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Biochemistry

Calcium-binding protein of chorioallantoic membrane: Identification and developmental expression

(calcium transport/embryonic skeletal formation/chick embryo)

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ABSTRACT **A calcium-binding protein (CaBP) which has a molecular weight of 100,000 and an isoelectric point of 8.0 has been isolated from the chorioallantoic membrane (CAM) of the chick embryo. Expression of the CaBP occurs simultaneously with the onset of calcium absorption from the egg shell by the CAM, and the temporal increase in CaBP activity is coincident with calcium deposition in the embryo. The CAM CaBP differs in properties from the CaBPs of the adult organs, including the vitamin-D-dependent protein of intestine.**

Vertebrate embryos must obtain large amounts of calcium from extra-embryonic sources during skeletal formation. The embryos of oviparous animals, such as the chicken, offer advantages for studying calcium translocation associated with bone mineralization in that the function is carried out by a simple cellular membrane, the chorioallantoic membrane (CAM). From the thirteenth day of incubation until hatching at day 21, the CAM transfers over 100 mg of calcium from the egg shell to the embryo via its circulatory system (1, 2). The CAM lines the interior of the shell after the ninth day and is situated against the noncellular shell membranes. Structurally, the CAM is highly vascularized and consists of three distinct layers of cells with the ectoderm adjacent to the inner shell membrane.

Because of its availability and simple morphology, the chick embryonic CAM has been utilized extensively for studies of calcium transport. Experiments with the CAM mounted in an Ussing-type apparatus have provided direct evidence that the CAM actively transports calcium uni-directionally from its ectodermal to endodermal side *in vitro* (3–5). The calcium transport function of the CAM was found to be age-dependent and expressed concomitantly with the onset of calcium accumulation by the embryo. Microscopic observations (6–8) further revealed a close correlation between the differentiation of the CAM and embryonic ossification, suggesting that the CAM is the regulatory site for calcium mobilization in the chick embryo. With this background, we recently began a comparative study of transporting and nontransporting CAM isolated from chick embroyos at ages prior to and after the onset of calcium deposition. In this report, we show that extracts of transporting CAM exhibit enhanced calcium-binding activities that result from the expression of a high-molecular-weight calcium-binding protein (CaBP).

MATERIALS AND METHODS

Embryos. Fertilized White Leghorn eggs were purchased from Shamrock Poultry and incubated at 37.5° in a humidified egg incubator.

Isolation of CAM and Preparation of CAM Extracts. The CAM was dissected from embryos of appropriate ages in the following manner. The shell was removed from the blunt end of the egg and a circular opening was cut in the underlying shell membrane and adherent CAM. The embryo together with the yolk and albumen sacs was decanted through the opening. The remaining CAM, which lines the inside of the egg shell, was rinsed with physiological saline, dissected away from the shell membrane, and again rinsed.

Extracts of the CAM were prepared by homogenizing the tissue in a Waring blender at 4° for four 15 sec intervals with four volumes (vol/wt) of TAD buffer (13.7 mM Tris·HCl, pH 7.4/0.12 M NaCl/4.74 mM KCl/98.5 μM glucose/0.71 mM dithiothreitol/0.02% sodium azide). After centrifugation at 31,000 × *g* for 30 min, the endogenous calcium of the supernatant fraction was removed by dialysis against the same buffer.

Assay for Calcium-Binding Activity. Calcium-binding activity was measured by the Chelex 100 ion-exchange method (9). Assay mixtures consisted of 0.2 ml of a suspension of Chelex 100 resin (Bio-Rad) in TAD buffer (1:1, vol/vol) and 1.0 ml of sample. The reaction was started by the addition of $^{45}CaCl_2$ (carrier-free, 0.5 μCi) and carried out with agitation for 30 sec at room temperature. After centrifugation for 10 min, the radioactivity in a 0.5 ml aliquot of the supernatant was determined by liquid scintillation counting. Units of calcium-binding activity are calculated as

$$\frac{\text{radioactivity in supernatant}}{\text{radioactivity retained by resin}}$$

and expressed as a percentage. We have confirmed the linearity of the assay with respect to the amount of calcium-binding material as originally shown by Wasserman *et al.* (10).

