

# Intestinal Calcium-Binding Protein in the Developing Rat Duodenum

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## Summary

An immunoassay for rat intestinal calcium-binding protein (CaBP) employing antibody raised in chickens using purified rat intestinal CaBP has been used to measure immunoreactive CaBP in the duodenum of suckling and weanling rats. A 5-fold increase in immunoreactivity, associated with a 2½-fold increase in calcium-binding activity occurred at weaning, a period during which duodenal mucosal mass increased 2-fold and supernatant protein concentration increased 1½-fold.

## Speculation

The ontogeny of calcium-binding protein in the duodenum of young rats parallels the maturation of cholecalciferol hydroxylases and represents maturation of the intestine as an end organ responsive to the vitamin D endocrine system.

The exact mechanism by which vitamin D stimulates calcium transport remains unclear (10). A major theory holds that 1,25-dihydroxycholecalciferol, the vitamin D metabolite most active in stimulating active calcium transport, acts analogously to other steroid hormones (19). Following this analogy, 1,25-dihydroxycholecalciferol is bound by specific receptors in the cytoplasm of the intestinal epithelial cell (6). The receptor-hormone complex subsequently binds nuclear chromatin (5), resulting in the synthesis of messenger RNA, (29, 33) and ultimately, specific proteins responsible for the effect of the hormone on the end organ cell.

The protein most convincingly demonstrated as a result of the interaction between 1,25-dihydroxycholecalciferol and the intestinal epithelial cell is the vitamin D-dependent intestinal calcium-binding protein (CaBP) (31). A role for the CaBP in vitamin D-dependent active intestinal calcium transport has not yet been clearly defined, but its identification as a specific product of the interaction of the vitamin D endocrine system and the intestinal mucosa (12) makes it useful as a molecular marker of the vitamin D-dependent intestinal calcium transport mechanism.

Functional maturation of the intestinal mucosa in the suckling animal is characterized by enhancement of mucosal enzyme activities and active transport systems (20), and localization of these digestive and transport functions to the site of their maximal activity in the mature intestine (2). Calcium absorption occurs throughout the small intestine and colon of the suckling rat (2) and is not vitamin D-dependent (11). This widespread absorption of calcium decreases with maturation, (2, 32) and is replaced at weaning by active (1), vitamin D-dependent (11) calcium transport localized to the duodenum (2), the site of its maximal activity in the intestine of the adult animal (28, 30).

DeLorme, *et al.* (9) and Bruns, *et al.* (7) have shown a corresponding increase in intestinal CaBP, measured by immunochemical techniques, between days 17 and 22 after birth.

The purpose of this investigation was to further define the ontogeny of CaBP in the developing duodenum, relating it to the hypertrophy and functional maturation that characterize the in-

testine at weaning. We therefore developed an immunoassay for the purified rat intestinal CaBP (15) and, using this assay, measured immunoreactive CaBP in homogenates of duodenal mucosa from suckling and weanling animals, comparing such changes to changes in mucosal weight, supernatant protein concentration and calcium-binding activity.

## MATERIALS AND METHODS

One and one-half mg of rat intestinal CaBP purified to homogeneity in our laboratory by heat treatment, gel filtration chromatography, and ion exchange chromatography (15), were dispersed in complete Freund's adjuvant and injected subcutaneously into the footpads of each of two young adult Leghorn hens (Al Blum Rabbitry, San Antonio). Each animal received a booster dose of 0.5 mg intramuscularly 4 wk later. Six wk after the original injection, 10 ml of blood was obtained from the wing vein of each animal. Serum was separated and stored at -20°C for further testing.

The chicken antiserum was tested for antibody by Ouchterlony double immunodiffusion (27) in 1% agarose containing 8% NaCl (Fig. 1). Ten  $\mu$ liters of the chicken antiserum were pipetted into the center well. The outer wells contained aliquots from preparations of the rat intestinal CaBP of varying degrees of purity: the crude, heated supernatant of the mucosal homogenate (well A), the pooled peak of calcium-binding activity eluted from a gel filtration column (well B) and the purified CaBP (well C).

