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Isolation and characterization of a 25-hydroxyvitamin D binding protein from rat enterocyte cytosol

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In this study, we have purified and partially characterized a unique protein in rat enterocyte cytosol that is capable of binding 25-hydroxyvitamin D_3. The protein was purified using ammonium sulfate precipitation and gel permeation chromatography, followed by two anion exchange chromatography steps. The protein has an apparent molecular weight of 68,000 assessed by gel permeation chromatography, and 58,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid composition and N-terminal amino acid sequence were determined. Differences in chromatographic behavior, molecular weight, amino acid composition, and sequence suggest that the protein is distinct from serum vitamin D binding protein and serum albumin. It differs from the 1,25-dihydroxyvitamin D receptor as well, in its stability during chromatography, cytosolic location, molecular weight, and sequence and differs from the putative basal-lateral membrane receptor in its cytosolic location. The protein isolated is a candidate cytosolic vitamin D-binding protein, which may be involved in absorption or in intracellular metabolism of vitamin D metabolites. (J. Nutr. Biochem. 8:195–200, 1997) © *Elsevier Science Inc. 1997*

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Introduction

The small intestine plays a key role in vitamin D metabolism. Vitamin D_3 (D) is absorbed by both chylomicron-dependent and -independent routes, reflecting the fact that D and its metabolites are slightly water-soluble neutral lipids.[1,2] In addition, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) is presented to the intestine from the plasma. A $1,25(OH)_2D$ receptor has been identified in the nuclear fraction of enterocytes that mediates genomic actions of the metabolite to enhance calcium absorption.[3,4] It appears that $1,25(OH)_2D$ also has nongenomic effects on the intestinal cells in enhancing calcium absorption.[5,6] $1,25(OH)_2D$ treatment in $CaCO_2$ cells causes rapid increases in inositol 1,4,5-triphosphate and diacylglycerol levels, as well as translocation of protein kinase C-alpha activity to the membrane.[6,7] A putative basal-lateral membrane protein has also been partially purified and found associated with rapid changes in calcium transport, termed transcaltachia.[8] Thus, it is important to understand the mechanism of intracellular transport of vitamin D and its metabolites through the enterocyte cytosol. A priori, a binding protein for vitamin D might be expected to have a role in directing the movement of this lipid and its metabolites through the enterocyte. The mechanism of $1,25(OH)_2D$ intracellular transport to the sites of action, however, has not been clearly defined. Binding proteins have been found for other lipids absorbed by the small intestine. These proteins include fatty acid binding protein[9–11] and retinol binding protein.[12] In addition, tissues other than the intestine (i.e., kidney and liver) that metabolize D have been found to have cytosolic D binding proteins, but have not been characterized.[13,14] A protein that binds vitamin D metabolites has not been found in the intestine cytosol.

Therefore, we sought a binding protein for D or its metabolites in enterocyte cytosol. In this article, we will describe a candidate protein in rat enterocyte cytosol (cDBP) that binds 25-hydroxyvitamin D_3 (25OHD), and that is distinct from serum vitamin D-binding protein

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(sDBP), albumin, the putative basal lateral $1,25(OH)_2D$ membrane receptor and the $1,25(OH)_2D$ receptor.

Methods and materials

Chemicals were all of reagent grade or better. Chemicals for sequencing were of Sequanal grade (Pierce Chemicals, Rockford, IL USA) or from Applied Biosystems (Foster City, CA USA). Protein standards used with gel electrophoresis were from BioRad (Hercules, CA USA).[15] DEAE-Sephacel was from Pharmacia Fine Chemicals (Piscataway, NJ USA). Agarose-hexylamine was from P-L Biochemicals, Inc. (Milwaukee, WI USA). The ToyoSoda G3000SW HPLC gel permeation column was from BioRad, and the Mono Q anion exchange column was from Pharmacia Fine Chemicals. Radioactive compounds were from Amersham (Arlington Heights, IL USA). Male rats weighing 250 to 400 g, used for purification of the serum and cytosolic DBPs, were from Sprague-Dawley.

