Rat skin calcium-binding protein is parvalbumin

John P. MACMANUS, David C. WATSON and Makato YAGUCHI

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6

(Received 29 October 1984/15 February 1985; accepted 4 March 1985)

Only one major low- M_r calcium-binding protein could be isolated by h.p.l.c. procedures from aqueous extracts of homogenized adult rat skin. This was shown by tryptic peptide mapping and independent amino acid sequence analysis to be identical in all 109 residues with the parvalbumin from rat skeletal muscle. This calcium-binding protein was not in skin epidermis, but was confined to the dermal layer. Skin calcium-binding protein is therefore parvalbumin.

Rat skin is reputed to contain two calcium-binding proteins of M_r 11500. One is shown by immunohistochemistry to be present in the dermal muscle layer, panniculus carnosus, and to have chromatographic properties identical with those of the parvalbumin of skeletal muscle (Berchtold *et al.*, 1984). The anti-parvalbumin sera used do not cross-react with other calcium-binding proteins, such as calmodulin, but have been reported to react with non-muscle parvalbumin-like proteins in rat kidney (Berchtold *et al.*, 1984), and human tumour cells (Pfyffer *et al.*, 1984). The amino acid sequence of the rat muscle parvalbumin is known (Berchtold *et al.*, 1982; MacManus *et al.*, 1983a).

The other skin calcium-binding protein is reported to be the major M_r 11 500 calcium-binding material in rat skin (Laouari *et al.*, 1980), and is restricted to the basal layer of the epidermis (Saurat *et al.*, 1981; Pavlovitch *et al.*, 1983). This epidermal skin CaBP has the same M_r , and similar amino composition, as the skeletal muscle parvalbumin, but a partial amino acid sequence analysis of residues 75–94 shows two differences between these two proteins (Rinaldi *et al.*, 1982). Antisera to the skin CaBP do not cross-react with muscle parvalbumin, or other related proteins such as calmodulin, S-100, or the vitamin D-dependent intestinal CaBP (Laouari *et al.*, 1980; Saurat *et al.*, 1981; Pavlovitch *et al.*, 1983, 1984). Immunohistochemistry also shows the skin CaBP to be present in normal human skin, and to be increased in skin carcinoma (Pavlovitch *et al.*, 1984).

Because of our interest in parvalbumin-like proteins in neoplasia (MacManus & Whitfield, 1983), we undertook to isolate both of these skin

calcium-binding proteins, and compare their complete amino acid sequences.

Materials and methods

The skin samples were from male Sprague-Dawley rats (approx. 200g body wt.) bred in this laboratory. The samples were shaved, frozen in liquid N₂, pulverized and homogenized in 13.7 mM-Tris/119 mM-NaCl/4.7 mM-KCl (pH 7.4) as described by Laouari *et al.* (1980). The 10000g supernatant was brought to 65% saturation with (NH₄)₂SO₄ (430 g/l), and the precipitated material was removed by centrifugation. The supernatant was dialysed exhaustively against distilled water, and lyophilized. Samples of skin from 20-day-old rat fetus were similarly treated. In separate experiments the epidermis was dissected free of the underlying dermis (Marrs & Voorhees, 1971) and both preparations were extracted in the manner described.

The lyophilized powder (50 mg) was dissolved in 0.1% trifluoroacetic acid, and injected onto a SynChropak RP-P (C-18) h.p.l.c. column (4.1 mm × 250 mm; SynChrom Inc., Linden, IN, U.S.A.), and eluted with a gradient of propan-1-ol (Burdick and Jackson, Muskegon, MI, U.S.A.). Calcium-binding proteins were located by the Chelex-binding assay (Wasserman *et al.*, 1968).

An alternative procedure was used for preparative purification of amounts of material sufficient for further analysis. The lyophilized powder (20 g) was dissolved in 20 mM-imidazole, pH 6.5, and chromatographed by h.p.l.c. ion-exchange on an AX300 column (10 mm × 250 mm; SynChrom Inc.) by using described procedures (Durkin *et al.*, 1983). The only detectable calcium-binding entity

Abbreviations used: CaBP, calcium-binding protein.