Immunoelectrophoresis was carried out to further establish the specificity of the chicken anti-rat CaBP (Fig. 2). Thirty  $\mu$ g of protein from the heated supernatant of the rat intestinal mucosal homogenate and 10  $\mu$ g of the ion exchange purified CaBP were electrophoresed in a polyacrylamide disc gel electrophoresis system using 7% polyacrylamide at a running pH of 8.9 in the presence of 0.3 mM  $\text{CaCl}_2$  (8). After electrophoresis, the gels were placed on a film of 1% agarose in 8% NaCl. A trough was cut in the agarose, parallel to the gel, and 50  $\mu$ liters of the chicken antiserum was pipetted into the trough.

Quantitative measurement of immunoreactive CaBP was carried out using radial immunodiffusion as described by Mancini, *et al.* (24). Twenty  $\mu$ liters of the chicken antiserum was added to each ml of 1% agarose in 8% NaCl and the mixture was poured onto Gel Bond plates (FMC Corp., Rockland, ME). After solidification of the agarose, wells were cut and 2.5-10  $\mu$ liters of a standard solution of the purified rat intestinal CaBP was pipetted into each well.

The agarose plates were incubated at room temperature for 48 h, then washed twice in distilled deionized water, dried and stained by immersion in 0.1% thiazine red in 0.1% acetic acid for all of the above immunochemical techniques. After washing in 50% methanol:10% acetic acid, they were again dried. For calculation of immunoreactive CaBP, the diameter of the zone of immunoprecipitation in the radial immunodiffusion system was measured. The area of the zone of immunoprecipitate was calculated and plotted against the log of the amount of CaBP added to the well. A linear relationship existed between 0.5-25  $\mu$ g of CaBP.

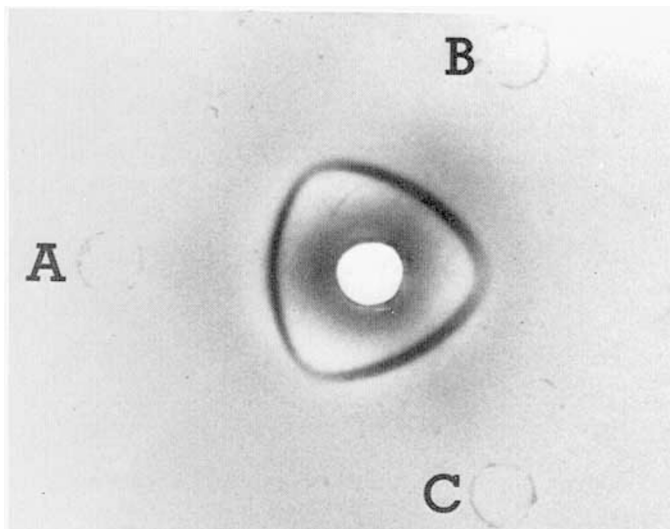


Fig. 1. Double immunodiffusion of the chicken anti-rat calcium-binding protein (CaBP). Ten  $\mu$ liters of the chicken anti-rat CaBP was placed in the center well. Well A contains 22  $\mu$ g of protein from the crude, heat-treated intestinal mucosal supernatant. Well B contains 4  $\mu$ g of protein from the pooled fractions within the gel filtration peak of calcium-binding activity. Well C contains 0.6  $\mu$ g of the purified rat intestinal CaBP.

Female Sprague-Dawley rats were mated, housed in nesting boxes, and allowed *ad libitum* access to chow containing 1.2% calcium, 0.99% phosphorus and distilled, deionized water. Births of pups were noted, and the pups were weighed at weekly intervals. The pups were suckled as their only source of nourishment but had free access to water.

The pups were sacrificed at from 1–7 weeks of age. Duodenal segments, anatomically defined as beginning at the pylorus and ending at the duodenal ligament corresponding to the ligament of Treitz in man, were removed, rinsed in ice cold 0.9% NaCl, blotted, and weighed. After weighing, the duodenal segments were homogenized in 3 volumes of 0.0137 M Tris-HCl, pH 7.4, with 0.12 M NaCl, 0.003 M KCl. The homogenates were heated at 65°C for 5 min, incubated for 5 min at 4°C, and centrifuged at 30,000  $\times g$  for 30 min. The resulting heat-treated supernatant was assayed for calcium-binding activity using a modification of the Chelex-100 competitive radiocalcium-binding assay (14). For each determination duodenal segments were pooled from four 1-week-old, four 2-week-old, and two 3-week-old pups.

