Identification and characterization of a calcium-binding protein in the mouse chorioallantoic placenta

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Mouse chorioallantoic placenta contains a specific calcium-binding protein (M_{Ca}BP). A procedure involving gel filtration and ion-exchange chromatography was developed to purify the M_{Ca}BP. The M_{Ca}BP activity increased as a function of embryonic gestation and was highly specific for Ca²⁺. The M_{Ca}BP is a monomeric protein of *M_r* 57000, with pI 4.7. Specific antibodies were prepared against the M_{Ca}BP and were used to localize the M_{Ca}BP to syncytiotrophoblasts of the chorionic villi of mouse chorioallantoic placenta. These properties suggest that the M_{Ca}BP may be involved in transplacental calcium transport.

INTRODUCTION

During mammalian embryonic development, the fetoplacental structure transfers nutrients from the maternal to the fetal circulation. Although some of the nutrients are translocated passively (e.g. gases) or diffuse in a facilitated manner (e.g. sugars) by virtue of concentration gradients, most of the nutrients are probably transported actively, i.e. in an energy-dependent fashion, across the placental barrier (Miller *et al.*, 1976; Hill & Longo, 1980; Truman & Ford, 1984).

For calcium, it has been shown that its concentration is higher in the fetal blood than in the maternal blood, and the experimental evidence available in the literature (Delivoria-Papadopoulos *et al.*, 1967; Twardock & Austin, 1970; Tan & Raman, 1972; McIntosh & Lutwak-Mann, 1974; van Kreel & van Dijk, 1983; Sweiry & Yudilevich, 1984) are all strongly suggestive of an active up-gradient calcium-transport mechanism in the mammalian placenta. However, the inherent tissue complexity of the placenta, a result of intimate interactions between maternal and fetal cell types, has greatly limited the understanding of the basic mechanism of active calcium transport in the placenta.

We have been investigating transplacental calcium transport by identifying specific macromolecules associated with the transport function. The systems we have studied include the chick embryonic chorioallantoic membrane (CAM) (Tuan & Scott, 1977) and the human placenta (Tuan, 1982). The avian CAM, derived from the fusion of the chorionic and allantoic membranes during early development, lines the internal surface of the eggshell and is responsible for the mobilization and translocation of shell calcium into the embryonic circulation throughout embryonic development (Terepka *et al.*, 1976). The relative simplicity of the CAM, owing to the absence of maternal tissues, makes it an attractive experimental model for the study of placental calcium transport. Our previous studies have clearly demonstrated the involvement of a specific calcium-binding protein (CaBP) which is expressed in the chick CAM concomitantly with the onset of calcium-transport activity (Tuan & Scott, 1977; Tuan & Zrike, 1978). The chick CAM

CaBP has been purified and characterized (Tuan *et al.*, 1978a), and localized to specific calcium-transporting cells of the CAM (Tuan *et al.*, 1978b). Subsequently we have identified a specific Ca²⁺-activated ATPase, which also appears to be associated with CAM calcium transport (Tuan & Knowles, 1984). In the human placenta, our study has also indicated the presence of a specific, high-*M_r*, calcium-binding protein (H_{Ca}BP) (Tuan, 1982), and our most recent findings strongly suggest its functional involvement in placental calcium uptake (Tuan, 1985). Thus the identification of these specific marker molecules, such as CaBPs, has provided valuable experimental means for the biochemical analysis of transplacental calcium transport.

In the present study, prompted by our previous findings, we present data showing the presence of a specific, high-*M_r*, calcium-binding protein (M_{Ca}BP) in the mouse chorioallantoic placenta. The M_{Ca}BP has been purified to homogeneity and is different from a low-*M_r* calcium-binding protein previously discovered in rodent placentae (Marche *et al.*, 1978; Bruns *et al.*, 1978). The M_{Ca}BP has been characterized with respect to its physicochemical and immunochemical properties and its cellular location.

