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Calcium-binding proteins in rat skin

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Skin Ca^{2+}-binding protein (SCaBP) was reported to be distinct from the Ca^{2+}-binding parvalbumin (PV), however, more recently its amino acid sequence was shown to be identical to PV. We purified a protein (M_r 12000; p*I*4.5) from isolated epidermis (free of other cell layers) of adult rats and whole skin (containing no PV) of newborn rats. This protein is referred to as epidermal protein (EP-12), distinct from PV in its hydrophobicity, amino acid composition and immunological properties. Previously isolated SCaBP was shown to be a mixture of EP-12 and PV. The localization and possible functions of EP-12 and of PV in skin of adult and newborn rat are discussed.

Ca^{2+}-binding protein; Parvalbumin; (Skin, Epidermis)

1. INTRODUCTION

Ca^{2+} and Ca^{2+}-binding proteins (e.g. calmodulin) may be involved in regulating the proliferation of many somatic cells [1,2]. The epidermis is one of the most rapidly proliferating normal tissues and therefore was chosen to study the involvement of Ca^{2+} and Ca^{2+}-binding proteins in the control of cell proliferation.

To date, two Ca^{2+}-binding proteins have been described in the epidermis: S-100 proteins are localized in melanocytes and Langerhans cells [3] and calmodulin in all keratinocytes [4,5]. There is, however, a controversy about a third skin Ca^{2+}-binding protein (SCaBP) [6–8] reported to be present in the proliferating layer of the epidermis [9] and suggested to be a marker for tumors of epidermoid origin [10].

SCaBP was reported to be biochemically and immunologically distinct from the Ca^{2+}-binding parvalbumin (PV) [6], however, more recently its amino acid sequence was shown to be identical to PV [8]. Originally, these proteins were isolated from whole rat skin, composed of epidermis, dermis and subcutaneous tissues (mainly muscle). We purified a protein (M_r 12000; p*I* 4.5) from isolated epidermis (free of other cell layers) of adult rats and from whole skin (containing no PV) of newborn rats. This protein is referred to as epidermal protein (EP-12), distinct from PV in its hydrophobicity, amino acid composition and immunological properties. Previously isolated SCaBP was shown to be a mixture of EP-12 and PV. EP-12 bound ^{45}Ca in a Ca/transblot assay only when high protein concentrations were applied,

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Abbreviations: p*I*, isoelectric point; HPLC, high performance liquid chromatography; 1D/2D PAGE, one-dimensional/two-dimensional polyacrylamide gel electrophoresis; PAP, peroxidase anti-peroxidase; PMSF, phenylmethylsulfonyl fluoride

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suggesting a lower affinity for Ca^{2+} than PV. The localization and possible functions of EP-12 and of PV in skin of adult and newborn rats are discussed.

2. MATERIALS AND METHODS

2.1. *Isolation of proteins*

Parvalbumin was purified from rat muscle [11] and calmodulin (CaM) from ox brain [12]. SCaBP was prepared from whole skin [6] of adult male Wistar rats (250–300 g). Final purification was done by reverse-phase HPLC fitted with an Aquapore RP-300 column (particle size, 10 μm; pore size, 300 Å; Brownlee Labs, Santa Clara, CA) [11]. Proteins were eluted with a linear gradient of acetonitrile (Fluka) in 25 mM Tris-HCl (pH 7.4), 0.1 mM EGTA. The fractions under each peak were pooled and stored at −80°C.

EP-12 was isolated from the epidermis of adult male rats prepared free of underlying tissue layers. Epidermis was separated from the dermis by heating whole skin in 0.9% NaCl for 1 min at 52°C. It was then homogenized in 13 mM Tris-HCl, 4 mM PMSF, pH 7.8, in a Polytron homogenizer (2 min, max. speed), passed through a molecular size membrane (fluoropolymer, 0.2 μm, Gelman Sciences Inc.), and subjected to HPLC as described above.

