Identification of A Basic Protein of M_r 75,000 as an Accessory Desmosomal Plaque Protein in Stratified and Complex Epithelia

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Abstract. Desmosomes are intercellular adhering junctions characterized by a special structure and certain obligatory constituent proteins such as the cytoplasmic plaque protein, desmoplakin I, and the transmembrane protein, desmoglein. Desmosomal fractions from bovine muzzle epidermis contain, in addition, a major polypeptide of $M_r \sim$ 75,000 ("band 6 protein") which differs from all other desmosomal proteins so far identified by its positive charge (isoelectric at pH $\sim$8.5 in the denatured state) and its avidity to bind certain type I cytokeratins under stringent conditions. We purified this protein from bovine muzzle epidermis and raised antibodies to it. Using affinity-purified antibodies, we identified a protein of identical SDS-PAGE mobility and isoelectric pH in all epithelia of higher complexity, including representatives of stratified, complex (pseudostratified) and transitional epithelia as well as benign and malignant human tumors derived from such epithelia. Immunolocalization studies revealed the location of this protein along cell boundaries in stratified and complex epithelia, often resolved into punctate arrays. In some epithelia it seemed to be restricted to certain cell types and layers; in rat cornea, for example, it was only detected in upper strata. Electron microscopic immunolocalization showed that this protein is a component of the desmosomal plaque. However, it was not found in the desmosomes of all simple epithelia examined, in the tumors and cultured cells derived thereof, in myocardiac and Purkinje fiber cells, in arachnoideal cells and meningiomas, and in dendritic reticulum cells of lymphoid tissue, i.e., all cells containing typical desmosomes. The protein was also absent in all nondesmosomal adhering junctions. From these results we conclude that this basic protein is not an obligatory desmosomal plaque constituent but an accessory component specific to the desmosomes of certain kinds of epithelial cells with stratified tissue architecture. This suggests that the M_r 75,000 basic protein does not serve general desmosomal functions but rather cell type-specific ones and that the composition of the desmosomal plaque can be different in different cell types. The possible diagnostic value of this protein as a marker in cell typing is discussed.

INTERCELLULAR adhering junctions are prominent, symmetrically organized plasma membrane domains involved in two kinds of functions. Their outer surface components form sites of relatively stable intercellular contacts, and their inner, i.e., cytoplasmic surface is covered by a plaque of densely woven filamentous material that mediates the site-specific membrane-anchorage of two different kinds of cytoskeletal filaments (8, 16, 19, 28, 31, 73). Two major groups of adhering junctions with plakoglobin as a common constituent can be distinguished by morphological and biochemical criteria. These are the desmosomes (*maculae adhaerentes*) and the nondesmosomal junctions, the latter often being collectively referred to as "intermediate junctions" (*zonulae* and *fasciae adhaerentes, puncta adhaerentia*).

The junctions of the "intermediate" group are characterized by an $\sim$20 nm virtual membrane-to-membrane space and a relatively loose-packed and indistinct plaque which anchors actin microfilaments and is characterized by several constitutive plaque proteins such as vinculin, α-actinin and plakoglobin (10, 21, 31, 32). In addition, some accessory cell type-specific components have recently been described such as the ZA-ITJ antigen which has been localized to the *adhaerens* plaque region subjacent to the *zonula occludens* of polar epithelial cells only (46; for a general *zonula occludens* marker that is also found in polar epithelial as well as in endothelial and Sertoli cells see reference 75). Moreover, some cell adhesion proteins such as L-CAM (uvomorulin; 4, 13, 38, 56, 59) and A-CAM (82, 83) have been reported to occur in association with certain subtypes of intermediate junctions.

The desmosome is usually characterized by a virtual intercellular space of $\sim$30 nm, which often appears to be bisected

by a midplate structure, and a pair of cytoplasmic plaques associated with intermediate-sized filaments (IFs)[1]. The advanced state of knowledge on the molecular composition of desmosomes is largely due to the fact that several protocols are available for the isolation of these structures from suitable tissues such as bovine muzzle epidermis (16, 22, 28, 36, 71, 72). The development of antibodies to several of the proteins found in such desmosomal fractions has provided direct evidence for the location of certain desmosomal proteins in diverse tissues of a wide range of species (7-12, 20, 25, 28, 35, 37, 51, 52, 54, 57, 68, 69, 74, 79).

In addition to plakoglobin, which is a plaque component (polypeptide M_r ~83,000) of all types of adhering cell junctions (10, 21, 44), the plaques of all desmosomes studied so far, whether they occur in epithelial, myocardial, arachnoideal cells, or lymphoid follicles, contain some other major constitutive proteins, notably a nonglycosylated polypeptide of M_r ~250,000, termed desmoplakin I (8, 9, 24, 25, 36, 54) and a transmembrane glycoprotein of M_r ~165,000 termed desmoglein ("band 3 polypeptide") whose cytoplasmic portion projects into the plaques (5, 36, 45, 51, 68, 69). In addition, a minor component of $M_r \geq 200,000$ ("D1 antigen") has been localized to the plaques of epithelial and myocardial desmosomes (28). Two further plaque proteins have so far been identified only in desmosomes of stratified epithelia: desmoplakin II (9, 25) of M_r ~215,000 which is immunologically and biochemically very closely related to desmoplakin I (9, 45, 54) and desmocalmin, a calmodulin-binding protein of M_r ~240,000 (81). The cell type distribution of another group of glycopolypeptides of M_r 130,000 and 115,000 ("bands 4a and b"; desmocollins I and II; 7, 11, 12, 35, 57, 58) is less clear.

