Vitamin D_3–Induced Calcium Binding Protein in Bone Tissue

Abstract. *As detected by radioimmunoassay with antiserums against chick intestinal calcium binding protein (CaBP), administration of vitamin D_3 to rachitic chicks causes a 25- to 100-fold increase in immunoreactive CaBP in chick bone. The bone CaBP has a higher molecular weight (approximately 34,000 daltons) than intestinal CaBP (28,000 daltons), is concentrated principally in the spongiosa and cartilage plate regions of tibia, and responds adaptively to reflect the level of dietary calcium.*

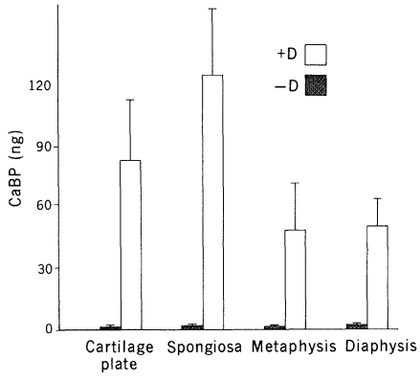
Vitamin D–dependent calcium binding protein (CaBP) was first found in the duodenal mucosa of the chick by Wasserman and Taylor in 1966 (*1*). Since then much interest has been generated concerning the role of CaBP in the intestinal absorption of calcium and its relation to the action of vitamin D (*2–4*). Similar vitamin D–dependent calcium binding proteins have been identified in the duodenal mucosa of the rat, pig, and human (*5, 6*), and in the kidney of the chick, pig, and human (*7–9*). A CaBP that cross-reacts with highly specific antiserums to duodenal CaBP has also been reported in chick brain (*10*). Also, Arnold *et al.* (*8*) reported finding a CaBP with immunochemical properties similar to those of pig intestinal CaBP in pig serum, liver, thyroid, and pancreas. We now report the presence of a vitamin D–dependent CaBP in bone.

The most widely used methods for quantitating CaBP have been the relatively insensitive Chelex ion exchange binding assay (*1*) and the radial immunodiffusion assay (*11*). Recently we have developed a radioimmunoassay for chick vitamin D–dependent intestinal CaBP (*12*) and have used this assay to detect CaBP in several tissues including bone. Briefly, the assay involves iodinating highly purified chick intestinal CaBP by the chloramine-T method (*13*). Antiserum to highly purified chick intestinal CaBP was prepared in rabbits by standard techniques (*14*) with Freund's adjuvant. Fifty percent of the iodinated CaBP was bound to the antibody in a final serum dilution of 1:55,000. For all the radioimmunoassays we used a double antibody technique which reduced background to a minimum [nonspecific binding, 10 ± 2 percent (± standard error)] and maximized sensitivity (assay sensitvity, 1 ng of CaBP). After ^{125}I-labeled CaBP, antibody, and sample or standard CaBP were incubated for 48 hours at 4°C, 50 μl of sheep antiserum to rabbit γ-globulin was added to each assay tube and incubated at 4°C for a further 24 hours. The assay tubes were next centrifuged at 1500*g* for 30 minutes at 4°C. The supernatant solution was decanted and the radioactivity present in the precipitate was determined in a Beckman gamma counter. The percentage of total counts precipitated by the samples derived from bone tissue was compared to the percentage of total counts precipitated with the CaBP reference preparation (*12*).

Chick tibias were sliced longitudinally and homogenized in a blender in 0.15*M* NaCl (saline) (20 percent, weight to volume). This extract was subsequently homogenized with a Potter-Elvehjem homogenizer fitted with a Teflon pestle and then centrifuged at 38,000*g* at 4°C for 30 minutes. Appropriate dilutions of the bone supernatant fluid were added directly to the radioimmunoassay. Protein was measured by the method of Lowry *et al.* (*15*).

As shown in Table 1, tibias from 4-week-old rachitic chicks contained an average of 4.4 ng of CaBP per milligram of protein (experiment A). After giving 1.3 nmole of vitamin D_3 daily for 2 weeks to 4-week-old rachitic chicks, the level of CaBP increased to 109 ng/mg, indicating that the CaBP activity detected by radioimmunoassay in bone was indeed responsive to the administration of vitamin D. In these same chicks the concentration of CaBP in the duodenum increased from 36 to 25,000 ng and serum CaBP increased to 49 ng/ml or 1.4 ng per milligram of protein. Thus the concentration of CaBP in bone was approximately 80 times greater than that of CaBP in serum.