EP-12 was also isolated from the skin of newborn rats, according to [6] with the following modifications: gel filtration of the initial extract on Sephadex G-75 rather than Sepharose CL6B followed by fractionation on Sephacryl S-200 and final purification on HPLC under conditions described above.

2.2. *Antibodies*

Antisera against rat muscle parvalbumin [18] and SCaBP were raised in rabbits [13]. Antibodies to EP-12 were prepared in rabbits: initial intradermal injection of 100 μg EP-12 in 1 ml of 0.9% NaCl emulsified with 1 ml Freund's complete adjuvant, followed by subcutaneous injections of 50 μg EP-12 in incomplete Freund's adjuvant at 2-week intervals. Specificity was checked by immunoblotting.

2.3. *Immunoblotting*

Proteins were separated by SDS-PAGE (15% gels) and transferred to nitrocellulose sheets [14]. All primary antisera were diluted 1:2000, the secondary antiserum, peroxidase-labeled goat anti-rabbit IgG (Miles) was diluted 1:1000. Proteins were located by reacting with chloronaphthol (0.01%) and hydrogen peroxide (0.001%).

2.4. *Dot-immunobinding assay*

This method is described in [15]. Primary antisera were diluted 1:2000 and peroxidase-labeled goat anti-rabbit IgG was diluted 1:1000. Incubation with the primary antibody was for 2 h at room temperature. Reactions were visualized with chloronaphthol as above.

2.5. *Two-dimensional SDS-PAGE*

Purified proteins and skin extracts were labeled in vitro by reductive methylation ([^{14}C]-formaldehyde, NEN, spec. act. 52 Ci/mol), separated by two-dimensional PAGE and visualized by fluorography.

2.6. *Amino acid analysis*

Analyses were performed on a Hitachi 835-50 analyser. EP-12 was hydrolyzed in trifluoroacetic acid/conc. HCl (1:2, v/v) containing 0.2% phenol at 170°C for 25, 35 and 45 min in evacuated sealed tubes [16]. The values for threonine and serine were obtained by extrapolation to zero time. All other values are the averages of three hydrolyses.

2.7. *^{45}Ca-overlay transblotting*

Calcium binding to proteins was demonstrated as described in [17]. Proteins were first separated by SDS-PAGE (15%) and then transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schüll membrane filter, 0.45 μm). The membrane was incubated with ^{45}Ca (NEN, spec. act. 1 mCi/mmol). Calcium-binding proteins were visualized by autoradiography.

2.8. *Immunohistochemistry*

Full details of the methods used are given in [18]. EP-12 distribution in newborn rat skin was studied after perfusion-fixation with Bouin's fluid and paraffin embedding. Adult rat skin was fixed by freeze substitution. Primary antisera were diluted 1:2500 (anti-PV) or 1:5000 (anti-EP-12). Affinity-purified anti-EP-12 was diluted 1:2000; goat anti-rabbit IgG (Miles) was diluted 1:300;

rabbit peroxidase-anti-peroxidase (PAP) complex (Sternberger Meyer, Inc.) was diluted 1:200. The peroxidase substrate was diaminobenzidine (0.04%, Polysciences) and H_2O_2 (0.004%): reaction time was 15–30 min at room temperature.

3. RESULTS

3.1. *Isolation of EP-12*

3.1.1. From SCaBP

HPLC of SCaBP produced two major peaks (not shown). One, eluting at 60% buffer B, was identified as PV by its elution position, 2D PAGE analysis and immunoblotting. The second protein, eluting at 50% buffer B, did not cross-react with antibodies to PV and was designated EP-12 as it has an apparent M_r on SDS-PAGE of 12000.

3.1.2. From newborn rat skin

EP-12 was prepared from the skin of newborn rats by gel filtration followed by HPLC on reverse-phase supports. The EP-12 showed the same elution characteristics and migrated in exactly the same position on SDS-PAGE as EP-12 prepared from SCaBP. It showed no cross-reactivity with antibodies to PV or CaM.

