Isolation and Characterization of a Rat Skin Parvalbumin-like Calcium-Binding Protein†

Murielle L. Rinaldi, Jacques Haiech, Jana Pavlovitch, Marthe Rizk, Concepcion Ferraz, Jean Derancourt, and Jacques G. Demaille*

ABSTRACT: A low molecular weight acidic calcium-binding protein was isolated from rat skin with a yield of 0.04% of the total soluble protein, by a combination of gel filtration and anion-exchange chromatographies. Rat skin calcium-binding protein (S-CaBP) comigrated with rat muscle parvalbumin upon isoelectric focusing and dodecyl sulfate–polyacrylamide gel electrophoresis, indicating a molecular weight of 12 000. The ultraviolet absorption spectra and the amino acid compositions of S-CaBP and parvalbumin were very similar, with the absence of prolyl, cysteinyl, tyrosyl, and tryptophyl residues and the presence of a single arginyl residue. The NH_2 terminus of the polypeptide chain was blocked in S-CaBP as in most parvalbumins. The limited tryptic cleavage of persuccinylated S-CaBP and parvalbumin allowed the automatic sequencing of 19 residues following arginine, presumably located at position 75 by homology with other parvalbumins. S-CaBP and parvalbumin were identical except for two positions. In one of them, the Y ligand of the EF Ca^{2+}-binding loop, S-CaBP exhibits a leucyl residue instead of an aspartyl residue. This explains in part the differences in the calcium-binding properties of S-CaBP and parvalbumin. The latter exhibits two characteristic high-affinity (Ca^{2+}–Mg^{2+})-binding sites with $k_{Ca^{2+}}$ = 1.2 and 1.9 nM. In contrast S-CaBP shows two lower affinity sites with $k_{Ca^{2+}}$ = 20 and 490 nM and $k_{Mg^{2+}}$ = 0.38 and 2.1 mM for sites 1 and 2, respectively. Rat epidermis, a rapidly proliferating tissue, therefore contains a low molecular weight acidic calcium-binding protein, closely related to but different from parvalbumin.

Calcium ions are now believed to control cell proliferation since Ca^{2+} entry into cytoplasm acts as a general mitogen (Durham & Walton, 1982). Epidermis is one of the most rapidly proliferating normal tissues, and calcium-binding proteins may play a role in the control of cell division. It is therefore not surprising that studies are now being conducted to establish the presence in skin of members of the family of the low molecular weight calcium-binding proteins, which includes calmodulin, troponin C, parvalbumin, the S-100 protein, and the vitamin D dependent intestinal Ca^{2+}-binding protein, as well as alkali and regulatory light chains of myosin (Goodman et al., 1979; Goodman, 1981). Among them, the best candidates are those proteins that have already been shown to be present in nervous tissue and may be neuroectodermal markers. In this respect, the S-100 protein, considered up to now to be unique to the nervous system, was recently identified in normal skin, where it is located specifically in melanocytes and in cells with morphological features of Langerhans cells (Cocchia et al., 1981).

Another Ca^{2+}-binding protein, parvalbumin, was initially described as mostly present in fast skeletal muscle (Pechère, 1977; Blum et al., 1977). It was also found in brain (Baron et al., 1975; Celio & Heizmann, 1981). Meanwhile, a low molecular weight, vitamin D dependent, calcium-binding protein was shown to be present in rat skin (Laouari et al., 1980), to be immunologically different from the intestinal CaBP,[1] and to be localized in the cytosol of the basal cell layer of both skin and malpighian mucosa (Saurat et al., 1981). The possible involvement of this skin calcium-binding protein (S-CaBP) in the control of the basal layer proliferation prompted its isolation. The pure protein was compared to homogeneous rat muscle parvalbumin, which it closely resembles. Differences were found in their partial amino acid sequence and in their calcium-binding properties. S-CaBP is the first example of an epidermal parvalbumin-like protein.

Materials and Methods

Materials. Sepharose CL-6B and Sephacryl S-200 were from Pharmacia, and DEAE-cellulose (Whatman DE-52) was from Reeve-Angel. Polyacrylamide gel electrophoresis reagents were from Bio-Rad, molecular weight markers were from Sigma, Protein sequencing reagents were from Pierce, and $^{45}CaCl_2$ (>20 mCi/g) was from CEA, Gif-sur-Yvette, France. TPCK–trypsin (278 units/mg) was purchased from Worthington and soybean trypsin inhibitor from Boehringer.

