

# Skin Calcium-Binding Protein Is a Parvalbumin of the Panniculus Carnosus\*

Pamela Hawley-Nelson, M.S., Martin W. Berchtold, Ph.D., Henrik Huitfeldt, M.D., Jack Spiegel, Ph.D., and Stuart H. Yuspa, M.D.

Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute (PH-N, HH, SHY), Bethesda, Maryland; Department of Cell Biology, Baylor College of Medicine (MWB), Houston, Texas; and Department of Biology, Catholic University of America (PH-N, JS), Washington, D.C., U.S.A.

Skin calcium-binding protein (SCaBP) is a calcium binding protein purified from whole rat skin. It has a molecular weight of approximately 12,000 daltons but migrates at  $M_r$  13,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gels. On nitrocellulose blots of SDS-polyacrylamide gels, 6 different antisera to SCaBP reacted equally well with SCaBP and parvalbumin (PV), an 11,500-dalton calcium-binding protein purified from rat skeletal muscle, which also migrates at  $M_r$  13,000 on SDS-polyacrylamide gels. Rabbit antiserum to muscle PV also recognized both PV and SCaBP, and either protein absorbed specific antibodies against either antigen from both types of antisera. Soluble protein extracts from whole adult rat and mouse skin contained a  $M_r$  13,000 protein which was recognized on nitrocellulose blots of SDS gels by both antisera. Blots of extracts from epidermis, dermis, whole skin, and skin scraped on the dermal side to remove hypodermal tissue revealed

that the  $M_r$  13,000 PV/SCaBP cross-reacting antigen was restricted to the hypodermal tissue removed by scraping. Immunofluorescent staining of Bouin-fixed skin sections with these antisera confirmed the localization of PV/SCaBP to the panniculus carnosus, a hypodermal muscle layer. Newborn mouse skin does not contain this antigen. Additional polypeptides of  $M_r$  10,500 and 12,000 on SDS gels of extracts from the epidermis of newborn and adult rats and mice were found to be immunoreactive with anti-SCaBP serum. These polypeptides were not recognized by the PV antiserum, and the reactivity of anti-SCaBP for these antigens was not absorbed by purified PV or SCaBP. Our results indicate that SCaBP is antigenically indistinguishable from PV and is localized in the adult rodent panniculus carnosus, and that antisera to SCaBP are poly-specific, recognizing epidermal proteins in addition to SCaBP/PV. *J Invest Dermatol* 86:157-162, 1986

Calcium ion concentration has been shown to control the differentiation of mouse epidermal keratinocytes in culture [1] and this regulation can be altered in carcinogen-treated or malignantly transformed keratinocytes [2,3]. It was of great interest, therefore, when a skin calcium-binding protein (SCaBP) was identified and purified from whole adult rat skin [4,5], and antisera to this protein were raised in rabbits. The localization of SCaBP antigen, determined by indirect immunofluorescence of frozen skin sections, was reported to be in basal cells of the epidermis [6]. Its molecular weight (12,000 daltons), isoelectric point (5.0), and amino acid composition were similar to another calcium binding protein, parvalbumin (PV) [5]. PV was known to be a major component of fast-twitch skeletal muscle [7] and has been shown to be present in relative abundance in adult rat skin [8], localized to the hypodermal muscle, the panniculus carnosus.

The present study was undertaken to compare SCaBP and PV, and to determine their tissue distribution by immunohistochemistry and immunoblot analysis, the latter technique having the advantage of providing information on molecular weight and antigenicity simultaneously. We present data demonstrating that PV and SCaBP are antigenically indistinguishable and that the PV/SCaBP antigen is localized in the panniculus carnosus of adult rodent skin. Additional antigens of unknown nature, with  $M_r$  10,500-12,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gels, are recognized by anti-SCaBP serum and are localized in the epidermis of newborn as well as adult rodent skin. These antigens may account for previous reports [6] of SCaBP in epidermis, and of regulated synthesis of low-molecular weight antigens in epidermal cell cultures [9,10].

## MATERIALS AND METHODS

**Proteins** Purified adult rat skin SCaBP was a gift of Dr. Jana Pavlovitch, Paris, France, and was prepared as described previously [4,5]. Purified adult rat muscle PV and bovine brain calmodulin were from Calbiochem. Adult Sprague-Dawley rat and BALB/c mouse skin was fractionated by the procedure of Kawamura et al [11]. Shaved skin was treated with Nair and then removed from the animal. The dermal side was scraped vigorously with a scalpel to remove the hypodermal material; the scraped skin was floated on 1% trypsin in saline for 1 h at 37°C. The epidermis was removed from the dermis by scraping on the epidermal side with a scalpel. Newborn mouse skin was not scraped but was floated on 0.25% trypsin overnight at 4°C, and the epidermis and dermis were separated with forceps. Cytosol extracts were prepared from full-thickness skin, skin from which the hy-

Manuscript received April 4, 1985; accepted for publication August 26, 1985.

