

CALCIUM-BINDING PROTEIN OF THE CHICK CHORIOALLANTOIC MEMBRANE

I. Immunohistochemical Localization

ROCKY S. TUAN, WILLIAM A. SCOTT, and ZANVIL A. COHN

From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021. Dr. Tuan's present address is the Department of Orthopaedic Surgery, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT

The preparation of a specific antiserum (anti-CaBP) against the calcium-binding protein (CaBP) of the chorioallantoic membrane (CAM) is described. The anti-CaBP appeared to be specific for the CaBP by immunodiffusion and immunoelectrophoresis. Application of the anti-CaBP in immunofluorescence histochemistry revealed that the CaBP is present in the CAM only at developmental ages corresponding with the expression of the calcium transport function of the membrane. Furthermore, the CaBP is localized to the ectoderm of the CAM, appears to be exposed to the entire external surface of the ectoderm, and can be shown to be associated with cells enzymatically dissociated from the CAM. These results are consistent with a functional role of the CaBP in the CAM calcium transport process.

KEY WORDS calcium transport · calcium-binding protein · chorioallantoic membrane · embryonic development · immunohistochemistry

In the chick embryo, the calcium required for skeletal calcification is mobilized from the egg shell by the chorioallantoic membrane (CAM)¹

¹ *Abbreviations used in this paper:* anti-CaBP, antiserum prepared against purified CaBP; anti-NT, antiserum prepared against whole extract of nontransporting CAM from 11-day-old embryos; anti-T, antiserum prepared against whole extract of transporting CAM from 19-day-old embryos; buffer A, 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, pH 7.4; buffer B, buffer A containing 0.38% (wt/vol) sodium citrate; CaBP, calcium-binding protein; CAM, chorioallantoic membrane; F-GAR, FITC-conjugated goat anti-rabbit antiserum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PCMBs, *p*-chloromercuribenzenesulfonate; 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.

(8, 14, 16). The calcium transport function of the CAM is energy-dependent (6) and is expressed in an age-dependent fashion during embryonic development (3, 17).

We have recently identified a calcium-binding protein (CaBP) associated with the CAM (19). The CaBP has been subsequently purified to homogeneity and its physical and chemical properties have been characterized (18, 20). Our studies revealed a close similarity between several properties of the CaBP and those of the calcium transport activity of the CAM (16), including high selectivity for calcium ions and sensitivity to sulfhydryl-binding agents. In particular, there exists an excellent correlation between the temporal

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expression of the calcium-binding (19) and calcium-transport (17) activities of the CAM during embryonic development. Levels of both activities are low before incubation day 12, rise rapidly thereafter, and reach maximal levels shortly before hatching on day 21. The parallel between these properties strongly suggests that the CaBP may play a functional role in the transport of calcium by the CAM.

The CAM consists of three distinct layers of cells, with the ectodermal epithelium facing the calcium source, the egg shell (1). Studies by Terepka et al. (16) have shown that the CAM ectoderm, which is the first cellular barrier to the entry of calcium from the egg shell into the embryonic circulation, comprises the calcium-transporting region of the CAM. Therefore, in order to ascertain the transport-related function of the CAM CaBP, it is essential to determine its cellular localization. We report here the preparation of a specific antiserum against the CaBP (anti-CaBP) and the immunohistological localization of the CaBP on the ectoderm of the CAM.

MATERIALS AND METHODS

Embryos

Fertilized white Leghorn eggs (Shamrock Farms, North Brunswick, N. J.) were incubated at 37.5°C in a humidified egg incubator for the desired period of time. Eggs were routinely examined by transillumination for fertility and normal development of the embryo.

Purification of the CaBP

This was carried out using CAM from 19- to 20-day-old chick embryos by the previously described procedure (18, 20) which involved four steps: preparation of CAM extract, ammonium sulfate precipitation, gel filtration, and isoelectric focusing. The homogeneity of the CaBP preparations was assessed as described elsewhere (18, 20).