Rat muscle parvalbumin was prepared from hind leg muscles by submitting the myogen to a heat treatment and trichloroacetic acid precipitation essentially as described by Blum et al. (1974). Parvalbumin was purified by filtration on a Sephacryl S-200 column (2 × 200 cm) equilibrated with 20 μM $CaCl_2$–65 mM ammonium bicarbonate. Parvalbumin- and Ca^{2+}-containing fractions, detected by atomic absorption spectrophotometry, were lyophilized, and final purification was achieved on DEAE-cellulose DE-52 as previously described (Haiech et al., 1979b). The final yield was 461 mg of parvalbumin/kg of starting material (wet weight).

Miscellaneous Methods. Calcium concentration of column eluates was determined by atomic absorption spectrophotom-

† From the Centre de Recherches de Biochimie macromoléculaire du CNRS, U-249 INSERM and Faculté de Médecine de Montpellier, 34033 Montpellier Cedex, France (M.L.R., J.H., C.F., J.D., and J.G.D.), and the Laboratoire des Tissus calcifiés CNRS, ER 126 and U-30 INSERM, Hôpital des Enfants malades, 75730 Paris Cedex 15, France (J.P. and M.R.). *Received April 5, 1982.* This work was supported in part by grants from CNRS (ATP modulation de l'action des hormones au niveau cellulaire), INSERM (CRL 80.5.032 and 81.3.023), DGRST (ACC Relations dynamiques entre macromolécules biologiques), Fondation pour la Recherche Médicale, and Muscular Dystrophy Association of America.

[1] Abbreviations: S-CaBP, skin calcium-binding protein; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Quadrol, *N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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etry with a Varian Model 1150 instrument, without correction for protein quenching. Protein determination was carried out by the Coomassie blue technique (Spector, 1978). Absorbance spectra were recorded on a Cary 118 instrument. Sodium dodecyl sulfate (0.1%)–15% polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Samples were treated with either $CaCl_2$ or EDTA as previously described (Autric et al., 1980). Two-dimensional gel electrophoresis was carried out according to O'Farrell (1975) as modified by Whalen et al. (1978).

Purification of Rat S-CaBP. All operations were carried out at 4 °C unless otherwise indicated. Rat skin samples were taken from male adult albino rats, frozen, and minced as previously described (Laouari et al., 1980). The skin mince was lyophilized and the powder (330 g) homogenized with 4 volumes of H_2O for three 30-s periods. The homogenate was centrifuged at 14000*g* for 15 min and the pellet reextracted as above. Supernatants were combined and lyophilized. Proteins were dissolved in 50 mL of 20 μM $CaCl_2$–65 mM ammonium bicarbonate, pH 8, and fractionated on a Sepharose CL-6B column (5 × 125 cm) equilibrated with this buffer (void volume 810 mL, total volume 2450 mL). The flow rate was 90 mL/h, and 8-mL fractions were collected and analyzed for their Ca^{2+} content and by dodecyl sulfate–polyacrylamide gel electrophoresis of 15-μL aliquots.

Fractions containing S-CaBP (fractions 230–288) were lyophilized, and the powder was dissolved in 25 mL of a 1 mM EDTA–20 mM imidazole buffer, pH 6.5 (buffer A), and dialyzed overnight vs. buffer A. The sample was then loaded onto a DEAE-cellulose DE-52 column (4.7 × 12 cm) equilibrated with buffer A. The flow rate was 60 mL/h, and 10-mL fractions were collected. Proteins were eluted by a linear NaCl gradient (0–0.4 M) in buffer A. Fractions were analyzed as above. To S-CaBP-containing fractions was added 1.5 mM $CaCl_2$, and the solution was lyophilized. The powder was dissolved in 1 mM EDTA–15 mM imidazole buffer, pH 6.5, dialyzed vs. this buffer, and loaded onto a 2.5 × 60 cm DE-52 column equilibrated with the same buffer. The flow rate was 50 mL/h, and 10-mL fractions were collected. S-CaBP was eluted by a shallow linear gradient from 20 to 50 mM NaCl in the 1 mM EDTA–15 mM imidazole buffer, pH 6.5. Fractions were analyzed as above, and the S-CaBP peak, neutralized with $CaCl_2$ as described above, was lyophilized.

Final purification and desalting were carried out by filtration on a 2 × 200 cm Sephacryl S-200 column equilibrated with 20 μM $CaCl_2$–65 mM ammonium bicarbonate, pH 8, and eluted at 20 mL/h. Fractions (8 mL) were collected and analyzed as above, and the S-CaBP peak was lyophilized. From 330 g of skin powder, 18 mg of pure S-CaBP was obtained.