This work was performed in partial fulfillment of the requirements for the Doctor of Philosophy degree of the Biology Department, Catholic University of America (PH-N). MWB is supported by a Swiss National Foundation Fellowship for Young Advanced Scientists (83.011.082).

\*A preliminary report of this work has been published (*J Cell Biol* 99:A430, 1984).

Reprint requests to: Pamela Hawley-Nelson, M.S., National Cancer Institute, Building 37, Room 3A23, Bethesda, Maryland 20892.

### Abbreviations:

PV: parvalbumin

SCaBP: skin calcium-binding protein

SDS: sodium dodecyl sulfate

podermal material was removed, the hypodermal material, epidermis, dermis, and leg muscle by homogenizing the tissue in 20 mM Tris, pH 7.4, with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, freezing and thawing 3 times and centrifuging at 100,000 *g* for 30 min, retaining the supernatant. Cell cultures of primary newborn mouse epidermal cells were prepared as described [12]. After 3 days of culture in 0.07 mM calcium medium, cells were scraped from the dish and homogenized in the same buffer as described for tissue fractions. All cytosol preparations were assayed for protein concentration by the BioRad protein assay kit.

**Antisera** Six different unfractionated rabbit anti-SCaBP sera were a gift of Dr. Jana Pavlovitch, Paris, France, and were prepared as previously described [4]. Except where otherwise noted, anti-SCaBP was from rabbit no. 3. Unfractionated antiserum to PV as well as anti-PV IgG purified from the antiserum on a PV affinity column were prepared as described [8]. Affinity-purified anti-PV IgG was reconstituted and used at 2.2  $\mu\text{g}/\text{ml}$  (final dilution). In the undiluted anti-PV serum the concentration of this specific IgG was 1.6 mg/ml. Absorbed antisera were prepared by incubating 50  $\mu\text{g}$  antigen with 1 ml antiserum followed by centrifugation at 15,000 *g* to remove immune complexes, repeating the incubations 3 times: first at 37°C for 1 h, then overnight at 4°C, then again at 37°C for 1 h.

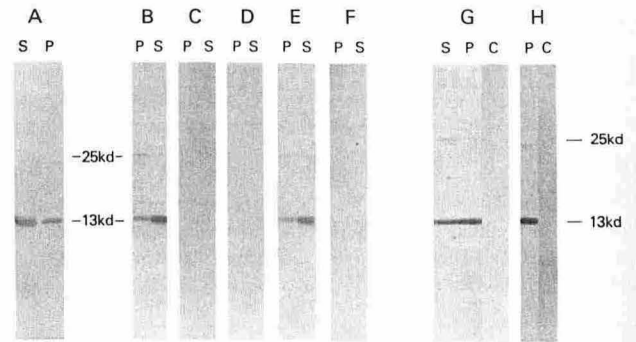
**Electrophoresis, Blotting, and Immunostaining** Electrophoresis was performed in the presence of 0.1% SDS and 2-mercaptoethanol (5.5% in sample buffer), in 15% polyacrylamide gels according to Laemmli [13]. Protein bands were electrophoretically transferred (blotted) to nitrocellulose (from Schleicher and Schuell, BA83) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol for 14 h at 60 V. Where indicated, nitrocellulose strips were stained for 1 min in 0.1% amido black in 25% isopropanol and 10% acetic acid [14]. Nitrocellulose strips were reacted with the unfractionated antisera at a standard 1:100 dilution or anti-PV IgG at 2.2  $\mu\text{g}/\text{ml}$ . Positive bands were visualized using the BioRad Immun-blot peroxidase assay kit.

**Immunohistochemistry** Full-thickness adult rat skin was fixed in Bouin's fixative (71% saturated aqueous picric acid, 24% formaldehyde, 5% acetic acid) as described for visualization of PV [7]. Tissue was embedded in paraffin, sectioned, mounted on slides, and deparaffinized [7]. Immunofluorescent staining was performed as described [15], except that an indirect technique was applied. Briefly, sections were incubated with unfractionated antisera diluted 1:100 or anti-PV IgG at 2.2  $\mu\text{g}/\text{ml}$ . After washing, sections were exposed to fluorescein-conjugated swine antirabbit Ig (Dakopatts) at 1:40 dilution, for 30 min at room temperature. All dilutions were made in phosphate-buffered saline with 12.5% bovine serum albumin and 0.01% thimerosal. Washed sections were mounted and examined on a Nikon Labophot microscope with an epifluorescent attachment (HMX-HBO 100 W Lamp-house) with a B2 filter block with 460–484 interference excitation filter and 515–545 barrier filter. Ektachrome ASA 400 daylight film was used.