Immunological Methods

Antisera were prepared against purified CaBP and CAM extracts from 12- and 19-day-old chick embryos. Emulsions (1:1, vol/vol) of these samples in 13.7 mM Tris-HCl, pH 7.4, 0.12 M NaCl, 4.74 mM KCl, 98.5 μ M glucose, 0.71 mM dithiothreitol, 0.02% sodium azide (TAD buffer) (CaBP, 200 μ g/ml; 12- and 19-day-old CAM extract, 2 mg protein/ml) were prepared in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Two rabbits (New Zealand White) were used for each sample, and each animal was immunized with 2 ml of the respective emulsion by injection into foot pads. The rabbits were bled 5 wk after the injection

and sera were collected. A second injection with the same amount of each antigen was performed 12 wk later, and sera were collected after one month.

The γ -globulin (IgG) fraction was obtained from the anti-CaBP anti-serum by a standard procedure involving ammonium sulfate precipitation (7) and diethylaminoethyl-cellulose ion exchange chromatography (5). The IgG fraction was reconstituted to the original volume of the serum and stored at 4°C until use.

Ouchterlony double immunodiffusion (11) was performed in diffusion plates purchased from Hyland Co. (Costa Mesa, Calif.). Immunoelectrophoresis was carried out in 1% agarose containing 0.05 M veronal buffer, pH 8.6, at 150 V for 1½ h according to Ouchterlony and Nilsson (11).

Enzymatic Dissociation of CAM into

Single Cells

The buffers used in the dissociation procedure were: (a) physiological saline, 0.9% NaCl; (b) calcium- and magnesium-free phosphate-buffered saline (PBS), 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 6.5 mM Na_2HPO_4 , pH 7.4 (buffer A); and (c) buffer A containing 0.38% sodium citrate (buffer B). All steps subsequent to dissection of the CAM from the embryo were carried out at 37°C unless indicated otherwise. The procedure was as follows: The CAM was dissected from embryos of the appropriate age, washed in physiological saline, weighed, and minced into 2-mm² pieces with scissorlike action of two scalpel blades. The tissue was washed at least five times with buffer A with final wash in buffer B. The minced CAM was then suspended in 10 ml of buffer B/gm tissue and placed in a siliconized Erlenmeyer flask. Collagenase (Sigma Chemical Co., St. Louis, Mo.) and hyaluronidase (bovine, Calbiochem, San Diego, Calif.) were added to a final concentration of 0.01 and 0.001%, respectively. The flask and its contents were shaken at ~150 rpm for 30 min. At the end of the incubation period, the dissociated cell suspension was carefully decanted, filtered twice through Nyltex filter (Newark Wire Cloth, Newark, N. J.) of 28- μ m mesh, sedimented by centrifugation at 650 g for 10 min, and resuspended in a small volume of buffer A. The tissue left in the flask was further dissociated by incubation for 30 min with shaking in the same volume of buffer B with collagenase and hyaluronidase as above. Isolated cells (fraction 1) were collected by centrifugation, and the whole procedure was repeated three times (fractions 2, 3, and 4) until the tissue appeared to be mostly dissociated.

Immunohistochemistry

SAMPLE PREPARATION: For the preparation of frozen sections, the CAM was first cut into strips of ~0.5 cm², placed in small polystyrene disposable beakers, embedded in I.C.T. Tissue-Tek (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) and frozen

by immersion into dry ice-ethanol. The frozen blocks were stored at -70°C until use. Sections, 4–6 μm thick, were cut on an IEC cryostat microtome (International Equipment Co., Needham, Mass.) at -10 to -20°C , placed on glass slides, and air-dried for at least 2 h at room temperature. The sections were fixed in acetone for 1 min and washed with PBS, containing 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 0.02% sodium azide, pH 7.65, for 5 min before staining.

Cells dissociated from the CAM as described above were smeared onto glass slides, fixed in acetone or 5% paraformaldehyde for 1 min, washed with PBS for 5 min, and used immediately for immunohistochemical staining.