Fig. 1. Reverse-phase h.p.l.c. on C-18 at pH 2.5 of extracts of adult and fetal skin
(a) Demonstration of the major CaBP protein eluted at 40.5 min; (b) lack of this calcium-binding entity in fetal skin (arrow); (c) and (d) elution at 40.5 min of the purified skin CaBP and muscle parvalbumin.

was in the flow-through fraction which was dialysed against 10mM-Tris/2mM-EGTA, pH 7.5, and injected onto a RPSC (C-3) h.p.l.c. column (4.6mm × 75mm; Beckman, Fullerton, CA, U.S.A.) equilibrated in Tris buffer and eluted with a gradient of Tris/EGTA in propan-1-ol (2:3, by vol.). The peaks were pooled, made 2mM in EGTA and dialysed against water. The sole calcium-binding peak (pool D, Fig. 2) was lyophilized, dissolved in 0.1% trifluoroacetic acid, and injected

Table 1. Amino acid composition of tryptic peptides of skin CaBP (S) and muscle parvalbumin (P) from Figs. 3 and 4

Peptide ... Residues ...	T1 69-75		T2 84-96		T3 45-54		T4 46-54		T5 14-28		T6 14-27	T7-1 1-13		T7-2 29-36		T8 55-68		T9 97-109	
Amino acid	S	P	S	P	S	P	S	P	S	P	S	S	P	S	P	S	P	S	P
Asx	1.1	1.0	3.3	3.0	2.2	2.0	2.2	2.1	2.3	2.2	2.2	2.3	2.1			1.1	1.1		
Thr			1.1	1.0					0.8	0.8	0.9	1.0	0.9					0.9	0.8
Ser	1.7	1.9							0.9	0.9	0.9	1.0	1.8			1.5	1.9	1.6	1.8
Glu												1.3	1.2	1.3	1.1	2.9	3.2	3.1	3.2
Gly	1.1	1.1	3.0	3.2					1.1	1.1	1.1	1.2		1.0	0.9	2.1	2.0	1.1	1.1
Ala	0.9	1.0	1.8	2.1					3.0	3.5	3.6	3.9	0.8					1.0	1.0
Val					0.9	0.9	1.0	1.0						0.9	0.9			2.0	2.0
Met			1.0	0.9															
Ile					1.0	1.0	1.0	0.9	1.2	1.1	1.0	0.9	1.0			1.9	1.8	1.0	0.9
Leu			1.1	1.0	1.0	1.0	1.1	1.1						1.0	1.1	2.2	2.0	1.1	1.1
Phe		1.1			1.2	1.4	1.2	1.2	2.2	2.4	2.4	2.3		2.1	1.9	1.0	1.2	1.0	1.2
His					0.9	0.8	0.8	0.9	1.0	1.1	0.9	0.8							
Lys			1.7	2.0	2.8	2.7	1.8	1.9	2.0	2.0	1.0	1.9	1.8	0.8	1.0	1.1			
Arg	1.1	0.9															0.8		

onto the RP-P column described above. The eluted calcium-binding peak (Fig. 1c) was the material used for further analysis.

Before cleavage by trypsin this skin calcium-binding material was decalcified by treating 1 mg, dissolved in 200 μ l of 50 mM- NH_4HCO_3 with trichloroacetic acid to a final concentration of 20% (w/v) (Haiech *et al.*, 1981). The protein was allowed to precipitate for 15 min at room temperature, and then collected by centrifugation. The pellet was washed with a further 200 μ l of 20% trichloroacetic acid, resuspended in 200 μ l of 60 mM- NH_4HCO_3 /1 mM-EGTA, and the pH was adjusted to 8.0 with NH_3 solution. A sample of parvalbumin from rat muscle (MacManus *et al.*, 1983a) was treated similarly. Trypsin cleavage and separation of the tryptic peptides by h.p.l.c. procedures was as previously described (MacManus *et al.*, 1983b).

Amino acid composition analysis, cleavage at methionine with CNBr followed by h.p.l.c. separation of the large peptides, and subcleavage by acid hydrolysis have been described (MacManus *et al.*, 1983a,b). The N-terminal sequence analysis of peptides by Edman degradation, followed by

amino acid derivative analysis by h.p.l.c., in addition to back hydrolysis and amino acid analysis, have also been reported in detail (MacManus *et al.*, 1983a,b).

Results and discussion

When the crude extract containing low- M_r material from adult skin was subjected to reverse-phase h.p.l.c., only one calcium-binding peak was observed at 40.5 min (Fig. 1a). No calcium binding was observed in any peak of the fetal skin extract, but particularly, no calcium-binding or absorbance peak was observed at 40.5 min (Fig. 1b). This calcium-binding activity in adult skin was eluted at the same retention time as genuine parvalbumin from rat muscle (Fig. 1d). Since the skin CaBP and parvalbumin are so similar (Rinaldi *et al.*, 1982), it was possible that the two reported skin calcium-binding proteins were co-eluting from this C-18 reverse-phase support.