3.1.3. HPLC of adult rat epidermis extracts

Aqueous extracts of rat epidermis were subjected to HPLC (fig.1). One peak eluted at the same position as purified EP-12, the second and third peaks were identified as calmodulin and PV by their elution positions on HPLC, their migration on SDS-PAGE and their immunoreactivity. The EP-12 peak showed no cross-reaction with antibodies to either PV or CaM.

3.2. *Characterization of EP-12*

3.2.1. Elution from HPLC

Fig.2 shows the elution patterns of two preparations of EP-12 compared to PV and CaM. EP-12 was well separated from PV, but ran close to CaM: EP-12 prepared from adult rat epidermis and whole skin of newborn rats eluted in identical positions. EP-12 appeared to be somewhat unstable, as indicated by the asymmetry of the HPLC peak. A second, minor band (M_r approx. 10000) was observed on SDS-PAGE (fig.3a) and the quantity of this component increased with time in storage.

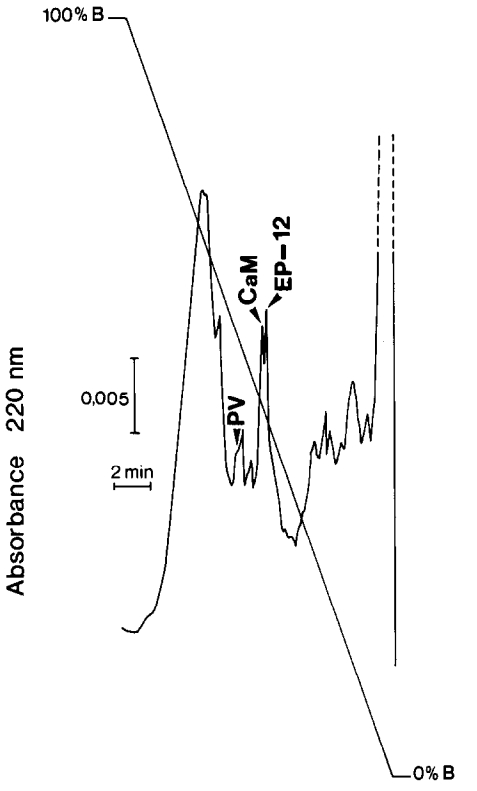

Fig.1. HPLC of a Tris-HCl extract of rat epidermis (500 μg). A Tris-HCl/EGTA, pH 7.4, buffer system was used. The gradient was 0–100% buffer B in 15 min. EP-12 was well separated from PV. EP-12, epidermal protein (M_r 12000); PV, parvalbumin; CaM, calmodulin.

3.2.2. SDS-PAGE

A one-dimensional gel of EP-12 is shown in fig.3a. EP-12 has an apparent M_r of 12000 when compared to PV (M_r 12000). Two-dimensional PAGE of EP-12 (not shown) indicated that EP-12 has an apparent isoelectric point at pH 4.5, slightly more acid than PV (p*I* 4.9).

3.2.3. Immunoblotting

Fig.3 shows that the anti-EP serum reacted only with EP-12 (fig.3d), and not with either PV (fig.3e) or Ca (fig.3f). The anti-PV serum (fig.3g–i) only reacted with parvalbumin. CaM reacted with neither antiserum (fig.3f,i). An antiserum raised earlier against SCaBP cross-reacted with both EP-12 and PV (not shown).