Assay for Calcium-Dependent ATPase. This activity was determined by the method of Pollard and Korn (11). In this method, the Ca^{2+}-dependent ATPase activity (ATP phosphohydrolase, EC 3.6.1.3) was obtained as the difference between ATPase activity levels measured in the absence of Ca^{2+} (2 mM EDTA added to assay mixture) and in the presence of additional 10 mM $CaCl_2$. The activity was expressed as nmol of phosphate per min.

Protein. Protein was estimated by the method of Lowry *et al.* (12) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

A newly hatched chick contains approximately 130 mg of calcium (12), all of which is derived from the egg shell and transported by the CAM to the circulation during embryonic development. Because calcium is toxic to cells and specific carrier proteins are often associated with membrane transport functions, it seemed likely that the transporting CAM contains a calcium-binding protein (CaBP). With this in mind, we assayed extracts of the CAM obtained from embryos between incuba-

Abbreviations: CAM, chorioallantoic membrane; CaBP, calcium-binding protein; TAD buffer, buffer containing 13.7 mM Tris·HCl at pH 7.4/0.12 M NaCl/4.74 mM KCl/98.5 μM glucose/0.71 mM dithiothreitol/0.02% sodium azide; EDTA, ethylenediaminetetraacetic acid.

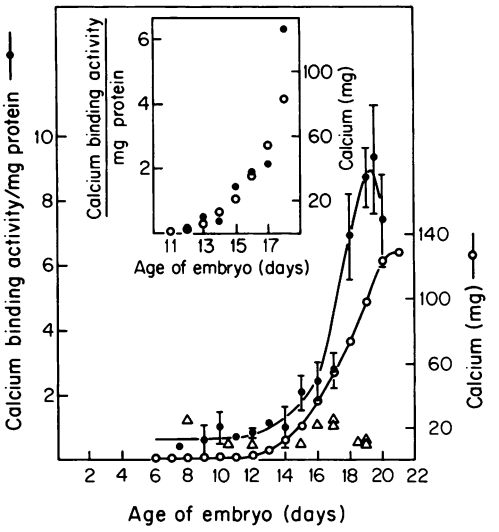

FIG. 1. Age profile of the specific calcium-binding activity of CAM extracts and the calcium content per chick embryo plotted from the data given by Romanoff (2). The specific calcium-binding activities (activity/mg protein) are the means ± SEM of at least five determinations on soluble extracts of pooled membranes from five or more embryos. The open triangles represent the specific activities in CAM extracts treated with Pronase (1:10, enzyme:CAM protein) at 37° for 15 min. (*Inset*) The net calcium-binding activity in extracts of transporting CAM and the calcium content of the embryo. Net activities were obtained by subtracting the background levels of calcium binding found in extracts of nontransporting CAM (membranes from embryos incubated for less than 12 days). The average basal activity was calculated to be 0.6 units/mg of protein from the data given in the activity-age profile.

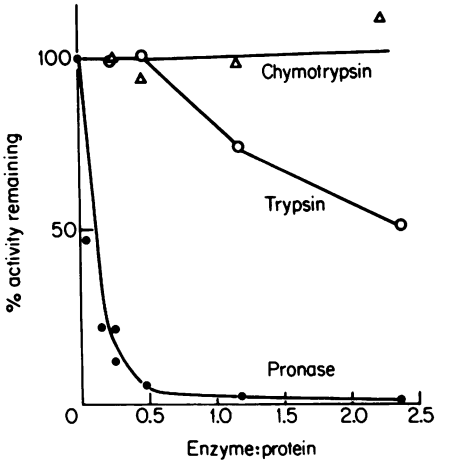

FIG. 2. Sensitivity of the CAM calcium-binding activity to proteolysis. The CAM extracts were prepared as in *Materials and Methods* from 18- to 20-day-old embryos. Pronase (Calbiochem), chymotrypsin (Boehringer-Mannheim), or trypsin (Worthington) was incubated with the CAM extracts at the indicated concentrations for 15 min at 37°. Calcium-binding activities of the CAM extracts after incubation were determined by the Chelex 100 assay (see *Materials and Methods*). Controls containing the proteases alone exhibited no calcium-binding activity.

tion days 7 and 20 for calcium-binding activity. As shown in Fig. 1, low levels of calcium binding are evident at early stages of development prior to day 12. Between incubation days 12 and 13, however, the activity increases, and it rises rapidly thereafter. The maximal level of calcium binding, which occurs on or about day 19, represents a greater than 10-fold increase over the basal level at day 11. The location of the calcium-binding activity in the CAM was determined by subcellular fractionation (Table 1). A homogenate of the CAM prepared in isotonic buffer was fractionated by differential centrifugation, and the various subcellular fractions were assayed for calcium-binding activity. Our results indicated that the calcium-binding activity is a soluble component of the CAM and is not associated with membranes or other particulate components.