Vitamin D binding assay

To trace cytosolic proteins capable of binding 25OHD, ^{3}H-25OHD was incorporated into a commercially available emulsion described below, and protein fractions were assayed for their ability to extract and bind 25OHD from the emulsion. 25OHD was used throughout these studies because it is the most abundant form of D in plasma, and preliminary experiments indicated that its polarity was most suitable to demonstrate transfer to serum and cytosolic vitamin D binding proteins in this assay. Liposyn (Abbott Laboratory, North Chicago, IL USA) was diluted using 0.9% sodium chloride with 0.05% EDTA (pH 7.40)(saline/EDTA) in a polyethylene tube, and then centrifuged at 65,000 × g for 1 hr at 2°C to remove nonemulsified components of the mixture. The lower aqueous fraction was drawn off with a long needle and discarded, and the lipid layer was resuspended and dispersed in saline/EDTA. This rinse was repeated once. To incorporate ^{3}H-25OHD into the emulsion the labelled material in ethanol was dried on No. 2 filter paper. The emulsion was incubated with the filter paper for 1 to 2 hr at 37°C and then centrifuged at 65,000 × g for 1 hr. The emulsion was then washed twice as described above and the radioactivity measured.

The emulsion was resuspended to approximately 1% total lipid concentration (w/v). To each 0.5 mL cytosol 0.1 mL of emulsion was added and dispersed with a needle and syringe. At various times, aliquots of the incubation mixture were removed and diluted 5 fold with saline/EDTA at 0°C. Samples were then centrifuged at 65,000 × g for 90 min at 4°C. The bottom of the tubes were punctured with a needle and 2 to 3 mL were drawn off and the radioactivity of an aliquot was measured.

Purification of cytosolic 25-hydroxyvitamin D binding protein

cDBP was isolated from male Sprague Dawley rats. The animal was anesthetized with ether and a PE60 catheter was placed in the mesenteric artery. 15 mL of cold saline was perfused to remove contamination of sDBP and albumin in the blood and the pale small intestine removed while saline was being perfused. The cells were isolated using a modification of a method by Stern, which uses citrate buffer to dissociate the enterocytes from the basement membrane.[16] The intestinal segment was rinsed with Krebs Ringer Phosphate buffer (KRP) and incubated for 3 min with 0.2 mol/L citrate buffer, pH 7.4. The segment was rinsed with KRP and the KRP was placed in the segment. The segment was massaged on a towel and rinsed with KRP. This incubation/massage procedure was repeated four times. The first KRP rinse after the massage was discarded and the remaining four rinses collected at 0°C. The cells were centrifuged at 12,000 × g for 10 min at 4°C and washed twice with KRP. The washed cells were then homogenized at 0°C with a Teflon pestle. The homogenate was centrifuged at 100,000 × g for 1 hr. The cytosol was defined as the supernatant obtained from the last centrifuged step.

Analytical procedures

Protein concentration was measured by several methods, including the Bradford method from BioRad and absorbance at A280. SDS-PAGE was as described by Laemmli,[17] a 0.75-mm thick gel containing 10% bis-acrylamide cross linker was used. The gels were stained using either Coomassie Blue or silver nitrate.[15,18] Amino acid analyses were prepared as described elsewhere.[19] Manual *N*-terminal amino acid sequencing was performed using diaminoazobenzene isothiocyanate on 86 pmol of purified cDBP.[20] The coupled amino acids were separated using a reverse phase HPLC column, using a modification of the method of Chang,[20] as described in the figure legend.

Purification of serum 25-hydroxyvitamin D binding protein

Rat sDBP was purified by a modification of the method of Haddad[21] to compare this protein with the cDBP. The modifications are described elsewhere.[22] Briefly, the sDBP was purified by ammonium sulfate precipitation, gel permeation ion exchange chromatography, and finally using a 25OHD affinity column. 25OHD binding activity was consistently found in the 40 to 60% saturated ammonium sulfate precipitate. The ammonium sulfate fraction containing sDBP was further purified by semipreparative gel permeation chromatography using a 30-cm ToyoSoda G3000SW column with 0.1 mol/L sodium chloride, 0.05 M sodium phosphate, pH 6.8 as the buffer. The sDBP was detected in a single peak of an apparent molecular weight 58,000. sDBP obtained from the gel permeation column containing sDBP was applied to a 25OHD affinity column prepared as described and the sDBP eluted with 1 M acetic acid.[21]

The purity of sDBP was assessed by SDS-PAGE using 10% (w/v) acrylamide gel, silver stained[18] and purity was assessed at greater than 95%. In addition, the integrity of the protein after purification was verified by HPLC gel permeation chromatography in which a molecular weight of 58,000 was again obtained.