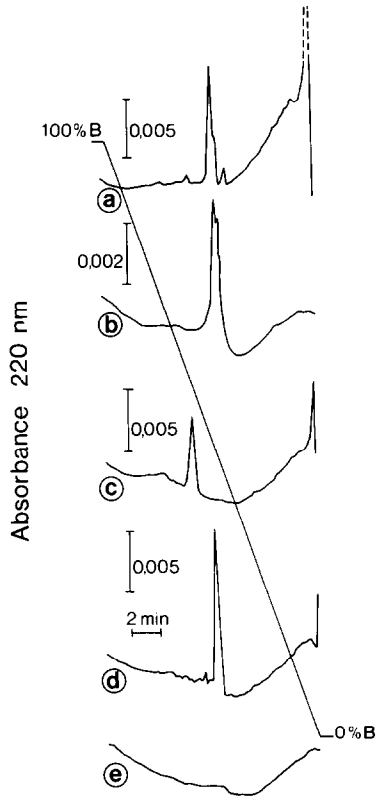

Fig.2. HPLC of purified EP-12 isolated from (a) the epidermis of the skin of adult rats (10 μg), (b) whole skin of newborn rats (10 μg), (c) rat muscle PV (6 μg), (d) ox brain calmodulin (6 μg) and (e) buffer blank. A Tris-HCl/EGTA, pH 7.4, buffer system was used. The gradient was 0–100% in 15 min.

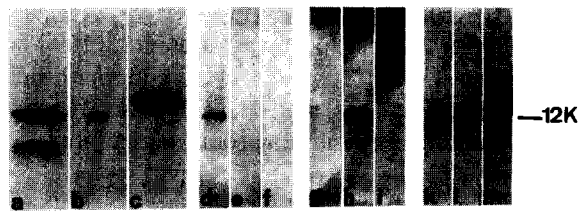

Fig.3. Immunological and ^{45}Ca binding properties. 1D SDS-PAGE (15%) of purified proteins: (a) EP-12 (3 μg) from newborn rats, (b) rat muscle PV (2 μg) and (c) calmodulin (2 μg) ^{14}C-labeled and visualized by fluorography. Immunoblotting: same order of proteins (but in concentrations of 2, 3 and 10 μg, respectively) were transferred to nitrocellulose followed by incubation with anti EP-12 serum (d–f) and with anti rat muscle PV serum (g–i). Immunoreaction was visualized by incubation with a second antibody (peroxidase-coupled goat anti-rabbit IgG) and the color reaction developed with chloronaphthol. ^{45}Ca-transblot: same order but concentrations of 5, 1 and 2 μg of protein were blotted to nitrocellulose and incubated with ^{45}Ca to detect calcium-binding proteins by autoradiography (k–m).

3.2.4. Calcium binding

EP-12 (fig.3k) binds ^{45}Ca when analyzed by the transblot method. The affinity appeared to be somewhat lower than that of PV (fig.3l) and Ca (fig.3m) as a 2–5-fold higher protein concentration was required for a visible ^{45}Ca signal.

3.2.5. Amino acid composition

The amino acid compositions of EP-12, PV and rat epidermal thiol proteinase inhibitor (TPI) are shown in table 1. There are a number of differences between EP-12 and PV, notably in their contents of Lys, Glu and Pro, but similarities to TPI.

3.2.6. Immunohistochemistry

EP-12 immunoreactivity was observed in the epidermis of rat skin, but the dermis and striated cutaneous muscle (panniculus carnosus) layers were unreactive (fig.4). Identical results were obtained with newborn (fig.4a) and adult (not shown) rat skin. Affinity-purified EP-12 antibodies gave the same results (not shown). Anti-PV serum stained neither the epidermis nor the muscle layer of newborn rat skin (fig.4b). However, the muscle layer and some nerve endings of adult rat skin were deeply stained with anti-PV serum, while the epidermis and dermis remained unstained (fig.4d). No staining was observed with anti-EP-12 serum in a number of other rat tissues, including brain,

Fig.4. Immunohistochemical localization. Newborn rat skin incubated with (a) anti-EP-12 serum and (b) anti-PV serum. Only the epidermis (E) displayed EP-12 immunoreactivity (a). When newborn skin was incubated with anti-muscle PV serum (b) the epidermis remained unstained. In adult skin (d) a thin layer of muscle tissue in the reticular layer of the cutis (panniculus carnosus) reacted. No immunoreactivity was found in newborn (c) skin incubated with preimmune sera. (a–c) Bouin-fixation, (d) freeze-substitution. E, epidermis; D, dermis; M, striated cutaneous muscle layer.