It is well documented that the level of intestinal vitamin D–dependent CaBP is regulated in an adaptive way to reflect the level of calcium present in the diet (*4*). To study the possible effects of dif-



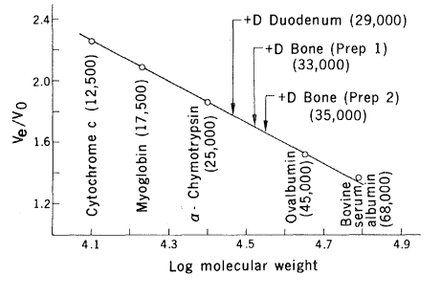


Fig. 1 (left). Total CaBP per part of chick tibia in −D chicks (maintained for 4 weeks on a rachitic diet) and +D chicks (maintained for 4 weeks on a rachitic diet and then given 1.3 nmole of vitamin D_3 daily for 2 weeks). Fig. 2 (right). The molecular weight of immunoreactive CaBP from duodenal and bone tissue extracts as determined by gel filtration (Sephadex G-100). The column was calibrated with known proteins and the ratio of the elution volume (V_e) to void volume (V_0) was plotted against the log of the molecular weight. The molecular weight of the immunoreactive CaBP from the tissue extracts was determined by comparing the measured V_e/V_0 ratio against this curve.

Table 1. Effects of vitamin D status and differing dietary levels of calcium on CaBP in chick bone. In experiment A, hatchling chicks were raised for 6 weeks on a rachitogenic diet (*16*). The +D group received for the last 2 weeks a daily oral supplement of 1.3 nmole of vitamin D_3. The −D group received no such supplement. In experiment B, hatchling chicks were raised on a standard rachitogenic diet (*16*). After 2 weeks the calcium composition was adjusted to 0.1 percent (low calcium) or 2.0 percent (high calcium) and the chicks were given a daily oral supplement of 1.3 nmole of vitamin D_3 for 2 weeks. The values reported are the mean ± the standard error. *N* is the number of samples.

Group	Bone CaBP (ng/mg protein)	Duodenal CaBP (ng/mg protein)	Serum CaBP (ng/ml)	Serum Ca^{2+} (mg/100 ml)	*N*
		Experiment A			
−D	4.4 ± 1.4	36 ± 0.014	0	5.4 ± 0.36	6
+D	109.0 ± 22*	25,000 ± 4,400	49 ± 8†	8.0 ± 0.26	6
		Experiment B			
Low calcium	460 ± 55	12,000 ± 900	65 ± 23	6.6 ± 0.40	7
High calcium	101 ± 19*	6,400 ± 1,100	9.8 ± 1.8	9.0 ± 0.42	6

*$P < .001$. †This concentration of CaBP in the serum is equivalent to 1.4 ng per milligram of serum protein.

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fering amounts of dietary calcium on the CaBP of bone, we placed chicks on a standard rachitogenic diet (*16*) for 2 weeks, and then for 4 weeks on either a high calcium (2 percent) or a low calcium (0.1 percent) diet with 0.4 percent phosphorus. During the final 2 weeks the chicks were given 1.3 nmole of vitamin D_3 daily. At 6 weeks of age the chicks were killed, the tibias were excised and extracted, and the supernatant solutions were assayed for CaBP by radioimmunoassay as described above. Table 1 shows (experiment B) that the tibias from chicks fed a low calcium diet had approximately a fourfold greater content of CaBP (460 ng per milligram of protein) than tibias from chicks fed a high calcium diet (101 ng/mg). Serum calcium and duodenal CaBP for the chicks on these diets are also shown in Table 1. Clearly, the concentrations of CaBP in both bone and intestine adapt inversely to reflect the level of dietary calcium.

When the individual segments of tibias obtained from rachitic (−D) or D-replete chicks were analyzed for CaBP, we found that, in the − D state, CaBP was evenly distributed at a very low level (2.0 to 4.5 ng) in the cartilage plate, spongiosa, metaphysis, and diaphysis (see Fig. 1). After 1.3 nmole of vitamin D_3 was given daily for 2 weeks, the greatest concentration of CaBP was in the spongiosa (125 ng) and in the cartilage plate (83 ng) (Fig. 1).