In addition to these plaque components, a major polypeptide of M_r ~75,000 has been described in desmosomal preparations from bovine epidermis (band 6 polypeptide [B6P]; 26, 28, 54) which is basic (pH ~8.5) and thus different from all desmosome and other junction proteins identified so far. Previously we have shown, by peptide map analysis and amino acid composition, that this polypeptide is different from all other desmosomal proteins and the cytokeratins and that it is a genuine translation product encoded by a distinct mRNA (26, 45, 54). From observations that this protein is reduced in "desmosomal core" fractions obtained after extraction in citric acid of pH ~2.5 and metrizamide gradient centrifugation Steinberg and colleagues (36, 74) have suspected that this protein might be a plaque component, but because of the lack of antibodies specific for this protein (also see 7, 15, 51, 76) its location and cell type distribution has remained unclear (35, 37, 76). Here we described antibodies specific for this protein which have enabled us to detect and identify it as a desmosomal plaque constituent in certain cells of stratified and complex epithelia but not in desmosomes of other cell types.

Materials and Methods

Preparation of Cytoskeletal Material

Bovine tissue samples were obtained from the local slaughterhouse (cf. 3, 27, 28). Rat tissues were from healthy, sexually mature animals of both sexes

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1. *Abbreviations used in this paper*: B6P, band 6 polypeptide; IF intermediate-sized filaments.

purchased from a commercial animal breeding company. Human tissue samples, including tumors, were obtained during surgery or at autopsy (cf. 24, 52). Cytoskeletal fractions were prepared from bovine muzzle epidermis, tongue and esophagus mucosa, cornea, trachea, lactating udder, liver, heart, and meninges, from rat tongue mucosa, liver, small intestine and heart, and from human thigh epidermis.

The epithelial layers collected by dissection with fine scalpels or by scraping off from luminal surfaces or small pieces from other tissues were incubated in ice-cold buffer A (cf. 9), and homogenized with a Polytron homogenizer for 1 min at setting 6. After filtration through gauze and centrifugation for 1 min at 500 g, the supernatant fraction was centrifuged at 2,000 g × 30 min. The resulting pellet was taken up in 20 mM phosphate buffer (pH 7.5) containing 1.5 M KCl, 1% Triton X-100, 0.5 mM (dithiothreitol) (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 45 min at 4°C with stirring. After centrifugation of the homogenate at 10,000 g × 20 min the pellet was washed twice by suspension in PBS and recentrifugation, and the final pellet was stored frozen at −20°C. Small samples of various human and animal tissues and tumors were used for microdissection of tissue regions under question as described (53). Brush border cytoskeletal preparations were prepared from rat small intestine as described (30). Rat and bovine liver cytoskeleton fractions were prepared as described (9, 14, 23). Plasma membrane fractions of rat liver were prepared as described (43).

Bovine cell culture lines from Madin–Darby bovine kidney (MDBK), mammary gland epithelium (BMGE+H, BMGE-H), calf lens, pulmonary artery endothelium (CPAE) and fibroblasts from skin were grown as described (10, 21, 27, 28, 62, 70). Origin and conditions for cell culture growth of human cell lines MCF-7 derived from a breast carcinoma, A-431 derived from a vulvar carcinoma, PLC cells derived from a hepatocellular carcinoma have been given elsewhere (9, 52, 53). Rat hepatoma cells of line MH₁C₁ have also been described (23). Cytoskeletal fractions resistant to high salt buffer and Triton X-100 were obtained from these monolayer cultures as previously described (1, 27).

Isolation of Desmosomes

Bovine muzzle tissue was obtained from a local slaughterhouse (cf. 25, 28), and desmosomes were prepared according to the method of Gorbsky and Steinberg (36) with the following modifications. The superficial strata were removed with a scalpel and slices of the underlying *stratum spinosum* (~20 g) from bovine muzzle epidermis were minced with scissors and homogenized using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) at setting 4 or 5 for 1 min in 500 ml "CASC A1 buffer," i.e., 0.1 M citric acid, 0.05% NP40, 1 mM DTT, 1 mM PMSF (Sigma Chemical Co., St. Louis, MO), 0.5 mM L-3-*trans*-carboxy-oxiran-2-carbonyl-l-leugmatin ("E64"; from the Peptide Institute, Osaka, Japan), 5 μg/ml of each leupeptin and pepstatin (Boehringer, Mannheim, FRG), final pH 2.3. After a 3-h incubation under rapid stirring at 4°C, the homogenate was filtered through fine-meshed gauze and then centrifuged at 13,000 g × 20 min. The pellet was resuspended in 200 ml of "CASC B1 buffer," i.e. 0.1 M citric acid (pH 2.3), 0.01% NP40, 1 mM DTT and protease inhibitors (as above) and sonicated with a Branson sonicator ten times at setting 7 for 15 s each, with 10-s intervals. The resulting, finely dispersed homogenate was centrifuged at 13,000 g × 20 min. The upper white layer was removed and resuspended in ~100 ml CASB B1 buffer with a motor-driven, glass-Teflon Potter-Elvejhem device. The pellet obtained after centrifugation at 13,000 g × 20 min was further extracted by four cycles of resuspension and sedimentation in the same buffer. The final pellet was washed with PBS and stored at −20°C.

Gel Electrophoresis

Conditions for SDS-PAGE and two-dimensional gel electrophoresis were as described (e.g., 9, 45, 54). For non-equilibrium pH gradient electrophoresis (NEPHGE), samples solubilized in SDS-containing sample buffer (50) were precipitated with methanol and chloroform (85), the precipitates air-dried, then solubilized in buffer containing 9.5 M urea, and processed essentially as described by O'Farrell et al. (55) as modified by Müller and Franke (54). Second dimension electrophoresis was performed in polyacrylamide (8%) gels using the buffer system of Laemmli (50).