MATERIALS AND METHODS

Animals

Mice (females, C57BL; males, SJL) were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). Mating was carried out randomly, and the first presence of a vaginal plug in the female was counted as day zero of gestation. New Zealand White rabbits were used for raising antibodies.

Extraction of placenta

At specific gestational time intervals, pregnant females were killed by cervical dislocation. The uterus was cut open and the chorioallantoic placental plugs were dissected clear from the amniotic sac and uterine wall, rinsed thoroughly in cold 0.9% NaCl, and were either used immediately or frozen at –70 °C until use. The placental

tissues were minced and homogenized in a buffer containing 13.7 mM-Tris/HCl, pH 7.4, 0.12 M-NaCl, 4.74 mM-KCl and 0.02% NaN_3 (Tuan, 1982). The soluble placental extract was defined as the supernatant obtained after centrifugation at 31 000 *g* for 30 min. All operations were carried out at 4 °C.

Assay for calcium-binding activity

(A) **Chelex assay.** Calcium-binding activity was measured as previously described (Tuan & Scott, 1977) by the Chelex 100 (Bio-Rad) ion-exchange method, with ^{45}Ca (Amersham) as a tracer. Units of calcium-binding activity were calculated as (radioactivity in supernatant)/(radioactivity retained by resin) and expressed as a percentage value. All samples were extensively dialysed against the above Tris buffer before assay for calcium-binding activity in duplicates.

(B) **Analysis of electrophoretogram.** Proteins separated electrophoretically on a native polyacrylamide-gel system (see below) were assayed for calcium-binding activity by the counter-ion electrophoresis method of Ueng & Bronner (1979) and the post-electrophoresis method of Schibeci & Martonosi (1980) (details described in the legend to Fig. 7).

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard.

Purification of MCaBP

The final procedure adopted for purifying MCaBP from extracts of mouse placenta included two steps of chromatography: gel filtration on a column of Bio-Gel A-1.5 m (Bio-Rad) and ion-exchange chromatography on a Mono Q anion-exchange column (FPLC System; Pharmacia). The experimental details of these steps are described in the legends to Table 1 and Figs 4 and 5.

Polyacrylamide-gel electrophoresis

(A) **Denaturing SDS gel.** The procedure was that of Laemmli & Favre (1973), with 4% and 10% acrylamide for the stacking and separating gels respectively. Gels were routinely stained with Coomassie Blue R-250.

(B) **Native gel.** The conditions were those of Davis (1964), with a tank buffer of Tris/glycine, pH 8.6. Separating gels ranged from 4 to 10% acrylamide concentration, and electrophoresis was carried out at 8 °C.

Isoelectric focusing

Flat-bed isoelectric focusing was carried out on the LKB Multiphor unit by using both polyacrylamide gel and granulated gel (Sephadex G-75, superfine; Pharmacia) according to the manufacturer's instructions: 25 W, 2 h for polyacrylamide gel and 8 W, 18 h for granulated gel. The pH gradient across the focused gel was determined by using a flat-surface combination pH-electrode (Phoenix Electrodes). The polyacrylamide gel was fixed in trichloroacetic acid and stained with Coomassie Blue; the granulated gel was fractionated with a steel grid (LKB) and eluted with Tris buffer.

Preparation of anti-MCaBP antibodies

Purified MCaBP (approx. 100 μg), cut out as a Coomassie-Blue-stained band from an SDS/polyacrylamide gel, was homogenized with 2 ml of a 1:1 (v/v) mixture of the Tris buffer and Freund's Complete Adjuvant (Cappel Laboratories) and injected subcutaneously into each of two rabbits. Sera were collected from the immunized rabbits 5 weeks after the injection. A second injection with approx. 20 μg of MCaBP in Freund's Incomplete Adjuvant was performed 8 weeks later, and sera were collected every 2 weeks afterwards.

Immunochemical procedures

(A) **Immunodiffusion.** Ouchterlony double immunodiffusion was performed as described previously (Tuan *et al.*, 1978b) in 1% agarose containing barbital buffer, pH 8.6.