Immunoreactive CaBP was quantitated in the heated supernatants from neonatal rat duodenum by radial immunodiffusion. Because the relationship between the area of the immunoprecipitate and the log of the CaBP concentration was linear between 0.5–25  $\mu$ g CaBP, samples were diluted and assayed in this range. Supernatant protein was determined using the method of Lowry, *et al.* (22). Data was analyzed using one way analysis of variance (4).

## RESULTS

The Ouchterlony double diffusion experiment revealed a single line of immunoprecipitate between the center well, containing the chicken anti-rat CaBP and the outer wells containing the crude, heat-treated rat intestinal mucosal supernatant, the partially purified preparation of the CaBP, and the purified CaBP (Fig. 1). This line formed a line of identity, with no spurs, between the outer wells. Immunoelectrophoresis revealed a single arc of immunoprecipitate, corresponding to the protein band with the characteristic mobility of the rat intestinal CaBP in all three preparations (Fig. 2).

The sensitivity of the chicken anti-rat CaBP, as determined by testing various concentrations of the purified CaBP by Ouchterlony double diffusion, was in the range of 0.1  $\mu$ g.

The pattern of gain in total body weight and weight of the duodenal segments is shown in Figure 3. Intestinal mucosal growth, as evidenced by increasing duodenal segment weight and supernatant protein concentration, showed the characteristic increase in rate of mucosal growth between wk 2 and 3, just before weaning (26). Soluble protein content of the mucosal supernatant increased 1½-fold between wk two and three and remained constant thereafter.

Small amounts of calcium-binding activity were seen in the heated intestinal mucosal supernatants from suckling animals (Fig. 4). These values increased 2½-fold in the wk before weaning. High levels of calcium-binding activity persisted to 5 wk of age, at which time they decreased to 1½ times that seen in the suckling