Purification of B6P from Bovine Muzzle Epidermis

Material of desmosome-enriched fractions from bovine muzzle epidermis was solubilized in 5 mM Tris-buffer (pH 8.5) containing 9.0 M urea, 5 mM DTT, and 1 mM PMSF, and incubated for 2 h at 15°C under magnetic stir-

ring. The extract obtained as the supernatant fraction after centrifugation at 20,000 g × 1 h was dialyzed against "chromatography buffer" (8 M urea, 30 mM Tris-HCl, pH 8.5, 2.5 mM DTE, 1 mM PMSF). After clearing by another centrifugation (15,000 g × 30 min) at 20°C, ~50 mg protein of the supernatant fraction was applied to a 20 ml DEAE-cellulose anion exchange column (2 × 6.5 cm DE-62, Whatman Chemical Separation Inc., Clifton, NJ) equilibrated with chromatography buffer at a flow rate of 20 ml/h. The proteins not firmly bound were washed off with the same buffer until the baseline was stable (monitored by absorbance at 280 nm). This protein fraction was further analyzed by Mono S cation exchange chromatography (Pharmacia, Uppsala, Sweden). The bound proteins were then eluted with a linear gradient of guanidinium hydrochloride (0–100 mM) in chromatography buffer. Usually, fractions of 4 ml were collected. The aliquots of the unbound and bound fractions were precipitated with methanol and chloroform (see above) and monitored by SDS-PAGE. Alternatively, polypeptides of the pooled unbound and washed fractions were separated by preparative SDS-PAGE and harvested essentially as described (45). The polypeptides from the acrylamide strips were eluted by overnight diffusion in 5 mM Tris-HCl buffer (pH 7.4) containing 0.05% SDS and 1 mM DTT. The suspensions of protein material were then dialysed and concentrated by vacuum dialysis against 5 mM Tris buffer (pH 7.4) and diluted 1:1 with complete Freunds adjuvant for immunization.

Antibodies

Guinea pig antibodies to plakoglobin and B6P were prepared as follows. The animals were given three injections of B6P either obtained after DEAE-ion exchange chromatography and SDS-PAGE separation or by preparative SDS-PAGE alone. The proteins were emulsified with complete Freunds adjuvant for the first time and with incomplete Freunds adjuvant for booster injections and were administered by subcutaneous injections at three sites (total ~300 µg) with 3-w intervals. Murine monoclonal antibodies to desmoplakin (9), desmoglein (68, 69) and plakoglobin (10) have been described. Monoclonal cytokeratin antibodies K_G8.13 (34), $K_x$panl-8.136 (from Progen Biotechnics, Heidelberg) and IFA antibody (60) were used for immunoblotting tests.

Affinity Purification of Guinea Pig Antibodies to Plakoglobin and B6P

IgG fractions of both sera were prepared either by DEAE-ion exchange chromatography (40) or ammoniumsulfate precipitation. Monospecific antibodies were prepared essentially according to Krohne et al. (48). 15–20 slots of 8% polyacrylamide gels (1.5-mm thick) were loaded either with total proteins of desmosomal fractions or with a B6P-enriched fraction as obtained after DEAE-ion exchange chromatography, and the electrophoretically separated polypeptides were transferred electrophoretically to nitrocellulose paper (BA 85, 0.45 µm pore size; Schleicher & Schuell, Dassel, FRG) according to Kyhse-Andersen (49). Horizontal strips (~2 mm) containing B6P were excised and incubated for 2 h in Tween-PBS-buffer under shaking. Each strip was incubated for 3 h at room temperature in a sealed plastic envelope with 2 ml of diluted sera (1:10 in PBS with 0.1% BSA). The nitrocellulose strips were then washed in PBS three times for 10 min each. For elution of antibodies, the nitrocellulose strips were incubated for 3 min at room temperature in a sealed plastic envelope containing 3 M KSCN in PBS (final pH 7.0). The eluted immunoglobulins were immediately dialysed and concentrated by vacuum dialysis against PBS using collodion bags (model No. SM 13200; Sartorius, Göttingen, FRG), with ten changes of dialysis buffer. Affinity-purified antibodies were either directly used for immunofluorescence microscopy or immunoblotting or were stored at −20°C.

Immunofluorescence Microscopy

Fresh tissue samples of bovine, murine (rat, mouse), *Xenopus laevis*, and human biopsy specimens were snap-frozen in isopentane, cooled in liquid nitrogen, and stored at −70°C. Sections (~5 µm) were prepared with a cryostat (Jung-Reichert, Nussloch, FRG), dried and fixed in acetone at −20°C for 10 min. Primary antibodies were applied for 30 or 45 min, followed by three 5-min washes in PBS for 5 min each. Goat antibodies to guinea pig or to mouse IgGs coupled with Texas Red or fluorescein were used as secondary antibodies (Dianova, Hamburg, FRG), usually at a dilution of 1:150, for 30 min. The slides were rinsed in PBS three times for 5 min each, briefly dipped into water, then in ethanol, and coverslips were mounted in Moviol (Hoechst, Frankfurt, FRG). The sections were examined using epifluorescence illumination with a Zeiss photomicroscope II

(Carl Zeiss, Oberkochen, FRG). For double-label immunofluorescence microscopy both primary antibodies were applied either simultaneously or successively. Cultured cells grown on coverslips were processed for single and double label immunofluorescence microscopy as described (for methodological refs. see 10, 25, 28, 33, 62).

Immunoelectron Microscopy

For postembedding immunolocalization on ultrathin sections conjugates with collodial gold were used. Lowicryl K4M-embedded samples were sectioned and processed essentially (cf. 10, 47, 69). Ultrathin sections were incubated with B6P antibodies overnight at 4°C, followed by three 5-min washes with PBS, and a 1-h reaction with rabbit antibodies to guinea pig IgGs coupled to collodial-gold particles of ~10 nm (Janssen, Beerse, Belgium). After three washes in PBS the sections were conventionally stained with lead citrate and uranyl acetate.