STAINING: The procedure employed the method of indirect staining with a fluorescein isothiocyanate (FITC)-conjugated secondary antiserum (goat anti-rabbit IgG, Boehringer Diagnostics, Somerville, N. J.). All antisera were used in 1:10 dilutions and incubations were carried out at room temperature in a humidified chamber. Tissue sections or smears of cells dissociated from the CAM, after the PBS wash, were overlaid with 10 μl of rabbit anti-CaBP antiserum and incubated for 25 min. They were then washed with two changes of PBS for 5 min each. 10 μl of the secondary antiserum were applied and the samples were incubated for 25 min. The sections were finally washed in three changes of PBS, 5 min each, and were mounted with one drop of glycerol-PBS (1:1, vol/vol; pH 8.0). The specimens were examined with a Zeiss photomicroscope equipped with a Zeiss HBC 50-watt high pressure mercury vapor lamp, BG-3 exciter filter, and 500/530 nm barrier filters. Photomicrographs were taken with either Kodak Ektachrome (High Speed) or Tri-X film.

RESULTS

Specificity of Anti-CaBP

The specificity of the anti-CaBP antiserum (anti-CaBP) was assessed by immunodiffusion and immunoelectrophoresis.

As shown in Figs. 1A, 2A, and B, upon immunodiffusion and immunoelectrophoresis, a single immunoprecipitin line was observed between anti-CaBP and purified CaBP, demonstrating the specificity of anti-CaBP. In addition, our results also indicated that the CaBP was present at a higher concentration in the extract of transporting, 19-day-old CAM than in the extract of nontransporting, 11-day-old CAM. Thus, antiserum prepared against the former (anti-T) reacted with purified CaBP (Figs. 1B and 2A), whereas antiserum against the latter (anti-NT) was not reactive (Figs. 1C and 2B). These findings were further substantiated by immunodiffusion of anti-

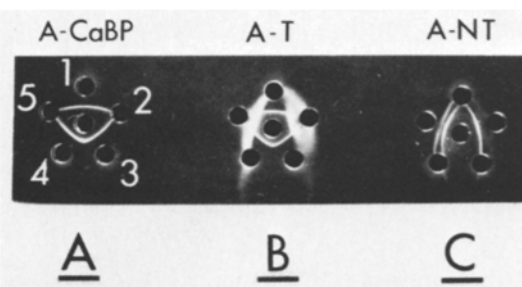


FIGURE 1 Ouchterlony double immunodiffusion of extracts of transporting (20-day-old) and nontransporting (10-day-old) CAM and purified CaBP with anti-CaBP (A-CaBP), anti-T (A-T), and anti-NT (A-NT). Samples were 10 μl each in TAD buffer and placed in the wells as follows: 1, 3, 4 = purified CaBP; 2 = extract of transporting CAM, and 5 = extract of nontransporting CAM. The CAM extracts contained 2 mg protein/ml and the CaBP preparation contained 0.5 mg of CaBP/ml. The antisera were placed in the respective center well as indicated and diffusion was allowed to proceed for at least 48 h at room temperature.



FIGURE 2 Immunoelectrophoresis of CaBP diffused against anti-T, anti-NT, and anti-CaBP. 10 μl of purified CaBP (0.5 mg/ml) was subjected to electrophoresis at 150 V for 90 min (11). After electrophoresis, 100 μl of the respective antiserum was placed in the troughs. Diffusion was allowed to proceed for at least 48 h at room temperature. See text and legend to Fig. 1 for explanation of symbols for antisera.

CaBP with extracts of CAM prepared from embryos of various developmental stages (Fig. 3). A single immunoprecipitin line, which revealed an age-dependent increase in the concentration of CaBP in the CAM extracts, was observed. The immunospecificity of anti-CaBP was also substantiated by the finding that the purified IgG fraction from anti-CaBP exhibited identical immunochemical reaction patterns.

These observations taken together demonstrated the specificity of the anti-CaBP and, furthermore, confirmed the homogeneity of the CaBP used to prepare the antiserum.

