The purification and complete amino acid sequence of the 9000- M_r Ca^{2+} -binding protein from rat placenta

Identity with the vitamin D-dependent intestinal Ca^{2+} -binding protein

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A 9000- M_r Ca^{2+} -binding protein was isolated from rat placenta and purified to homogeneity by h.p.l.c. procedures. The complete amino acid sequence was established for the 78-residue placental protein. A sequence analysis of a minor component of the rat intestinal Ca^{2+} -binding protein (residues 4–78) and a tryptic peptide (residues 55–74), both purified by h.p.l.c., showed both proteins to be identical. Thus this placental 9000- M_r Ca^{2+} -binding protein is the same gene product as the intestinal Ca^{2+} -binding protein whose synthesis is dependent on vitamin D.

INTRODUCTION

In mammals vitamin D controls the synthesis of a Ca^{2+} -binding protein of M_r approx. 10000 in the intestinal mucosa, this being regarded as the major Ca^{2+} -transport protein (Wasserman & Fullmer, 1983). The protein has been isolated from bovine (Fullmer & Wasserman, 1973), human (Davie, 1981; Staun *et al.*, 1983), pig (Hitchman *et al.*, 1973) and rodent tissue (Bruns *et al.*, 1977; Gleason & Lankford, 1981; Delorme *et al.*, 1982; Christakos *et al.*, 1984). The amino acid sequences of the bovine and pig proteins have been determined directly (Hofmann *et al.*, 1979; Fullmer & Wasserman, 1980, 1981). However, only a partial amino acid sequence of the rat intestinal protein has been presented, from studies of mRNA sequences (Desplan *et al.*, 1983) or translation *in vitro* (Leonard *et al.*, 1984). These reports established that the intestinal CaBP structure is highly conserved, and belongs to the multi-gene family of proteins that includes calmodulin, troponin C and parvalbumin (Goodman *et al.*, 1979; Klee *et al.*, 1980; Kretsinger, 1980; Baba *et al.*, 1984).

Antisera have been generated against the rodent intestinal CaBP, and used to show the presence of a cross-reactive material in extracts of the placenta and visceral yolk-sac of mice and rats (Bruns *et al.*, 1978, 1981; Marche *et al.*, 1978; Delorme *et al.*, 1979, 1982), and also in bone, kidney, lung and teeth (Thomasset *et al.*, 1982; Delorme *et al.*, 1983; Taylor *et al.*, 1984). The equivalence of vitamin D-dependent Ca^{2+} -binding proteins in various tissues on the basis of immunological cross-reactivity alone is further complicated by the presence in mammalian tissues, such as bone, brain, and kidney, of another protein, of M_r 28000, which may or may not be dependent on vitamin D, and which is akin to the intestinal Ca^{2+} -binding protein of chickens (Delorme *et al.*, 1983; Pansini & Christakos, 1984; Sonnenberg *et al.*, 1984).

Before accepting the identity of the rat intestinal and placental Ca^{2+} -binding proteins proposed on immuno-

logical grounds (Bruns *et al.*, 1978, 1981; Marche *et al.*, 1978; Delorme *et al.*, 1979), it was considered necessary to isolate each of these proteins from their respective tissues, especially because of the presence in placenta of several proteins that bind Ca^{2+} (Bruns *et al.*, 1978, 1981; Umeki *et al.*, 1981; Tuan, 1982; MacManus *et al.*, 1985a). The present paper describes the first isolation of a 9000- M_r rat placental protein, by the use of the h.p.l.c. procedures, and its amino acid sequence analysis in conjunction with that of the rat intestinal CaBP.

MATERIALS AND METHODS

Protein purification from placenta

The Ca^{2+} -binding proteins of placenta were purified from 200 g wet wt. of tissue dissected out of conceptus after 18–21 days of gestation in Sprague–Dawley rats

Table 1. Purification of the 9000- M_r Ca^{2+} -binding protein from rat placenta

For experimental details see the text.