Immunoblotting

Polypeptides separated by electrophoresis were transferred to nitrocellulose paper (see above), and the efficiency of the procedure was controlled by staining of the proteins with Ponceau S (Sigma Chemicals Co., St. Louis, MO). After reduction of nonspecific binding by incubation with 0.05% Tween 20 in PBS for 2 h, the nitrocellulose paper was incubated with the antibodies for at least 2 h, followed by four 20-min washes with Tween-PBS containing either 0.1% Triton X-100 (first wash) or 0.5% Triton X-100 (second wash), 0.5 M NaCl (thrid wash), and finally with Tween-PBS alone. Bound antibodies were detected by incubation of ^{125}I-labeled protein A (Amersham International, Amersham, U.K.) for 2 h followed by washes as described above.

In Vitro Binding of Cytokeratins

The binding reaction of proteins with purified cytokeratins was examined on SDS-PAGE-separated polypeptides after transfer to nitrocellulose paper using the method of Hatzfeld et al. (39). In most experiments, ^{125}I-labeled purified human cytokeratins 8 or 18 (~2 × 10^5 cpm/ml) were used. After incubation in 10 mM Tris-buffer (pH 7.4) containing 1.5% BSA, 0.05% Tween-20, and 140 mM NaCl for 5 h, unspecifically bound proteins were washed off under shaking with Tris-buffer (pH 7.4) containing 1.5 M KCl, 0.5% Triton X-100, 10 mM NaCl for 3 h, with several buffer changes. Specifically bound radioiodinated cytokeratins were autoradiographically detected as used for immunoblotting.

Peptide Map Analysis

Polypeptide spots separated by two-dimensional gel electrophoresis were excised, radio-labeled and digested with trypsin; fragments obtained were analyzed according to the method of Elder et al. (18) with some modifications (67).

Fractionation Studies of Soluble and Particle-Bound B6P from Tissues and Cultured Cells

Bovine muzzle epidermis was minced and homogenized with a Polytron device in various buffers, including physiological buffer A (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM DTT, 1 mM PMSF, 0.5 mM E64) and "buffer B" (2 mM NaHCO$_3$, pH 9.0, 2 mM DTT, and protease inhibitors as in buffer A). The homogenates were centrifuged at 100,000 g × 2 h at 4°C; pellets and supernatant fractions were analyzed separately. Other tissues were processed similarly.

Soluble proteins of BMGE-H monolayer cell cultures were prepared as follows: Physiological buffer A (see above) was used to rinse the monolayer cell cultures three times for 2–3 s each. Then, alternatively, 2 ml either of buffer A with 0.05% Triton X-100 or PBS with 0.05% Triton X-100 were added to each dish. In some experiments, cells were lysed directly in high salt buffer (1.5 M KCl, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM EDTA, 2 mM DTT, and protease inhibitors) as above. Cells were collected with a rubber policeman and manually homogenized by a series of strokes with a Dounce homogenizer. All homogenates were centrifuged; pellets and supernatant fractions were analyzed.

The polypeptides of the pellets and the supernatant fractions from cells and tissues were analyzed for the presence of B6P and plakoglobin by SDS-PAGE and immunoblotting. Alternatively, proteins of the 100,000 g × 2 h supernatant fractions from bovine muzzle epidermis were fraction-
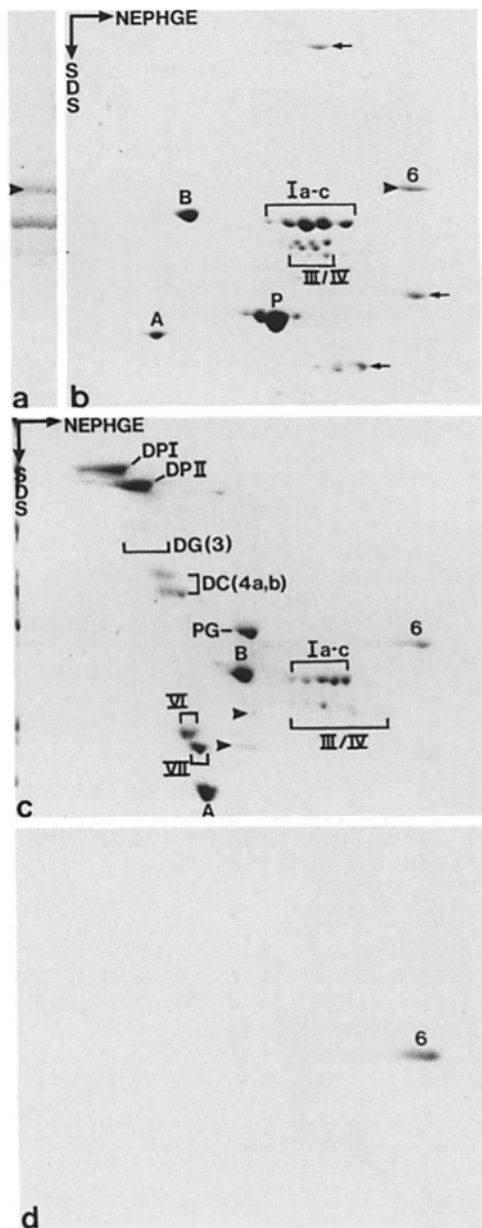
Figure 1. Identification and purification of B6P from bovine muzzle epidermis. (*a*) Coomassie Blue staining of SDS-PAGE-separated polypeptides from the unbound fraction after DEAE cellulose chromatography of desmosomal proteins in buffer containing 8 M urea. Note that B6P is one of the major proteins in this fraction (*arrowhead*). (*b*) Coomassie Blue staining of proteins of a fraction similar to that shown in *a*, separated by two-dimensional gel electrophoresis using nonequilibrium pH gradient electrophoresis (*NEPHGE*) in the first (basic polypeptides to the right) and SDS-PAGE (7.5% acrylamide) in the second dimension. Markers used for co-electrophoresis are rabbit skeletal muscle α-actin (*A*), BSA (*B*), and yeast phosphoglycerokinase (*P*). The horizontal arrows denote three as yet unidentified proteins of this fraction. Arrowhead indicates the position of B6P; cytokeratins I a–c, III and IV are denoted by brackets. (*c*) Coomassie Blue staining of total polypeptides of desmosomal fraction after separation by two-dimensional gel electrophoresis (as in *b*). Major desmosomal proteins are denoted: desmoplakins I and II (*DPI* and *DPII*), desmoglein (*DG[3]*), desmocollins (*DG4a* and *b*), plakoglobin (*PG*) and B6P (6) are denoted (for nomenclature see reference 8). Brackets denote cytokeratins I a–c, III/IV, VI, and VII (for nomenclature see 29, 67). Other symbols as in *b*. (*d*) Autoradiograph corresponding to *c*, showing the specificity of the immunoblot reaction with affinity-purified guinea pig antibodies to B6P.

ated by sucrose gradient centrifugation and analyzed for the presence of B6P by SDS–PAGE and immunoblotting as described (44).