Amino Acid Analysis and Partial Sequence of Rat S-CaBP and Rat Muscle Parvalbumin. Amino acid analyses were performed in triplicate on a Beckman multichrom analyzer, Model 4255, according to Moore & Stein (1963) after 24-, 48-, and 72-h hydrolysis in 6 N HCl at 110 °C under vacuum. A single-column (6 × 300 mm) methodology was adopted, with 0.4 M NaCl–0.2 M trisodium citrate, pH 7.0, as the last buffer (Autric et al., 1980). The NH_2-terminal residue was determined by the dansyl chloride technique, followed by separation of dansyl amino acids by two-dimensional chromatography on polyamide thin-layer sheets (Gray, 1972).

Persuccinylation of the protein (250 nmol) was carried out with a Radiometer pH stat in 6 M guanidinium chloride, 10 mM EGTA, and 10 mM sodium phosphate, at pH 8.5, by five additions of succinic anhydride dissolved in anhydrous dioxane, up to a 100-fold molar excess over the lysyl residues. Sodium hydroxide (4 M) was used to keep the pH constant. The succinylated protein was freed from salts by filtration on a 2.5 × 60 cm Sephadex G-25 fine column equilibrated with 65 mM ammonium bicarbonate, pH 8, and lyophilized. Then cleavage at the single arginyl residue was carried out by tryptic hydrolysis at 37 °C for 2 h in a 0.1 M *N*-ethylmorpholine buffer, pH 8.0, with TPCK–trypsin (E/S = 0.02]. At the end of the incubation, trypsin was inhibited by addition of the same weight of soybean trypsin inhibitor, and the digest was loaded into the cup of an automatic liquid-phase sequencer SOCOSI. The Edman degradation was carried out by using 0.2 M Quadrol as the buffer. Phenylthiohydantoin amino acids were separated and identified by high-performance liquid chromatography on μBondapak C_{18} column eluted with a methanol gradient (Bhown et al., 1978). The HPLC equipment was from Waters. The repetitive yield of the degradation was computed from the yield of phenylthiohydantoin alanine to be 91%.

Calcium-Binding Determination. Rat S-CaBP and parvalbumin were freed from Ca^{2+} by trichloroacetic acid precipitation, followed by complete renaturation as previously described (Haiech et al., 1981). Ca^{2+} binding was measured by flow dialysis experiments carried out at 25 °C essentially as described by Haiech et al. (1981). Protein concentration was determined by amino acid analysis. Rat parvalbumin (60 μM) and S-CaBP (20.6 μM) were dissolved in 0.15 M KCl–25 mM Hepes buffer, pH 7.5. $MgCl_2$ (Analar) was eventually added to a final concentration of 5 or 20 mM. The total Ca^{2+} was measured by atomic absorption spectrophotometry.

Experimental points were fitted to the equation

$$\nu = (K_1x + 2K_1K_2x^2)/(1 + K_1x + K_1K_2x^2)$$

where ν represents the number of moles of Ca^{2+} bound per mole of protein at the free Ca^{2+} concentration x. K_1 and K_2 refer to the equilibrium constants of the equilibria

$$\text{protein} + \text{Ca} \overset{K_1}{\rightleftharpoons} \text{protein·Ca} + \text{Ca} \overset{K_2}{\rightleftharpoons} \text{protein·Ca}_2$$

as defined in Haiech et al. (1979a). The BMDP 3R program (BMDP statistical software, University of California, Los Angeles, CA) was run on an IBM 3033 computer (Centre Universitaire de Calcul Sud).

A sequential binding model was used to fit the Ca^{2+}-binding data of S-CaBP as previously described for calmodulin (Reid & Hodges, 1980; Haiech et al., 1981). Such a model was not used for parvalbumin (see below).

Results

Purification and Properties of Rat Muscle Parvalbumin. Rat muscle parvalbumin was purified with a yield of 461 mg/kg wet weight. It exhibits the usual spectrum of parvalbumin lacking tyrosyl and tryptophyl residues, with the characteristic vibronic structures of phenylalanine at 253, 259, and 264 nm.[2] It migrated in dodecyl sulfate–polyacrylamide gel electrophoresis with a velocity indicatve of M_r ca. 12 000. Its mobility was only slightly higher in the presence of Ca^{2+} than in the presence of a Ca^{2+} chelator.[2] It migrated in two-dimensional gel electrophoresis with an approximate p*I* of 5.3 (Figure 1).

Its amino acid composition, shown in Table I, , is very similar to that of rabbit parvalbumin, the only major difference

[2] Not shown but submitted to reviewers for examination. The corresponding figures will be sent to interested readers upon request.