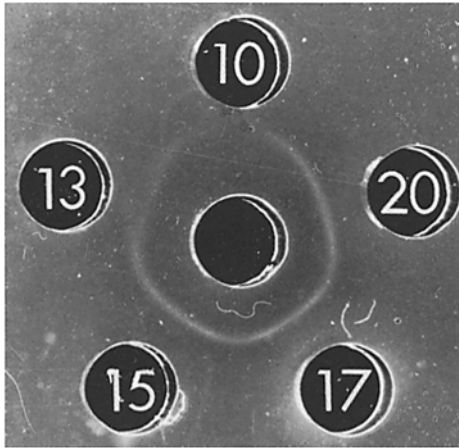


FIGURE 3 Ouchterlony double immunodiffusion of CAM extracts prepared from embryos of different ages against anti-CaBP. 10 μ l of each CAM extract (3.5 mg protein/ml) were placed in the outside wells. The embryonic ages (days) of the CAM extracts are as indicated. 10 μ l of anti-CaBP was placed in the center well and diffusion was allowed to proceed for at least 48 h at room temperature.

Immunohistochemical Localization of CaBP in CAM

In the indirect staining method used here, the anti-CaBP was employed as the primary antiserum. The localization of the bound anti-CaBP was then visualized with FITC-conjugated goat anti-rabbit antiserum (F-GAR).

CAM SECTIONS: Fig. 4 shows the immunohistochemical staining patterns of cryosections of transporting CAM from 20-day-old chick embryos. As shown in Fig. 4A, intense specific fluorescent staining of the CAM was observed, and was localized exclusively on the ectodermal epithelium of the CAM. Staining at the mesoderm and endoderm of the CAM was significantly less intense. With higher magnification (Fig. 4B), it was evident that the fluorescent staining on the ectoderm of the 20-day-old, transporting CAM was associated with the peripheral or cortical region of individual cells. The highest intensity of fluorescence was observed at the surface of the CAM ectoderm facing the shell membrane. In many fields, the staining pattern also appeared to trace the outline of the cells. Occasional intracellular fluorescence could be observed, but, in general, the cell cortex was more intensely stained. As shown in Fig. 4C, distinct staining was also observed in a capillary-covering region of the

CAM ectoderm. In general, however, the fluorescent staining appeared to be evenly distributed throughout the entire ectodermal layer of the CAM.

To test whether the CaBP is externally exposed on the surface of the ectodermal cells, samples of CAM were preincubated with heat-treated (56°C, 30 min, for complement inactivation) anti-CaBP (1:10 in PBS) for 15 min at room temperature before cryostat sectioning. The CAM sections were then fixed in acetone, washed in PBS, and stained. Intense cortical fluorescence of the ectodermal cells (not shown), similar to that obtained with the normal staining procedure, was observed, indicating that the CaBP of the CAM was accessible to externally added anti-CaBP.

The specificity of the immunohistochemical procedure was tested with the following controls: substitution of anti-CaBP with preimmune rabbit serum or PBS, and substitution of F-GAR with PBS in the staining procedure. In each case, the CAM sections revealed no fluorescent staining, indicating the requirement for both anti-CaBP and F-GAR in the procedure.

Furthermore, the fluorescent staining of the CAM was development-dependent. Sections of nontransporting CAM from 10-day-old CAM did not exhibit any specific and significant fluorescence at the ectodermal epithelium.

These results therefore indicated that the CaBP, present only in transporting CAM, was localized at its ectodermal layer and appeared to be at least partially exposed on the external surface of the ectoderm facing the egg shell.

DISSOCIATED CELLS: To investigate whether the CaBP is associated with any specific cell population of the CAM, immunohistochemistry for CaBP was also carried out with single cells dissociated from transporting, 20-day-old CAM.

