Biochemical characterization of mouse vitamin D-dependent calcium-binding protein

Evidence for its presence in embryonic life

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We compared immunochemical and biochemical properties of the vitamin D-dependent Ca^{2+} -binding protein (CaBP) from rat and mouse intestine. The two intestinal CaBP species were extensively purified by gel filtration and successive anion-exchange chromatographies. Both had a similar mol.wt. of 9000. Their pI values differed markedly, being 8.0 and 4.9 in rat and mouse CaBP respectively. Accordingly, mouse CaBP displayed more anodal migration in electrophoresis under non-denaturing conditions. Both mouse and rat CaBP only exhibited partial immunochemical similarities, but their amino acid compositions were very similar. Chromatofocusing was also found to be a good method of detecting calcium-dependent changes in their pI. We developed a sensitive radioimmunoassay for mouse CaBP enabling us to detect substantial amounts of CaBP in uterus, yolk sac and chorio-allantoic placenta. During normal mouse gestation, CaBP appeared on day 12 in the chorio-allantoic placenta but was already present on day 9 in the yolk sac, where its level rose sharply between days 9.5 and 10. CaBP may therefore be considered as a new marker for mouse yolk-sac differentiation.

Since the discovery of vitamin D-dependent Ca^{2+} -binding protein (CaBP) in the rat duodenal mucosa (Kallfelz *et al.*, 1967), several authors have specified that it is induced by the hormonal form of vitamin D_3 , 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$], in normal rats (Bruns *et al.*, 1979; Ueng *et al.*, 1979; Delorme *et al.*, 1980) or in vitamin D-deficient rats (Thomasset *et al.*, 1979). CaBP synthesis in the rat intestine is now known to require the presence of cytosolic and nuclear $1,25(\text{OH})_2\text{D}_3$ receptors (Kream & DeLuca, 1977; Weckslers *et al.*, 1979; Feldman *et al.*, 1979; Stumpf *et al.*, 1979) and to involve mRNA translation (Thomasset *et al.*, 1981). The exact physiological role of CaBP remains unclear. It is probably involved in the active process of calcium absorption, since its distribution along the intestine and its intestinal concentration are correlated with active

calcium transport (Krawitt & Schedl, 1968; Armbricht *et al.*, 1979; Delorme *et al.*, 1979; Pansu *et al.*, 1981). In addition, the subcellular localization of CaBP in the intestinal absorptive cells suggests that it may modulate calcium-mediated processes in the terminal web region (Marche *et al.*, 1979, 1980).

To date, only fragmentary information is available on the biochemical characterization of rat intestinal CaBP. The latter is a small (10000-mol.wt.), cytosolic, highly soluble protein with a high affinity for calcium (Drescher & DeLuca, 1971; Freund & Bronner, 1975; Bruns *et al.*, 1977; Marche *et al.*, 1977; Gleason & Lankford, 1981). We report here our recent attempt to quantify CaBP in mouse, a useful animal model for studying gestation. To obtain a radioimmunoassay for mouse CaBP, mouse intestinal CaBP had to be purified. During the purification steps, we observed that it behaved differently from rat CaBP. This dissimilarity in two close species led us to evaluate comparatively some of the biochemical properties of rat and mouse intestinal CaBP. The development of a radioimmunoassay for mouse CaBP enabled us to define its tissue distribution throughout normal gestation.

Abbreviations used: CaBP, calcium-binding protein; $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol; SDS, sodium dodecyl sulphate.

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Materials and methods

Tissue preparations

(a) *Duodenal mucosa from rats and mice.* Normal rats or mice, 4–6 weeks old, were bled by decapitation, and the duodena were quickly removed. All subsequent operations were carried out at +4°C. Duodena were opened longitudinally, rinsed in Tris buffer (13.7 mM-Tris/HCl, pH 7.4, containing 120 mM-NaCl and 3 mM-KCl) to which 4 mM-phenylmethanesulphonyl fluoride was added. The mucosa was scraped, weighed and suspended in Tris-buffer (1:4, w/v) containing 1% Trasylol (Sigma). The mucosa was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 100 000 g for 1 h. The supernatant without the fluffy lipid layer was taken as the cytosol fraction.