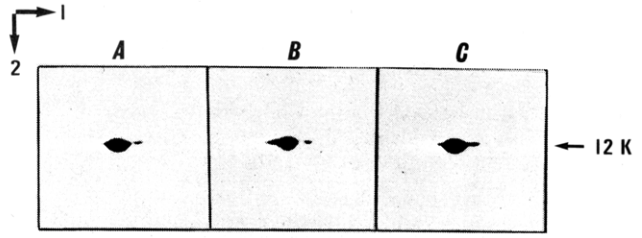

FIGURE 1: Two-dimensional gel electrophoresis of rat parvalbumin and rat S-CaBP: (A) rat parvalbumin (24 μg), (B) rat S-CaBP (20 μg), and (C) rat parvalbumin (24 μg) and rat S-CaBP (10 μg). Only the relevant portion of the gel is shown, e.g., between pH 4.5 and 6.9 in the isoelectric focusing dimension (→ 1), where the arrow points toward increasing pH. The second dimension (→ 2) was carried out in a 0.1% dodecyl sulfate–15% polyacrylamide slab gel. Note the comigration of the two protein species with some trailing due to protein overload. M_r of both proteins is 12 000 (12K).

Table I: Amino Acid Composition of Rat Skin CaBP and Rat Muscle Parvalbumin

residue	rat skin CaBP found	rat skin CaBP integer	rat parvalbumin found	rat parvalbumin integer	rabbit parvalbumin[a]
Asx	13.8 ± 1.0	14	14.9 ± 1.1	15	12
Thr[b]	5.3	5	4.9	5	5
Ser[b]	11.2	11	10.9	11	8
Glx	9.6 ± 0.6	10	9.4 ± 1.1	9	12
Pro	0	0	0	0	1
Gly	8.9 ± 0.6	9	8.7 ± 0.8	9	9
Ala	10.7 ± 0.7	11	11.1 ± 0.7	11	11
Cys[c]	0	0	0	0	0
Val[d]	5.0 ± 0.4	5	5.1 ± 0.2	5	5
Met	3.1	3	2.5	2–3	3
Ile[d]	5.8 ± 0.3	6	5.9 ± 0.3	6	6
Leu	9.4 ± 0.4	9	9.8 ± 0.5	10	9
Tyr	0	0	0	0	0
Phe	7.9 ± 0.4	8	7.8 ± 0.7	8	9
His	2.0 ± 0.1	2	1.9 ± 0.4	2	2
Lys	15.1 ± 1.0	15	15.1 ± 1.6	15	16
Arg	1.2 ± 0.1	1	1.1 ± 0.1	1	1
Trp[e]	0	0	0	0	0
total		109		109–110	109

[a] From the primary structure reported by Capony et al. (1976). [b] After extrapolation to zero time of hydrolysis. [c] After performic acid oxidation (Hirs, 1967). [d] From the 72-h hydrolysis values. [e] From spectroscopic evidence.

Table II: Purification of S-CaBP from 330 Grams of Lyophilized Skin

step	vol (mL)	protein (mg/mL)	total protein (mg)	protein yield (%)
extract (after lyophilization and redissolution)	50	861	43 050	
Sepharose CL-6B eluate	550	26.25	14 437	33
first DE-52 eluate	320	1.55	496	1.1
second DE-52 eluate	430	0.11	47.3	0.1
Sephacryl S-200 eluate	56	0.32	18.0	0.04

being the absence of proline. Also there is one more leucyl residue and one less phenylalanyl residue in the rat protein. Both parvalbumins share the absence of cysteinyl, tyrosyl, and tryptophyl residues and the presence of a single argininyl residue, presumably in the highly conserved position 75, where it forms an internal salt bridge with Glu-81 (Moews & Kretsinger, 1975).

Purification of Rat Skin CaBP. Rat S-CaBP was purified from lyophilized skin by a combination of anion exchange and gel filtration as shown in Table II. The overall yield was 5.5 mg/100 g of starting material. It is therefore much less abundant than parvalbumin is in muscle, and represents 0.04% of skin soluble proteins. The latter value, on the basis of an assumed 100% yield during the purification procedure, is obviously a minimal figure.

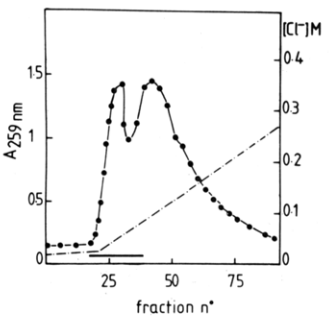

FIGURE 2: First anion-exchange chromatography of S-CaBP. S-CaBP-containing fractions were loaded onto DE-52 as described under Materials and Methods, and elution was carried out by a linear NaCl gradient (–·–). Fractions were analyzed by their absorbance at 259 nm (—) and by dodecyl sulfate–polyacrylamide gel electrophoresis, and fractions under the bar were pooled and processed for the second anion-exchange chromatography as described under Materials and Methods.