	Total protein (mg)	Sp. radioactivity (c.p.m./mg of protein)	Purification (fold)
Homogenate	6675	1800	1.0
Supernatant after heat treatment	2200	3000	1.7
65% satn. with $(NH_4)_2SO_4$	320	4400	2.4
DEAE-Sephacel	14	57100	31.7
Sephadex G-50	2.8	214200	119.0
H.p.l.c., reverse-phase, pH 7.6	1.6	250000	138.9
H.p.l.c. reverse-phase, pH 2.5	2.1*	—	—

*After freeze-drying.

Abbreviation used: CaBP, 9000- M_r Ca^{2+} -binding protein.

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Fig. 1. Purification of placental Ca^{2+} -binding protein

(a) DEAE-Sephacel anion-exchange of proteins remaining in solution after adjustment to 65% saturation with $(\text{NH}_4)_2\text{SO}_4$. X marks the place where the Chelex cation-exchange pre-column was disconnected and the start of the wash of the DEAE-Sephacel column with 100 mM-NaCl/5 mM-Tris/HCl buffer, pH 7.5. Y marks the place where gradient elution with NaCl (----) was started. —, A_{280} ; ●—●, ${}^{45}\text{Ca}$ radioactivity. (b) Gel filtration on a calibrated Sephadex G-50 column of the major Ca^{2+} -binding protein in (a). —, A_{280} ; ●—●, ${}^{45}\text{Ca}$ radioactivity. (c) Determination of the apparent M_r of this Ca^{2+} -binding protein. M_r standards were: a, ovalbumin (M_r 45000); b, chymotrypsinogen A (M_r 25000); c, ribonuclease (M_r 13500); d, oncomodulin (M_r 11700); e, aprotinin (M_r 6500); f, insulin B-chain (M_r 3500).

bred in this laboratory. The initial purification steps were based on the isolation of a 108-residue Ca^{2+} -binding protein, oncomodulin, found in rat hepatoma (MacManus, 1980), and also placenta (MacManus *et al.*, 1985a). A summary of the purification scheme is given in Table 1.

Briefly, the placental tissue was homogenised in 100 mM-NaCl/50 mM-KCl/0.5 mM-MgCl₂/100 mM-sodium acetate/1 mM-phenylmethanesulphonyl fluoride/0.1% 2-mercaptoethanol, pH 7.0 (buffer A). The temperature of the extract was raised in a water bath, and maintained between 65 and 80 °C for 5 min. The extract

was rapidly cooled and centrifuged at 10000 *g* for 30 min. The supernatant was adjusted to 65% saturation with $(\text{NH}_4)_2\text{SO}_4$ (43 g/100 ml) and stirred for 1 h. The precipitated proteins were removed by centrifugation, and the supernatant containing the low- M_r components was dialysed extensively against distilled water. This extract was freeze-dried, the resulting powder redissolved in 100 ml of 5 mM-Tris/HCl buffer, pH 7.5, and the solution dialysed overnight against this same buffer.

The dialysed material was passed first through a column (1.5 cm $\times$ 30 cm) of Chelex-100 (Bio-Rad Laboratories, Mississauga, Ont., Canada) cation-exchange resin

Fig. 2. Further purification by h.p.l.c. of the placental Ca^{2+} -binding protein from the purification step shown in Fig. 1(b)

(a) Reverse-phase h.p.l.c. on C-18 at pH 7.5. —, A_{280} ; ●—●, ${}^{45}\text{Ca}$ radioactivity. (b) Demonstration of purity of the Ca^{2+} -binding protein eluted at 34.5 min in (a) by reverse-phase h.p.l.c. on C-18 at pH 2.5. —, A_{280} . Amino acid analysis of this material is given in Table 2.

equilibrated with 5 mM-Tris/HCl buffer, pH 7.5, and then straight on to a tandem column (1.5 cm $\times$ 30 cm) of DEAE-Sephacel (Pharmacia, Dorval, P.Q. Canada). After the loading and washing of both columns with 5 mM-Tris/HCl buffer, pH 7.5, the cation-exchanger was disconnected, and elution of the adsorbed proteins on the DEAE column was commenced (Fig. 1a).

