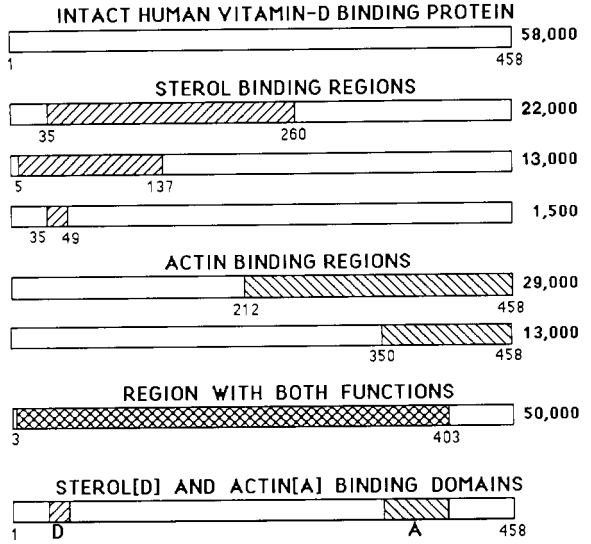


FIGURE 7: Graphic presentation of intact hDBP and its constituent peptides identified to have sterol-binding [indicated by slants (/) in the boxes], actin-binding [indicated by reverse slants (\) in the boxes], or both [criss-crossed boxes] binding properties. The domain assignments permitted by the present results are shown in the bottom panel. Values to the right are molecular weight estimates for the intact protein or identified segment.

By computer analysis, an $\alpha$-helix–turn–$\alpha$-helix conformation and a 3-AA residue $\beta$-sheet motif were predicted for the sterol-binding region. The sterol-binding domain is comprised of known amino acid sequences (Cooke & David, 1985) that premit their plotting every 100–110 °C consecutively around the helical wheel or spiral. A hydropathy plot of this region revealed two hydrophobic pockets. Secondary structure analysis of the actin-binding region predicted a mixture of helices and sheets in this relatively large region (350–403).

## DISCUSSION

The human DBP molecule is a single polypeptide chain with a single binding site for one molecule of a vitamin D related sterol per molecule of protein (Cooke & Haddad, 1989). The findings reported here assign the sterol-binding region to the amino terminus of the molecule. At equimolar incubation with hDBP, the 3$\beta$-(bromoacetoxy) ester of 25-hydroxycholecalciferol covalently bound to the vitamin D sterol-binding site. The specificity of this labeling with the radioactive ester was shown by its displacement by 25-$OHD_3$ but not by non-vitamin D steroids. Trypsin digestion yielded three overlapping peptides that were labeled with 3$\beta$-(bromoacetoxy)-[$^3$H]calcifidiol. The critical role of disulfide bonds in the binding of 25-$OHD_3$ to DBP was demonstrated when their reductive alkylation markedly reduced specific binding by DBP (Kawakami & Goodman, 1981). Our data indicate that the sterol-binding site resides in the region between the first disulfide bond at the amino terminus of the protein. Since, even in highly cross-linked polyacrylamide gels, diffusion of small peptides occurs (Huang & Matthews, 1990), the 1.5-kDa peptide could be of 3.4-kDa size. Analyses of the region between residues 35 and 70 predicted hydrophobic pockets consistent with binding regions for lipophilic ligands (Carlstedt-Duke et al., 1988). Secondary structure predictions (Chou & Fasman, 1978) indicated $\alpha$-helix–turn–$\alpha$-helix configuration in this region near the amino terminus of the molecule. Our findings reveal that ligand binding to DBP differs from the common C-terminus binding of sterols to their receptor proteins (Carlstedt-Duke et al., 1988; Rusconi & Yamamoto, 1987). In the superfamily of steroid receptors, hydrophobic regions are also important to the binding of steroids to their receptors, and these receptor domains (glucocorticoid estrogen, progestin, c-erb A) also display a transition from $\beta$-sheet to $\alpha$-helix. The 1,25-dihydroxyvitamin D receptor (VDR) is now recognized to belong to this receptor family (Ozono et al., 1991). Also, the androgen-binding domain of the human sex hormone-binding globulin was reported to have secondary structural features similar to those now reported for DBP (Danzo et al, 1991).