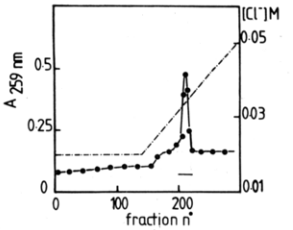

FIGURE 3: Second anion-exchange chromatography of S-CaBP. Conditions are as described under Materials and Methods. Elution of S-CaBP along the salt gradient (–·–) was followed by absorbance at 259 nm (—) and dodecyl sulfate–polyacrylamide gel electrophoresis. S-CaBP-containing fractions under the bar were pooled.

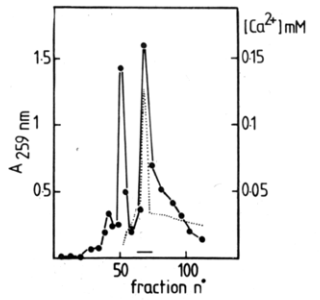

FIGURE 4: Filtration of S-CaBP on Sephacryl S-200. Conditions are as described under Materials and Methods. Fractions were analyzed for their absorbance at 259 nm (—) and their Ca^{2+} content (···). Fractions under the bar were pooled and lyophilized.

The first gel filtration step, which provided little purification, resulted in the removal of high molecular weight contaminants, mainly at M_r 68 000 (not shown). When loaded onto DEAE-cellulose, S-CaBP only bound in the presence of EGTA. It was eluted at the beginning of the salt gradient, between 0 and 80 mM NaCl (Figure 2). The corresponding fractions were again submitted to anion exchange in the absence of calcium, and S-CaBP was eluted as a sharp peak at 33 mM chloride along the shallow gradient (Figure 3). Final purification by removal of a higher molecular weight contaminant was achieved by gel filtration on Sephacryl S-200 (Figure 4).

The protein exhibited the characteristic vibronic structure of phenylalanine-rich proteins that lack tyrosine and trypto-

Table III: Partial Amino Acid Sequence of S-CaBP and Rat Muscle and Rabbit Muscle Parvalbumins[a]

ligand																	X		Y		Z
position	75					80					85						90				
rat S-CaBP	Arg-Asp-*Ala*-Ser-*Ala*-Lys-Glu-Thr-Lys-Thr-Leu-Met-Ala-Ala-Gly-Asp-Lys-*Leu*-Gly-Asp-																				
rat muscle parvalbumin	Arg-Asp-*Leu*-Ser-*Ala*-Lys-Glu-Thr-Lys-Thr-Leu-Met-Ala-Ala-Gly-Asp-Lys-*Asp*-Gly-Asp-																				
rabbit muscle parvalbumin	Arg-Asp-*Leu*-Ser-*Val*-Lys-Glu-Thr-Lys-Thr-Leu-Met-Ala-Ala-Gly-Asp-Lys-*Asp*-Gly-Asp-																				

[a] Rabbit muscle parvalbumin sequence from Capony et al. (1976). Only those residues that are not identical in the three proteins are italicized. The partial sequences were obtained by automatic Edman degradation after tryptic cleavage at the single Arg residue at position 75. X, Y, and Z are the first three ligands of the Ca^{2+} ion in the EF loop (Moews & Kretsinger, 1975).

phan, with maxima at 253, 259, and 264 nm. Peaks were, however, less sharp that those of parvalbumin, and absorbance (or diffusion) was noticeable above 270 nm,[2] due either to nonprotein contaminants or to a minor protein contaminant that was not detectable on Coomassie blue stained polyacrylamide gel electrophoretograms. S-CaBP migrated in dodecyl sulfate–polyacrylamide gel electrophoresis with an apparent M_r of ca. 12 000, irrespective of the absence or presence of Ca^{2+} ions. When mixed before electrophoresis, the rat skin and muscle proteins comigrated.[2] Upon two-dimensional gel electrophoresis, S-CaBP comigrated with rat parvalbumin, indicating similar p*I* and M_r values (see Figure 1). The amino acid composition of rat S-CaBP (see Table I) was almost identical with that of rat parvalbumin, with the same absence of prolyl, cysteinyl, tyrosyl, and tryptophyl residues and the presence of a single arginyl residue. Possible, though not conclusive, differences may be found in the content of aspartyl residues and perhaps of methionyl and leucyl residues. The N terminus of the protein was found to be blocked, by both the dansyl procedure and Edman degradation. This is also the case for most parvalbumins (Pechère et al., 1973).