Fractions containing Ca^{2+} -binding material were located by the Chelex assay (Wasserman *et al.*, 1968) and pooled for further purification by gel filtration on a column (1.5 cm $\times$ 90 cm) of Sephadex G-50 (Pharmacia) equilibrated in buffer A. The single Ca^{2+} -binding peak (Fig. 1b) was collected and freeze-dried.

The freeze-dried material from the Sephadex G-50 was dissolved in 2 ml of 10 mM-Tris/HCl buffer, pH 7.6, and injected on to a C-18 reverse-phase h.p.l.c. column

(4.1 mm $\times$ 250 mm; Synchrom, Linden, IN, U.S.A.) also equilibrated in 10 mM-Tris/HCl buffer, pH 7.6. The proteins were eluted with an increasing concentration (1% per min for 50 min) of propan-1-ol, and monitored for Ca^{2+} -binding activity. The peaks of interest were freeze-dried and dissolved in 0.1% trifluoroacetic acid. The dissolved material was injected on to another C-18 h.p.l.c. column equilibrated with 0.1% trifluoroacetic acid, and eluted with an increasing concentration of propan-1-ol in 0.1% trifluoroacetic acid. The single major absorbance peak, eluted at 40.5 min (Fig. 2b), was collected and freeze-dried.

Protein purification from intestinal mucosa

In a similar fashion, 50 g wet wt. of intestinal mucosa that had been scraped from the first 10 cm of rat

duodenum was extracted, and the proteins were separated by DEAE-Sephacel anion-exchange, gel-permeation and h.p.l.c. steps as described above.

Peptides produced by trypsin and acid cleavage

The materials that had been freeze-dried after h.p.l.c. in 0.1% trifluoroacetic acid from placenta or intestine were dissolved (1 mg/200 μ l) in 50 mM-NH₄HCO₃, pH 8.0, and decalcified by addition of saturated trichloroacetic acid to a final concentration of 20% (w/v). The precipitated protein was kept on ice for 15 min, and collected by centrifugation. The pellet was washed in 20% trichloroacetic acid, resuspended in 200 μ l of 50 mM-NH₄HCO₃/1 mM-EGTA, pH 8.0, and the pH adjusted to 8.0 by dropwise addition of aq. NH₃. Without this decalcification these, and other, Ca²⁺-binding proteins are very resistant to the action of trypsin (Klee *et al.*, 1980; MacManus *et al.*, 1983). The cleavage by trypsin and the method of separation of the resulting peptides by h.p.l.c. has been described previously (MacManus *et al.*, 1983).

Cleavage at aspartate residues was performed by mild acid hydrolysis by incubation of protein in 0.25 M-acetic acid at 100 °C *in vacuo* for 10 h as described previously (MacManus *et al.*, 1983).

Amino acid composition and sequence analysis

Amino acid composition analysis was performed after hydrolysis in 6 M-HCl at 100 °C *in vacuo* for 24, 48 and 72 h (MacManus *et al.*, 1983).

Sequence analysis of tryptic peptides by liquid-phase methods was as described previously (MacManus *et al.*, 1983). Other peptides were sequenced by automated gas-phase methods with a Applied Biosystems 470 A sequencer by dissolving 1–5 nmol in 30 μ l of distilled water and applying the sample to a cartridge filter containing 1 mg of precycled Polybrene. Identification of amino acid derivatives was by h.p.l.c. (MacManus *et al.*, 1983).

Analysis of the N-terminal tripeptide

The putative N-terminal tripeptide was obtained from tryptically cleaved protein as described above, but with isocratic elution of the h.p.l.c. column (detailed results not shown) to resolve the peptides eluted in the first 4 min of Fig. 4. Amino acid analysis identified the putative N-terminal tripeptide (Ala₁, Lys₁, Ser₁). A 10 nmol portion of this peptide was dissolved in 50 mM-NH₄HCO₃, pH 8.0, and subjected to digestion with carboxypeptidases A and B (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a final concentration ratio of 1:25 (w/w) at 37 °C (Ambler, 1972). Samples (50 μ l) were removed at 1, 2, 4, 6 and 24 h, and, after the addition of 10 μ l of acetic acid, were then freeze-dried. After several cycles of dissolution in 300 μ l of water and re-freeze-drying, the samples were subjected to amino acid analysis.