When bone extracts were assayed at several dilutions the immunodisplacement curve was always parallel to that of pure intestinal CaBP. According to Berson and Yalow (*17*) this suggests apparent identity of immunochemical reactivity between bone and highly purified CaBP. The molecular weight of immunoreactive CaBP in bone was estimated by gel filtration on a calibrated Sephadex G-100 column (1.5 by 85 cm) equilibrated with 0.1*M* sodium phosphate buffer, *p*H 7.4. Two separate preparations of bone were homogenized (50 percent, weight to volume) in 0.1*M* phosphate buffer, *p*H 7.4, and the extracts were centrifuged at 38,000*g* for 30 minutes. Two milliliters of the supernatant solution were applied to the column and 1.2-ml fractions were collected. The concentration of CaBP in the various fractions was determined by radioimmunoassay. The peak of immunoreactive CaBP from the bone extracts consistently eluted earlier than the immunoreactive CaBP peak from duodenal extracts. The bone CaBP (Fig. 2) had an estimated molecular weight of 34,000 daltons as compared to duodenal CaBP which is 29,000 daltons on our column. [Taylor and co-workers reported a molecular weight of 28,000 for intestinal CaBP (*3*).] The different molecular weights suggest that the bone CaBP is not identical to the intestinal CaBP. Also, the calcium binding property of this protein was demonstrated through use of the Chelex ion exchange binding assay (*18*).

Neither the role of vitamin D in bone metabolism nor the function of intestinal CaBP in intestinal calcium translocation is clear. Thus the bone CaBP might be involved in any number of metabolic activities in bone including bone formation and resorption. Certainly the identification of the existence of a bone CaBP enables us to initiate new biochemical studies on the effect and mechanism of action of vitamin D and its metabolites on bone, both in normal development and in conditions of bone disease. To our knowledge this represents the first unequivocal demonstration of a vitamin D–dependent protein in bone.

SYLVIA CHRISTAKOS
ANTHONY W. NORMAN
Department of Biochemistry, University of California, Riverside 92521

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18. A modification of the Chelex ion exchange assay of Wasserman and Taylor (*1*) was used to evaluate the calcium binding properties of the putative bone CaBP. The immunoreactive peaks from the Sephadex G-100 column chromatography of bone supernatant (see Fig. 2) were pooled, dialyzed against H_2O, lyophilized, and suspended in 1.0 ml of buffer (1.0 mg of protein per milliliter). To this test sample or 1 ml of buffer, or 1 ml of buffer with bovine serum albumin at the same concentration (1 mg/ml), as well as a sample of duodenal supernatant from a +D chick, was added 0.2 ml of Chelex −100 resin (Calbiochem) suspension (containing 0.1 ml of packed resin) followed by carrier-free ^{45}Ca (223,000 count/min). The samples were vigorously vortexed for 15 seconds and centrifuged at 1500 rev/min for 10 minutes. The ^{45}Ca in the supernatant phase in a 0.2-ml portion was determined by liquid scintillation counting. The percentage of total ^{45}Ca in the supernatant phase of the resin assay system is a measure of the CaBP in that phase. With buffer alone, 4.2 percent of the counts remained in the supernatant. With bovine serum albumin (1 mg/ml) in the buffer, 7.2 percent of the total counts remained in the supernatant; the supernatant of the sample of bone CaBP had 12.4 percent of the total added counts present and the + D duodenal supernatant had 52.8 percent. The results demonstrate that the protein present in the bone supernatant has a capability to bind calcium.
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Grand Banks and J-Anomaly Ridge

Based on two lines of evidence, Gradstein *et al.* (*1*) interpreted the J-anomaly ridge in the vicinity of Deep Sea Drilling Project (DSDP) site 384, which bottomed in basalt (*2*), as having been part of the Grand Banks continental block in the Early Cretaceous. The first line of evidence is that the total subsidence of the Puffin and Tern wells on the Grand Banks is similar to that observed at site 384. The second is based on extrapolation of seismic data measured on the southern Grand Banks to the drill site locations.

In the absence of other data, these lines of evidence might imply that the magnetic J-anomaly is situated on continental crust. However, they are insufficient to dispel what is now a well-established sequence of magnetic anomalies created by sea-floor spreading processes (*3, 4*). Similarity between the subsidence at site 384 and that on the Grand Banks continental block does not necessarily imply that the DSDP site is situated on continental crust. Indeed, rather large amounts of subsidence of other aseismic ridges that are situated on oceanic crust are well documented (*5*). Furthermore, the seismic profile shown in figure 5 of Gradstein *et al.* (*1*) does not traverse site 384 but lies close to the strike of the Newfoundland Ridge. Any controversy with respect to the nature of