Results

We observed that when an emulsion containing ^{3}H-25OHD was incubated with rat enterocyte cytosol, the label was transferred to cytosol. Under conditions described in Materials and methods, 29% of the 25OHD in the emulsion transferred to the cytosol after incubation for 1 hr at 37°C; in contrast, only 4% of the 25OHD was lost from the emulsion to buffer under the same conditions. The amount of label transferred was constant after 30 min of incubation at 37°C.

We purified the ^{3}H-25OHD binding activity from cytosol, first by fractional ammonium sulfate precipitation, followed by HPLC gel filtration and two anion exchange chromatographic procedures (*Figure 1*). Experiments using ammonium sulfate in 20% concentration increments demonstrated that binding activity was in the 60 to 80% saturated precipitate, but varied somewhat among various cytosol preparations, depending on protein concentration. The final preparation used the fraction of ammonium sulfate

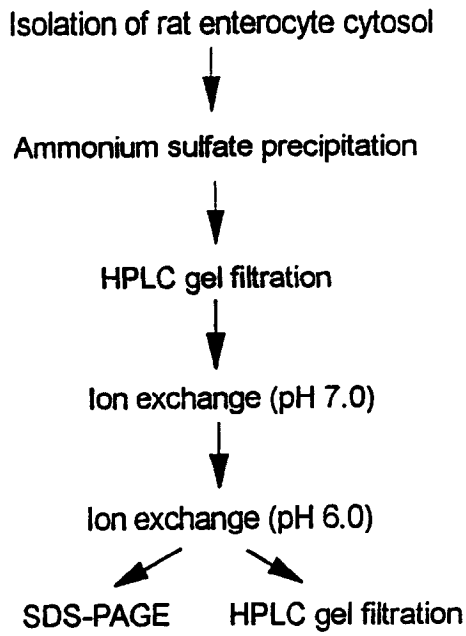

Figure 1 Flow-chart of cDBP purification.

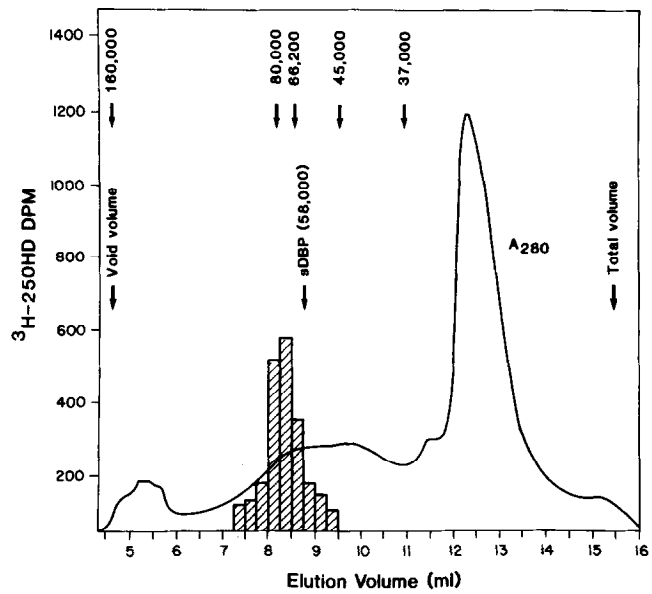

Figure 2 Molecular weight determination of cDBP from cytosol. The molecular weight of cDBP and sDBP after ammonium sulfate precipitation was estimated using a ToyoSoda G3000SW HPLC gel permeation column. The elution buffer was 0.1 mol/L sodium chloride, 0.05 mol/L sodium phosphate, pH 6.8. The flow rate was 1 mL/min. and fractions were 0.5 mL. The radioactivity in an aliquot of the fractions is shown by the *hatched bars* and the A_{280} by the *solid line*. Protein standards were also chromatographed to estimate the molecular weight of the cytosolic and sDBP, and the elution positions of the standards are indicated in the figure.

precipitation from 45 to 75% saturation to ensure recovery of all binding activity.