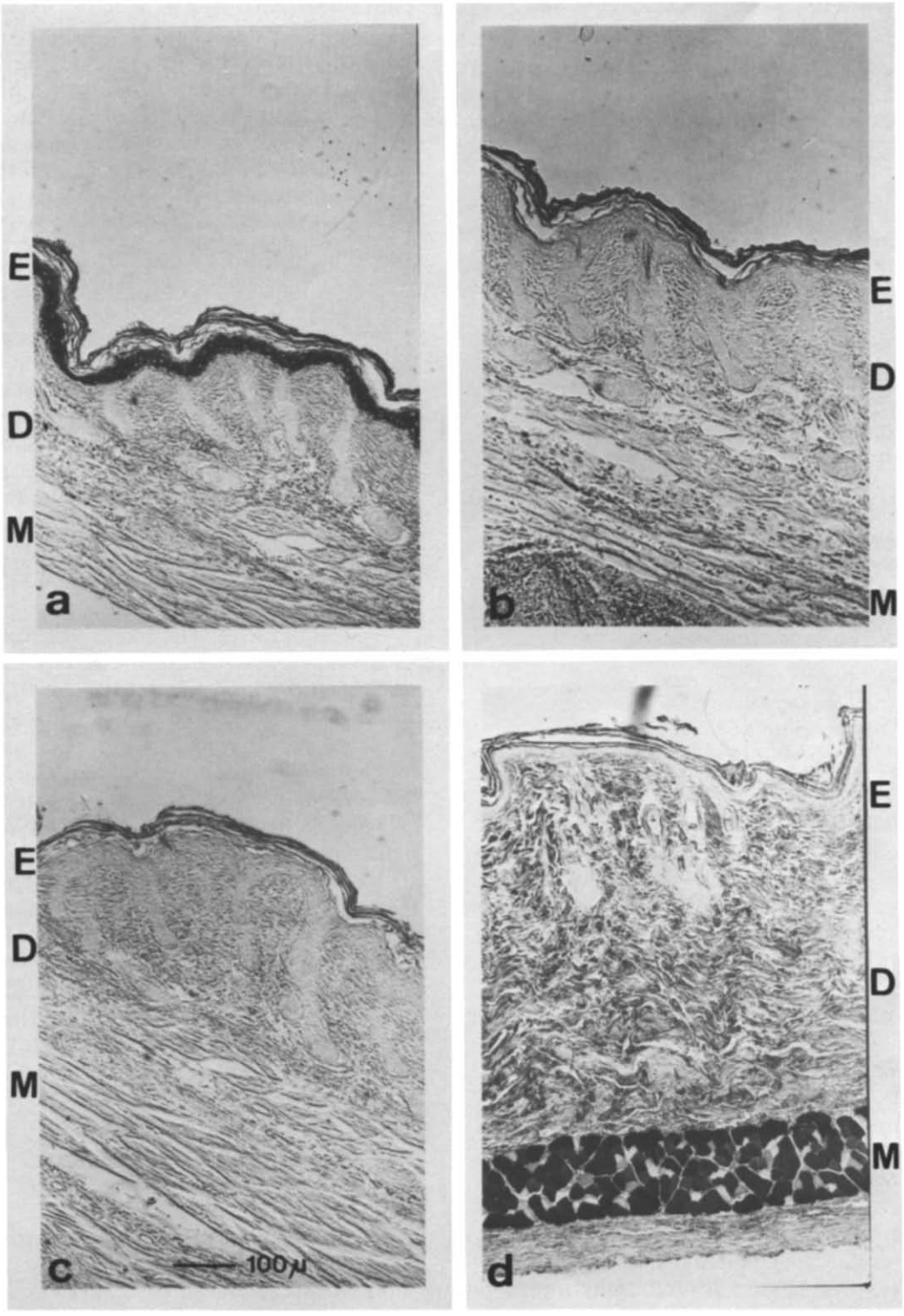
E
D
M
a
E
D
M
b
E
D
M
c
100 µ
E
D
M
d

Table 1

Amino acid composition of rat epidermal protein EP-12, parvalbumin (PV) and thiol proteinase inhibitor (TPI)