Comparison of Partial Sequences of Rat Muscle Parvalbumin and Rat S-CaBP. Since the scarcity of S-CaBP prohibited the complete determination of its primary structure, advantage was taken of the presence of a single arginyl residue to specifically cleave the succinylated proteins by trypsin and determine the amino acid sequence of the residues following arginine-75. The results of the Edman degradation are shown in Table III, which compares residues 75–94 of rat skin and muscle proteins to those of rabbit parvalbumin. Both muscle parvalbumins were identical except for the conservative substitution Val ↔ Ala at position 79.

In contrast, rat muscle parvalbumin and rat S-CaBP differed in two positions. A conservative substitution was observed at position 77 (Ala ↔ Leu), and, more important, the calcium ligand at position Y (residue 92) of the EF binding loop (Moews & Kretsinger, 1975) was Asp in parvalbumins and Leu in the rat S-CaBP. Such a substitution in the EF binding loop is expected to modify the Ca^{2+}-binding properties of S-CaBP when compared to those of parvalbumin.

Comparison of Calcium-Binding Properties of Rat S-CaBP and Parvalbumin. Binding isotherms were determined for both proteins in the absence of Mg^{2+} or in the presence of either 5 or 20 mM Mg^{2+} (Figure 5 and Table IV). Both S-CaBP and parvalbumin exhibited two calcium-binding sites. Their Ca^{2+}-binding properties are, however, strikingly different. In the absence of Mg^{2+}, the affinity of S-CaBP for Ca^{2+} was higher than 10^7 M^{-1}, and the values of the binding constants K_1 and K_2 should only be taken as preliminary estimates. Since $4K_2 < K_1$ at 5 and 20 mM Mg^{2+}, calcium binding at the two sites exhibits an apparent negative cooperativity; i.e., the binding polynomial $1 + K_1x + K_1K_2x^2$ has two real roots.

In contrast, calcium binding at the two Ca^{2+}-binding sites of rat parvalbumin shows an apparent positive cooperativity at 5 and 20 mM Mg^{2+}, with $4K_2 > K_1$; i.e., there are no real

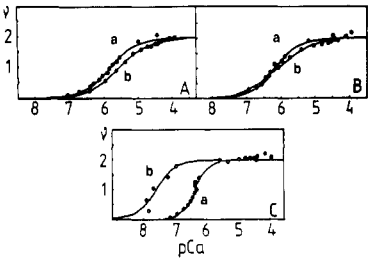

FIGURE 5: Calcium-binding isotherms of rat parvalbumin (curve a) and rat S-CaBP (curve b). Conditions are as described under Materials and Methods. Experimental points were fitted to the theoretical curve drawn from the equation $\nu = (K_1x + 2K_1K_2x^2)/(1 + K_1x + K_1K_2x^2)$ with K_1 and K_2 values from Table IV. Ca^{2+} binding was measured in the presence of (A) 20 mM Mg^{2+} and (B) 5 mM Mg^{2+} and (C) in the absence of Mg^{2+}. pCa = −log [free Ca^{2+}].

roots to the binding polynomial (Haiech et al., 1981). When studied with a sequential binding model, the intrinsic dissociation constants of S-CaBP were 20 and 490 nM for the dissociation of Ca^{2+} from sites 1 and 2, respectively, and 0.38 and 2.1 mM for the dissociation of Mg^{2+} from sites 1 and 2, respectively.

In contrast, the intrinsic dissociation constants of parvalbumin for Ca^{2+}, computed with the assumption that Mg^{2+}-binding properties of rat and rabbit parvalbumin (Haiech et al., 1979a) are identical, are 1.2 and 1.9 nM for sites 1 and 2, respectively, i.e., quite similar to constants already reported to be for α and β parvalbumins 2.2–7.8 nM (Haiech et al., 1979a).

Discussion

The present report describes the isolation of a minor protein from rat skin, which shares many of the properties of muscle parvalbumins, namely, the same molecular weight of 12 000 and the same acidic p*I*, a very similar amino acid composition with a high phenylalanine content, a striking homology in the partial sequence, and the capability of binding two calcium ions with high affinity.

There is therefore no doubt that S-CaBP is homologous to parvalbumin and therefore belongs to the evolutionary family of intracellular low molecular weight Ca^{2+}-binding proteins (Goodman et al., 1979). That S-CaBP is distinct from parvalbumin was already suspected on immunological grounds, since anti-S-CaBP antibodies are unable to stain skeletal muscle. In fact S-CaBP was shown by indirect immunofluorescence to be present only in the basal proliferative cell layer of all malpighian epithelia and related tissues (epidermis, cornea, esophagus, and vagina) as well as in brain ependyma and lens epithelia. In contrast no immunoreactivity was found in fibroblasts, muscle, cartilage, blood vessels, nerve tissue, liver, endocrine glands, urogenital tract, and intestinal and respiratory epithelia (J. H. Pavlovitch, L. Didierjean, M. Rizk, S. Balsan, and J. H. Saurat, unpublished results).