(B) **Immunoblotting.** Electrophoretically separated proteins were electrotransferred on to nitrocellulose in an ElectroBlot Apparatus (E-C Apparatus) by using the manufacturer's protocol. For immunodetection, the protein blot was then sequentially reacted with anti-MCaBP antiserum and peroxidase-labelled goat anti-rabbit IgG (Sigma) by the procedure of Towbin *et al.* (1979), and developed histochemically with 4-chloro-1-naphthol as the peroxidase substrate (Hawkes *et al.*, 1982).

Fluorescence immunohistochemistry

The MCaBP was localized by indirect immunofluorescence histochemistry by a procedure similar to that used previously for the chick CAM (CaBP, Tuan *et al.*, 1978b; carbonic anhydrase, Tuan, 1984) and the human placenta (HCaBP; Tuan, 1985). Immunostaining was carried out on cryosections (6–8 μm) of mouse placenta by using rabbit-derived anti-MCaBP antiserum (1:25 dilution) as the primary antibody and fluorescein isothiocyanate-labelled sheep anti-rabbit IgG (1:25 dilution, Behring Diagnostics) as the secondary antibodies. Controls included exclusion of the anti-MCaBP antiserum or its replacement with pre-immune serum. The sections were observed under phase and fluorescence optics with an Olympus BH2 photomicroscope equipped with a 100 W mercury lamp. All photography was done on Kodak Tri-X film.

RESULTS

Identification of calcium-binding activity

In initial experiments, an extract of mouse placenta obtained on day 19–20 of gestation was tested for calcium-binding activity by the Chelex assay. The results in Fig. 1 clearly demonstrated that the extract contained a heat-labile and proteinase-sensitive (Fig. 1, inset) calcium-binding activity. The validity of the assay was indicated by the linearity of the activity (up to 15 units) with respect to sample size.

Activity during gestation

We next determined whether placental calcium-binding activity showed any temporal correlation with the increasing calcium need of the developing embryo (Bruns *et al.*, 1978; Greizerstein & Abel, 1979). It was assumed that, if the calcium-binding activity were involved in

Fig. 1. Identification of calcium-binding activity in mouse placental extract

The soluble extract (9.4 mg of protein/ml) was prepared from gestation-day 19–20 chorioallantoic placentae, and calcium binding was assayed by the Chelex method, where 1 activity unit = (supernatant radioactivity)/(resin radioactivity) expressed as a percentage (see the Materials and methods section). The inset shows sensitivity of placental calcium-binding activity to proteinases and heat. Incubation of the extract with proteinases (trypsin and Pronase) was carried out at enzyme concentrations of 1 mg/ml for 15 min at 37 °C. Thermal treatment was at 100 °C for 5 min. All activities were expressed as percentages of control. (Note: incubation of the extract at 37 °C for 15 min in the absence of added proteinases resulted in no change of activity.)

Fig. 2. Developmental profile of calcium-binding activity in mouse placental extract

Chorioallantoic placentae were obtained at the indicated gestation times, and the specific activities (activity/mg of protein) of calcium binding in the extracts (means $\pm$ S.E.M. for two or three separate determinations) are plotted as a function of gestational age.

transplacental calcium transport, it ought to increase as a function of gestation. As shown in Fig. 2, the specific activity of calcium binding in placental extracts indeed exhibited an age-specific increase.

Characterization of calcium-binding activity

The proteinaceous nature of the calcium-binding activity was indicated above (Fig. 1) by its sensitivity to specific (trypsin) and non-specific (Pronase) proteinases and was also consistent with its thermal lability. It was observed that a minor residual calcium-binding activity, representing approx. 20% of the total, remained after heating (100 °C) and was probably due to non-proteinaceous component(s). As demonstrated below, the calcium-binding activity in the placental extract is primarily due to a high- M_r calcium-binding protein (MCaBP).