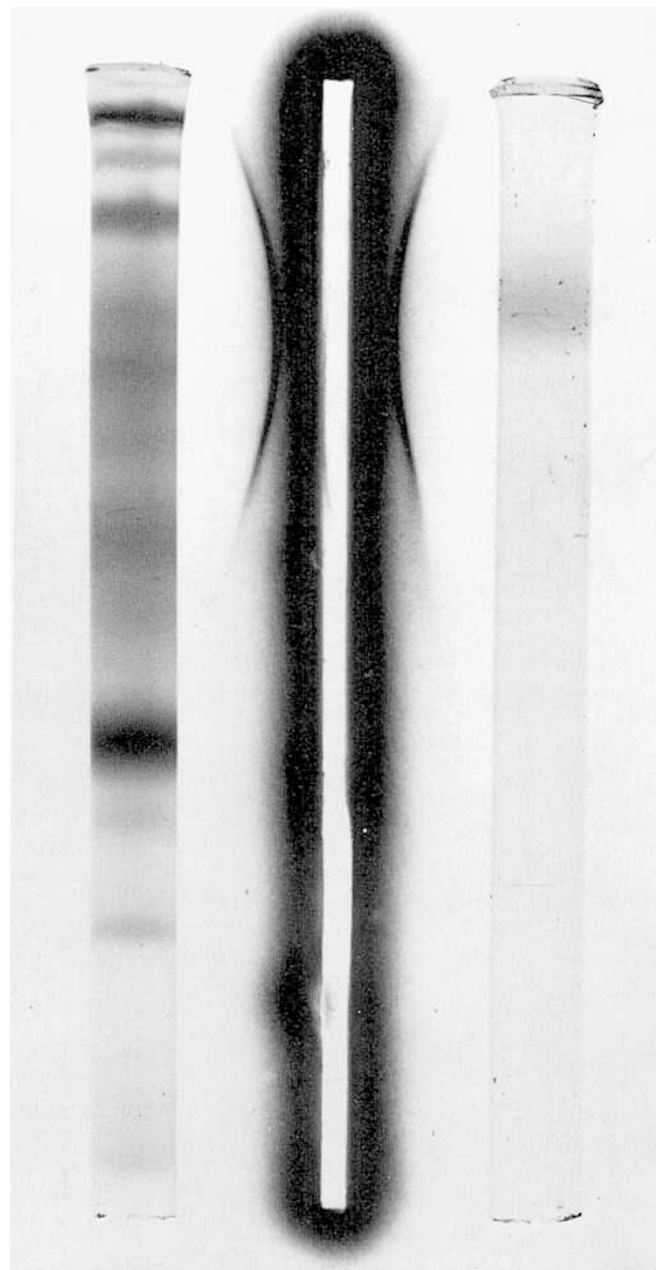


Fig. 2. Immunoelectrophoretic analysis of the chicken anti-rat calcium-binding protein (CaBP). The trough in the center contains 50  $\mu$ liters of the chicken anti-rat CaBP. The gel on the left shows the electrophoretic pattern of 30  $\mu$ g of protein from the heated supernatant from the rat intestinal mucosal homogenate. The gel on the right, 10  $\mu$ g of purified rat intestinal CaBP. The arc of immunoreactivity corresponds to the electrophoretic position of the CaBP.

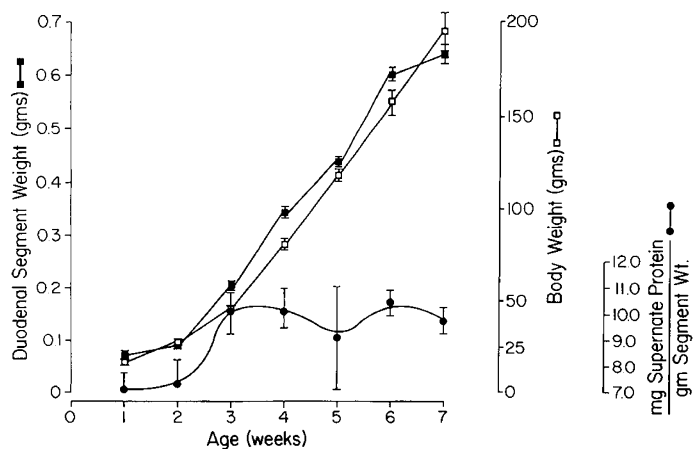


Fig. 3. Pattern of gain in body weight, duodenal segment weight and protein content of the mucosal supernatant during suckling and weanling periods.

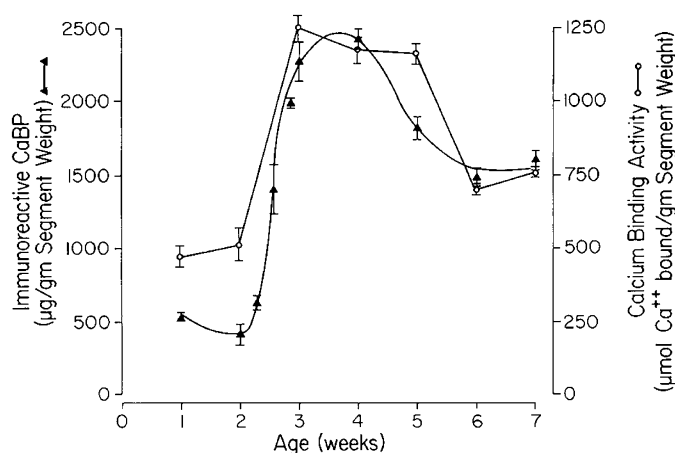


Fig. 4. Calcium-binding activity and calcium-binding protein immunoreactivity in heat-treated supernatants of duodenum during the suckling and weanling periods.

intestine. Changes in immunoreactive CaBP paralleled the changes in calcium-binding activity but were more dramatic. The peak of immunoreactive CaBP concentration occurred at weaning and resulted in a 5-fold increase over immunoreactive CaBP in the suckling intestine. A similar decrease in CaBP concentration was seen after weaning, beginning at 4 wk and resulting in a net 3-fold increase in CaBP concentration between the suckling period and the second month of life.

Because of the marked change in calcium-binding activity and immunoreactive CaBP concentrations between wk 2 and 3, the change was more closely monitored by sampling duodenal segments from suckling animals at days 16, 18, and 20 of age. The additional points indicate that the increase in CaBP begins promptly at 15 to 16 days of age, and results in a rather abrupt increase in CaBP, tripling in 4 days and peaking within less than a wk.

#### DISCUSSION

This paper reports the production of a specific chicken anti-rat CaBP and its use in immunochemical quantitation of CaBP in the developing duodenum. Repeated attempts at producing an antibody by immunizing rabbits with up to 3 mg of the purified rat intestinal CaBP were unsuccessful in our hands. We therefore selected the chicken, because of its phylogenetic distance from the rat, and because of the successful experience of others in producing antibodies using this animal (3, 18).