(b) *Mouse embryonic and foetal organs.* The pregnant mice were of the Swiss strain. The day on which the vaginal plug was found was designated as day 0. On the appropriate days the pregnant mice were killed by cervical dislocation and the uteri were rapidly excised. Under a binocular lens the conceptuses were carefully dissected in Tris buffer. At days 9 and 9.5, the yolk sac was freed from the decidua and the ectoplacental cone, but not dissected apart from the embryo proper. From day 10, yolk sacs were obtained free of contamination by the embryo proper. From day 12, placentas were separated into their maternal and foetal parts. All the tissues were frozen in liquid N₂. After thawing, cytosol was prepared as described for duodenal mucosa.

Uteri and mammary glands were also excised from normal pregnant or lactating females and prepared in the same manner, except that uteri were homogenized for 45 s with a Polytron homogenizer at setting 5.

Purification of duodenal CaBP

CaBP was purified by a procedure modified from that described by Marche *et al.* (1977). During the successive steps, CaBP was monitored by the ⁴⁵Ca²⁺-Chelex assay for Ca²⁺-binding activity (Freund & Bronner, 1975) and by radioimmunoassay. Briefly, the purification began by gel filtration with Sephadex G-75 (Pharmacia), which yielded what will be referred to as 'partially purified CaBP'. From this fraction, pure CaBP was obtained by successive DEAE-cellulose chromatographies (Pharmacia) in the presence of 1 mM-EDTA or 1 mM-CaCl₂ in buffers.

CaBP radioimmunoassay

The preparation of anti-CaBP immunoserum, the iodination of pure CaBP and the radioimmunoassay procedure were performed as described by Marche *et al.* (1977). Immunoreactivity was expressed as:

$$\frac{^{125}\text{I-labelled CaBP bound to the immunoserum in the presence of standard pure CaBP or of sample (B)}}{^{125}\text{I-labelled CaBP bound to the immunoserum in the absence of CaBP (B}_0\text{)}} \times 100$$

CaBP concentrations are expressed as μg of CaBP per mg of cytosolic protein. Protein was determined by a modified Lowry method (Bensadoun & Weinstein, 1976).

Immunological studies

Ouchterlony double immunodiffusion was performed in plates that contained 1.5% (w/v) agarose in phosphate-buffered saline, pH 8.0 (Melay Laboratories, Springfield, VA, U.S.A.) and were incubated overnight at +4°C. Immunoprecipitates were stained by 0.6% Amido Black in 2% (v/v) acetic acid.

Immunofilms from Sebia Issy les Mx, France, were used for immunoelectrophoresis. The buffer was 50 mM-sodium veronal/10 mM-veronal/60 mM-Tris, pH 9.2. The buffer also contained either 1 mM-EDTA or 1 mM-CaCl₂, according to the conditions under which electrophoresis was performed. A voltage of 20 V/cm of gel was applied at +10°C for 45 min. Immunodiffusion was allowed to proceed overnight at +4°C. Plates were stained with 0.5% Amido Black in methanol/water/acetic acid (5:4:1, by vol.).

Chromatofocusing

For chromatofocusing, partially purified CaBP was first equilibrated with the appropriate start buffer by gel filtration with Sephadex G-25 (Pharmacia). Chromatofocusing was then carried out by using Polybuffer exchanger (Pharmacia) packed in a column 0.9 cm $\times$ 30 cm, and the pH gradients (10.5–7; 8–5 and 7–4) were produced with the appropriate Polybuffer (Pharmacia). Collected fractions were monitored for pH with a Tacussel pH-meter and for CaBP by immunoreactivity.

Isoelectrofocusing

Isoelectrofocusing was conducted on LKB PAG (polyacrylamide-gel) plates, pH 3.5–9.5, at 1500 V for 1.5 h. Bovine serum albumin (pI 4.9) and myoglobin (pI 7.0) (Serva) were run as markers. The pH gradient was measured across the plate at the end of the focusing period. Plates were then fixed and stained with Coomassie Blue R 250 in ethanol/water (1:3, v/v), to which 8% acetic acid was added. In another set of experiments, plates were cut into 2 mm-wide slices after isoelectric focusing. Slices were incubated overnight at +4°C in phosphate buffer containing 0.5% bovine serum albumin. The presence of CaBP in the slice extracts was checked by radioimmunoassay.