RESULTS

Protein purification

The purification of the major low-*M_r* rat placental Ca²⁺-binding protein is outlined in Table 1. Two Ca²⁺-binding entities were observed after DEAE-cellulose chromatography, in tubes 14–18 and 27–29 (Fig. 1a). The latter, minor, Ca²⁺-binding material was oncomodulin, a

Table 2. Amino acid composition of the 9000-*M_r* placental Ca²⁺-binding protein

Residues were analysed from protein hydrolysed for 24, 48 and 72 h. Corrections were made for hydrolytic loss by extrapolation of values to zero time.

	Amino acid composition (mol of residue/mol)			
	Determined			Assumed
	24 h	48 h	72 h	
Asx	7.21	7.30	7.18	7
Thr	1.06	0.97	0.89	1
Ser	9.64	8.22	7.42	10
Glx	14.81	14.76	14.81	15
Pro	3.09	3.15	3.14	3
Gly	3.10	3.18	3.13	3
Ala	4.05	4.10	4.02	4
Cys	0	0	0	0
Val	1.98	2.03	2.24	2
Met	0.95	0.93	0.90	1
Ile	2.02	2.02	2.07	2
Leu	10.17	10.14	10.30	10
Tyr	2.10	2.09	1.90	2
Phe	6.12	6.19	6.08	6
His	0	0	0	0
Lys	11.84	11.87	11.76	12
Arg	0	0	0	0
Trp	—	—	—	0*
Total				78

* From sequence.

108-residue protein found originally in tumours (MacManus *et al.*, 1983, 1985a). The fractions containing the former, major, Ca²⁺-binding material were pooled, and further purified by gel filtration (Fig. 1b) on a calibrated column of Sephadex G-50 (Fig. 1c). A single Ca²⁺-binding peak eluted at an apparent *M_r* of 9000.

When this 9000-*M_r* Ca²⁺-binding material was subjected to h.p.l.c. at neutral pH, a single peak of Ca²⁺-binding activity was found, co-eluted with a major 280 nm-absorbance peak at 34.5 min (Fig. 2a). This Ca²⁺-binding protein was collected and further purified by h.p.l.c. in 0.1% trifluoroacetic acid, resulting in a single major 280 nm-absorbance peak at 40.5 min (Fig. 2b). The amino acid composition of this placental CaBP (Table 2) bore some resemblance to the estimates reported for rat intestinal CaBP in the high proportion of acidic amino acids, and the absence of cysteine, histidine and arginine (Christakos *et al.*, 1984; Delorme *et al.*, 1983).

The isolation of Ca²⁺-binding material from rat intestinal mucosa yielded a more complicated picture than that from placenta. The 9000-*M_r* Ca²⁺-binding material from Sephadex G-50 yielded more than one Ca²⁺-binding entity when chromatographed by reverse-phase h.p.l.c. at neutral pH (Fig. 3a). Three 280 nm-absorbance peaks were observed that had the ability to bind Ca²⁺, the first being eluted at 33.0 min (peak I), the second at 34.0 min (peak II) and the third at 35.5 min (peak III). Each peak was collected and chromatographed separately by reverse-phase h.p.l.c. in 0.1% trifluoroacetic acid. Under these conditions peak I material was eluted at 38.0 min (result not shown), peak II material, the major

Fig. 3. Purification by h.p.l.c. of intestinal CaBP obtained from the Sephadex G-50 step (see the Materials and methods section)

(a) Reverse-phase h.p.l.c. on C-18 at pH 7.5, showing three peaks of Ca^{2+} -binding activity. —, A_{280} ; ●—●, ^{45}Ca radioactivity.
 (b) Further purification on C-18 at pH 2.5 of peak II from (a). —, A_{280} . (c) Peak III from (a) at pH 2.5. —, A_{280} .