## RESULTS

### Immunochemical Cross-Reactivity of PV and SCaBP

Purified preparations of PV and SCaBP were electrophoresed in SDS polyacrylamide gels, transferred to nitrocellulose, and stained with amido black. Both proteins yielded identical bands at *M*, 13,000 (Fig 1A). Faint bands at *M*, 25,000 can be seen in these preparations, possibly representing dimers of the *M*, 13,000 proteins. When identical nitrocellulose blots of electrophoresed PV or SCaBP were exposed to antisera to PV or SCaBP and visualized using a peroxidase-conjugated second antibody, both proteins were recognized equally well by both antisera (Fig 1B,E). Normal rabbit serum did not react with PV/SCaBP (not shown). The specific antibodies in these antisera were absorbed by exposure

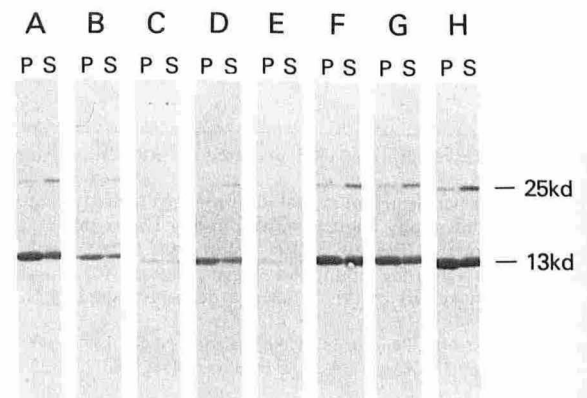


**Figure 1.** Parvalbumin and SCaBP cross-react antigenically. One microgram each of purified calcium-binding proteins parvalbumin (P), SCaBP (S), and calmodulin (C) were electrophoresed on reducing SDS 15% polyacrylamide gels and transferred (blotted) to nitrocellulose. Panel A was stained with amido black. SCaBP and PV comigrate at *M*, 13,000. Panels B–H were reacted with equal dilutions of antisera and visualized with a peroxidase reaction as described in *Materials and Methods*. B = anti-SCaBP, C = anti-SCaBP which had been absorbed with PV, D = anti-SCaBP which had been absorbed with SCaBP, E = anti-PV, F = anti-PV which had been absorbed with PV, G = anti-SCaBP, H = anti-PV. The *M*, 25,000 band seen in this and subsequent figures probably represents aggregates or proteins dimers.

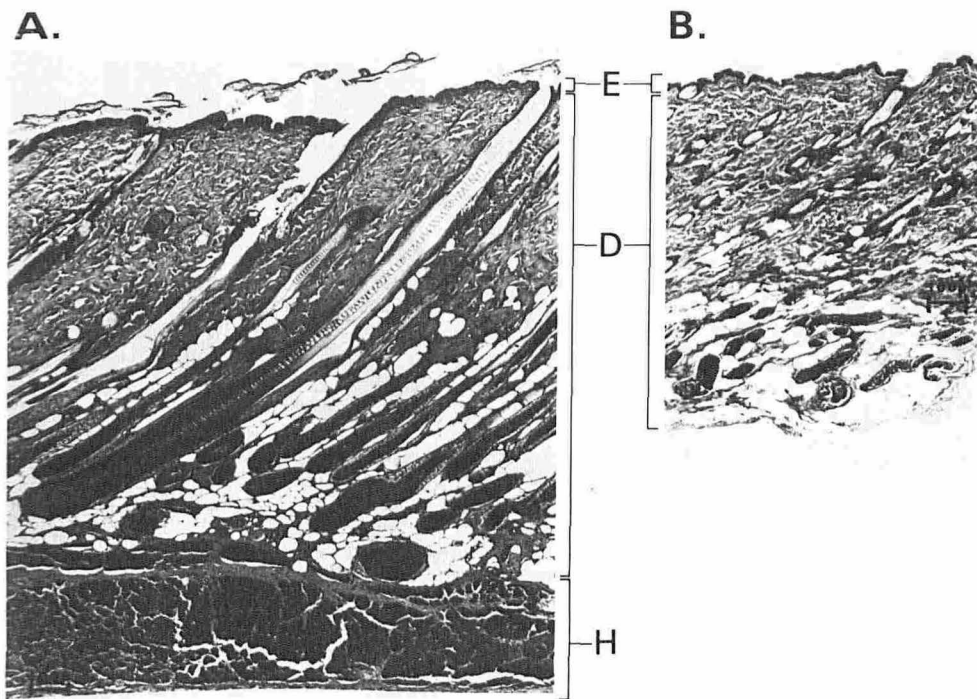
of the sera to PV or SCaBP. After absorption by either protein, reactivity toward both antigens was reduced (Fig 1C,D,F). These antisera were not recognizing the whole class of calcium binding proteins, as both failed to react with purified calmodulin (Fig 1G,H), and also failed to react with PV from rabbit (not shown). Antisera to SCaBP produced in 6 different rabbits all reacted equally well with SCaBP and PV (Fig 2). Affinity-purified antiserum to PV also reacted with both PV and SCaBP. Complete cross-reactivity was also seen with purified PV and SCaBP spotted directly onto nitrocellulose without denaturation by exposure to SDS (not shown).

