Placental Calcium Binding Protein in Rats

APPARENT IDENTITY WITH VITAMIN D-DEPENDENT CALCIUM BINDING PROTEIN FROM RAT INTESTINE*

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Rat placenta contains a calcium binding protein (CaBP) which is closely related or identical with the vitamin D-dependent CaBP in rat intestine. Placental CaBP was detected using antiserum prepared in rabbits against the intestinal form of the protein purified as previously described (Bruns, M. E. II., Fliesher, E. B., and Avioli, L. V. (1977) J. Biol. Chem. 252, 4145-4150). The antiserum was monospecific as indicated by double immunodiffusion and immunoelectrophoresis. The amount of immunoreactive intestinal material was vitamin D-dependent (rachitic 63 ± 10 μg; vitamin D replete 462 ± 17 μg). Using 40,000 × g supernatant fractions of total tissue homogenates, immunoreactive CaBP was detectable by double immunodiffusion only in the proximal 12 cm of the small intestine and in placenta. The placental and intestinal proteins were immunologically identical and demonstrated no significant difference in molecular weight (10,500), electrophoretic mobilities in the presence or absence of EDTA, or apparent Ca⁺⁺ binding constants \( K_r = 0.12 \pm 0.03 \mu M \) and \( 0.3 \pm 0.1 \mu M \), n = 2.3 ± 0.12 and 2.4 ± 0.4 Ca⁺⁺ bound per mol, respectively, for placental and intestinal forms.

The concentration of immunoreactive CaBP in maternal intestine and placenta was dependent upon gestational age. Maximal concentrations of CaBP in both placenta and maternal intestine were observed after Days 18 to 19 and coincided with the period of most rapid growth of the rat fetus. These results, consistent with the previously documented time course of fetal skeletal mineralization and with the calcium absorption adaptations during pregnancy, suggest that a placental CaBP may condition or regulate maternal-fetal calcium exchange in the rat.

The availability of calcium in utero is dependent upon

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Placental Calcium Binding Protein

Rat intestinal CaBP was purified as previously described (8). Antiserum was produced in a 4-month-old male white rabbit by first injecting 0.18 mg of CaBP emulsified in complete Freund's adjuvant into footpads and subcutaneous sites on the back. Second and third injections (0.1 mg of CaBP each) in Freund's incomplete adjuvant were given 4 and 7 weeks later. The presence of antibody was detected after 7 weeks by Ouchterlony double diffusion technique (10) in 0.8% agarose, 10 mM sodium phosphate, pH 7.6, and 0.14 M NaCl with 0.02% thimerosal (PBS buffer) using 5 to 10 µl of samples per well.

For immunoelectrophoretic analyses, protein samples were first fractionated by disc gel electrophoresis according to the method of Davis (11) in a 7% polyacrylamide gel at pH 8.2 for 6 cm. The gels were sliced longitudinally and then embedded in PBS buffer 0.8% agarose on a glass microscope slide. A thin longitudinal trough was cut about 7 mm from the agarose-embedded gel. Antiserum was pipetted into the trough and immunodiffusion was allowed to proceed at room temperature for 18 to 24 h. The immunoreactants were stained by standard techniques with Buffalo black (10).

Immunochromatographic quantitation of CaBP antigen was performed by single radial immunodiffusion (RID) (12). The CaBP standard used in these assays was prepared as described previously (8) and then subjected to second preparative slab gel electrophoresis; in this step 0.1 mM EDTA was incorporated in the electrode buffer to change the % of the protein to 0.70 to 0.80 (8). Agarose plates were used at 1.0% agarose in PBS buffer with diluted antiserum. Each 2.0-mm well contained a 4-µl sample; standards and unknowns were randomized in duplicate throughout the agarose plate. Equilibrium was achieved by 18 h incubation at room temperature and a linear relationship was achieved between diameter of immunoprecipitate and CaBP concentration (25 to 200 µg/ml).

Preparation of Tissue Extracts—Tissue samples were obtained from (fed ad libitum) female rats under ether anesthesia. Intestinal segments (5 or 12 cm) were obtained starting distal to the pyloric valve. All subsequent analyses were performed at 4°C. Segments were washed with 5 ml of Buffer A (10 mM Tris/HCl, pH 7.2, 1.0 mM EDTA containing 0.02% NaN₃, 5% glycerol, and 5 mM benzamidin). Mucosal tissue, obtained by squeezing the gut with a spatula, was homogenized in a glass tissue grinder in 2 ml of Buffer A. The homogenate was centrifuged at 40,000 x g for 20 min and the supernatant fraction designated S-1. Other rat tissues studied were processed similarly except that when necessary they were homogenized and parathyroid tissue sonicated before centrifugation. Cartilage was obtained from the tibial epiphysis.