The specificity of the calcium-binding activity in mouse placental extract was estimated by determining the relative ability of various metal cations to compete with ^{45}Ca in the Chelex-binding assay (Fig. 3). It was observed that non-radioactive ^{40}Ca was capable of effectively displacing ^{45}Ca , whereas other bivalent (or trivalent) cations were either totally ineffective or only partially effective as competitive ligands. The following ion affinity series was apparent: $\text{Ca} > \text{Sr} > \text{Cd}$, Co , $\text{Fe(III)} > \text{Sn} > \text{Ni}$, Mg , $\text{Mn} > \text{Zn}$. These results strongly suggest that a specific ionic structure, that of calcium, is probably required for binding to the MCaBP of the mouse placenta.

Further characterization of the placental calcium-binding activity with respect to pH and ionic-strength dependence showed that optimal activity occurred at

Fig. 3. Ion specificity of mouse placental calcium-binding activity

The 19–20-day placental extract contained 9.4 mg of protein/ml. The Chelex 100 calcium-binding assay mixtures each contained 1 mM of the respective cations indicated. The activity values represent means of duplicates, are each expressed as a percentage of that in the absence of additional non-radioactive cations, and are plotted as a function of the ionic radius of the respective cation (Weast, 1975).

pH 7–8.4 and in the presence of 40–120 mM-NaCl (results not shown).

Purification of the MCaBP

In initial experiments, we used a purification scheme involving sequential $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration and preparative isoelectric focusing, which was similar to that previously used for the CaBP of human placenta (Tuan, 1982) and of chick CAM (Tuan *et al.*, 1978a). Although satisfactory purification was occasionally obtained, the length of time required for the procedure caused significant aggregation and degradation of the preparation, as revealed by chromatography and electrophoresis. Consequently, a shorter and more efficient

procedure was devised and adopted as described below and in the legends to Figs 4 and 5. All steps were carried out at 4 °C.

Step 1: gel filtration. Freshly prepared mouse placental extract was concentrated in a dialysis sac by immersion in Aquacide III (Calbiochem) for 3–5 h (10–15 g of tissue routinely yielded approx. 10 ml of concentrated extract, with 10–20 mg of protein/ml). The extract was then fractionated by gel filtration in a Bio-Gel A-1.5 m column (2.5 cm $\times$ 95 cm). The chromatogram in Fig. 4 showed that two distinct calcium-binding activity peaks (I and II) were resolved. Since the smaller peak II most probably consisted of the low- M_r CaBP previously identified

Fig. 4. Gel-filtration chromatography of mouse placental calcium-binding activity

Concentrated day-18–19 placental extract (10 ml, containing approx. 25 mg of protein/ml) was loaded on a Bio-Gel A-1.5 m (Bio-Rad) column (2.5 cm $\times$ 90 cm), which was eluted with the Tris buffer at a flow rate of 20 ml/h; 4.4 ml fractions were collected and protein contents were monitored as A_{280} . Calcium-binding activities were assayed by the Chelex 100 method (see the Materials and methods section). Peaks I and II were consistently observed to be the major calcium-binding components in the chromatogram, whereas the middle minor peak was only occasionally observed.

Fig. 5. Isolation of MCaBP

(a) Ion-exchange chromatography of mouse placental calcium-binding activity. The fractions constituting Peak I of the A-1.5 m gel-filtration chromatogram (Fig. 4) were pooled and dialysed exhaustively against 20 mM-Tris/HCl, pH 7.4, and loaded on a Mono Q anion-exchange column of the Pharmacia FPLC system. The chromatogram was developed as described in the inset [buffer A, 20 mM-Tris/HCl (pH 7.4)/0.02% NaN_3 ; buffer B, buffer A with 0.5 M-NaCl] at a flow rate of 1 ml/min, and 1 ml fractions were collected. Protein contents were monitored as A_{280} , and calcium-binding activities were measured by the Chelex 100 method (see the Materials and methods section). (b) Purification of MCaBP as analysed by SDS/polyacrylamide-gel electrophoresis. Lane A, total placental extract; lane B, peak I after Bio-Gel A-1.5 m chromatography; lane C, purified MCaBP after Mono Q chromatography. Protein M_r standards are indicated.