Four fractions of cells were obtained from the dissociated CAM by the procedure described in Materials and Methods. The isolated cells appeared viable, as indicated by more than 97% trypan blue exclusion. Fractions 1 and 2 consisted largely of erythrocytes and other blood cells, and exhibited no observable immunohistochemical staining with anti-CaBP. On the other hand, fraction 3, and particularly fraction 4, contained a population of cells which appeared, in general, large (about two to three times the average size of the erythrocytes), round, and highly vacuolated (Fig. 5A). These cells stained intensely with anti-CaBP (Fig. 5B). Fluorescence was associated

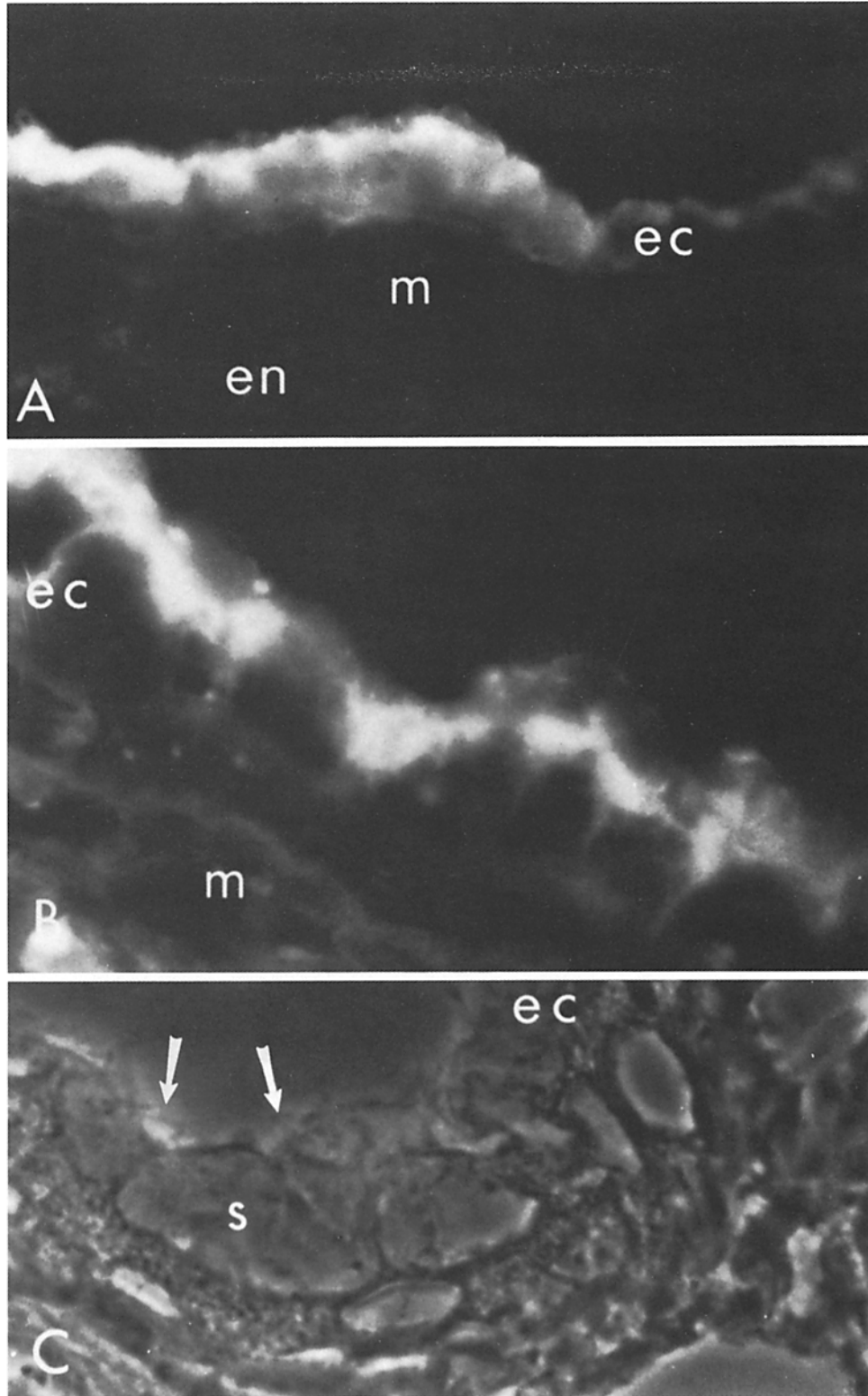


FIGURE 4 Fluorescence immunohistochemistry of CAM from 20-day-old embryo. Cryosections (4-6 μm) of the CAM were stained by the procedure described in Materials and Methods. (A) Section showing ectoderm (*ec*), mesoderm (*m*), and endoderm (*en*) under fluorescence optics. $\times 770$. (B) Higher magnification of the ectoderm, revealing intense fluorescence at the cortical region of the ectodermal cells. $\times 1,650$. (C) Capillary-covering region of the ectoderm showing sinusoid space (*s*), observed with epifluorescence. $\times 1,800$.