Molecular-weight determinations

SDS/15% (w/v)-polyacrylamide-gel electro-

phoresis was performed in 250 mM-Tris/HCl buffer, pH 8.7. The electrophoresis buffer contained 50 mM-Tris/380 mM-glycine, pH 8.7. All solutions contained 0.1% SDS (Laemmli, 1970).

SDS/12.5% (w/v)-polyacrylamide-gel electrophoresis was also performed in the presence of 8 M-urea as described by Swank & Munkres (1971). The molecular-weight markers were albumin (68 000), ovalbumin (45 000), chymotrypsinogen (25 000), cytochrome *c* (12 500), aprotinin (6500) and the insulin B-chain (3400) (all from Boehringer). Proteins mobilities were measured in relation to the mobility of Bromophenol Blue.

Gel-filtration experiments were performed with Sephadex G-50 in a 1.6 cm $\times$ 100 cm column, which was calibrated with chymotrypsinogen (mol.wt. 25 000), ribonuclease (13 700) and aprotinin (6500) (detected by their u.v. absorbance at 280 nm). The void volume of the column was determined by Blue Dextran exclusion. The partition coefficient between the liquid phase and the gel phase (K_{av}) was plotted against log (molecular weight) of the protein.

Amino acid composition

For amino acid composition, pure CaBP species were hydrolysed in sealed degassed tubes in 6 M-HCl in the presence of 0.2% β -mercaptoethanol for 24 h at 120°C. The Beckman standard was treated under the same conditions. Amino acids were chromatographed in a Biotronic LC 2000 auto-analyser. They were detected by fluorimetry after reaction with orthophthalaldehydicarboxaldehyde (Merck) in the presence of β -mercaptoethanol.

1,25(OH)₂D₃ cytosolic receptors

To check the presence of 1,25(OH)₂D₃ receptors

in mouse yolk-sac cytosol, we used sucrose-gradient centrifugation as we did previously for rat yolk sac (Danan *et al.*, 1981).

Results

Immunochemical comparison of rat and mouse CaBP

Fig. 1(a) shows that the anti-(rat duodenal CaBP) immunoserum used was monospecific, since a single precipitation line was observed when it reacted with cytosol from rat duodenal mucosa. A single precipitation line was also found when the immunoserum was incubated with cytosol from mouse duodenal mucosa. In addition, both precipitation lines from rat and mouse immunocomplexes completely fused, indicating the presence of apparently identical antigenic determinants in rat and mouse proteins.

Immuno-electrophoresis also enabled comparison of rat CaBP and mouse immunoreactive protein. A single precipitation line was obtained for both rat and mouse samples. When cytosols from rat and mouse duodenal mucosa were deposited in the same well of immuno-electrophoresis, the precipitin lines fused without any spur (Fig. 1b), thus confirming the apparent immunochemical identity of rat CaBP and mouse protein. However, the mouse immunoreactive protein exhibited more anodal mobility than rat CaBP. The mouse protein was probably able to bind calcium, since its electrophoretic mobility, like that of rat CaBP, was affected by as little as 1 mM-CaCl₂. The anodal mobilities of both rat CaBP and mouse protein were smaller in the presence of calcium than of EDTA. This was particularly striking in the case of rat CaBP, which migrated towards the cathode in the presence of calcium, indicating

Fig. 1. Immunochemical comparison of rat and mouse CaBP

(a) Ouchterlony immunodiffusion. The centre well contained undiluted rabbit anti-(rat CaBP) immunoserum. Well A contained undiluted non-immune rabbit serum. Wells B and D respectively contained 100 and 200 μg of cytosolic protein (2 or 4 μg of CaBP) from rat duodenal mucosa. Wells C and E respectively contained 100 and 200 μg of cytosolic protein from mouse duodenal mucosa. (b) Agarose immunoelectrophoresis. By using the anti-(rat CaBP) immunoserum, immunoelectrophoresis of 50 μg (1 μg of CaBP) of cytosolic protein from rat duodenal mucosa (R), and 50 μg of cytosolic protein from mouse duodenal mucosa (M) was performed in the presence of CaCl₂ or EDTA. In one experiment, a 1:1 mixture of rat and mouse duodenal mucosa cytosols (M + R) was run in the presence of CaCl₂.

that its calcium-bound form had a net electropositive charge at pH 9.2.

Mouse immunoreactive protein was considered as mouse duodenal CaBP, since, like rat CaBP, it bound $^{45}\text{Ca}^{2+}$ and had a mol.wt. of 9000 (see below).