Fig. 4. Tryptic peptide map by h.p.l.c. of placental (a) and intestinal (b) CaBPs

The amino acid compositions of these peptides are given in Table 3.

entity, was eluted at 40.5 min (Fig. 3b), and peak III material, the minor entity, was eluted at 41.5 min (Fig. 3c). This minor intestinal Ca^{2+} -binding entity proved to be very useful in the elucidation of the primary structure of these proteins (see below).

Amino acid sequence analysis

The h.p.l.c.-pure placental Ca^{2+} -binding protein (Fig. 2b) was subjected to several cycles of Edman degradation with no resulting amino acid phenylthiohydantoin

Table 3. Amino acid compositions of tryptic peptides of placental (P) and intestinal (I) CaBPs (see Fig. 4)

Residues ...	Amino acid composition (mol of residue/mol)											
	16-19		29-32, 76-78		4-10		20-28		11-15		20-32	
Peptide ...	P1-1*	I1-1†	P1-2*	I1-2†	P2	I2	P3	I3	P4	I4	P5‡	
Asx	—	—	—	—	—	—	1.8	1.9	—	—	2.0	
Thr	—	—	—	—	—	—	—	—	—	—	—	
Ser	—	—	0.9	1.0	1.0	1.1	1.1	1.0	1.1	1.1	1.0	2.1
Glx	—	—	2.8	2.9	1.9	2.1	1.9	2.2	0.8	0.9	3.8	1.9
Pro	—	—	—	—	1.0	1.2	1.0	1.0	—	—	1.0	1.1
Gly	—	—	—	—	—	—	1.4	1.1	—	—	0.9	—
Ala	2.1	1.9	—	—	—	—	—	—	—	—	—	1.2
Val	—	—	—	—	—	—	—	—	—	—	—	—
Met	—	—	—	—	0.9	0.8	—	—	—	—	—	—
Ile	—	—	—	—	—	—	—	—	1.1	1.0	—	—
Leu	—	—	—	2.1	—	—	1.2	1.0	—	—	2.0	2.0
Tyr	1.0	0.9	—	—	—	—	—	—	1.2	1.1	—	1.1
Phe	—	—	—	—	—	—	—	—	—	—	—	0.7
His	—	—	—	—	—	—	—	—	—	—	—	3.2
Lys	1.0	1.1	1.0	1.0	2.2	2.2	1.0	0.9	0.9	1.1	2.0	1.8

* These peptides were separated from peptide P1 (Fig. 4a) by h.p.l.c. at pH 7.0 (MacManus *et al.*, 1985b)

† These peptides were separated from peptide I1 (Fig. 4b) by h.p.l.c. at pH 7.0.

‡ No equivalent of peptide P5 was found in the tryptic hydrolysate of intestinal CaBP (Fig. 4b).

derivative, showing that the *N*-terminus was blocked. Tryptic peptides were generated from this placental protein, and also from the intestinal peak II material (Fig. 3b). These tryptic peptides were separated by h.p.l.c. (Fig. 4), and the amino acid compositions of these peptides were obtained (Table 3). The tryptic peptide profiles in Figs. 4(a) and 4(b) were similar but not identical. However, when the amino acid compositions were examined these differences were seen to be more apparent than real. Peptide P5 from the placental material appeared to have an uncleaved lysine residue, which was totally cleaved in the possibly equivalent intestinal peptide, leading to the relatively larger amounts of peptides I1 and I3. The amino acid sequences of these placental tryptic peptides were obtained, but other cleavages of the intact protein were required before alignment was possible. Tryptic peptide P1-2 gave two sequences corresponding to residues 29–32 and 76–78, as determined by overlap with peptides A3 and A4 (see Fig. 6).