Table 1. Purification of mouse placental MCaBP

Protein was determined by the method of Lowry *et al.* (1951). Calcium-binding activity was determined by the Chelex 100 assay (see the Materials and methods section). The placental extract was prepared from the chorioallantoic placenta obtained at day 17–18 of gestation. The gel-filtration step fractionated the high- M_r MCaBP from the low- M_r vitamin D-dependent CaBP, and their activities were approx. 55% and 45%, respectively, of the total activity in the original extract (Fig. 4). The values for % yield in parentheses represented an estimation of the actual recovery of the MCaBP by taking the above into consideration.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (Fold)
Placental extract	14	116	690	5.9	100	1
Gel filtration	44	14	164	11.6	24(44)	2
Anion-exchange chromatography	16	0.18	55	305.6	8(15)	52

(Bruns *et al.*, 1978, 1981; Delorme *et al.*, 1982; also see the Discussion section), only the fractions constituting the major, higher- M_r , activity peak were pooled for further fractionation.

Step 2: ion-exchange chromatography. This was carried out on a Mono Q anion-exchange column of the Pharmacia FPLC system (Fig. 5a). A single peak of calcium-binding activity was eluted coincidentally with an A_{280} peak at approx. 75% buffer B (0.35 M-NaCl) and represented the purified MCaBP. The efficacy of the purification procedure and homogeneity of the MCaBP preparation was routinely monitored by SDS/polyacrylamide-gel electrophoresis (Fig. 5b). Representative yield and recovery of the MCaBP purification scheme are shown in Table 1.

The purified MCaBP in Tris buffer rapidly lost its activity with storage at either 4° or -70 °C. The cause of this instability was not known; various manipulations, such as addition of reducing agents (e.g. dithiothreitol), glycerol and/or proteinase inhibitors, or varying the buffer ionic strength, failed to stabilize the MCaBP. The lability of the MCaBP probably contributed to the low recovery indicated in Table 1. For this reason, most of the experiments on characterization of the MCaBP were carried out on gel-filtration-enriched preparations.

M_r of MCaBP

The M_r of native MCaBP was determined by (1) h.p.l.c. gel-permeation chromatography and (2) native gel electrophoresis.

Gel-permeation chromatography of the purified MCaBP preparation was carried out on a TSK G3000SW column (Alltech Associates, Deerfield, IL, U.S.A.) with the Pharmacia FPLC system. The chromatogram (Fig. 6) revealed a single protein peak corresponding to a M_r of 56 000–58 000.

The M_r of the native MCaBP was also determined from its electrophoretic mobility in polyacrylamide gels of different percentages of cross-linking. The MCaBP was localized in the gel by means of: (1) counter-ion electrophoresis (Ueng & Bronner, 1979) in which ^{45}Ca placed in the anode-buffer tank migrated upward towards the cathode during electrophoresis and was chelated to the downward-moving MCaBP; or (2) post-electrophoresis incubation of the gel in ^{45}Ca -containing buffer (Schibeci & Martonosi, 1980) to allow

Fig. 6. H.p.l.c. gel-permeation chromatography of purified MCaBP

The purified MCaBP (19 μg in 200 μl) was loaded on a TSK G3000SW column (7.5 mm $\times$ 30 mm; Alltech Associates) and eluted at 0.7 ml/min at a pressure of 1 MPa. The chromatogram was monitored at 280 nm for protein and was calibrated with the following M_r ($\times 10^3$) standards: bovine serum albumin, dimer (130) and monomer (65); ovalbumin (45); α -chymotrypsinogen (23).

binding of ^{45}Ca by the MCaBP. Subsequent autoradiography of the dried gels permitted observation of the MCaBP (Fig. 7). These data were analysed by Ferguson plots (Chrambach, 1980), on the basis of which the retardation coefficient (K_r) of the MCaBP was compared with those of other globular proteins of known M_r (Fig. 8). This analysis yielded a value of 55 000 for the M_r of native MCaBP. Since denatured MCaBP also consisted of a single electrophoretic band of M_r 57 000 (under both reduced and non-reduced conditions), the MCaBP is therefore most likely a monomeric protein of M_r 55 000–58 000.