We tested the ability of mouse duodenal cytosol to compete for binding of rat ^{125}I -labelled CaBP to anti-(rat CaBP) immunoserum (Fig. 2). Under radioimmunoassay conditions (Marche *et al.*, 1977), mouse duodenal cytosol was able to displace rat pure ^{125}I -labelled CaBP from the immunoserum. However the immunodilution curve was not parallel with that obtained with pure standard rat CaBP. Our results indicate that, by contrast with the immunodiffusion methods, radioimmunoassay was able to detect an only partial immunological identity between rat and mouse CaBP.

Comparison of biochemical characteristics in rat and mouse CaBP

The partially purified mouse CaBP was analysed with two successive anion-exchange gels. Fig. 3(a) shows that the anionic charge of this CaBP was

decreased when the protein bound to calcium, since the NaCl concentration at which mouse CaBP was eluted changed from 0.21 M to 0.15 M when EDTA was replaced by CaCl_2 . When partially purified rat CaBP was analysed in exactly the same way, its net electric charge also changed (Fig. 3b). When the pH 7 buffer contained CaCl_2 , rat CaBP was excluded in the void volume of the anion-exchange column. This meant that rat CaBP associated with calcium was positively charged and that its pI was above 7. Fig. 3 also indicates that rat and mouse CaBP differed in their respective affinities for the ion-exchanger. Mouse CaBP was always retained on the column, which confirmed that its anionic charge exceeded that of rat CaBP, as shown by immunoelectrophoresis.

To further investigate the changes in the electric charge of CaBP as a function of the presence or absence of calcium, we used chromatofocusing, a convenient tool for determining the protein pI. When partially purified mouse CaBP was applied to a column of Polybuffer Exchanger 94 with a descending gradient of pH from 7 to 4 formed by Polybuffer 74, immunoreactivity was eluted in a single sharp peak at pI 4.9 (Fig. 4b). In the presence of

Fig. 2. Radioimmunoassay of mouse CaBP

The anti-(rat CaBP) immunoserum and ^{125}I -labelled rat CaBP were incubated with increasing dilutions of mouse duodenal 100000g supernatants (●) or pure rat CaBP (x). Pure mouse CaBP was iodinated and incubated with the anti-(rat CaBP) immunoserum in the presence of increasing amounts of pure mouse CaBP (○). The insert shows the immunodilution curves obtained with the anti-(rat CaBP) immunoserum incubated with mouse ^{125}I -labelled CaBP in the presence of increasing dilutions of mouse 100000g supernatants from duodenal mucosa (■), placenta (▲), yolk sac and uterus (▲) and of pure mouse CaBP (○).

Fig. 3. Elution profiles of mouse and rat CaBP on DEAE-Sephadex A-25 columns

(a) Partially purified mouse duodenal CaBP (14 mg of protein) was applied on a 1.6 cm $\times$ 18 cm DEAE-Sephadex A-25 column equilibrated in buffer (20 mM-imidazole/20 mM-NaCl/1 mM-EDTA, pH 7). The column was first eluted with the same buffer and then with 200 ml of a linear gradient from 0.02 to 0.7 M-NaCl in imidazole/EDTA buffer. The flow rate was 10 ml/h. Fractions (1.2 ml) were collected. The gradient was monitored by conductometry. CaBP was detected by immunoreactivity (▲). The immunoreactive peak obtained was desalted and applied on a DEAE-Sephadex A-25 column in the same manner, except that EDTA was replaced by 1 mM- CaCl_2 in the imidazole/NNaCl buffer (△). (b) The same experiments as in (a) were performed with partially purified rat duodenal CaBP (27 mg of protein) in imidazole/EDTA buffer (●) or in imidazole/ CaCl_2 buffer (○).