Amino acid	EP-12[a]	PV[b]	TPI[c]
Lysine	8.7	16	13
Histidine	1.7	2	1
Arginine	3.5	1	3
Aspartic acid	14.0	14	13
Threonine	5.7	5	7
Serine	7.7	11	3
Glutamic acid	17.3	8	14
Proline	4.1	0	3
Glycine	8.1	9	9
Alanine	6.6	11	4
Valine	8.0	5	11
Methionine	3.0	3	3
Isoleucine	5.1	6	3
Leucine	8.0	9	9
Tyrosine	1.5	0	3
Phenylalanine	4.8	8	4
Tryptophan	not determined	0	
Cysteine	not determined	0	

[a] M_r of 12000 was used for calculations
[b] Taken from sequence data [29]
[c] Taken from sequence data [26]

muscle and kidney. Some staining was seen in liver and intestine. In contrast, anti-PV serum specifically stained muscle (fast-twitch fibres), kidney (part of the distal tubule and proximal collecting duct) and brain (subpopulation of neurons), as was reported previously [18].

4. DISCUSSION

This report describes for the first time the purification of an epidermal protein EP-12 (M_r 12000) from the epidermis of adult rats and from whole skin of newborn rats. EP-12 is distinct from PV in several biochemical and immunological properties. In contrast to a variety of other Ca^{2+}-binding proteins, including PV, this protein gave a weak signal in the ^{45}Ca transblot electrophoresis, suggesting a lower affinity for Ca^{2+}. The contradictory results obtained previously are now clarified by the present data. SCaBP, isolated previously from total skin of adult rats, was a mixture of EP-12 (located in the epidermis) and PV (located in the striated cutaneous muscle). This explains why previous anti-SCaBP sera cross-reacted with EP-12 and PV and stained epidermis, neurons in the brain and fast-twitch fibres in skeletal muscle, as an anti-PV serum. The specific anti-EP-12 serum only stained the epidermis of adult and newborn rats, but not muscle and brain. Other epithelial cells, e.g. in intestine and tongue, were also EP-12 immunoreactive indicating that this protein may not be specific to the epidermis. The present results will allow one to reinvestigate whether the synthesis of EP-12 in the skin may depend on vitamin D, as has been reported for the SCaBP [19].

The identity of EP-12 is presently not known. There are however, several low molecular mass proteins which have been described in the epidermis, e.g., epidermal cysteine-rich proteins [20,21], keratolin [22], a phospholipase A_2 [23], retinol binding proteins [24], as well as lymphokines [25]. The properties of most of these components are distinct from EP-12. The amino acid composition of EP-12, however, closely resembles that of rat epidermal thiol proteinase inhibitor (TPI, M_r 10000–12000) [26]. The amino acid sequence of rat epidermal TPI was found to be homologous (but not identical) to either rat liver [27], or human leucocyte TPI [28], and it was suggested that rat epidermal TPI may represent a new type of low molecular mass TPI [26]. The strongest immunostaining of the anti EP-12 serum was observed in the epidermis, however, a specific staining was also observed in liver and intestine. These two tissues also contain suitable amounts of the low molecular mass TPIs which may be immunologically related to EP-12 or TPI from rat epidermis. The elucidation of the amino acid sequence of EP-12 will be undertaken for a direct comparison with the sequence of rat epidermal TPI.

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