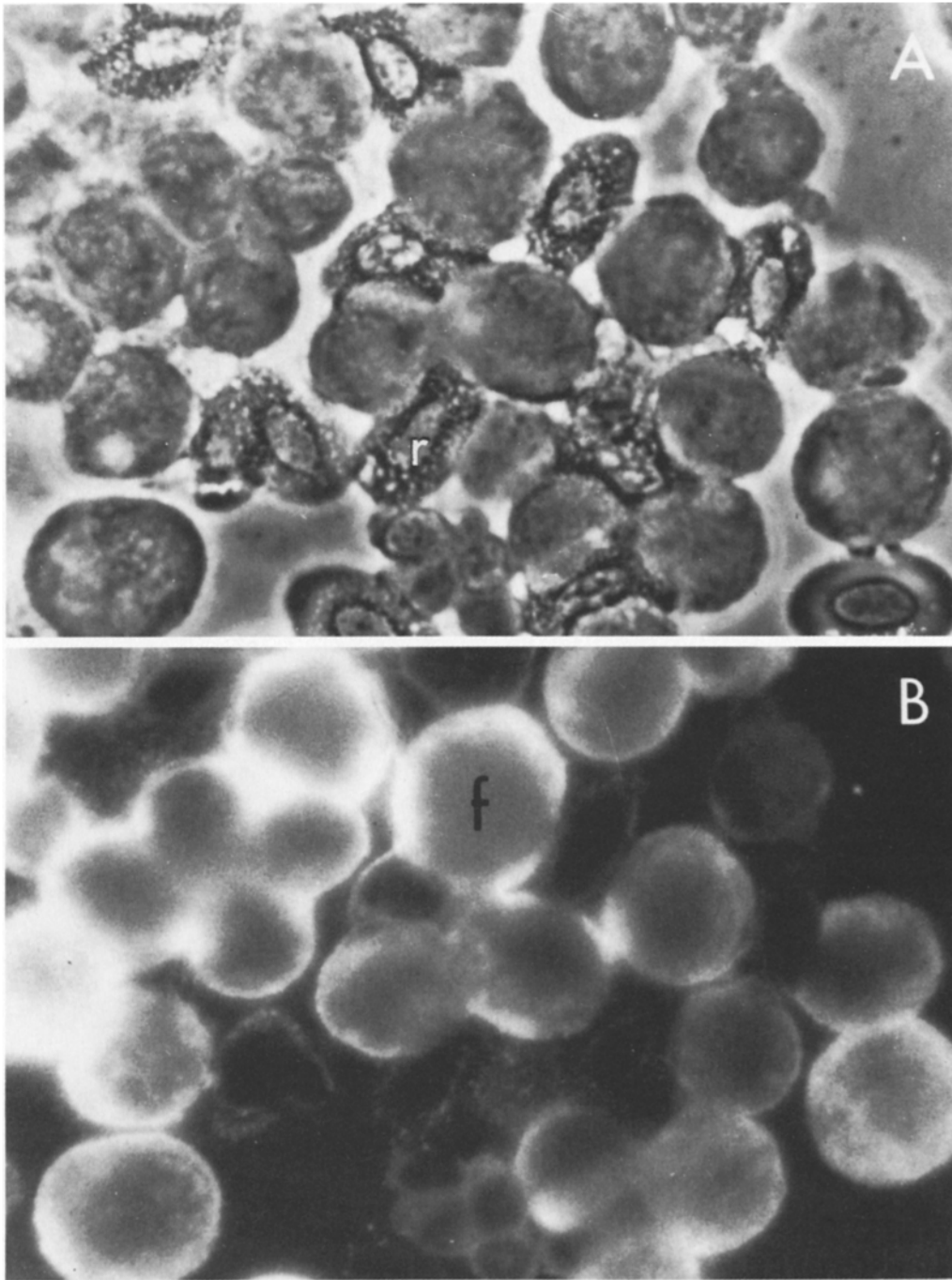


FIGURE 5 Fluorescence immunohistochemistry of cells dissociated from 20-day-old CAM. The cells (fraction 4) were dissociated from the CAM and stained for CaBP as described in Materials and Methods. (A) Phase micrograph showing erythrocytes (*r*) and a population of large, round cells. $\times 1,400$. (B) The same field under fluorescence optics, revealing intense cortical staining pattern of the large, round cells (*f*). Erythrocytes were not fluorescent. $\times 1,400$.

mainly with the cortical regions of these cells, and intracellular fluorescence was relatively weaker. Occasionally, these cells appeared to possess microvilluslike processes which were also fluorescent. The fluorescent staining pattern observed in these cell smears did not appear to be an artifact of the dehydrating effect of acetone fixation, since fixation in 5% paraformaldehyde yielded similar pattern. Controls similar to those used for CAM sections, as well as cells dissociated from nontransporting CAM (12-day-old), exhibited no fluorescence.

DISCUSSION

We have previously shown that calcium-binding activity (19) and CaBP (20) are both expressed in the CAM in a developmentally dependent fashion. Calcium-transporting CAM from embryos older than 15 days exhibits higher levels of calcium-binding activity and CaBP than nontransporting CAM from embryos incubated for <12 days. We have confirmed these findings in the present study. Immunodiffusion showed that the concentration of CaBP in the CAM is age-dependent, detectable only after incubation day 13. Immunofluorescence histochemistry also revealed that the CaBP is present only in transporting, 20-day-old CAM, but not in nontransporting, 10-day-old CAM.

Our results further indicate that the CaBP is localized at the ectodermal layer of the CAM and suggest that the CaBP may be associated with the external surface of the ectoderm. As mentioned in the Introduction, Terepka and co-workers (16) have demonstrated that the ectoderm comprises the calcium-transporting region of the CAM. The location of the CaBP at the ectodermal surface, along the pathway of calcium mobilization from the egg shell by the CAM, is therefore consistent with a functional role for the CaBP in the transport process.