The preparative procedure (see the Materials and methods section) yielded calcium-binding activity only in the flow-through fraction from AX300 ion-exchange h.p.l.c. (results not shown),

Fig. 2. Large scale purification by h.p.l.c. on C-3 at pH 7.5 of skin CaBP in the flow-through fraction of the ion-exchange column (see the Materials and methods section)

Small arrows, injection of 2 ml samples; large arrow, start of gradient elution. Peak D was the only calcium-binding peak, and was eluted at 40.5 min when rechromatographed at pH 2.5 on C-18 (Fig. 1c).

and only one calcium-binding peak when this flow-through material was chromatographed on a C-3 reverse-phase h.p.l.c. column (Fig. 2, peak D). This calcium-binding entity, when rechromatographed under the same conditions as the crude skin extract (Fig. 1a), was eluted with a retention time of 40.5 min (Fig. 1c).

It was still considered possible that the two putative skin calcium-binding proteins were so similar as to be inseparable by reverse-phase h.p.l.c. under diverse conditions. Therefore, the calcium-binding material eluted at 40.5 min (Fig. 1c) was subjected to tryptic cleavage, and subsequent separation of the tryptic peptides was expected to give a more complex elution profile than the tryptic peptides derived from muscle parvalbumin alone. This was not the case. The tryptic h.p.l.c. profile of the skin material was identical with that of muscle parvalbumin (Fig. 3). This identity was supported by amino acid analysis of these tryptic fragments (Table 1). Tryptic

peptide T7 could be separated by h.p.l.c. at pH 7.5 into two distinct peptides T7-1 (residues 1–13) and T7-2 (residues 29–36) (Fig. 4).

The skin calcium-binding material was also subjected to CNBr cleavage followed by peptide separation (results not shown). The elution profiles of CB1 (residues 87–109), CB2 (residues 1–32) and CB3 (residues 33–86) were identical with those reported before for rat muscle parvalbumin (MacManus *et al.*, 1983a). The amino acid compositions of these peptides was also similar to those from rat parvalbumin (MacManus *et al.*, 1983a).

Amino acid sequence analysis of these large peptides, and the tryptic peptides described above, gave the sequence of the skin CaBP shown in Fig. 5. The *N*-terminus was blocked, presumably by acetylation (Berchtold *et al.*, 1982). Subcleavage at aspartate of the CNBr-generated peptide CB2 (residues 1–32), CB3 (residues 33–86), and the tryptic peptide T7-1 (residues 1–13) was achieved with mild acid and separation was as before (MacManus *et al.*, 1983b). This complete independent sequence of the major skin calcium-binding

Fig. 3. Similarity of the elution profile of the tryptic peptides of skin CaBP (a) and muscle parvalbumin (b). The amino acid composition of these peptides is given in Table 1.

Fig. 4. Separation at pH 7.0 of the components of peptide T7 from Fig. 3(a).

The amino acid composition of these peptides is given in Table 1.

protein was identical with that of rat muscle parvalbumin (Fig. 5; Berchtold *et al.*, 1982; MacManus *et al.*, 1983a). We have been unable to find Ala-77 or Leu-92 as previously reported (Rinaldi *et al.*, 1982), in either the trypsin- or CNBr-generated peptides. The reason for this discrepancy is unknown.

The skin CaBP has been localized immunohistochemically only in the basal cell layer of the epidermis (Saurat *et al.*, 1981; Pavlovitch *et al.*, 1983). In a final effort to obtain this skin CaBP, thought to be different from parvalbumin (Rinaldi *et al.*, 1982), the epidermis was dissected free of the dermis which is thought to contain parvalbumin (Berchtold *et al.*, 1984). The crude extracts of these preparations were separated on h.p.l.c. as described above for whole skin (Fig. 1a), and examined for calcium binding (Figs. 6a and 6b). Only the dermis contained a calcium-binding protein, which eluted as before at 40.5 min, and is genuine parvalbumin (Fig. 5).

The inescapable conclusion of these studies is that rat skin contains only one major M_r 11 500 CaBP, and this is the same molecule known as parvalbumin from skeletal muscle (Berchtold *et al.*, 1982; MacManus *et al.*, 1983a). This finding

proves the presence of parvalbumin in skin, as already suggested by immunological and chromatographic studies (Berchtold *et al.*, 1984). However there are unanswerable questions concerning the differences described in the literature between cross-reactivity of the anti-(skin CaBP) serum with parvalbumin, and the lack of immunostaining of muscle sections (Pavlovitch *et al.*, 1983, 1984). Also a puzzle is the evidence of the epidermal location of the anti-(skin CaBP) immunostaining compared with the dermal residence of the genuine parvalbumin (Saurat *et al.*, 1981; Berchtold *et al.*, 1984; Figs. 6a and 6b).