The present study shows unequivocal differences in the primary structure and in the calcium-binding properties of S-CaBP and parvalbumin. In particular, the presence of a

Table IV: Ion-Binding Properties of Rat S-CaBP and Parvalbumin

(A) Macroscopic Calcium-Binding Constants (M^{-1})

conditions	S-CaBP K_1	S-CaBP K_2	parvalbumin K_1	parvalbumin K_2
no Mg^{2+}	2.9×10^7	5.2×10^7	[a]	2.4×10^6
5 mM Mg^{2+}	3.8×10^6	4.4×10^5	2.0×10^6	1.4×10^6
20 mM Mg^{2+}	9.3×10^5	2.0×10^5	1.1×10^6	5.6×10^5

(B) Intrinsic Dissociation Constants (M)

conditions	S-CaBP site 1[b]	S-CaBP site 2[b]	parvalbumin site 1[c]	parvalbumin site 2[c]
$k_{Ca^{2+}}$	2.0×10^{-8}	4.9×10^{-7}	1.2×10^{-9}	1.9×10^{-9}
$k_{Mg^{2+}}$	3.8×10^{-4}	2.1×10^{-3}	nd	nd

[a] In this experiment, data were fitted to the theoretical curve $\nu = 2K_2^2x^2/(1 + K_2^2x^2)$. [b] The sequential binding model was applied to S-CaBP, allowing the computation of intrinsic binding constants for Ca^{2+} and Mg^{2+} by using the equation $1/K_i = k_{Ca^{2+}}(1 + [Mg^{2+}]/k_{Mg^{2+}})$. [c] The rat parvalbumin intrinsic $k_{Ca^{2+}}$ was computed assuming that $k_{Mg^{2+}}$ is identical in rat and rabbit parvalbumins, i.e., 16 μM (Haiech et al., 1979a): $1/K_i = k_{Ca^{2+}}(1 + [Mg^{2+}]/k_{Mg^{2+}})$.

leucyl residue in the coordinating position Y of the EF loop of S-CaBP (Moews & Kretsinger, 1975) may provide the rationale for the weaker binding properties of S-CaBP site 2.

Rat parvalbumin exhibits two characteristic high-affinity Ca^{2+}–Mg^{2+} sites as previously reported for other parvalbumins of both α and β genetic lineages, in line with the parvalbumin function of soluble relaxing factor (Birdsall et al., 1979; Haiech et al., 1979a; Gillis, 1980). In contrast, S-CaBP shows no positive cooperativity, and the two sites behave as if they were independent, with a second lower affinity site. Mg^{2+} competes with Ca^{2+} at both sites, as shown to be the case for calmodulin (Haiech et al., 1981). Even though it is premature to speculate about the explanation for such differences before the entire primary structure of S-CaBP becomes available, S-CaBP may differ from parvalbumin in either the connecting peptide between CD and EF domains or in the structure of the loops as shown for instance here for the Y liganding position of the EF loop or in the Ca^{2+}-induced movement of amino acid side chains (Reid & Hodges, 1980), or any combination of the above factors. Another parvalbumin-like protein has already been purified from chicken leg muscle by Heizmann et al. (1977) and Heizmann & Strehler (1979). It differs, however, from S-CaBP in that it exhibits a ubiquitous distribution, whereas S-CaBP is only present in malpighian epithelia, brain ependyma, and lens epithelia. Moreover, the chicken leg muscle parvalbumin-like protein exhibits a very low affinity for Ca^{2+}, with $K_d > 10^{-3}$ M, which suggests that it is not involved in a Ca^{2+}-dependent process or regulation (Heizmann & Strehler, 1979). In this respect, S-CaBP is considerably closer to parvalbumin than the chicken leg muscle parvalbumin-like protein.

The presence of S-CaBP in normal malpighian epithelia, which are rapidly proliferating tissues (J. H. Pavlovitch, L. Didierjean, M. Rizk, S. Balsan, and J. H. Saurat, unpublished results), raises the intriguing possiblity that it is related to another 11 500-dalton acidic calcium-binding protein recently isolated from Morris hepatomas (MacManus, 1980). Oncomodulin, the name coined for the latter protein, exhibits, however, marked differences in its amino acid composition and in its interaction with anion exchangers, from which it was eluted by only 0.25–0.35 M salt. The presence of oncomodulin in neoplastic cells, as well as the increase in calmodulin during growth of normal and cancerous liver (MacManus et al, 1981), points to the importance of Ca^{2+}-binding in the control of cell proliferation. There is, however, no evidence as yet for involvement of S-CaBP in cell growth and division, though it may be a reasonable working hypothesis.