Other workers have utilized photoaffinity vitamin D analogs to label DBP and intestinal VDR (Kutner et al., 1986; Ray et al., 1986, 1991a,b; Link et al., 1987). In the study of Ray et al. (1991b), an 11.5-kDa peptide of DBP, liberated by cyanogen bromide, contained the radiolabel of an azido analogue of 25-$OHD_3$, tentatively identifying the binding site for sterol to reside between N-terminal residues 1 and 108. We elected to use the 3$\beta$-(bromoacetoxy) ester of 25-$OHD_3$ for chemical labeling of the binding site, as this electrophilic agent was useful for DBP studies (Haddad et al., 1981) and in other binding systems (Khan & Rosner, 1977; Sweet et al., 1972; Warren et al., 1975). The linkages formed were known to be relatively stable, and specific amino acid residues in enzymes bound to (bromoacetoxy)steroids have been identified (Penning et al., 1987, 1991). This linkage stability permits complete hydrolysis of peptides with amino acid analyses to identify covalently labeled residues. With the 3$\beta$-(bromo[$^{14}$C]acetoxy)-calcifidiol (Figure 2A) preparation, nonhydrolyzable labeing of nucleophilic residues will permit specific residue identifications in the future. Candidate nucleophilic residues in the sterol-binding domain presently delineated included Cys-58, -59, -67, Lys-35, -49, Arg-71, Ser-38, -45, -53, and Thr-40, -55, -70. The other nucleophilic residues (His, Met) do not appear in this region. Since the in vitro synthesized carboxy-terminal-truncated peptide (Figure 6) also exhibited specific sterol binding, the sterol-binding region delineated by the ligand affinity-labeled peptide analyses was confirmed.

The G-actin-binding property of DBP is recognized to be of high affinity and eliminated by chaotropic buffer or ionic detergent (Cooke et al., 1979; McLeod et al., 1989). Comparisons of its behavior toward actin forms indicate that DBP is a monomer sequestrant of actin (Lees et al., 1984). Our results indicate that the actin-binding region of DBP is near the carboxy terminus of the protein, which contains a binding region similar to those reported in other actin-binding proteins (Tellam et al., 1989). A loose actin-binding consensus

sequence is found within hDBP residues 385–400, which represents the carboxy terminus of the region identified to have the actin-binding property in our present studies (Figure 7).

Sequence similarities to two other G-actin sequestrants, DNase I, and profilin, are most relevant. Unlike profilin, which appears to share with DBP its actin-recognition site, DNase I can bind to the actin–DBP complex (Haddad, 1982; McLeod et al., 1989). The binding region on G-actin for DBP and profilin, therefore, must differ from that of DNase I. Multiple actin-binding sites exist for other proteins that are capable of severing and capping actin, as revealed by site-directed mutagenesis experiments (Eichinger et al., 1991; Wang et al., 1991).

In our confirmatory studies of the regional assignment of the sterol-binding domain (Figure 6, Table II), we also attempted to examine the carboxy-terminus-truncated peptide for loss of actin-binding activity. The $^{35}S$-labeled products of intact and truncated rDBP translated in vitro appeared to be bound to high molecular weight products and did not enter native polyacrylamide gels. Since the reticulocyte lysate translation system is likely to be contaminated with actin and other actin-binding proteins, future studies might include translation in cells capable of secreting the products into media and away from intracellular actin moieties.

In a comparison of the primary amino acids in the predicted sterol-binding region (AA 35–49) among human, mouse, and rat DBPs, a high degree of sequence conservation was noted. Between mouse and human DBP there is only one nonconservative amino acid difference, located at residue 39. Between rat and human DBP there are no nonconservative differences. In a comparison of the predicted actin-binding domain (AA 373–403), there are no nonconservative substitutions. This is in marked contrast to a previous comparison of this region among the three members of the DBP gene family: DBP, albumin, and $\alpha$-fetoprotein (Cooke, 1986). The predicted actin-binding domain lies within the region 373–404 which contains the lowest sequence similarity in full comparison with all three proteins. Only two residues, Cys-391 and Tyr-394 are conserved in this region, despite more than 20% amino acid identity overall. DBP, but neither albumin nor $\alpha$-fetoprotein, contains an actin-binding consensus sequence in this region (Tellam et al., 1989). The region 373–403 also corresponds precisely to exon 10 of the rDBP gene (Ray et al., 1991). As has been suggested from analyses of exon-encoded domains of other molecules (Gilbert, 1978; Sudhof et al., 1985), exon 10 of DBP may encode a major functional domain of this protein, and this domain is likely to reside on the surface of the molecule.

At present, there is no evidence to indicate that these two binding functions of DBP are functionally related. Sterol binding by DBP is not altered by G-actin occupancy and actin binding by DBP is not altered by sterol occupancy. Such studies have been carried out with these ingredients in solution and have not included analyses of the cell-surface binding or associations of DBP (Guoth et al., 1990; Petrini et al., 1985; Yamamoto & Homma, 1991) or possible relationships of DBP with oligomeric actin and G-actin–DNase I complexes. Since another member of the DBP/albumin/$\alpha$-fetoprotein family has been shown to gain specific cellular entry (Torres et al., 1991) and sensitive techniques have demonstrated DBP mRNA in several tissues other than liver (McLeod & Cooke, 1989), it is attractive to speculate that the sterol occupancy of DBP might regulate DBP–actin and/or DBP–cell associations and functions.

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