Results

Purification of B6P and Preparation of Antibodies

In our protocol for the enrichment and purification of B6P we took advantage of the basic nature of this protein. Desmosomal proteins solubilized in "buffer B" containing 9 M urea were dialyzed into 8 M urea buffer and separated by ion exchange chromatography. While most of the negatively charged desmosomal proteins were bound to DEAE cellulose, the flow-through and wash fractions contained B6P as one of the major polypeptides, together with the basic (type II) bovine epidermal cytokeratins I–IV (for details see 27, 29, 42) and three as yet unidentified polypeptides (Fig. 1 *a* and *b*). The unbound fraction was then further fractionated by Mono S column chromatography or was directly subjected to preparative SDS–PAGE. B6P was excised, eluted, and processed for immunization in guinea pigs. From the antisera obtained, immunoglobulins were prepared, B6P antibodies were affinity-purified and their specificity was examined by immunoblotting on total epidermal proteins or on proteins of the desmosomal fraction separated by two-dimensional non-equilibrium pH gradient electrophoresis. Only B6P reacted with these antibodies (Fig. 1, *c* and *d*).

Identification of B6P in Tissues and Cultured Cells by Immunoblotting

Using immunoblot analysis of SDS–PAGE-separated polypeptides of desmosomal and cytoskeletal fractions from diverse tissues and cells of different mammalian species we have identified a single polypeptide band of almost identical M_r value of ~75,000 (Figs. 2 and 3). Immunoblotting with plakoglobin antibodies was performed in parallel for control. Strong B6P reactions were noted in all stratified epithelia examined, including bovine muzzle epidermis, cornea, tongue, and esophagus, in normal human epidermis and in wart tissue, in several squamous cell carcinomas such as of the skin, the tongue, the cervix, the esophagus, and the larynx, and in rat tongue mucosa. B6P was also detected in bovine transitional epithelium, i.e., urothelium of bladder, and in bovine trachea, a complex (pseudostratified) epithelium, and in the human cell culture line A-431 derived from an epidermoid carcinoma of the vulva. Simple epithelia examined such as rat and cow liver and intestine, lactating cow's udder, adenocarcinoma of human colon, several cultured epithelial cell lines derived from bovine mammary gland (lines BMGE-H, BMGE+H) or kidney (MDBK), from human mammary gland (MCF-7) and liver (PLC), and from rat liver (MH_1C_1), myocardiac and meningeal tissue were all negative, and so were all nondesmosome bearing tissues and cells examined. The presence of plakoglobin in these cells and tissues was monitored by positive immunoblotting results with plakoglobin antibodies from the same species (Figs. 2 and 3).
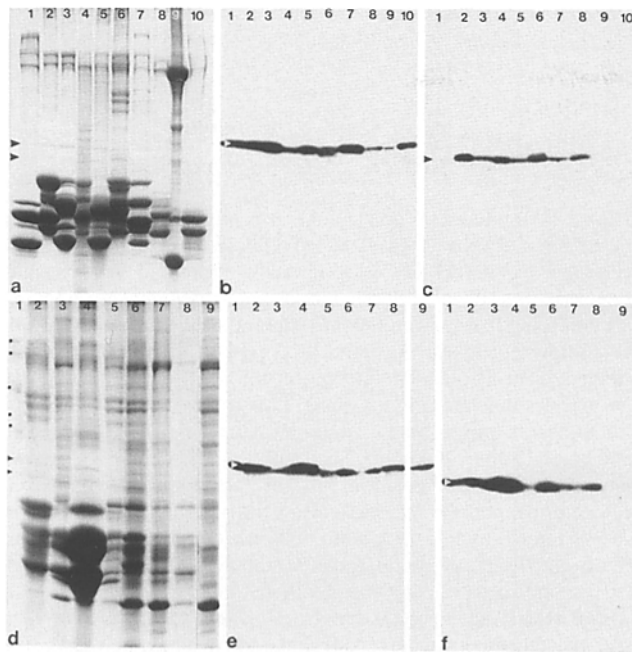
Figure 2. Detection of desmosomal B6P in different tissues and species by SDS–PAGE and immunoblotting. (*a*) Coomassie Blue staining of SDS–PAGE–separated polypeptides of total cytoskeletal fractions from cultured bovine mammary gland epithelial cells of line BMGE-H (lane *1*), bovine muzzle epidermis (lane 2), bovine tongue mucosa (lane *3*), bovine cornea (lane *4*), bovine esophagus (lane *5*), human epidermis (lane *6*), cultured human carcinoma cells of line A-431 (lane *7*), rat tongue mucosa (lane *8*), rat heart (lane *9*) and rat liver (lane *10*). The two horizontal arrowheads on the left margin denote the positions of bands 5 (plakoglobin) and 6 (B6P). (*b* and *c*) Autoradiographs corresponding to *a*, showing the immunoblot reactions with guinea pig antibodies to plakoglobin (*b*) and B6P (*c*). The specific positions of plakoglobin (*b*) and B6P (*c*) are indicated by arrowheads. Note positive reactions of B6P antibodies only in material from stratified epithelia whereas simple epithelia and heart tissue are negative (*c*, lanes 2–8) and that the plakoglobin controls detect their antigen in all tissues and cultured cell lines (*b*). (*d*) Coomassie Blue staining gel of SDS–PAGE–separated polypeptides of the desmosomal fraction from bovine muzzle epidermis (lane *1*), in comparison with cytoskeletal fractions of human tissues such as epidermis (lane 2), squamous cell carcinoma of the tongue (lane *3*), epidermal wart tissue (*verruca vulgaris*, lane *4*), basal cell carcinoma of the skin (lane *5*), cervix carcinoma (lane *6*), squamous cell carcinomas of the esophagus (lane *7*) and of the larynx (lane *8*), and adenocarcinoma of the colon (lane *9*). Arrowheads on left margin as in *a*; dots denote, from top to bottom, positions of desmoplakins I and II, desmoglein (band 3) and desmocollins (bands 4 *a* and *b*). (*e* and *f*) Autoradiographs corresponding to *d*, showing the immunoblot reaction with guinea pig antibodies to plakoglobin (*e*) and to B6P (*f*). The positions of plakoglobin (*e*) and B6P (*f*) are indicated by arrowheads. Note the positive reaction of B6P antibodies in all stratified tissues and tumors derived thereof, whereas the adenocarcinoma of the colon (lane *9*) is negative. In contrast, plakoglobin is positive in all samples.