1 mM- CaCl_2 in buffers, the apparent pI of mouse CaBP rose to 6.9 (Fig. 4a). These changes in pI completely fitted the variations in the electric charges observed by other analytical methods like immunoelectrophoresis (Fig. 1b), native-polyacrylamide-gel electrophoresis (results not shown) and anion-exchange chromatography (Fig. 2a). In the rat CaBP experiments performed without calcium, we never obtained a definite peak of immunoreactive CaBP, for unknown reasons. Conversely, a sharp peak was obtained for rat CaBP when calcium was added to the buffers (Fig. 4c). In this case, rat CaBP had an apparent pI of 9.8, entirely in agreement with

Fig. 4. Chromatofocusing of mouse and rat CaBP

(a) Partially purified mouse duodenal CaBP (12 mg of protein, containing 2.5 mg of CaBP) was applied on a column Polybuffer Exchanger 94. The start buffer was 25 mM Tris/acetate, pH 8.3, containing 1 mM- CaCl_2 ; the elution buffer was a mixture of Polybuffer 96 (30%) and Polybuffer 74 (70%), adjusted to pH 5 with acetic acid, containing 1 mM- CaCl_2 . The flow rate was 13 cm/h. Fractions (1.6 ml) were collected and tested for immunoreactive CaBP (●). (b) Partially purified mouse duodenal CaBP (8 mg of protein, containing 0.9 mg of CaBP) was applied on a column of Polybuffer Exchanger 94. The start buffer was 25 mM-imidazole/HCl, pH 7.4; the elution buffer was Polybuffer 74/HCl, pH 4.0. The flow rate was 21 cm/h. Fractions (2.2 ml) were collected and tested for immunoreactive CaBP (●). (c) Partially purified rat duodenal CaBP (7.5 mg of protein, containing 1.5 mg of CaBP) was applied on a column of Polybuffer Exchanger 118. The start buffer was 25 mM-triethylamine/HCl, pH 11, containing 1 mM- CaCl_2 ; the elution buffer was Pharmalyte/HCl, pH 7, containing 1 mM- CaCl_2 . The flow rate was 23 cm/h. Fractions (1.1 ml) were collected and tested for immunoreactive CaBP (●).

its cathodic migration in immunoelectrophoresis (Fig. 1b) and its exclusion from anion-exchange gel, pH 7 (Fig. 3b). In addition, chromatofocusing clearly revealed a marked difference between the pI of rat and mouse CaBP.

Isoelectric-focusing analysis of mouse and rat CaBP species confirmed the difference in their pI. Fig. 5 shows the pattern of both pure CaBP species. After staining, each CaBP gave a single band which exactly corresponded to the immunoreactivity. Mouse CaBP had a pI of 5, a value similar to that

Fig. 5. Isoelectrofocusing of pure mouse and rat CaBP

Isoelectric focusing was performed on LKB PAG plates, pH 3.5–9.5. A sample (6 μ g) of pure mouse CaBP (M) and 6 μ g of pure rat CaBP (R) were submitted to isoelectric-focusing analysis on LKB PAG plates, pH 3.5–9.5. Myoglobin, pI 7 (Myo) and ovalbumin, pI 4.6 (Ov) were run simultaneously as markers. The proteins were detected by staining with Coomassie Blue (a). (b) shows the immunoreactive pattern for mouse (▲) and rat (●) CaBP after elution of the sliced polyacrylamide-gel plate.

determined by chromatofocusing in the absence of calcium. Rat CaBP had a pI of 8.

Two methods were used to estimate the molecular weight of both rat and mouse pure CaBP species (Fig. 6). One was gel filtration of partially purified rat or mouse CaBP on a precalibrated Sephadex G-50 column, and gave a single identical peak for immunological and ^{45}Ca -binding activities. The elution volume of this peak corresponded to a mol.wt. of 9000 for mouse and rat CaBP (Fig. 6a). The second method, using SDS/polyacrylamide gel, gave a single band with identical mobility for both rat and mouse pure CaBP species (Fig. 6b). However, the precise determination of their common molecular weight was not possible, since, as expected, the wide molecular-weight range of the markers (68 000–3400) led to a calibration curve with two

Fig. 6. Molecular-weight determinations

(a) Partially purified rat or mouse CaBP (15 mg of protein containing 1 mg of CaBP) was chromatographed through a Sephadex G-50 column (1.6 cm × 100 cm). Fractions (2.3 ml) were collected and assayed for $^{45}\text{Ca}^{2+}$ binding by Chelex resin (○) and immunoreactive CaBP (●). Arrows indicate the elution volume of molecular-weight markers. The insert shows the calibration straight line, from which the mol.wt. of 9000 was obtained for CaBP species (*). (b) A sample (6 μ g) of pure mouse or rat CaBP was submitted to SDS/15%-polyacrylamide-gel electrophoresis. Molecular-weight markers were run simultaneously. The arrow indicates mobility of both CaBP species in relation to Bromophenol Blue mobility.

slopes. Depending on the part of the curve used, the estimated molecular weight ranged from 5800 to 9000. Addition of 8 M-urea to the SDS/polyacrylamide gels (Swank & Munkres, 1971) did not improve the precision of the results, demonstrating that SDS/polyacrylamide-gel electrophoresis is not suitable for unambiguous characterization of CaBP.