The next cleavage of the placental Ca²⁺-binding protein was with mild acid, and the resulting peptides were separated by h.p.l.c. (Fig. 5). Both their amino acid compositions (Table 4) and sequences were obtained. The sequence analysis of these large peptides A1, A3 and A4, in conjunction with the sequences of the tryptic peptides, and referral to the published bovine and pig intestinal CaBP sequences (Hofmann *et al.*, 1979; Fullmer & Wasserman, 1980, 1981), permitted the alignment shown in Fig. 6 for residues 4–78 of the placental protein.

A comparison of the amino acid compositions of peptide A2 (Table 4), the tryptic peptides (Table 3) and the intact protein (Table 2) indicated that a remaining putative *N*-terminal tripeptide of composition (Ala₁Lys₁Ser₁) was missing. Such a peptide was recovered by repeating a tryptic peptide separation such as that illustrated in Fig. 4, but with isocratic elution for the first 6 min (detailed results not shown). When it was subjected to carboxypeptidase digestion, lysine was liberated first, followed by serine (Fig. 7). No alanine was detected, even after 24 h digestion. The conclusion of these experiments was that the *N*-terminal sequence was blocked (presumably acetylated) Ala-Ser-Lys (Fig. 6).

Because of the similarity of composition of the tryptic peptides of the intestinal CaBP to those of the placental CaBP (Table 3), it was decided that sequence analysis was redundant except for peptide I8. However, use was made of the minor (peak III) intestinal Ca²⁺-binding entity (Fig. 3c). This polypeptide was analysed in the gas-phase sequencer for 57 cycles, at which point the yield of the amino acid derivative fell below detectable levels. However, this analysis yielded information for residues 4–61, which, in conjunction with the sequence of tryptic peptide I8, gave overlap with all the mild-acid cleavage points, and most of the tryptic cleavage points (Fig. 6). This analysis unequivocally showed that the intestinal and placental Ca²⁺-binding proteins were identical for residues 4–74. The inclusion of evidence from the identical h.p.l.c. retention time of tryptic peptides from both proteins (P1-2 and I1-2), and their identical amino acid compositions (Table 3), in conjunction with the composition of the presumptive intestinal *N*-terminal tripeptides (residues 1–3) (Fig. 6), strongly indicate the identity of both proteins in all 78 residues.

Fig. 5. Peptides purified by h.p.l.c. from placental CaBP cleaved by mild acid

The amino acid compositions of these peptides are given in Table 4.

Table 4. Amino acid compositions of peptides generated from placental CaBP by mild acid hydrolysis (see Fig. 5)

Residues ... Peptide ...	Amino acid composition (mol of residue/mol)			
	51-56 A1	1-21 A2	62-78 A3	23-49 A4
Asx	1.0	—	—	1.2
Thr	—	—	—	0.9
Ser	—	2.9	1.9	4.7
Glx	1.1	4.3	4.8	5.4
Pro	—	1.1	—	1.9
Gly	—	1.4	1.3	—
Ala	—	2.9	—	1.0
Val	—	—	1.8	—
Met	—	0.9	—	—
Ile	—	0.8	—	0.8
Leu	2.1	—	1.3	6.8
Tyr	—	0.9	0.8	—
Phe	0.9	1.1	2.9	1.2
Lys	1.0	5.1	2.1	3.3

DISCUSSION

The primordial gene of the Ca^{2+} -binding protein multigene family is regarded as having consisted of coding sequences for one domain: a helix, a Ca^{2+} -binding loop of oxygen-donating side chains, and another flanking helix (Goodman *et al.*, 1979; Kretsinger, 1980;

Baba *et al.*, 1984). By gene duplication and deletion the separate genes for the four-domain calmodulin and troponin C, the three-domain parvalbumin and oncomodulin and the two-domain intestinal CaBP and S-100 protein are said to have arisen (Goodman *et al.*, 1979; Kretsinger, 1980; Baba *et al.*, 1984). The primary and secondary structures of these proteins are very similar, consisting of units of these 30-residue helix-loop-helix domains jointed by hinge sections (Goodman *et al.*, 1979; Kretsinger, 1980; Gariepy & Hodges, 1983). Moreover, the tertiary structures estimated by X-ray diffraction of parvalbumin and the intestinal CaBP also show similarities (Kretsinger, 1980; Szebenyi & Moffat, 1983).