### Immunohistochemical Localization of PV/SCaBP Antigen

Adult rat skin contains a muscular component, the panniculus carnosus, associated with the underside of the dermis. Fig 3 shows a section of whole adult rat skin including the panniculus carnosus (labeled H for its hypodermal location) and a section of adult rat skin which had been scraped with a scalpel on the dermal side to remove the hypodermal material. Full-thickness adult rat



**Figure 2.** Anti-PV and 6 different anti-SCaBP sera react equally well with parvalbumin and SCaBP. Purified proteins were electrophoresed and blotted as described for Fig 1. Proteins are parvalbumin (P) and SCaBP (S). Panels A–H were reacted with antisera as in Fig 1: A = anti-PV serum, B = affinity-purified anti-PV IgG, C = anti-SCaBP from rabbit no. 2, D = anti-SCaBP no. 3, E = anti-SCaBP no. 5, F = anti-SCaBP no. 6, G = anti-SCaBP no. 7, H = anti-SCaBP no. 8.



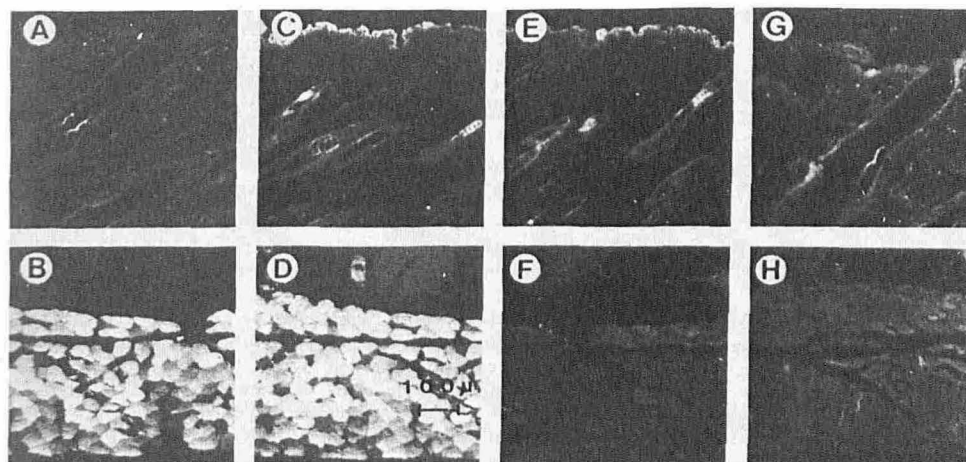
**Figure 3.** Histologic sections of adult rat skin fractions. *Panel A* is full-thickness skin, fixed in Bouin's fixative and stained with hematoxylin and eosin. Note presence of panniculus carnosus (labeled *H* due to its hypodermal location). *Panel B* is adult rat skin after scraping of the dermal side with a scalpel, fixing and staining as in *A*. Note absence of the panniculus carnosus, and presence only of dermis (*D*), epidermis (*E*), and a thin layer of fat from the hypodermis.

skin, fixed in Bouin's fixative, was sectioned, mounted on slides, and reacted with affinity-purified anti-PV IgG diluted to 2.2  $\mu\text{g}/\text{ml}$  (Fig 4A,B). The staining pattern of the panniculus carnosus (Fig 4B) is similar to that previously reported for skeletal muscle [7]. No staining is seen in epidermis (Fig 4A). Sections stained with anti-SCaBP serum diluted 1:100 showed a similar pattern of staining in the panniculus carnosus (Fig 4D), which was diminished by absorption with SCaBP (Fig 4F). Staining of the living layers of the epidermis is seen with both anti-SCaBP and absorbed anti-SCaBP (Fig 4C,E). Normal control rabbit serum diluted 1:100 shows no staining (Fig 4G,H).