Figure 3. Identification of desmosomal B6P in various tissues and cell culture lines. The arrowheads indicate the positions of plakoglobin (*upper arrowhead* in *a*, *d* and *g*; *b*, *e* and *h*) and B6P (*lower arrowhead* in *a*, *d*, and *g*; (*c*, *f*, and *i*). (*a*) Coomassie Blue staining of SDS–PAGE–separated polypeptides of bovine muzzle desmosome fraction (as in Fig. 2 *d*, lane *1*), in comparison with cytoskeletal material of bovine liver (lane 2), bovine bladder urothelium (lane *3*), and a desmosome-enriched canalicular plasma membrane fraction from bovine liver (lane *4*). (*b* and *c*) Autoradiographs of immunoblots corresponding to a gel in parallel to *a*, showing reactions with guinea pig antibodies to plakoglobin (*b*) and B6P (*c*). Note that B6P can only be detected in the epidermis and the bladder urothelium but not in the two liver fractions (lanes 2 and 4). (*d*) Coomassie Blue staining of proteins of cytoskeletal fractions from bovine trachea (lane *1*), and rat intestine (lane *3*), in comparison with a desmosomal fraction of bovine muzzle epidermis (lane 2) after separation by SDS–PAGE (the system of reference 78 was used here). Horizontal arrowheads in lane 2 denote the positions of plakoglobin and B6P. (*e* and *f*) Corresponding autoradiographs of immunoblot reactions with guinea pig antibodies to plakoglobin (*e*) and to B6P (*f*). Note the reaction in the complex epithelium of the trachea (lane *1*). Rat intestine (lane *3*) is negative for B6P (*f*) but not for plakoglobin (*e*). (*g*) Coomassie Blue stained gel of proteins of a bovine muzzle desmosomal fraction (as in *a*; lane *1*) and of a cytoskeletal fraction from cultured bovine endothelial cells of line CPAE (lane 2). Arrowheads as in previous figures. (*h* and *i*) Autoradiographs of the corresponding immunoblot reactions with guinea pig antibodies to plakoglobin *h* and to B6P (*i*). Note that B6P is not found in the cytoskeletal fraction of cultured CPAE cells which, however, is strongly positive for plakoglobin.

Co-electrophoresis of B6P from Different Tissues

The results described above suggested that B6P represents a single polypeptide identical in diverse epithelial and cultured cells. To further examine the relationship of this protein in different cell type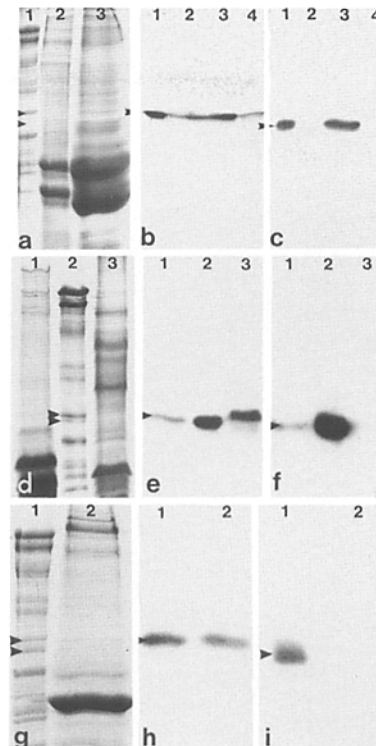s, we separated the proteins of isolated bo-vine muzzle desmosomes and those of cytoskeletal fractions of other cells by two-dimensional co-electrophoresis, followed by immunoblot analysis with the B6P antibodies. Fig. 4, for example, presents the results obtained with cytoskeletal proteins from bovine bladder urothelium and des-
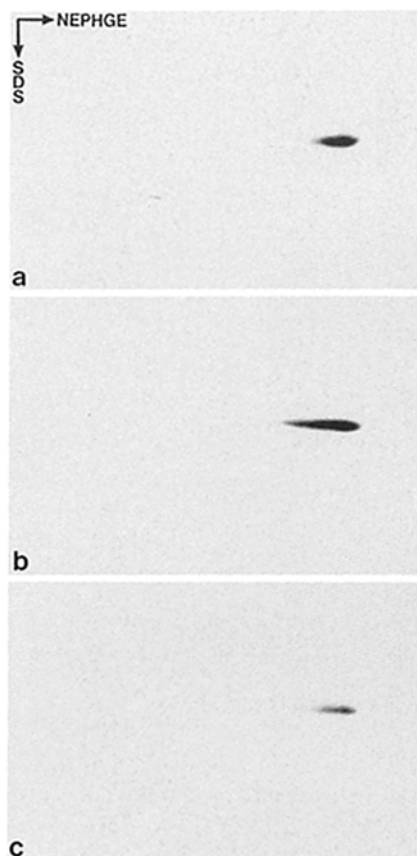
Figure 4. Identification of B6P from different bovine tissues by co-electrophoresis and immunoblotting. Desmosomal proteins from muzzle epidermis obtained after the citric acid method and cytoskeletal proteins from bladder urothelium were separated by two-dimensional gel electrophoresis (as in Fig. 1, *b* and *c*). (*a*) Autoradiograph showing the immunoblot reaction of B6P from bladder urothelium with guinea pig antibodies to B6P. (*b*) Autoradiograph showing the immunoblot reaction of B6P among the polypeptides of a mixture of desmosomal proteins from epidermis (as in Figs. 1 *c* and 4 *c*) and cytoskeletal proteins from urothelium (as in *a*). (*c*) Autoradiograph showing the immunoblot reaction of B6P among the desmosomal proteins from bovine muzzle epidermis.