Table 1. Subcellular localization of CaBP in CAM homogenate*

Fraction	% recovery of calcium-binding activity†
CAM homogenate	100
2,000 × g, 10 min (nuclear)	2.5
12,000 × g, 20 min (mitochondrial)	0
105,000 × g, 60 min (microsomal)	0
Supernate (soluble)	74.5

* The CAM was homogenized with a Ten Broeck homogenizer in isotonic sucrose-containing buffer (0.25 M sucrose/0.02 M Tris·HCl/0.01% mercaptoethanol, pH 7.4). The homogenate was then separated into the various fractions by differential centrifugation similar to the procedure of Saleuddin *et al.* (8). All operations were carried out at 4°.

† Calcium-binding activities were measured by the Chelex 100 method (*Materials and Methods*). The subcellular fractions other than the supernatant fraction were first solubilized in 0.1% Triton X-100 and then assayed for calcium-binding activity. Controls with 0.1% Triton X-100 alone exhibit no detectable calcium-binding activity.

The age-dependent onset of the calcium-binding activity in CAM extracts is coincident with that of the calcium transport capacity of the tissue. Studies on *in vitro* calcium transport by the CAM (3–5) have detected an increase in transport activity between incubation days 12 and 13. Because the solubilized calcium of the egg shell is ultimately deposited in the embryonic skeleton (1, 2), the physiological role of the CAM calcium-binding activity is better assessed by comparison with the calcium content of the embryo. Calcium deposition in the chick embryo has been studied in detail by Romanoff and others (2). These data are also shown in Fig. 1. In embryos between the ages of 12 and 17 days, calcium accumulation correlates well with the calcium binding of CAM extracts (see *inset* of Fig. 1). After day 17, deposition of calcium continues at a linear rate while the CAM calcium binding activity increases more rapidly. However, prior to hatching, the calcium content per embryo reaches a maximum, which is preceded by a decline in the specific calcium-binding activity of CAM extracts. Overall, these profiles demonstrate a relationship between the two parameters.

With the Chelex 100 ion-exchange assay, we have characterized the factor(s) responsible for the enhanced calcium-binding activity of transporting CAM. The proteinaceous nature of the material is indicated from its sensitivity to heat and proteolytic action. The activity is destroyed by heating CAM extracts for 15 min at 60°. As shown in Fig. 2, the activity is also lost on treatment of these extracts with Pronase or trypsin, but not with chymotrypsin. At an enzyme:CAM protein ratio of 2:1, calcium-binding activity is totally abolished by Pronase, and approximately 50% inactivation is obtained with trypsin. Furthermore, as shown in Fig. 1, Pronase digestion reduces the calcium-binding activity in extracts of transporting CAM to levels similar to those of untreated extracts of nontransporting CAM. This is true at all developmental ages of the embryo after the onset of calcium mobilization (incubation day 12–13). These

Table 2. Ammonium sulfate fractionation of CAM extract*

Fraction	% protein	% calcium-binding activity	% calcium-ATPase
I. Precipitate (40% salt saturation)	22.7	23.9	65.0
II. Precipitate (50% salt saturation)	9.6	57.0	5.4
III. Supernate (50% salt saturation)	66.1	15.6	20.1
Recovery	98.4	96.5	90.5

* The CAM extracts were prepared as in *Materials and Methods*. Crystalline enzyme-grade ammonium sulfate (Schwarz/Mann) was added slowly to CAM extracts with stirring to achieve 40% saturation. After complete dissolution of the salt, the sample was then allowed to stir for 30 min. The precipitated protein fraction I was obtained by centrifugation at 13,000 × *g* for 20 min. Additional ammonium sulfate was added with stirring to reach 50% salt saturation. After stirring for at least 2 hr, fraction II was obtained by centrifugation at 13,000 × *g* for 20 min. Fractions I and II were dissolved in minimal volumes of TAD buffer and, together with the supernatant fraction III, were dialyzed extensively against TAD buffer. All operations were carried out at 4° and the pH was maintained at 7.4 throughout the fractionation procedure. After dialysis, the samples were assayed for calcium-binding activity, calcium-dependent ATPase, and protein by procedures in *Materials and Methods*.

results suggest that a CaBP(s) is responsible for the enhanced calcium binding of transporting CAM.