The 45 to 75% saturated ammonium sulfate precipitate was redissolved in distilled water and a tracer amount of tritiated 25OHD in ethanol added. 25OHD binding activity was assayed by measuring protein-associated radioactivity during the purification. The sample was applied to a Tacite G3000SW HPLC gel permeation column. The radioactivity from the cytosol was found to be associated with a protein of apparent molecular weight of approximately 68,000 (*Figure 2*). Rat serum preincubated with tritiated 25OHD was injected onto the same column and the radioactivity eluted with a protein of an apparent molecular weight of 58,000, i.e., with the sDBP. For preparative purposes, the buffer used was of low ionic strength, to allow for the subsequent purification using ion exchange chromatography. Accordingly, 0.02 mol/L Tris-HCl, pH 6.8 was used. Much of the protein eluted in the void volume, but all of the 25OHD binding activity eluted in a single peak in the same position as with the high ionic strength buffer as shown in *Figure 2*. The cDBP containing fractions from the gel permeation chromatography column were further purified using an HPLC Mono Q anion exchange column equilibrated with 0.02 mol/L Tris, pH 7.00, and then eluted with a 0 to 1 mol/L sodium chloride gradient. The fractions from the gel permeation column containing radioactivity were applied directly to the anion exchange column without the need for any change of buffer. A single peak of radioactivity eluted at 0.27 mol/L sodium chloride concentration (*Figure 3*). In another experiment, purified rat sDBP eluted at a slightly, but not significantly, different position in the gradient, 0.26 mol/L sodium chloride. Rat serum albumin labelled with tritiated 25OHD were applied to this column and eluted at 0.07 mol/L sodium chloride, well separated from that of the cDBP. Though the sDBP eluted in a fraction distinct from cDBP, the separation was not sufficient to ensure that sDBP contamination of cDBP was completely eliminated. The ^{3}H-25OHD fractions obtained from the Mono Q column at pH 7.00 were concentrated, desalted, and changed to another buffer, 0.02 mol/L Tris, pH 6.00. The protein was then rechromatographed using the Mono Q column equilibrated in the same buffer at pH 6.00, and the protein was eluted using a 0 to 1 mol/L sodium chloride concentration (*Figure 4*). The purified sDBP was injected on the column and now eluted at a sodium chloride concentration of 0.15 mol/L, in fractions clearly distinct and well separated from the cDBP.

An aliquot of the cDBP containing fractions was analyzed by 10% SDS PAGE. The major band had an apparent molecular weight of 58,000 (*Figure 5*). Thus, there was a discrepancy in the apparent molecular weight obtained by gel permeation chromatography and SDS-PAGE; therefore, another aliquot of the fraction from the Mono Q pH 6.00 column was re-injected onto the HPLC gel permeation column to verify that the protein was still intact by the criterion of eluting with an apparent molecular weight of 68,000 (*Figure 6*). In separate experiments, molecular weight standards and purified sDBP were also injected onto the column. The chromatographic profile of the radioactive 25OHD was identical to that of the primary protein peak as assessed by the A280, and both had an apparent molecular weight of 68,000. In contrast, the [^{3}H]25OHD associated with sDBP eluted with an apparent molecular weight of 58,000. The results of the preceding experiments indicate 1) that the purity of cDBP was greater than 95%, 2) retained binding activity associated with an apparent molecular

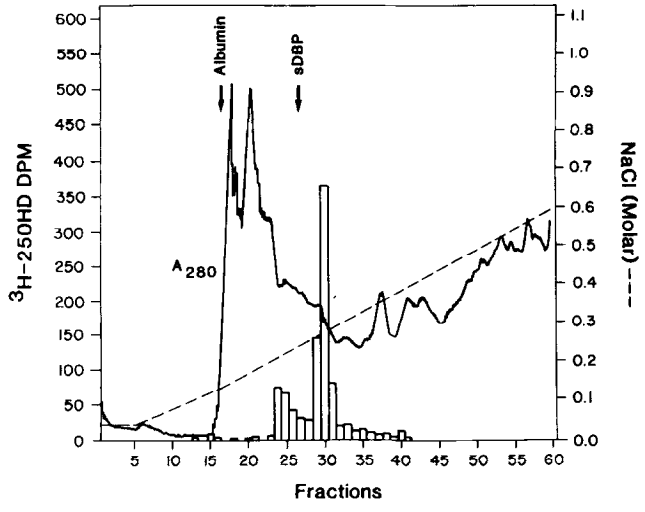

Figure 3 Anion exchange chromatography of cDBP at pH 7.00. Fractions containing ^{3}H-25OHD obtained from the gel permeation chromatography were applied directly onto a Mono Q HPLC column. The column was equilibrated with 0.02 mol/L Tris, pH 7.00. The protein was eluted using the sodium chloride gradient shown in the figure by *hatched line*; flow rate was 1 mL/min. the elution of protein was assessed by A_{280} shown by the *solid line* and the radioactivity is shown by the *bars*.