Other Methods—Ca binding assays by a Chelex resin method, Sephadex G-100 chromatography, and polyacrylamide disc gel electrophoresis were performed as previously described (8). Plasma calcium was determined using a fluorimetric assay (13).

RESULTS

Characterization of Antibody to Rat CaBP—The specificity of the rabbit antibody was analyzed by Ouchterlony double immunodiffusion described under "Experimental Procedures." Fig. 1A indicates that a single precipitin line was obtained with purified high molecular weight rat intestinal CaBP and the crude supernatant fraction (S-1) from rat mucosa of the proximal small intestine (first 12 cm) when reacted against the rabbit antiserum. No precipitation lines were obtained against pooled high molecular weight intestinal protein from S-1 which contain other CaBPs or when purified CaBP was incubated against nonimmunized rabbit serum.

Immunoelectrophoresis of CaBP was performed with Ca²⁺ bound to the CaBP and under conditions in which Ca²⁺ was removed from the protein. As previously reported (8, 14) the electrophoretic migration of CaBP was dependent on the amount of cation bound to the molecule. In either case, a single immunoprecipitate arc was observed opposite the purified CaBP band in the disc gel system; no spur formation was seen (Fig. 1B). Similar results were obtained using other less purified intestinal fractions including the pooled low molecular weight protein fraction (Fig. 4). The change in the mobility of the precipitin arc and the lack of spur formation or other

![Fig. 1. Characterization of antibody. A, Ouchterlony double immunodiffusion as described under "Experimental Procedures." The center well contains the undiluted antiserum. The outer well labeled A contains 1.5 µg of purified intestinal CaBP. The D-1 well contains about 0.12 µg of crude duodenal supernatant fraction (S-1). The D-2 well contains about 0.1 mg of high molecular weight proteins (>40,000) pooled from a G-100 chromatography of S-1. B, immunoelectrophoresis as described under "Experimental Procedures." Experiment 1 represents the protein stain of purified CaBP (12 µg) on a polyacrylamide disc gel; and Experiment 2, the immunodiffusion pattern of the embedded unstained disc gel in agarose. Both Experiments 1 and 2 were run in electrode buffer with 0.1 mM EDTA. Experiments 3 and 4 were processed the same as Experiments 1 and 2 except that EDTA was omitted from the electrode buffer. Experiment 3 is the immunodiffusion pattern of the disc gel embedded in agarose whereas Gel 4 is the protein stain pattern of the gel. The electrophoretic migration of the samples was from top to bottom.

areas indicate that the antiserum reacted only with CaBP. The antibody reaction to CaBP appeared to be independent of the amount of cation bound to CaBP suggesting that the antigenic determinants were equally accessible in the calcium-free and calcium-bound conditions of the protein.

Storage of rat CaBP at -20°C resulted in the appearance of minor electrophoretic bands similar to those reported for bovine CaBP (15). These bands appeared to represent minimally altered CaBP since the electrophoretic mobilities of the bands was altered by calcium ion concentration in the same way as was the major CaBP band. The presence of these bands explains the skew of the single immunoprecipitate are in Fig. 1B and the absence of spurs indicates that these minor bands are immunologically related to the major CaBP material. These data support the conclusion of Fullmer and Wasserman that the minor bands which appear after storage of bovine CaBP represent degradation products of CaBP (15).

Vitamin D Dependence of Immunoreactive CaBP—The purified low molecular weight intestinal CaBP which was used as the immunizing antigen was assumed to be the vitamin D-dependent form of rat CaBP based upon its molecular weight, electrophoretic mobility, and Ca²⁺ binding properties (8). To further verify this, the amount of immunoreactive CaBP in rat intestine as a function of dietary vitamin D₃ content was measured (Table I). Two groups of female rats are compared. The diets were identical with the exception of vitamin D₃ and were randomized in duplicate throughout the agarose plate. Equilibrium was achieved by 18 h incubation at room temperature and a linear pattern of the embedded unstained disc gel in agarose. Both Experiments 1 and 2 were run in electrode buffer with 0.1 mM EDTA. Experiments 3 and 4 were processed the same as Experiments 1 and 2 except that EDTA was omitted from the electrode buffer. Experiment 3 is the immunodiffusion pattern of the disc gel embedded in agarose whereas Gel 4 is the protein stain pattern of the gel. The electrophoretic migration of the samples was from top to bottom.