Some of the properties of the CaBP have been studied. On fractionation of CAM extracts with ammonium sulfate, the bulk of the calcium-binding activity is contained in the protein fraction precipitated between 40 and 50% salt saturation (Table 2). In contrast, 70% of the calcium-dependent ATPase activity is precipitated between 0 and 40% ammonium sulfate saturation. Furthermore, our data indicated that the specific activity levels of calcium-dependent ATPase do not exhibit an age-dependent increase similar to that of the CaBP. These findings strongly suggest that the CaBP of the CAM is not likely to be associated with a calcium-dependent ATPase activity. It may be pointed out that Saleuddin *et al.* (8) have recently shown that *in vitro* calcium uptake by transporting CAM is not accompanied by an increase in calcium-dependent ATPase activity.

Isoelectric focusing of the ammonium-sulfate-enriched AcBP revealed a single peak of activity with an isoelectric point of 7.9–8.1 (Fig 3*A*). The size of the CaBP has also been estimated from its elution characteristics on Sephadex G-200 (Fig. 3*B*). It elutes as a single sharp peak at a volume corresponding to an approximate molecular weight of 100,000. In contrast, elution profiles of extracts from nontransporting CAM are characterized by the absence of a CaBP activity peak. These results taken together support the notion that the enhanced calcium-binding activity of transporting CAM results from the expression of a single species of CaBP. It should be pointed out that the high isoelectric point of the CAM CaBP is not necessarily inconsistent with its cation binding activity because there is no correlation between the acidity of various proteins and their calcium affinity (13). Furthermore, enzyme activities such as a calcium-dependent ATPase are not obligatory properties of calcium-binding proteins in general (13).

By employing a procedure that involves ammonium sulfate precipitation, filtration on Sephadex G-200, and isoelectric

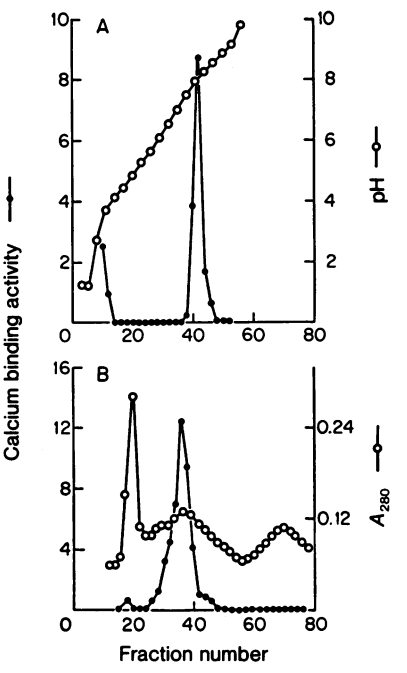

FIG. 3. Characterization of the CaBP in extracts of CAM from 19-day chick embryos. An enriched preparation of CaBP was obtained from the CAM extract by precipitation at 50% saturation of ammonium sulfate (see footnotes to Table 2). The precipitate was redissolved in TAD buffer and used as the source of CaBP for the following analysis. (*A*) Isoelectric focusing profile. The CaBP sample (9.8 mg of protein) was applied to a 110 ml column (LKB) containing 2% ampholytes, pH 3–10. Electrofocusing was carried out for 40 hr at 4° and 700 V. Fractions of 1.8 ml were collected. The increase in binding activity near the anode is an artifact due to the presence of phosphoric acid. (*B*) Elution profile from a Sephadex G-200 column. A 40 × 2.6 cm column was eluted with TAD buffer at a flow rate of 7.5 ml/hr after the addition of 4.6 mg of protein. Fractions of 3.6 ml were collected. The protein content of each fraction was estimated from the absorbance at 280 nm. The column was calibrated with the following molecular weight markers: ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (45,000), and aldolase (158,000). In both experiments, calcium-binding activity was determined by the Chelex 100 ion-exchange method (see *Materials and Methods*).

focusing, we have achieved a 170-fold purification of the CaBP.* These preparations of CaBP appear to be homogeneous on polyacrylamide gel electrophoresis. Specific antisera have been prepared against the purified CaBP. With these antisera, we have obtained further evidence that the CaBP is present, as a non-blood-borne component, only in transporting CAM.*