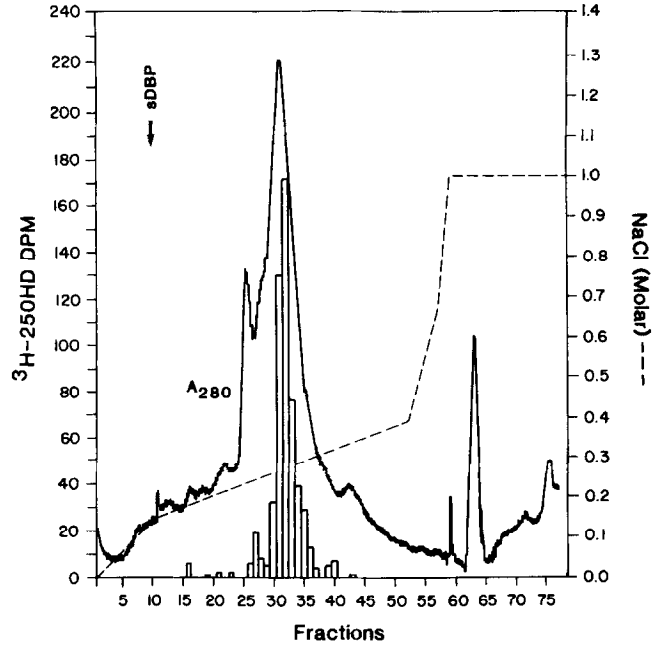

Figure 4 Anion exchange chromatography of cDBP at pH 6.00. Fractions from the Mono Q column at pH 7.00 were concentrated, desalted, and applied to the same column equilibrated in 0.02 mol/L Tris, pH 6.00. The column was eluted using a sodium chloride gradient as shown in the figure by the *hatched line*; flow rate was 1 mL/min/fraction. The A_{280} was assessed as shown by the *solid line* and the radioactivity recovered in an aliquot of the fractions is shown by the *bars*.

weight of approximately 68,000 assessed by gel permeation and, 3) is thus distinct from sDBP. The differences in chromatographic behavior of cDBP and sDBP using three separate columns—gel permeation, ion exchange pH 7.00, and 6.00—all suggest that these are not the same proteins.

The amino acid composition of both cDBP and sDBP were determined as previously described (*Table 1*).[19] There are small differences in the amino acid composition of these two proteins. cDBP has a higher content of arginine, valine, leucine, and isoleucine than sDBP and sDBP is enriched in acidic residues compared to cDBP. The recovery of cDBP from the enterocytes of 12 rats was estimated to be 15 to 20 μg or 350 pmol assessed by amino acid composition.

We were able to identify five of the six amino acids at the *N*-terminus of the protein as NH_2-Thr-Thr-x-His-Ala-Phe/Pro using approximately 5 μg of the protein (*Table 2*). The cDNA clone sequence of rat sDBP, rat albumin and the rat 1,25$(OH)_2$D receptor have been published and demonstrate the differences with cDBP.[23–25] The sequence obtained in our experiments is not present in sDBP, albumin, the 1,25$(OH)_2$D receptor, or other proteins.

Discussion

In this study we have described the purification and the initial characterization of a protein from rat enterocyte cytosol which binds vitamin D metabolites. The cDBP was purified by ammonium sulfate fractionation, gel permeation chromatography, and two anion exchange chromatographic procedures. Cytosolic binding activity for 25OHD was exploited to follow the binding activity throughout purification of the protein. SDS-PAGE demonstrated a primary band at an apparent molecular weight of 58,000. Using analytical gel permeation chromatography, the purified cDBP was observed to have an apparent molecular weight of 68,000. The fractions containing the 25OHD binding activity corresponded with the major protein peak as assessed by absorbance of the eluant at 280 nm, and was a single symmetrical peak.