These similarities of different small Ca^{2+} -binding proteins suggest that antibodies directed against sequence or conformational antigenic determinants on any one of the family could also cross-react with similar epitopes on other family members. Indeed, several independent anti-calmodulin sera also cross-reacted with troponin C (Chafouleas *et al.*, 1979; MacManus *et al.*, 1981; Van Eldik & Watterson, 1981) and some bacterial proteins (Harmon *et al.*, 1985), anti-parvalbumin sera with another parvalbumin-like protein in human tumour cells (Pfyffer *et al.*, 1984), and anti-(S-100 protein) sera with both protein S-100 α and protein S-100 β , which are separate gene products (Hidaka *et al.*, 1983; Van Eldik *et al.*, 1984). Confusion can also arise in reverse fashion. Antisera generated separately were supposed to have reacted with a skin Ca^{2+} -binding protein (Saurat *et al.*, 1981; Pavlovitch *et al.*, 1983). However, when isolated,

Fig. 6. Identity of the amino acid sequences of rat placental and intestinal CaBPs with overlapping peptides used for analysis

The continuous lines indicate sequences obtained by automated degradation of placental peptides, and the broken lines indicate sequences assumed from composition analysis and overlap. The broken arrowed line is the sequence from carboxypeptidase digestion. The continuous arrowed line is the sequence obtained from the minor peptide of the intestinal CaBP (peak III in Fig. 3c). Abbreviations for cleavage points are: A, mild acid; T, trypsin.

Fig. 7. Release of amino acids from the *N*-terminal tripeptide of placental CaBP by carboxypeptidase digestion

For details see the text.

skin Ca^{2+} -binding protein turned out to be parvalbumin (MacManus *et al.*, 1985b).

Anti-(intestinal CaBP) sera had been used to show the presence of a Ca^{2+} -binding entity in rodent placenta, and the identity of both proteins was suggested (Bruns *et al.*, 1981; Delorme *et al.*, 1982). But other members of the Ca^{2+} -binding protein family occur in placenta, e.g. calmodulin (Umeki *et al.*, 1981) and oncomodulin (MacManus *et al.*, 1985a). In addition, a large

Ca^{2+} -binding protein has also been described (Tuan, 1982). Any one of these proteins, their breakdown products or an unknown protein have the potential of cross-reaction with antisera directed against the small intestinal CaBP.

In the present work this immunoreactive Ca^{2+} -binding entity has been isolated and purified to homogeneity for the first time from rat placenta. It has an M_r of 9000 and cross-reacts with both a rabbit and a chicken anti-(intestinal CaBP) serum (J. P. MacManus, unpublished work). It has a primary structure of 78 residues with *N*-acetylalanine as the probable *N*-terminal amino acid. The rat intestinal CaBP has also been isolated and its primary structure also directly established. Both proteins are identical for residues 4–74, and probably in entirety for residues 1–78. The helix-loop-helix Ca^{2+} -binding domains can easily be discerned (Fig. 8), and are homologues to the domains in the bovine and pig molecules. The rat protein has 62 of 78 residues identical with the bovine molecule, and 67 identical with the pig molecule. A partial sequence of the rat protein has been presented from studies using intestinal nucleic acids (Desplan *et al.*, 1983; Leonard *et al.*, 1984). Several differences in this translated sequence can now be seen, most obviously residue 60 being a glycine in the Ca^{2+} -binding loop, not an aspartate. Also, residue 54 is a lysine residue, not a glutamate (Fig. 8). This extra lysine residue in the rat protein compared with both bovine and pig proteins means tryptic peptide mapping would not be a conclusive fingerprint of homology. The *N*-terminal amino acid is shown to be an alanine residue, not a serine. Finally, the

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