Calcium-binding proteins have been implicated in a number of calcium-mediated processes (13). However, the role of CaBP in calcium transport remains controversial (14). In the CAM, the onset of calcium transport is reminiscent of the vitamin-D-mediated stimulation of intestinal calcium absorption in rachitic chicks (15). Synthesis of a CaBP in the intestine also occurs on the stimulation of calcium transport. Although the specific calcium-binding activities measured by the Chelex 100 assay method in CAM and intestinal (16) extracts are similar, the properties of the CAM CaBP differ significantly from those of the intestinal protein. The intestinal CaBP has a molecular weight of 25,000, is not inactivated at 60°, and has an isoelectric point of 4.4 (15). These differences are of particular interest in that both tissues originate from embryonic hindgut and subsequently develop calcium transport functions to be expressed

* Details of these results will be presented in subsequent communications.

at different developmental stages. In the adult, calcium absorption by the ileum is responsible in part for the homeostatic regulation of intracellular calcium and bone metabolism (17). Calcium mobilization in the embryo, in contrast, occurs largely for the purpose of ossification and is carried out primarily by the CAM. The dissimilar physiological functions of the intestinal and CAM calcium absorption processes may account for the development-specific features of the two CaBPs. On the other hand, the properties of CaBPs in calcium-transporting tissues may be a reflection of the developmental stage of the organism. In support of this hypothesis, various tissues of the adult chicken, including the kidney (18), shell gland (19), brain (20), and pancreas (21), have been found to contain CaBPs with properties similar or identical to those of the intestinal CaBP. As yet, similar comparative studies of embryonic tissues are not available.

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1. Johnston, P. M. & Comar, C. L. (1955) *Am. J. Physiol.* **183,** 365–370.
2. Romanoff, A. (1967) *Biochemistry of the Avian Embryo. A Quantitative Analysis of Prenatal Development.* (Wiley Interscience, New York), p. 39.
3. Garrison, J. C. & Terepka, A. R. (1972) *J. Membr. Biol.* **7,** 128–145.
4. Moriarty, C M. & Terepka, A. R. (1969) *Arch. Biochem. Biophys.* **135,** 160–165.
5. Terepka, A. R., Stewart, M. E. & Merkel, N. (1969) *Exp. Cell Res.* **58,** 107–117.
6. Coleman, J. R., Dewitt, S. M., Batt, P. & Terepka, A. R. (1970) *Exp. Cell Res.* **63,** 216–220.
7. Narbaitz, R. (1972) *Rev. Can. Biol.* **31,** 259–267.
8. Saleuddin, A. S. M., Kyriakides, C. P. M., Peacock, A. & Simkiss, K. (1976) *Comp. Biochem. Physiol.* **54,** 7–12.
9. Wasserman, R. H. & Taylor, A. N. (1966) *Science* **152,** 791–793.
10. Wasserman, R. H., Corradino, R. A. & Taylor, A. N. (1968) *J. Biol. Chem.* **243,** 3978–3986.
11. Pollard, T. D. & Korn, E. D. (1973) *J. Biol. Chem.* **248,** 4682–4697.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Kretsinger, R. H. (1976) *Annu. Rev. Biochem.* **45,** 239–266.
14. Spencer, R., Charman, M., Wilson, P. & Lawson, E. (1976) *Nature* **263,** 161–163.
15. Wasserman, R. H., Taylor, A. N. & Fullmer, C. S. (1974) in *The Metabolism and Function of Vitamin D.* (Biochemical Society Special Publication No. 3), ed. Fraser, D. R. (Biochemical Society, London), pp 55–74.
16. Wasserman, R. H. & Taylor, A. N. (1968) *J. Biol. Chem.* **243,** 3987–3993.
17. Omdahl, J. L. & De Luca, H. F. (1973) *Physiol. Rev.* **53,** 327–372.
18. Taylor, A. N. & Wasserman, R. H. (1967) *Arch. Biochem. Biophys.* **119,** 536–540.
19. Fullmer, C. S., Brindak, M. E., Bar, A. & Wasserman, R. H. (1976) *Proc. Soc. Exp. Biol. Med.* **152,** 237–241.
20. Taylor, A. N. (1974) *Arch. Biochem. Biophys.* **161,** 100–108.
21. Zolock, D. T. & Morrisey, R. L. (1976) *Fed. Proc.* **35,** 1609.