**Tissue Localization of PV/SCaBP Antigen** Cytosol extracts were prepared from whole adult rat skin, adult rat skin scraped to remove hypodermal material, the hypodermal material, dermis, epidermis, or leg muscle. Equal amounts of these cytosol protein preparations were electrophoresed and transferred to nitrocellulose along with standards of purified PV. Fig 5 shows the distribution of the *M<sub>r</sub>* 13,000 cytosol antigen in adult rat skin fractions recognized by affinity-purified anti-PV IgG. In the low-molecular weight region only a *M<sub>r</sub>* 13,000 band stained, and it

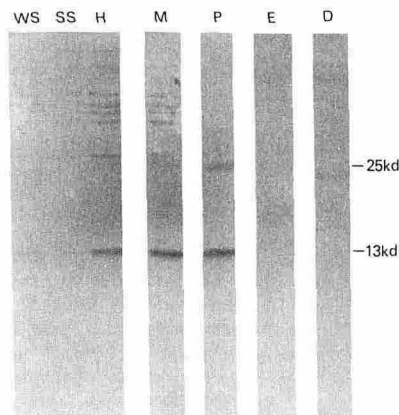
stained only in extracts from whole skin, hypodermal material, and adult leg muscle. Extracts from skin lacking the hypodermal material, as well as trypsin-separated adult rat epidermis and dermis were negative for reactivity with affinity-purified anti-PV IgG. Lanes containing cytosol preparations often exhibited stained bands of high molecular weight, possibly due to nonspecific staining.

**Localization of Additional Antigens Recognized by SCaBP Antiserum** Nitrocellulose strips similar to those used in Fig 5 were blotted from gels of cytosol extracts from adult rat skin fractions or muscle and were reacted with antiserum to SCaBP (Fig 6). This antiserum recognized the *M<sub>r</sub>* 13,000 band extracted from whole skin, hypodermal material, and muscle, as was observed with anti-PV IgG. In addition, this antiserum recognized a *M<sub>r</sub>* 12,000 antigen which was present in cytosol extracts of whole skin, scraped skin, and epidermis. It was not apparent in extracts of hypodermal material, muscle, or dermis. Adult mouse skin fractions reacted with PV and SCaBP antisera in a pattern similar to that of rat, although the adult mouse epidermis stained to a lesser extent with anti-SCaBP (not shown).



**Figure 4.** PV/SCaBP cross-reacting antigen is localized in the panniculus carnosus. Bouin-fixed adult rat skin sections were prepared and deparaffinized as described in *Materials and Methods*. Sections were reacted with antisera as follows: *A and B* = affinity-purified anti-PV IgG, *C and D* = anti-SCaBP, *E and F* = anti-SCaBP absorbed with SCaBP, *G and H* = normal rabbit serum control. All sera were diluted 1:100. Anti-PV IgG was used at 2.2  $\mu\text{g}/\text{ml}$ . Pairs of figures were present in the same tissue section but were photographed separately (i.e., *A and B*, *C and D*, etc.) Photographs were all at the same magnification (original magnification 25 $\times$ ). Epidermis and dermis were photographed in *A*, *C*, *E*, and *G* while panniculus carnosus was photographed in *B*, *D*, *F*, and *H*. Exposure times were 8 s for *A*, *B*, *G*, and *H* and 4 s for *C*, *D*, *E*, and *F*.