Two other studies have suggested that a surface-associated, calcium-binding moiety of the ectoderm is essential for the transport of calcium by the CAM. First, *p*-chloromercuribenzenesulfonate (PCMBs) and other sulfhydryl-binding agents, such as iodoacetate and mersalyl, applied to the ectoderm of the CAM, were found to inhibit calcium transport and/or calcium-stimulated oxygen consumption by the CAM (4, 6). Since PCMBs reacts specifically with sulfhydryl groups on proteins and does not readily penetrate the plasma membrane, these results suggest (6)

that a sulfhydryl-dependent component(s) on the surface of calcium-transporting cells in the CAM may be involved in the active transport process. Second, the *in vitro* calcium transport studies of Terepka et al. (16) showed that, before transport, there is a rapid initial binding of calcium to the CAM within 10 s. This binding of calcium by the CAM is not affected by the presence of metabolic inhibitors, such as dinitrophenol, in the transport buffer (16), and may therefore represent an energy-independent step of calcium binding to a surface-associated component(s) of the CAM ectoderm before uptake and transport. Our findings reported elsewhere (20) on the sulfhydryl requirement and the high calcium affinity of the CaBP are thus consistent with its possible role as the ectodermal surface-associated, calcium-binding moiety hypothesized by these investigators. This supposition is further strengthened by the present finding that the CaBP is indeed localized to the ectodermal layer of the CAM. It needs to be pointed out that, though our results also suggest that the CaBP may be externally exposed on the ectodermal surface, additional analysis by techniques with higher resolution, such as electron microscopy, is necessary to elucidate the exact subcellular location of the CaBP.