Figure 5. Immunoblot and cytokeratin binding analyses of desmosomal and other cytoskeletal proteins from various tissues and cell lines, using SDS-PAGE, followed by nitrocellulose paper blotting and reaction with different monoclonal antibodies reacting with a broad range of cytokeratins of different species or with radiolabeled bovine cytokeratins 8 and 18 as probes. (*a*) Coomassie Blue stained, SDS-PAGE-separated polypeptides of crude bovine epidermal desmosome fraction (lane *1*, as in Fig. 3 *a*), cytoskeletal fraction from human breast carcinoma cells of line MCF-7 (lane *2*), and bovine kidney epithelial cells of line MDBK (lane *3*). Arrowhead denotes the B6P position, brackets the region of the epidermal cytokeratins; dots denote cytokeratins 8 (*A*), 18 (*D*) and 19 (in lane *2*) but only 8 and 18 in lane *3*. Asterisk denotes the position of vimentin. (*b-d*) Autoradiographs of immunoblot reactions of gels corresponding to *a* using various monoclonal antibodies reacting with a broad range of cytokeratins: (*b*) K$_s$pan 1-8.136 which reacts with most members of the type II subfamily of cytokeratins, (*c*) IFA-antibody which reacts with members of all classes of proteins, and (*d*) K$_G$ 8.13 which reacts with all members of the type II cytokeratin subfamily as well as with the type I cytokeratin polypeptide 18. None of these antibodies reacts with the B6P component (*arrowhead*) but K$_s$pan 1-8.136 reacts with several epidermal cytokeratins as well as human and bovine cytokeratin 8 (*b*), IFA reacts with all IF proteins in the gel; and K$_G$ 8.13 reacts with all type II cytokeratins in the gel plus human and bovine cytokeratin 18. (*e*) Coomassie Blue stained polypeptides of a crude desmosomal fraction from bovine muzzle epidermis (as in Fig. 3 *a*). Arrowhead denotes B6P, dots on the left margin bovine epidermal cytokeratins I *a-c* and III/IV, dots on the right margin cytokeratins VI and VII (for nomenclature see references 29, 67). (*f*) Autoradiograph corresponding to *e*, after transfer of the proteins to nitrocellulose paper and incubation with ^{125}I-radiolabeled cytokeratin 18 (blot strip 1) and cytokeratin 8 (blot strip 2). Note that cytokeratin 18 has bound to B6P (*arrowhead*) as well as to epidermal cytokeratins I *a-c* and III/IV (lane *1*) whereas cytokeratin 8 has only bound to cytokeratins VI and VII (lane *2*).

mosomal proteins from muzzle epidermis. The immunoblot reaction showed a single component in both urothelium and muzzle epidermis (Fig. 4, *a* and *c*), and these polypeptides comigrated in the co-electrophoresis of a mixture of both fractions (Fig. 4 *b*), indicative of the identity of B6P in both tissues. Identical positions on two-dimensional co-electrophoresis were also found in comparisons of muzzle epidermal B6P with the corresponding protein of bovine cornea, esophagus and tongue (not shown).

Two-dimensional peptide map comparisons of the B6P material obtained after two-dimensional gel electrophoresis have also given very similar, probably identical patterns in the various tissues examined (data not shown; see also 43, 54).

Biochemical Characterization of B6P by Protein and Antibody Binding Assays

B6P is a positively charged protein both in the urea-denatured and in the non-denatured state ("renatured," soluble B6P
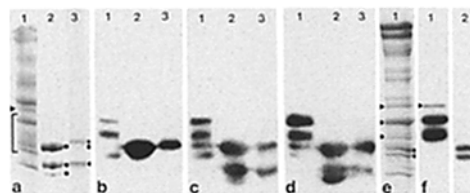
is isoelectric at pH ~8.0 when examined by isoelectric focusing in the absence of denaturing agents; data in ref. 43), similar to the large type II cytokeratins of stratified tissues. Recently, some unusually large (M_r ⩾70,000) type II cytokeratins have been described in certain epidermal locations of the mouse (63). Therefore, we considered the possibility that B6P might be an unusual cytokeratin and subjected the proteins of bovine muzzle desmosomal and several cytoskeletal fractions of human and bovine origin to SDS-PAGE and immunoblotting with diverse broad range cytokeratin antibodies, including K$_G$8.13, K$_s$pan1-8.136 and antibody IFA which reacts with different types of IF proteins from vertebrates and invertebrates (2, 6, 60). The results showed that none of these antibodies reacts with B6P (Fig. 5, *a-d*). Vice versa, antibodies to B6P did not cross-react with cytokeratins (Figs. 2 and 3). Other biochemical criteria such as peptide maps and determinations of amino acid compositions also showed that B6P is not closely related to any of the known cytokeratin polypeptides (data not shown; cf. 26, 43, 45, 54).