Both the SDS-PAGE and the amino acid sequence data suggest that a single protein species accounts for the preponderance of protein in this peak. The discrepancy in molecular weights obtained by SDS-PAGE and gel permeation chromatography may be explained as follows. The protein might be asymmetrical and thus elute anomalously on gel permeation chromatography. Alternatively, the protein might contain two subunits, one having a molecular weight of 58,000, and the other with a molecular weight of 10,000. In addition, the possibility existed, despite care to minimize contamination of cytosol by serum, that the two vitamin D-binding proteins in the serum, sDBP and albumin, were present in small quantities in the cytosol. Our evidence suggests that the former alternative is correct. Only one major band was seen in SDS-PAGE; a second subunit of 10,000 was not observed, and would, presumably, have been present in a 1:1 stoichiometry to a larger subunit if the protein contained two subunits. In addition, only one *N*-terminus, was identified by the sensitive method used for sequencing, and this sequence is not present in sDBP or albumin, nor the 1,25$(OH)_2$D receptor.[23,25] Thus, we infer that the protein is a single subunit polypeptide, and that the discrepancy in apparent molecular weights is because of an asymmetrical shape of the protein.

The differences in gel filtration and ion exchange chro-

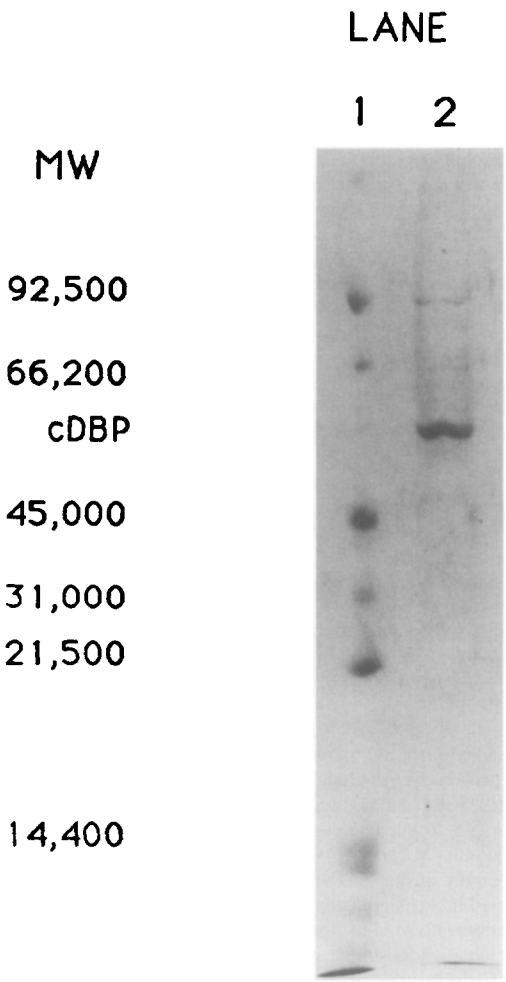

Figure 5 SDS-page of purified cDBP. The assessment of the purity of cDBP on a denaturing 10% SDS-PAGE. The proteins were stained with silver.[15] *Lane 1* is the protein standards with their molecular weights shown in the left column. *Lane 2* is the purified cDBP after elution on the anion exchange column with a buffer of pH 6.00.

matographic behavior of cDBP compared with those of rat sDBP and rat serum albumin also indicate that the three proteins are distinct. Several other compositional differences were noted between cDBP and other vitamin D binding proteins, but cDBP protein was not purified to homogeneity. Some differences exist between cDBP and sDBP in the amino acid compositions determined in our studies. The amino acid compositions of the two proteins are quite similar, perhaps suggesting some sequence homology between the cDBP and sDBP.

Another vitamin D metabolite binding protein in the enterocyte is the $1,25(OH)_2D$ receptor. The cDBP identified in our studies differs from the receptor in several ways; the former is stable during the chromatographic procedures, is cytosolic, and has a molecular weight of 58,000. In contrast, $1,25(OH)_2D$ receptor is unstable during chromatography, is localized in the nucleus under conditions described in the purification of the protein in this study, and has a molecular weight of 55,000 using both gel filtration and SDS-PAGE methods.[25–27] In addition, the sequence of the rat $1,25(OH)_2D$ receptor, deduced from cDNA, has been published and does not contain the N-terminal sequence determined for cDBP.[22,23] These observations suggest that cDBP is distinct from the $1,25(OH)_2D$ receptor protein from the intestine.