An interesting finding in the present study is the lack of specificity of the CaBP-immunostaining with respect to cell types of the CAM ectoderm. As shown in Fig. 4A, fluorescent staining was evenly distributed over all regions of the CAM ectoderm. Morphologically, the CAM ectoderm consists of two major cell types, the capillary-covering and villus-cavity cells (1), designated according to their location and morphology, respectively. On the basis of the specific localization of calcium by electron probe analysis, Coleman and Terepka (2) have considered the capillary-covering cells as the calcium-transporting cells of the CAM. On the other hand, several other workers (10, 12) have observed the morphological resemblance of villus-cavity cells to other secretory cells and contended instead that the villus-cavity cells are calcium-absorbing cells. The findings in this investigation, therefore, do not lend exclusive support to either contention. It is, however, conceivable that the CaBP is synthesized by a certain population of cells of the CAM ectoderm and is subsequently secreted and deposited onto the surface of the other ectodermal cells. Such a mechanism for the generation of CaBP on the ectoderm would therefore result in a uniform

staining pattern over a large fraction of the ectodermal cell population, i.e., CaBP-secreting as well as calcium-transporting cells. This model for the appearance of a CaBP has been proposed in another calcium-transporting epithelium, the chick intestinal mucosa, where the vitamin D-dependent CaBP exhibits a similar immunostaining pattern (15). Staining for the intestinal CaBP was observed at the secretory goblet cells as well as the brush border surface of the intestinal mucosa. Taylor and Wasserman (15) conjectured that the intestinal CaBP is secreted by the goblet cells into the mucus which is then deposited on the brush border. Though the properties of the embryonic CAM CaBP (18-20) differ significantly from those of the adult intestinal CaBP (21), and the CAM ectoderm is not known to be associated with major secretory functions (9), it remains possible that the CaBP's of the two calcium-transporting epithelia are generated in a similar fashion involving a cellular secretory mechanism. It needs to be pointed out that the CAM ectoderm has previously been postulated to secrete inorganic acids (e.g. carbonic acid) for the solubilization of the shell calcium (1, 9).

In the present study, we have shown that the CaBP is associated with a certain population of cells dissociated from the transporting CAM. Although the origin of these cells has not been ascertained, it is apparent that they were not fibroblastlike or derived from the blood. Since immunostaining for CaBP is present exclusively on the ectoderm of CAM sections, it is likely that these cells were derived from the CAM ectoderm. That the CaBP was not destroyed during enzymatic dissociation of the CAM indicates that the procedure employed here may be useful for the study of cellular aspects of the CAM CaBP.

Terepka et al. (16) have recently proposed a pinocytic model for calcium transport by the CAM. In this model, calcium solubilized from the egg shell is sequestered in pinocytic vesicles which then traverse the cell body of the ectodermal cells, subsequently fuse with the plasma membrane on the serosal side, and discharge their calcium content into the sinusoid space. Since a selective sequestration of calcium is carried out by the CAM during the transport process (4), the adsorptive pinocytic model necessarily postulates the presence of a calcium-specific receptor on the cell surface for the selective binding of calcium before pinocytosis (13). The results reported here on the localization of the CaBP on the CAM ectodermal

surface therefore suggest a possible receptor role for the CaBP in the transport process, and lend support for the pinocytosis model.

There is, at present, no conclusive evidence for any transport model for the calcium transport function of the CAM. The developmental expression of the CaBP (19) and its localization in the CAM reported here are strongly suggestive that the CaBP may be involved in the transport process. Further investigation to localize the CaBP of the CAM at an ultrastructural level is certainly needed to elucidate its possible functional role and to understand the mechanism of transcellular calcium transport by the CAM.

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