Amino acid composition was determined on CaBP species whose purity was assessed by electrophoresis in native polyacrylamide gels and by

isoelectric focusing. Table 1 shows that amino acid compositions were almost identical for mouse and rat CaBP. They were also very close to those already reported in other species (Fullmer & Wasserman 1975; Dorrington *et al.*, 1974). However, methionine seemed peculiar to rat and mouse CaBP.

Radioimmunoassay of mouse CaBP

The partial cross-reactivity exhibited by mouse CaBP with the anti-(rat CaBP) immunoserum hindered precise measurements of mouse CaBP in biological samples. To quantify mouse CaBP, a specific radioimmunoassay was developed and pure mouse CaBP was iodinated for this purpose. When mouse ¹²⁵I-labelled CaBP was incubated with the anti-(rat CaBP) immunoserum in the presence of increasing amounts of pure standard mouse CaBP, the displacement curve became parallel to that for the rat homologous CaBP system (Fig. 2). The affinity of mouse CaBP for the anti-(rat CaBP) immunoserum remained lower than that of rat CaBP. This was shown by the immunoserum titres (final immunoserum dilutions of 1:150 000 and 1:45 000 for rat and mouse CaBP tracers respectively, to obtain a *B*₀ of 45%). Nevertheless, the use of a mouse CaBP tracer with the anti-(rat CaBP) immunoserum yielded a highly sensitive immunoassay for mouse CaBP measurements and enabled

effective quantification of mouse CaBP. This proves once again that the nature of the tracer is crucial when elaborating radioimmunoassays. This radioimmunoassay allowed us to investigate immuno-reactive CaBP in various mouse tissues, some of them known to be vitamin D target organs and/or involved in calcium transport. CaBP was found in intestine, placenta, yolk sac, and uterus. Only negligible CaBP concentrations (less than 100 ng/mg of cytosolic protein) were detected in skin, mammary glands, pancreas, cerebellum, spleen, liver, lungs and striated and unstriated muscle.

Gestational changes in mouse CaBP

CaBP concentrations were monitored during gestation in the chorio-allantoic placenta, the yolk sac and the uterus (Fig. 7). In the chorio-allantoic placenta, low levels of CaBP were detected on day 12. A 2-fold increase in placental CaBP was observed on day 13. Thereafter the placental CaBP concentration increased slowly until day 19, the last day of gestation. In the yolk sac, low levels of CaBP were measured on day 9 and day 9.5. These concentrations were certainly underestimated, since, at this time the yolk sac was not dissected from the embryo. A sudden increase in the yolk-sac concentration of CaBP was observed on day 10. By day 13, the yolk-sac CaBP level reached its maximum and tended to decrease up to day 19. Therefore only yolk sac contained CaBP before day 12 of gestation, since none was found in either

Table 1. Amino acid composition of pure rat and mouse CaBP

A sample (4 µg) of each CaBP was hydrolysed with 6M-HCl in the presence of β-mercaptoethanol. The amino acids were analysed on a Biotronic LC 2000 Autoanalyser. Proline and cystine may be present, but, for technical reasons, they were not estimated.

Amino acid	CaBP ...	Content (mol/100 mol of amino acid residues)	
		Rat	Mouse
Aspartic acid		9.7	10.1
Threonine + serine*		6.6	6.6
Glutamic acid		23.1	20.1
Glycine		4.0	4.7
Alanine		5.4	8.6
Valine		3.2	2.3
Methionine		1.9	1.7
Isoleucine		2.8	3.0
Leucine		14.7	14.6
Tyrosine		2.5	2.6
Phenylalanine		7.8	7.9
Lysine		18.1	17.9
Arginine		0	0
Histidine		0	0

* Containing about 7% threonine.