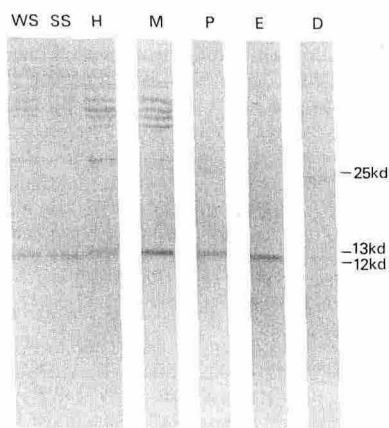




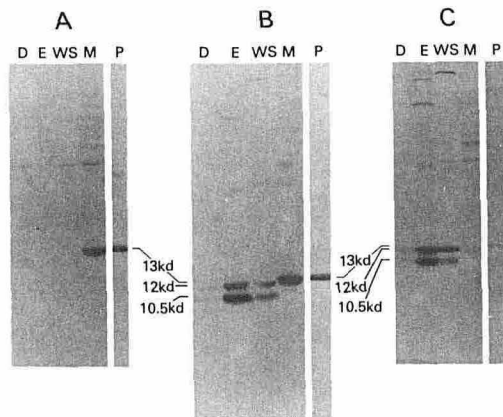
**Figure 5.** PV in adult rat skin is limited to the hypodermal material. Equal aliquots of 100  $\mu$ g cytosol protein or 1  $\mu$ g purified PV were electrophoresed and blotted as in Fig 1. Strips were reacted with affinity-purified anti-PV IgG diluted to 2.2  $\mu$ g/ml, and visualized with peroxidase. Tissue fractions were prepared as described in *Materials and Methods*. Lanes were as follows: *WS* = whole adult rat skin, *SS* = adult rat skin scraped on the dermal side with a scalpel to remove the hypodermal material, *H* = hypodermal material, *M* = adult rat leg muscle, *P* = purified parvalbumin, *E* = epidermis removed by scraping the epidermal side of adult rat skin after flotation on trypsin, *D* = dermis from scraped, floated skin. Some higher-molecular weight proteins stain faintly in lanes containing cytosol extracts, but this staining may be due to high amounts (100  $\mu$ g) of protein per lane staining nonspecifically.

#### Absence of SCaBP and Presence of Low-Molecular Weight Antigens in Newborn Skin and Cultured Newborn Keratinocytes

Newborn (less than 3 days) mouse epidermal cells have been shown to contain antigens recognized by anti-SCaBP sera [9,10]. PV is not present in rat muscle until later than 3 days after birth [7]; therefore it was of interest to determine whether these antigens were PV/SCaBP or the other low-molecular weight epidermal antigens. Cytosol extracts were prepared from newborn mouse skin fractions and adult mouse leg muscle. Equal amounts of protein from these extracts were electrophoresed and transferred to nitrocellulose. Fig 7A shows that PV antiserum does not recognize antigens in cytosol extracts of newborn mouse skin although it does recognize adult mouse muscle *M*, 13,000 antigen. Anti-SCaBP reacts with *M*, 10,500–12,000



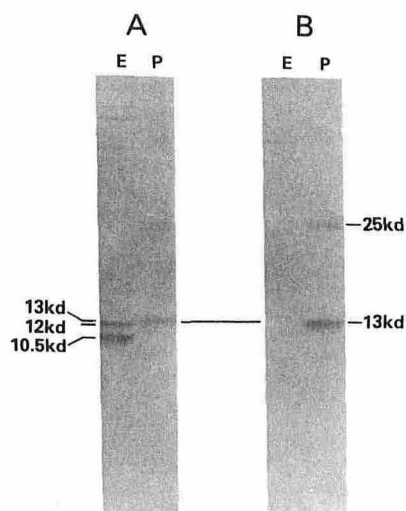
**Figure 6.** Anti-SCaBP serum recognizes the panniculus carnosus antigen PV/SCaBP and also an epidermal antigen in adult rat skin. Extracts and experimental conditions were as described in Fig 5, except that the anti-SCaBP serum was used. Note the distribution of the *M*, 13,000 band is identical to that seen with anti-PV serum, and that an additional band at *M*, 12,000 is present in whole skin, scraped skin, and epidermis.



**Figure 7.** Newborn mouse skin has epidermal antigens at *M*, 10,500–12,000 and lacks the PV/SCaBP *M*, 13,000 antigen. Experimental conditions were as described in Fig 5. Cytosol extracts were prepared from dermis (*D*) and epidermis (*E*) of newborn (0–3 day) mouse skin after flotation on trypsin. Skins were not scraped to remove hypodermal material. Extract of whole newborn mouse skin (*WS*) was also included. Adult mouse muscle (*M*) extracts and purified rat parvalbumin (*P*) were included as controls. *Panel A* = anti-PV serum; *Panel B* = anti-SCaBP serum; *Panel C* = anti-SCaBP serum which had been absorbed with SCaBP.

antigens extracted from newborn skin, and these antigens are enriched in epidermis (Fig 7B). It also recognizes the *M*, 13,000 antigen in adult mouse muscle cytosol. When anti-SCaBP is absorbed with purified SCaBP (Fig 7C), the reactivity with PV and muscle *M*, 13,000 antigen is reduced, but the reactivity to the lower-molecular weight antigens is not affected. These antigens are, therefore, distinct from the PV/SCaBP antigen in molecular weight, tissue distribution, immunoreactivity, and developmental regulation.