The antibody produced in these studies appears to be highly specific for rat intestinal CaBP. A single line of immunoprecipitate appears when it is tested by immunodiffusion against the crude heat-treated rat intestinal mucosal supernatant, the peak of calcium-binding activity eluted off the gel filtration column, and the purified CaBP (Fig. 1). The line of identity formed indicates that the antibody recognizes an immunologically similar protein in each. Absence of spurs indicates that the antibody recognizes only one protein species in each of the three mucosal supernatants of varying purity. Further study of the antibody's specificity using immunoelectrophoresis (Fig. 2) revealed that the antibody recognizes only one protein species in the crude heat-treated intestinal mucosal supernatant whose electrophoretic mobility matches that of the purified protein (15).

Although our finding of a 5-fold increase in immunoreactive CaBP occurring between days 14 and 21 in the suckling rat intestine confirms similar observations by DeLorme, *et al.* (9) and Bruns, *et al.* (7), subtle but important differences should be pointed out.

The antiserum used in our studies is clearly different from that used by DeLorme, *et al.* in that the protein used to generate it was purified to homogeneity (15), and the specificity of the antigen for the CaBP is indicated by the immunodiffusion and immunoelectrophoresis experiments here reported. The CaBP antiserum used by DeLorme, *et al.* was raised to a CaBP preparation that has two distinct protein bands when studied by polyacrylamide gel electrophoresis (25). The only evidence for the specificity of its recognition of CaBP is the coelution of CaBP immunoreactivity with calcium-binding activity from a gel filtration column (25). Additionally, our antibody recognizes porcine intestinal CaBP (16) whereas theirs reportedly does not (25).

Unlike the study of Bruns *et al.* (7), duodenal segments sampled in this study were defined anatomically as the segment between the pylorus and the ligament corresponding to the ligament of Treitz in man, rather than by sampling the proximal 20 cm of small intestine. This allowed standardization of the size of duodenal segments sampled across a broad range of duodenal segment lengths associated with rapid growth in the length of the intestine that occurs during this period (26). Because the duodenum is the site of maximal vitamin D-dependent calcium transport and CaBP concentration in the mature intestine (28) and this aboral gradient of CaBP has been shown to be present in fetal intestine (9), we anticipated that the changes in CaBP concentration and calcium-binding activity would be most marked in the duodenum.

Finally, the biologic activity of the CaBP-calcium-binding activity was measured and correlated with immunoreactive CaBP measurements.

The reasons for the sudden and dramatic increase in immunoreactive CaBP concentration and calcium-binding activity seen before weaning in the suckling rat intestine are unknown. The 5-fold increase in immunoreactive CaBP concentration and 2½-fold increase in calcium-binding activity (Fig. 4) between wk 2 and 3 occur during a period of rapid growth and maturation of the intestinal mucosa. Supernatant protein concentration increases only 1½-fold and mucosal weight doubles during the same period. It is unlikely, therefore, that the increase in CaBP is a nonspecific result of the mucosal hypertrophy and protein synthesis characterizing the preweaning intestine.

Dietary changes, particularly dietary restriction of calcium (14) and phosphorus (13) result in a vitamin D-dependent increase in duodenal CaBP levels; thus, changes in dietary calcium and phosphorus content during transition from suckling to weanling may be important in initiating the increase in duodenal CaBP seen in these studies. Although the calcium and phosphorus content of rat's milk increases slightly during the first wk of lactation and remains essentially stable thereafter through weaning (23, 21), a net decrease in dietary calcium or phosphorus could result from initiation of small amounts of solids in the diet as weaning begins.

Although other lines of evidence indicate that the vitamin D endocrine system is relatively unimportant in the regulation of

calcium homeostasis in the suckling animal (11, 17), the parallel development of pathways of vitamin D metabolism and intestinal responsiveness to 1,25-dihydroxycholecalciferol as manifest by the CaBP may represent the development of the vitamin D endocrine system as it exists in the adult animal. Further studies examining intestinal development in general and development of CaBP in vitamin D-deficient rat pups are necessary to clarify the role of the vitamin D endocrine system in intestinal development in general and development of active calcium transport specifically.

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