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Weanling female rats at 20 days old were placed on low calcium diet with or without vitamin D supplement as described under "Experimental Procedures." After 4 weeks on the diet, blood samples were obtained by cardiac puncture and the plasma was assayed for calcium (13). Proximal intestinal segments were obtained, Segment A, 0 to 6 cm, and Segment B, 6 to 12 cm, from the pyloric valve and mucosa processed as described under "Experimental Procedures." The S-1 fraction was assayed for CaBP by the radial immunodiffusion assay. The data were calculated as the mean (± S.E.) and represents six animals for each group.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Plasma calcium</th>
<th>Segment mucosal protein</th>
<th>Segment CaBP</th>
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<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Minus D</td>
<td>7.9 (±0.3)</td>
<td>39.0 (±2.8)</td>
<td>28.8 (±2.4)</td>
</tr>
<tr>
<td>Plus D</td>
<td>10.2 (±0.2)</td>
<td>39.0 (±4.3)</td>
<td>27.8 (±1.1)</td>
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-a Trace amounts too low to be accurately measured.

calcium concentration (7.9 ± 0.3 mg/dl), the immunoreactive CaBP was markedly reduced in both segments. In Segment A, CaBP levels in the "minus-D" rats were approximately 20% those of the "plus-D" group. In the "minus-D" Segment B, trace amounts of CaBP were detected which were too low to be accurately quantitated. Although immunoreactive CaBP was markedly reduced in the "minus-D" group, no change in total mucosal protein was observed when compared to the "plus-D" group. These data confirm that the antiserum was directed against the vitamin D-dependent form of CaBP.

Cross-reactivity with Rat Tissues—Tissues from normal female rats were screened for cross-reactivity with the antiserum to intestinal CaBP using a double-immunodiffusion system. The 40,000 × g supernatant fractions of total tissue homogenates were prepared as described under "Experimental Procedures." No immunologically detectable material was found in liver, kidney, brain, fat, red blood cells, serum, parathyroids, epiphyseal cartilage, or skeletal muscle. The protein concentration of each tissue fraction was adjusted to 15 to 20 mg/ml prior to assay. In this immunodiffusion assay (sensitive to approximately 25 μg of CaBP/ml), only the proximal 12 cm of the small intestine and the placenta demonstrated cross-reactivity to the CaBP antibody. The placental material cross-reacted with complete identity as shown by a single precipitin line which completely coalesced with the antibody-antigen precipitate of intestinal CaBP (Fig. 2).

Characterization of Placental Calcium Binding Protein—The 40,000 × g supernatant of rat total placental homogenate was fractionated by Sephadex G-100 chromatography and 4Ca binding was studied as described under "Experimental Procedures." The chromatographic pattern was similar to that of small intestinal mucosa, showing two major 4Ca binding peaks (Fig. 3). Aliquots from each fraction were concentrated and analyzed for immunoreactivity with anti-(intestinal) CaBP in a quantitative radial immunodiffusion assay. Detectable immunoreactive material was present only in the low molecular weight 4Ca binding peak (Fig. 3). The immunoreactive peak was pooled and rechromatographed on a Sephadex G-100 column which had been calibrated for molecular weight. The estimated molecular weight for both the 4Ca binding and immunoreactive material was 10,500 similar to previously reported values for rat intestinal CaBP which have ranged between M, = 8,000 and 13,000 (8, 9, 16). The calcium binding characteristics of the low molecular weight placental CaBP were measured by 4Ca binding with Chelex resin under conditions described previously (8). The dissociation constant (Kd) for Ca2+ binding placental CaBP and the molar ratio of calcium binding to the amount of immunoreactive protein were calculated (mean ± S.E. of three determinations) as 0.12 (±0.03) μM and 2.5 (±0.12) mol of Ca2+ bound/mole of immunoreactive protein, respectively. These values compare favor-
ably with the Ca\(^{2+}\) binding characteristics of purified rat CaBP with a \(K_d\) of 0.3 (±0.1) \(\mu\)M and a calcium binding maximum of 2.4 (±0.4) mol of Ca\(^{2+}\)/mol of CaBP (8).

Immunoelectrophoretic studies of the placental protein revealed further similarities to the intestinal CaBP. Fig. 4 illustrates the immunoelectrophoresis protein staining pattern of low molecular weight \(^{45}\)Ca binding peaks from intestine and placenta. In the EDTA buffer system (Fig. 4A), both intestinal and placental CaBP migrated as fast moving bands and cross-reacted with the antiserum in this position. In the absence of EDTA (Fig. 4B), with a total Ca\(^{2+}\) concentration approximating 50 \(\mu\)M, the intestinal CaBP band migrated more slowly but was still identified by the intestinal CaBP-antibody by the immunoprecipitation reaction. In this system, the placental CaBP band could not be discerned within the large amount of protein-staining material at the top of the gel but the presence of the protein was indicated by the immunoprecipitate arc directly opposite the intestinal CaBP band (Fig. 4B).