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Characterization of Intermediates up to Lipid-Linked Heptasaccharide Implicated in the Biosynthesis of *Saccharomyces cerevisiae* Mannoproteins†

Chandra Prakash and Inder K. Vijay*

ABSTRACT: The lipid-linked oligosaccharide $Glc_3Man_9(GlcNAc)_2$ serves as a precursor for the biosynthesis of the inner core portion of the asparagine-linked polysaccharide of *Saccharomyces cerevisiae* mannoproteins. As a prelude to establishing its detailed structure and assembly, lipid-linked oligosaccharides belonging to the general structure $Man_n(GlcNAc)_2$, n = 1-5, and presumably serving as intermediates in the assembly sequence were isolated from an in vitro incubation of *S. cerevisiae* microsomes with UDP-*N*-acetylglucosamine and GDP-[^{14}C]mannose. On the basis of size, elution characteristics on a column of concanavalin A-Sepharose, exo- and endoglycosidase digestions, acetolysis, and methylation analysis, the major species within the tri- through heptasaccharides had the following structures: Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Manα1→2Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, and Manα1→2Manα1→2Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc. These structures are identical with those of the major intermediates involved in the biosynthesis of asparagine-linked glycoproteins in animal tissues. Additionally, minor isomers were also observed in the tetra- through heptasaccharides and structurally characterized. The inner core of *S. cerevisiae* mannoproteins has been shown to have some structural differences from the high mannose glycoproteins of animal origin, notably in terms of terminally linked Manα1→3 residues in the side chains attached to the α1,6-linked polymannose backbone [Ballou, C. E. (1976) *Adv. Microb. Physiol. 14*, 93-158]. The initial studies reported here indicate that the lipid-linked assembly of the precursor unit for the inner core of *S. cerevisiae* mannoproteins might be similar to that in animal systems and modifications of the protein-linked polysaccharide occur that would give the final structure. The precise role of the minor isomers within the lipid-linked oligosaccharides in the assembly of the precursor oligosaccharide is presently unclear; it is possible that these arise due to a lack of specificity of the mannosyltransferases for acceptor substrates during the assembly process.

The mannoproteins of *Saccharomyces cerevisiae* consist of polymannose chains attached to both asparagine and hydroxy amino acid (serine and/or threonine) residues in the underlying polypeptides. The structure of the polysaccharide component of these glycoproteins, as proposed by Nakajima & Ballou (1974, 1975a,b), is shown in Figure 1. In this, about 10% of the mannose is attached to serine and threonine residues as short oligosaccharides of up to tetrasaccharide in size; the asparagine-linked polysaccharide, constituting nearly 90% of the total mannose, is present as two genetically differentiated sections. These are the inner core containing 12-17 mannose residues linked at the reducing end to an *N,N'*-diacetylchitobiose unit and a somewhat similar outer region of 150 or more mannose units. The entire structure is constructed on an α1,6-linked backbone with α1,2 and α1,3 linkages in the branches. The outer region also has occasional mannobiose phosphate side chains attached to the primary branches.

The biosynthesis of the inner core of the asparagine-linked polysaccharide of *S. cerevisiae* mannoproteins is somewhat analogous to the biosynthesis of similar units for animal glycoproteins (Lehle et al., 1980; Parodi, 1981; Trimble et al., 1980). A lipid (dolichol)-linked tetradecasaccharide, $Glc_3Man_9(GlcNAc)_2$,[1] is assembled within a membrane component of the cell and transferred en bloc to the polypeptide acceptors. This is followed by the excision of glucosyl residues by processing glucosidases (Lehle, 1980; Parodi, 1979a,b, 1981). At least one of the mannosyl residues of this newly transferred core portion also appears to be cleaved as it is elongated to build the outer region (Parodi, 1981). The mannose residues for the latter segment have been shown to be transferred directly from GDP-mannose to the inner core, catalyzed by several mannosyltransferases (Nakajima & Ballou, 1975b; Lehle, 1980; Parodi, 1979a,b). Additionally, a mannose phosphate transferase incorporates the phosphorylated mannose residues, which may be subsequently elongated by the addition of an α1,3-linked mannose unit (Karson & Ballou, 1978).

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[1] Abbreviations: DP, degree of polymerization; C/M, $CHCl_3$/CH_3OH; C/M/W, $CHCl_3/CH_3OH/H_2O$; CHO, Chinese hamster ovary; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_2$, *N,N'*-diacetylchitobiose; $GlcNAc_{OH}$, *N*-acetylglucosaminitol; Man, mannose; endo, endo-β-*N*-acetylglucosaminidase; Tris, tris(hydroxymethyl)aminomethane; Con A, concanavalin A.

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