Fig. 7. Changes in CaBP concentrations during gestation in the mouse

Cytosols of yolk sac (●), foetal placenta (■) and decidua (▲) were assayed for CaBP by radioimmunoassay, with mouse ¹²⁵I-labelled CaBP as tracer and pure mouse CaBP as standard. Data represent means ± s.e.m. for four to six pregnant mice on each day of gestation, except for days 9 and 9.5, when yolk sacs from five pregnant mice were pooled for a single determination. On days 9 and 9.5, yolk sacs were not dissected apart from the embryo proper (Emb.)

decidua including the ecto-placental cone or in the embryo. As regards to uterine CaBP, its concentration was $0.99 \pm 0.05 \mu\text{g}/\text{mg}$ of cytosolic protein ($n = 8$) in the non-pregnant normal mouse. This level did not change during gestation until the last day, when it rose to $1.66 \pm 0.05 \mu\text{g}/\text{mg}$ of cytosolic protein ($n = 3$).

Discussion

Our study specifies and compares immunochemical and biochemical characteristics of rat and mouse duodenal CaBP species. The pI values for rat and mouse CaBP differ markedly by 3 pH units, as clearly shown by isoelectric focusing and chromatofocusing. Mouse CaBP has a pI of 4.9, as observed for several acidic calcium-binding proteins (Kretsinger, 1976). By contrast, rat CaBP possesses a slightly basic pI, which explains both the low anodal mobilities on immunoelectrophoresis observed by ourselves and others (Bruns *et al.*, 1981) and the elution profile obtained for rat CaBP with carboxymethyl ion-exchange gel (Drescher & DeLuca, 1971). The difference of pI between rat and mouse CaBP species indicates a difference in their structures also reflected by their partial immunoidentity. Since the amino acid composition of both CaBP species is very similar, a difference in the amidation of dicarboxylic amino acids probably accounts for the difference in their pI. Mouse duodenal CaBP, whose mol.wt. of 9000 is similar to that of rat CaBP, also has a high affinity for calcium. This was indicated here by the Chelex assay and by the calcium-dependent electrophoretic mobilities observed. These changes in the mobility of the calcium-protein complex and the apoprotein are obviously related to changes in pI, well defined here by chromatofocusing. Removal of calcium from the complex appears to release negative charges, thus lowering the apparent pI from 6.8 to 4.9. Chromatofocusing is therefore a useful new tool for studying the pI variations induced by ligand binding to specific proteins.

In the second part of this work the development of a sensitive radioimmunoassay for mouse CaBP has enabled us to study its tissue distribution and its vitamin D-dependency. Of the mouse organs investigated, intestine, uterus, yolk sac and chorio-allantoic placenta were found to possess substantial amounts of CaBP, which underlines its selective tissue distribution. The presence of material which cross-reacts with rat CaBP does not imply a vitamin D-dependency. The apparent vitamin D-dependency of mouse intestinal CaBP was assessed by the rise in the intestinal CaBP concentration after injection of $1,25(\text{OH})_2\text{D}_3$ to suckling mice (A. C. Delorme, unpublished work), in agreement with the results obtained in the rat pups (Bruns *et al.*, 1979;

Delorme *et al.*, 1980). The vitamin D-dependency of mouse intestinal CaBP is also supported by the presence of $1,25(\text{OH})_2\text{D}_3$ receptors in mouse intestine (Colston *et al.*, 1980).

Our most significant results for CaBP studies during gestation essentially concerned two points: the presence of CaBP in the uterus and the evolution of CaBP in the yolk sac. As regards the mammalian uterus, the presence of CaBP reported here, to our knowledge, for the first time, suggests that $1,25(\text{OH})_2\text{D}_3$ acts directly on this organ. The nature of this action requires further investigation. With respect to the yolk sac, this is the only tissue which was found to contain CaBP soon after implantation. As differentiation proceeds, a drastic increase in yolk-sac CaBP occurred between days 9.5 and 10. It is noteworthy that this abrupt change may be related to structural and functional yolk-sac modifications associated during this phase with the initiation of vitelline circulation (Haar & Ackerman, 1971*a,b*). Thus CaBP may be considered as a new marker for yolk-sac differentiation. In other respects the fact that mouse yolk sac possesses both $1,25(\text{OH})_2\text{D}_3$ receptors (results not shown) and CaBP lends support to the concept that $1,25(\text{OH})_2\text{D}_3$ may play a part in embryonic development.

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