Fig. 7. Detection of MCaBP on native polyacrylamide gel on the basis of ^{45}Ca binding: (a) counter-ion electrophoresis with ^{45}Ca , and (b) post-electrophoresis incubation with ^{45}Ca

Total extracts from day-18–19 mouse placentae were used in these studies. Counter-ion electrophoresis (a) was carried out by the procedure of Ueng & Bronner (1979). A tracer amount of ^{45}Ca (0.5×10^6 c.p.m./ml) was included in the bottom buffer tank, and electrophoresis was then carried out in a discontinuous buffer system (Davis, 1964). Lanes A and B represented two different preparations of 18–19-day mouse placental extract. Calcium-binding activity was also detected after electrophoresis (Schibeci & Martonosi, 1980) by incubating the gel strip in Tris/glycine electrophoresis buffer (Davis, 1964) containing ^{45}Ca (2.5×10^6 c.p.m./ml). The gel was then washed in buffer and water to remove excess unbound ^{45}Ca . In both procedures, the ^{45}Ca -labelled gel was dried under vacuum at room temperature and autoradiography was done with Kodak XAR-Omat film at -70°C . [Note: In counter-ion electrophoresis (a), 1 mM-EDTA was included in the sample buffer to decrease the background of ^{45}Ca in the gel, which was vacuum-dried without washing. This accounted for the high radioactive exposure in non-sample-lane areas of the gel, whereas the sample lanes were relatively clear of radiolabel in order to reveal the ^{45}Ca -bound MCaBP.]

Isoelectric point of MCaBP

The elution of the MCaBP at 0.35 M-NaCl from the anion-exchange Mono Q column strongly suggested its highly acidic nature. Initial isoelectric-focusing experiments using wide-range (pH 3–10) ampholytes in agarose or polyacrylamide gel localized the MCaBP to a zone corresponding to a pI of 4.6–4.8 (results not shown). Further experiments using narrow-range ampholytes (pH 4–6) yielded a value of 4.70 for the pI of the MCaBP (Fig. 9), based on the analysis of more than five different batches of MCaBP preparations.

Anti-MCaBP antiserum and immunological properties

The specificity of the rabbit-derived anti-MCaBP antiserum was tested by means of Ouchterlony double immunodiffusion (Fig. 10a). A single immunoprecipitin line was observed between the placental extract (well A) and the anti-MCaBP antiserum (well B), demonstrating the specificity of the antiserum. Further confirmation of

Fig. 8. Determination of M_r of MCaBP by native polyacrylamide-gel electrophoresis

Mouse placental extract (day 18–19) was subjected to electrophoresis under native conditions in polyacrylamide gels of different percentage cross-linking (% T, percentage concentration of acrylamide and *NN'*-methylenebisacrylamide) as described in the Materials and methods section. The MCaBP was detected by ^{45}Ca autoradiography (see Fig. 7) and the electrophoretic mobilities were plotted against % T (Ferguson plots). The slope of the Ferguson plot represented the retardation coefficient, K_r . The plot of M_r versus K_r was made based on the Ogston theory (Chrambach, 1980) calculated for globular proteins, which defines a linear relationship between K_r and $(M_r)^{1/3}$. The protein M_r markers were: ovalbumin (M_r 45000); bovine serum albumin monomer, (65000); bovine serum albumin dimer (130000); isoenzymic forms of human carbonic anhydrase (30000). Arrow indicates MCaBP.

immuno-specificity was obtained from post-electrophoresis immunoblotting of total mouse placental extract (Fig. 10b), which revealed a single band of immunoreactive protein with M_r 57000 (lane C).