Effects of Gestational Age on CaBP in Placenta and Maternal Intestine—Increases in the concentrations of CaBP in placenta and maternal intestine were observed during gestation and were related to growth of the fetus. Fig. 5A illustrates fetal and placental growth during gestation. The increase in placental weight was rapid between Days 13 and 19 and preceded the rapid and large changes in fetal body weight which occurred between Day 18 and term (21 to 22 days). As noted in Fig. 5B, undetectable or small amounts of CaBP were present at Days 13 to 18. Between Days 18 and 19, a 4-fold increase in placental CaBP content occurred which coincided with the initiation of rapid fetal growth. These changes were attended by a significant increase in the total amount of maternal intestinal CaBP at the 18th gestational day, an increment which persisted through Day 20.5 (Fig. 5C). The concentration of maternal intestinal CaBP near the end of the third trimester was approximately 60% greater than that seen in nonpregnant female rats of identical age. Total mucosal protein in the small intestines of nonpregnant female rats, age-matched to the pregnant rats, between 17 and 18 weeks old. The data points for the pregnant animals represent three to seven intestines between Days 13 and 19 of gestation and 11 intestines at Day 20.5.
The development of a monospecific rabbit antiserum and the utilization of a radial immunodiffusion assay for quantitative measurements of CaBP allowed us to confirm the localization of a CaBP in the proximal small intestine of the rat (18) as well as its dependence on vitamin D (4-6, 9).

Moreover, using a wide variety of rat tissues, cross-reactivity with an antibody to intestinal CaBP was observed only in the placenta. Gel filtration analysis revealed a molecular weight of the rat placental CaBP which was similar to that of the intestinal CaBP (Fig. 3). Ca binding characteristics and the electrophoretic properties of placental CaBP in the presence or absence of EDTA were identical with the intestinal protein (Fig. 4). Based upon these results, we conclude that the rat placenta contains a protein with apparent identity to the vitamin D-dependent intestinal CaBP. In terms of relative concentrations in a 40,000 g supernatant fraction, the specific activity of CaBP (micrograms of CaBP/mg of protein) in term placenta was approximately 20% that of the intestinal CaBP isolated from a gestational age-matched maternal intestine.

The reported tissue distributions of CaBP are different in birds and mammals. In birds, proteins with immunochemical similarity to the avian intestinal CaBP have been described in kidney, shell gland, and brain (19-21) with the kidney and shell gland CaBPs being dependent upon the vitamin D status of the animal and the brain CaBP independent. In a mammalian system, Arnold et al. (22), using a radioimmunoassay developed with pig intestinal CaBP, observed undetectable cross-reactivity in brain while kidney concentrations of immunoreactive CaBP were 0.1% of those in proximal small intestine. In the present study, CaBP was not detected in rat kidney, but the limited sensitivity of the radial immunodiffusion assay would have precluded its detection if the rat kidney/intestine CaBP ratio approximates that of the pig.

To our knowledge this is the first report of the isolation and characterization of a placental protein which is similar to the vitamin D-dependent intestinal CaBP. The effects of gestation on both immunoreactive placental CaBP and maternal intestinal CaBP are consistent with the hypothesis that CaBP is associated with adaptation to transcellular calcium movement (4-7). In rate, ossification of fetal bones commences between Days 15 and 18 of gestation (23) and the net calcium transfer to fetal bones parallels total body growth which is rapid during the last 4 days of gestation (24). An active transport system for calcium across the placenta has been proposed to account for the transfer of calcium from mother to fetus (2, 3). In man and rat, the majority of the fetal bone calcium is accumulated during the last trimester of pregnancy, a process which in the rat is associated with an increase in calcium absorption by the maternal intestine (25). Serial quantitation of both maternal intestine and placental CaBP (Fig. 5) and their relationship to each other as well as to the growth of the fetus suggest the idea that the placenta and maternal intestine are cybernetically linked in order to satisfy the needs of the mineralizing fetal skeleton. Recent studies have shown increased serum levels of the active vitamin D metabolite 1,25-dihydroxycholecalciferol during reprodution in egg-laying hens and pregnant humans at term (26). Since it has been repeatedly established that vitamin D metabolite(s) are of primary importance in regulating intestinal calcium absorption in the rat, these metabolites may also control the transcellular transport of calcium across the rat placenta. The isolation and identification of a placental CaBP with apparent identity to the vitamin D-dependent CaBP of intestinal tissue supports this contention.

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REFERENCES
Placental calcium binding protein in rats. Apparent identity with vitamin 
D-dependent calcium binding protein from rat intestine.
M E Bruns, A Fausto and L V Avioli


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