Cytosol extracts prepared from cultured newborn mouse epidermal cells were electrophoresed and transferred to nitrocellulose. Fig 8 shows that anti-SCaBP recognizes *M*, 10,500 and 12,000 antigens in epidermal cell cultures. No band is seen at *M*, 13,000 and, in addition, affinity-purified anti-PV IgG does not bind any



**Figure 8.** Cultured newborn mouse epidermal cells contain *M*, 10,500–12,000 antigens but not *M*, 13,000 PV/SCaBP antigen. Experimental conditions were as described in Fig 5. Cytosol extracts were prepared from newborn mouse epidermal cells after 3 days in culture (*E*). Purified parvalbumin (*P*) was included as a control. *Panel A* = anti-SCaBP, *panel B* = affinity-purified anti-PV IgG.

bands. Thus, PV/SCaBP cannot be identified in extracts of cultured newborn mouse epidermal cells.

#### DISCUSSION

We have shown that the proteins PV and SCaBP are immunologically indistinguishable. By immunoblot analysis, PV and SCaBP both react equally well with a series of 7 polyclonal antisera, 6 raised against SCaBP and 1 raised against PV. The reactivity of these antisera toward PV or SCaBP can be diminished by absorption with either purified protein. Didierjean and Saurat (personal communication) have also observed immunologic cross-reactivity between PV and SCaBP. Previously [5], skeletal muscle PV and SCaBP have been reported to differ in the affinity of their calcium-binding sites for calcium. Such differences, if confirmed, could be due to minor differences among the proteins not detected by the antisera we have used. The PV and SCaBP antisera do not react with a very closely related protein from a different species, rabbit muscle PV; hence the PV proteins from skin and muscle in rat must be very similar, if not identical, in structure. In agreement with these findings MacManus et al [16] have recently determined that the amino acid sequence of rat SCaBP is identical to that of PV from rat skeletal muscle.

Both PV and SCaBP have identical distributions within the skin, both are localized to the panniculus carnosus. Affinity-purified anti-PV serum reacts only with protein in the panniculus carnosus of skin sections or in cytosol extracts of scraped hypodermal material. The presence of PV in skin had not been reported at the time of the original purification of SCaBP. It has since been shown with quantitative high-performance liquid chromatography techniques that PV is present in adult rat skin in relatively high concentration compared with other tissues, and that it is localized by immunohistochemistry in Bouin-fixed skin sections to the musculature of the dermis [8]. No PV was detected in the epidermis. Using a rat cDNA probe for PV, Berchtold and Means [17] found that PV mRNA is present in whole skin with the same molecular size (700 and 1000 bp) as it was found in the muscle, indicating identity of muscle and skin PV. However, no hybridization signals were detected in epidermis or several cell lines of epidermal origin (Berchtold and Hawley-Nelson, unpublished observations). MacManus et al [16] have also reported the dermal localization of PV/SCaBP.

Previous reports [6] had failed to detect SCaBP antigen in dermal tissue or muscle by immunofluorescence. However Celio and Heizmann [7] have shown that fixation techniques which result in protein cross-linking are required for immunohistochemical visualization of PV in tissue sections. The published reports localizing SCaBP to the epidermis utilized immunofluorescence of frozen sections [6]. It is therefore likely that the PV/SCaBP antigen in the panniculus carnosus was not detected at all. Our fixed tissue immunohistochemistry demonstrates that PV/SCaBP antigen is present in the panniculus carnosus of adult rats.

SCaBP antisera but not PV antisera recognize low-molecular weight epidermal antigens in addition to PV/SCaBP. By immunohistochemistry these antigens appear in all the living epidermal cell layers. In skin fractions, antigens are similarly localized to the epidermis. PV/SCaBP is not detectable in cultured mouse keratinocytes or in newborn rodent skin, but anti-SCaBP serum recognizes the low-molecular weight antigens in cultured keratinocytes and in newborn as well as adult epidermis. In separate studies we have found that antigen synthesis is confined to proliferating cultured keratinocytes in medium containing 0.07 mM calcium, and synthesis stops when differentiation is induced by raising the calcium concentration to 1.2 mM for 48–72 h [9]. The tumor promoter 12-O-tetradecanoyl phorbol-13-acetate, which also induces differentiation in keratinocyte cultures [18], suppressed synthesis of the antigens. These results are consistent with the low-molecular weight antigens being exclusively synthesized in basal cells. Using immunofluorescence, Didierjean et al [10] showed regulated distribution of the "SCaBP" antigens in epidermal cell cultures.