By means of double immunodiffusion, the MCaBP was also examined with respect to its immunological cross-reactivity with other CaBPs previously identified in this laboratory, i.e. the HCaBP of the human placenta (Tuan, 1982) and the CaBP of the chick embryonic CAM (Tuan *et al.*, 1978a). Specifically, the reactivity of each of the specific antisera was tested against each tissue extract. The results (not shown) indicated that there was significant cross-reactivity between the HCaBP and MCaBP, but that neither one shared any common properties with the chick CaBP. In addition, it was found that the MCaBP was also detectable in the mouse choriovitelline placenta (yolk-sac) of day-17 mouse embryo, although the specific activity was significantly (≥ 8 -fold) lower than that in the chorioallantoic placenta.

Immunohistochemical localization of MCaBP

The chorioallantoic placenta of the mouse is classically defined as haemochorial in nature, with an internal

Fig. 9. Determination of the isoelectric point of MCBP by isoelectric focusing in Sephadex G-75 using pH 4-6 ampholytes (Isolab)

The procedure was carried out as described in the Materials and methods section and the sample was the pooled Peak I (approx. 8 mg of protein) obtained after gel filtration (Fig. 4). After focusing, the pH across the gel was measured with a surface pH electrode. The gel was then fractionated and assayed for calcium-binding activity as described in the Materials and methods section. The high activity in the acidic fractions was an artifact owing to the presence of H_3PO_4 in the anode solution (see Tuan & Scott, 1977).

labyrinth structure consisting of interwoven fetal and maternal vascular structures lined by fetal trophoblastic cells (Ramsey, 1975; see Fig. 11a). As shown in Figs. 11(b) and 11(c), the immunofluorescent staining of MCBP was primarily associated with the syncytiotrophoblast layer. Moreover, the fluorescent staining appeared to be most intense in the cortical regions of the trophoblasts, often outlining the cell borders. This distribution of the MCBP was suggestive of a plasma-membrane association, although the limited resolution of the light-microscope did not permit a definitive localization. Finally, controls omitting anti-MCBP serum or replacing it with pre-immune serum yielded no detectable fluorescence (Figs 11c and 11d).

DISCUSSION

Our investigation has shown that the mouse chorioallantoic placenta contains a specific high- M_r calcium-binding protein (MCaBP), which is expressed as a function of gestational age. A relatively simple three-step procedure has been devised to permit the isolation of homogeneous preparations of the MCBP. In particular, the anion-exchange-chromatography step using the FPLC Mono Q column represented an efficient and quick (< 1 h) method of fractionation.

Cation-binding by the MCBP displays a high degree of stereospecificity (Fig. 3a) and therefore does not appear to be a result of simple electrostatic or ionic interactions (Diamond & Wright, 1969). On the basis of

Fig. 10. Specificity of anti-MCaBP antiserum

(a) Ouchterlony double immunodiffusion. The centre well contained anti-MCaBP antiserum, and surrounding wells (except well 1, which was empty) contained increasing amounts of total mouse 18-day placental extract (well 2, 1.5 μ g; well 3, 3 μ g; well 4, 4.5 μ g; well 5, 6 μ g; well 6, 7.5 μ g). A single immunoprecipitin line was observed, indicating the specificity of anti-MCaBP. (b) Post-electrophoresis immunoblotting. Total mouse placental extract was subjected to SDS/polyacrylamide-gel electrophoresis, electro-blotted on to nitrocellulose, and treated immunohistochemically with anti-MCaBP (see the Materials and methods section). Lane A, Coomassie-Blue-stained gel of total mouse placental extract; lane B, nitrocellulose electroblot of total mouse placental extract stained for proteins by the India Ink procedure of Hancock & Tsang (1983); lane C, immunohistochemically stained nitrocellulose blot, revealing a single band of M_r 57000, which was identical with that of purified MCBP (Fig. 5b). Protein M_r standards (Std.) were from Sigma.

its molecular properties, the MCBP does not appear to resemble any of the previously known calcium-binding proteins (Kretsinger, 1976; Van Eldik *et al.*, 1982), although some degree of cross-immunoreactivity exists with the human placental HCBP (Tuan, 1982).