The major skin CaBP was originally described as being synthesized only in the presence of vitamin D (Laouari *et al.*, 1980). This can now be interpreted as an intriguing vitamin D-dependence of parvalbumin expression in adult skin. The failure to find a CaBP in extracts of fetal skin (Fig. 1b) suggests that irradiation after birth to produce vitamin D may be necessary for expression of skin parvalbumin. Thus, besides the M_r 7500-9000 rat intestinal CaBP (Wasserman & Fuller, 1983), and a placental protein (Bruns *et al.*, 1981), vitamin D may influence other calcium-binding proteins of the same multigene family.

Fig. 5. Amino acid sequence of rat skin CaBP with the overlapping peptides used for analysis

The solid lines indicate sequences obtained by automated degradation, and the broken lines indicate sequences assumed from composition analysis and overlap. This sequence is identical in all 109 residues with that of rat muscle parvalbumin (Berchtold *et al.*, 1982; MacManus *et al.*, 1983a). Abbreviations for cleavage points are: A, mild acid; CB, CNBr; T, trypsin. In analysis of CNBr peptides, 45nmol of CB1 were used with an initial yield of Ala of 38.7nmol and a repetitive yield of 96.3%; 60nmol of CB3 were used with an initial yield of Val of 51 nmol and a repetitive yield of 96.5%.

Fig. 6. Demonstration that the major skin CaBP is absent from epidermis (a) and present in the dermis (b)

After this paper was submitted a corroborative report appeared which concluded that skin CaBP is antigenically identical with parvalbumin (Hawley-Nelson & Yuspa, 1984).

Our thanks are due to B. M. Braceland for provision of the various skin extracts, to Dr. L. M. Brewer for histological examination, to D. G. Gillan for the graphics, and to D. Piché for typing the manuscript. The help and comments of Dr. J. Fairley, Department of

Dermatology, University of Michigan Medical School, Ann Arbor MI, U.S.A. and of Dr. P. Nelson, National Cancer Institute, NIH, Bethesda, MD, U.S.A., are gratefully acknowledged. This publication is N.R.C.C. no. 24172.

References

- Berchtold, M. W., Heizmann, C. W. & Wilson, K. J. (1982) *Eur. J. Biochem.* **127**, 381–389

- Berchtold, M. W., Celio, M. R. & Heizmann, C. W. (1984) *J. Biol. Chem.* **259**, 5189–5196
- Bruns, M. E. H., Vollmer, S., Wallshein, J. & Bruns, D. E. (1981) *J. Biol. Chem.* **256**, 4649–4653
- Durkin, J. P., Brewer, L. M. & MacManus, J. P. (1983) *Cancer Res.* **43**, 5390–5394
- Haiech, J., Klee, C. B. & Demaille, J. G. (1981) *Biochemistry* **20**, 3890–3897
- Hawley-Nelson, P. & Yuspa, S. H. (1984) *J. Cell Biol.* **99**, 430a
- Laouari, D., Pavlovitch, H., Deceneux, G. & Balsan, S. (1980) *FEBS Lett.* **111**, 285–289
- MacManus, J. P. & Whitfield, J. F. (1983) *Calcium Cell Funct.* **4**, 411–440
- MacManus, J. P., Watson, D. C. & Yaguchi, M. (1983a) *Biosci. Rep.* **3**, 1071–1075
- MacManus, J. P., Watson, D. C. & Yaguchi, M. (1983b) *Eur. J. Biochem.* **136**, 9–17
- Marrs, J. M. & Voorhees, J. J. (1971) *J. Invest. Dermatol.* **56**, 174–181
- Pavlovitch, J. H., Didierjean, L., Risk, M., Balsan, S. & Saurat, J. H. (1983) *Am. J. Physiol.* **244**, C50–C57
- Pavlovitch, J. H., Delezoide, A. L., Didierjean, L., Saurat, J. H. & Pfister, A. (1984) *Am. J. Pathol.* **114**, 454–460
- Pfytter, G. E., Haemmerli, G., Strauli, P. & Heizmann, C. W. (1984) *Cell Calcium* **5**, 309
- Rinaldi, M. L., Haiech, J., Pavlovitch, J., Rizk, M., Ferraz, C., Derancourt, J. & Demaille, J. G. (1982) *Biochemistry* **21**, 4805–4810
- Saurat, J. H., Didierjean, L., Pavlovitch, J. H., Laouari, D. & Balsan, S. (1981) *J. Invest. Dermatol.* **76**, 221–223
- Wasserman, R. H. & Fullmer, C. S. (1983) *Annu. Rev. Physiol.* **45**, 375–390
- Wasserman, R. H., Corradino, R. A. & Taylor, A. N. (1968) *J. Biol. Chem.* **243**, 3978–3986