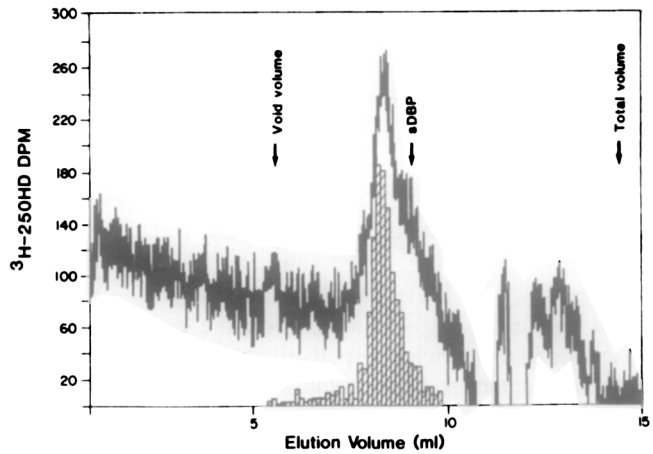

Figure 6 Analytical gel permeation chromatography of purified cDBP. The fractions eluted from the anion exchange column in pH 6.00 buffer was applied onto the ToyoSoda G 3000SW column. The proteins were eluted with 0.1 mol/L sodium chloride, 0.05 mol/L sodium phosphate pH 6.8 at a flow rate of 0.5 mL/min, and 0.125 mL fractions were collected. The radioactivity recovered in the fractions is shown by the *bars* and the A_{280} by the *solid line*.

A putative $1,25(OH)_2D$ basal-lateral membrane receptor has also been partially purified from chick intestine.[8] The presence of the protein in the basal-lateral membranes is associated with treatments where transcaltachia has also been documented.[8] This previously described protein could not be eluted from membrane fractions with washing using buffer or 0.3 to 0.5 mol/L KCl salt concentrations and therefore would not be present in the cytosol of our preparations. The protein described in the current study was clearly associated with the cytosolic fraction and suggests that it is distinct from the basal-lateral membrane $1,25(OH)_2D$ binding protein described previously.

Table 1 Amino acid composition of cytosol and serum vitamin D-binding proteins

Amino Acids	cDBP Residues	sDBP Residues
Asx	42	37
Glx	44	56
Ser	57	39
Gly	69	42
His	18	23
Thr	61	51
Ala	63	113
Arg	60	18
Pro	32	23
Tyr	30	14
Val	47	24
Met	18	6
Ile	43	11
Leu	68	38
Phe	33	18
Lys	53	67

Table 2 *N*-Terminal sequence of vitamin D binding proteins

Protein	*N*-Terminal Sequence
cDBP	Thr-Thr-X-His-Phe/Pro
sDBP	Leu-Glu-Arg-Gly-Arg-Asp
Albumin	Phe-Ser-Arg-Gly-Val-Phe
$1,25(OH)_2D$ receptor	Met-Glu-Ala-Thr-Ala-Ala

At this early stage of studies on the candidate cDBP described in this article, the potential physiologic role of this protein is a matter of speculation. cDBP may bind other vitamin D metabolites than 25OHD, and thus cDBP may be a transport protein for the vitamin D metabolites during absorption. Vitamin D and vitamin D metabolites are absorbed across the enterocyte partially via a chylomicron-independent route.[1,2] The vitamin D metabolites must, therefore, partition from a lipid environment, a prechylomicron particle or the brush border membrane, to the aqueous phase, the cytosol. The amount of the metabolite that could be transported across the enterocyte via the binding protein would be dependent on the partitioning of the metabolite between the lipid phase of the membrane or chylomicron and the aqueous phase as well as the affinity of the binding protein for the particular metabolite. A cDBP might help direct vitamin D or its metabolites to particular subcellular sites. Second, because the intestine is a target organ for $1,25(OH)_2D$, a cellular binding protein might have a role in mediating the effects of the metabolite within the enterocyte. A cellular vitamin D binding protein could be involved in the movement of the metabolite from the basolateral membrane across the cytosol to the site of action within the enterocyte, such as the membrane or the nucleus. Further work is necessary to elucidate in greater detail both the structure and the function of the candidate cDBP in the enterocyte.

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