The development- and tissue-specific onset of the appearance of the MCBP in the mouse chorioallantoic placenta is highly suggestive of its possible functional involvement in transplacental calcium transport. In rodents, such as the mouse, two placentations are operative to bring about nutrient transfer from the mother to the fetus (Ramsey, 1975). In the first half of gestation, before gestation day 13-14, the choriovitelline (yolk sac) placenta is principally involved, whereas during late gestation the chorioallantoic placenta is the active tissue. Since embryonic calcium demand is highest during late gestation, when rapid skeletal growth and mineralization are taking place (Garel, 1983), it is reasonable to presume that the chorioallantoic placenta is the major calcium-transporting placenta. Our finding that the MCBP is primarily associated with the chorioallantoic placenta is thus consistent with the MCBP being associated with placental calcium transport. Further circumstantial support for this notion is provided by immunohistochemistry. Our observations revealed that, within the haemochorial labyrinth of the mouse placenta, the MCBP is principally associated with the syncytiotro-

Fig. 11. Fluorescence immunohistochemical localization of MCaBP in the mouse chorioallantoic placenta

Cryosections ($6\ \mu\text{m}$) of frozen-embedded mouse placenta (day 17 of gestation) were stained as described in the Materials and methods section. (a) and (c), phase contrast; (b) and (d), fluorescence optics. (a) Structure of the labyrinth-like villi of the mouse chorioallantoic placenta, showing the interwoven nature of the maternal (MC) and fetal (FC) circulations separated by the syncytiotrophoblast (ST) layer. Arrows correspond to some of the regions of MCaBP immunofluorescence observed in (b). In most cases, the MCaBP staining appeared to outline the cellular cortex, suggesting a likely plasma-membrane association. (c) and (d), Cryosection treated with pre-immune serum instead of anti-MCaBP serum, showing the absence of specific fluorescence staining. Magnifications: (a) and (b), $\times 1320$; (c) and (d), $\times 1280$.

phoblastic layer, which constitutes the cellular boundary between maternal and fetal circulations. We have obtained evidence, based on an assay system of membrane vesicles *in vitro*, that the HCaBP of the human placenta is indeed functionally involved in transmembrane calcium uptake (Tuan, 1985). Specifically, we found that anti-HCaBP antibodies inhibited calcium uptake by human placental microsomal vesicles. A similar *in vitro* assay system should be applicable for investigating the

functional involvement of the MCaBP in mouse placental calcium transport.

A low- M_r (10000) CaBP has been previously found in mouse and rat placentae (Bruns *et al.*, 1978; Marche *et al.*, 1978). This CaBP is identical with the vitamin D-dependent CaBP of the rodent intestine (Bruns *et al.*, 1977) and is present in both the yolk sac and chorioallantoic placentae during embryonic development (Delorme *et al.*, 1982). The vitamin D dependence of the

expression of this CaBP and of placental calcium transport is still controversial (Halloran & DeLuca, 1979, 1981; Garel *et al.*, 1981; Bruns *et al.*, 1982; Durand *et al.*, 1983; Miller *et al.*, 1983; Brommage & DeLuca, 1984), and its functional role, if any, in transplacental calcium transport remains to be established. During MCaBP isolation, as reported here, the presence of the low- M_r vitamin D-dependent CaBP is consistently observed, and it is fractionated away from the MCaBP on the basis of their different biochemical properties. Furthermore, using the anti-MCaBP antiserum, we have observed no immunoreactivity in the low- M_r fraction of the placental extract. It is therefore highly unlikely that the two CaBPs are related, although it should be of great interest to elucidate the interrelationship between their functional roles (if any) in transplacental calcium transport.

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