Our results contradict aspects of previous reports on SCaBP. The original report using anti-SCaBP serum localized SCaBP to the basal layer of the epidermis [6]. It now seems likely that the antigens visualized in that study were the low-molecular weight antigens identified in epidermis cytosol extracts with anti-SCaBP serum but these antigens were not SCaBP itself. The best explanation for the results which have been previously and currently reported is that various SCaBP preparations were primarily PV from the panniculus carnosus but also contained minor contaminating proteins with molecular weights similar to PV. The antigens recognized by the various anti-SCaBP sera must have been present in the preparations of SCaBP used to immunize rabbits. Purified SCaBP used to absorb SCaBP antiserum no. 3 in our studies may have been more highly purified, or contained different contaminants, since it did not absorb the activity recognizing the additional low-molecular weight epidermal proteins. In studies using labeled epidermal cell culture extracts, immunoprecipitation with different SCaBP antisera yielded several distinct patterns of electrophoretic bands of molecular weight 8,000–16,000 proteins (data not shown). Hence different epidermal proteins may have been present in the protein preparations used to inoculate the different rabbits.

Further study of the low-molecular weight regulated epidermal antigens is in progress in order to determine their identity and relationship to epidermal cell proliferation and differentiation.

---

*The authors gratefully acknowledge the help of Drs. Jana Pavlovitch, Anthony Means, Claude Klee, and John R. Stanley. The authors also appreciate the cooperation of Drs. Liliane Didierjean, J. H. Saurat, and John MacManus in consultation and comparison of data prior to publication.*

---

#### REFERENCES

- Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH: Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19:245–254, 1980
- Kulesz-Martin MF, Koehler B, Hennings H, Yuspa SH: Quantitative assay for carcinogen altered differentiation in mouse epidermal cells. *Carcinogenesis* 1:995–1006, 1980
- Yuspa SH, Hawley-Nelson P, Koehler B, Stanley JR: A survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res* 40:4694–4703, 1980
- Laouari D, Pavlovitch H, Deceneux G, Balsan S: A vitamin D-dependent calcium-binding protein in rat skin. *FEBS Lett* 111:285–289, 1980
- Rinaldi ML, Haiech J, Pavlovitch J, Rizk M, Ferraz C, Derancourt J, Demaille JG: Isolation and characterization of a rat skin parvalbumin-like calcium-binding protein. *Biochemistry* 21:4805–4810, 1982
- Saurat JH, Didierjean L, Pavlovitch JH, Laouari D, Balsan S: Skin calcium-binding protein is localized in the cytoplasm of the basal layer of the epidermis. *J Invest Dermatol* 76:221–223, 1981
- Celio MR, Heizmann CW: Calcium-binding protein parvalbumin is associated with fast contracting muscle fibers. *Nature* 297:504–506, 1982
- Berchtold MW, Celio MR, Heizmann CW: Parvalbumin in non-muscle tissues of the rat. *J Biol Chem* 259:5189–5196, 1984
- Hawley-Nelson P, Pavlovitch J, Yuspa SH: Regulation of skin calcium-binding protein synthesis in normal and malignant epidermal cell cultures. *J Invest Dermatol* 80:304–305, 1983
- Didierjean L, Pavlovitch JH, Fusenig NE, Saurat JH: Extracellular calcium concentration regulates the expression of skin calcium binding protein (SCaBP) in mouse keratinocyte cultures. *Arch Dermatol Res* 275:202–203, 1983
- Kawamura H, Strickland JE, Yuspa SH: Association of resistance to terminal differentiation with initiation of carcinogenesis in adult mouse epidermal cells. *Cancer Res*, 45:2748–2752, 1985
- Yuspa SH, Hawley-Nelson P, Stanley JR, Hennings H: Epidermal cell culture. *Transplant Proc* 12(suppl 1):114–122, 1980

13. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970
14. Gershoni JM, Palade GE: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal Biochem* 124:396-405, 1982
15. Brandtzaeg P: Prolonged incubation time in immunohistochemistry: effects on fluorescence staining of immunoglobulins and epithelial components in ethanol- and formaldehyde-fixed paraffin-embedded tissues. *J Histochem Cytochem* 29:1302-1315, 1981
16. MacManus JP, Watson DC, Yaguchi M: Rat skin calcium-binding protein is parvalbumin. *Biochem J* 229:39-45, 1985
17. Berchtold MW, Means AR: The  $\text{Ca}^{2+}$  binding protein parvalbumin: Molecular cloning and developmental regulation of mRNA abundance. *Proc Natl Acad Sci USA* 82:1414-1418, 1985
18. Yuspa SH, Ben TB, Hennings H, Lichti U: Phorbol ester tumor promoters induce epidermal transglutaminase activity. *Biochem Biophys Res Commun* 97:700-708, 1980