The specific binding of a given cytokeratin to other cytokeratins or to non-keratinous proteins, including des-
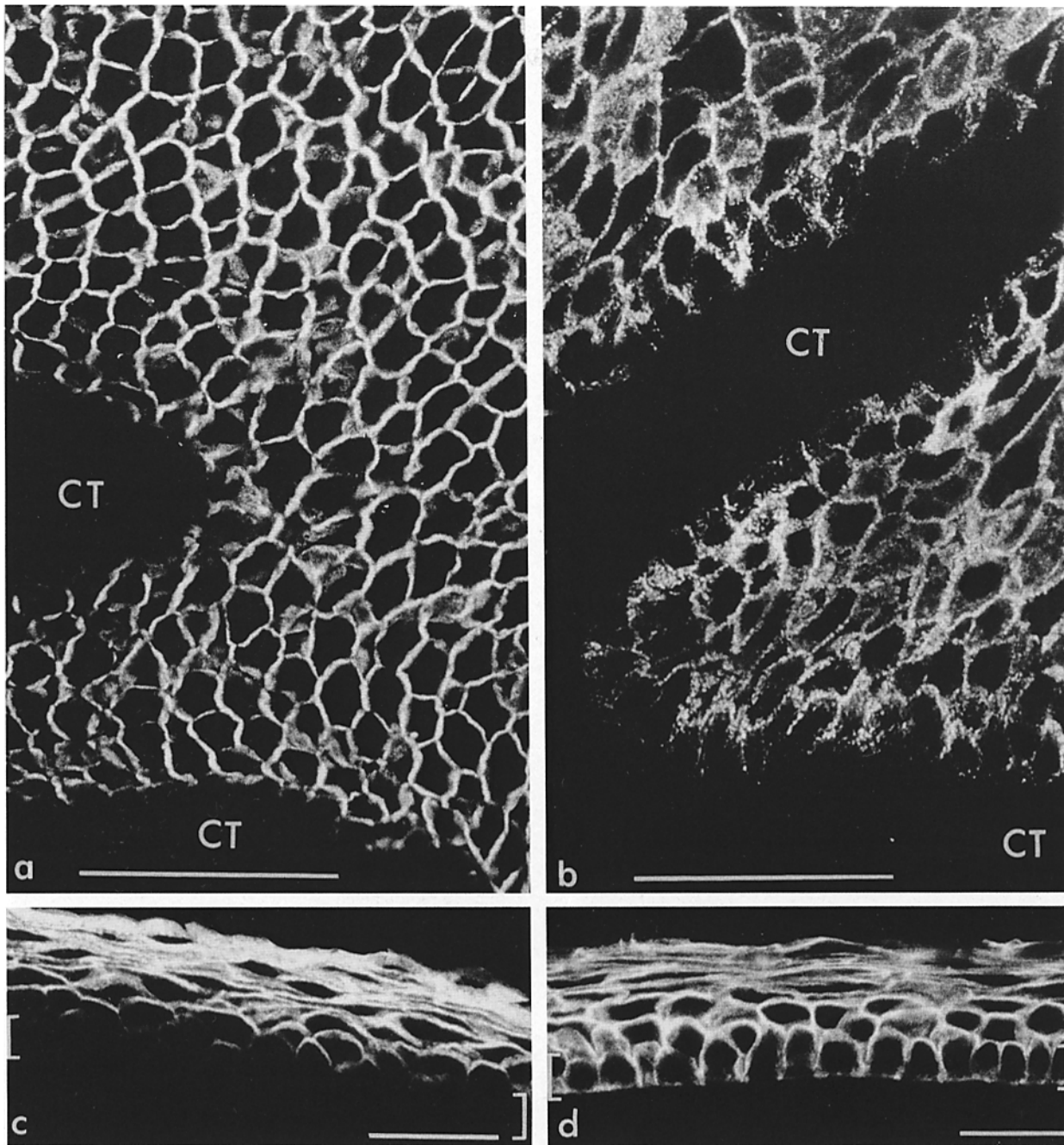
Figure 6. Immunofluorescence microscopy of bovine and rat stratified epithelia with affinity-purified B6P antibodies (*a*, *b*, and *d*) and monoclonal antibody DP1 and 2-2.15 to desmoplakin (*c*). (*a*) Muzzle epidermis, showing intense staining at cell boundaries in the epithelium but not in the connective tissue. (*CT*). (*b*) Tongue mucosa, showing cell boundary staining that is locally resolved into typical punctate desmosomal arrays. (*c*) Rat cornea, demonstrating strong B6P staining of upper layers, whereas the desmosomes of the basal layer (demarcated by brackets) do not show significant immunofluorescence with B6P antibodies. (*d*) Rat cornea, parallel section to that shown in *c*, showing strong and uniform staining of desmoplakin in all layers, i.e., incuding the basal ones (*brackets*). Bars: (*a* and *b*) 100 µm; (*c* and *d*) 25 µm.

mosomal proteins, can be conveniently assayed in nitrocellulose paper blot tests in which SDS–PAGE–separated, blot-transferred polypeptides are incubated with ^{125}I-labeled purified cytokeratin polypeptides (39). Such in vitro binding experiments revealed a specific binding of type I cytokeratins such as cytokeratin 18 to B6P (Fig. 5 *f*, lane *1*), although with a somewhat lower activity than to the type II cytokeratins present in the same blot. When the type II cytokeratin 8 was used in this assay, no significant binding was noticed (Fig.

5 *f*, lane *2*), whereas the bovine epidermal type I cytokeratins band VI and VII, which are equivalent to human cytokeratins 10 and 14 (cf. 53, 67), reacted strongly.

Immunolocalization of B6P in Epithelial Cells at the Light Microscopic Level

The localization and tissue distribution of B6P were examined by immunofluorescence microscopy on sections of fro-
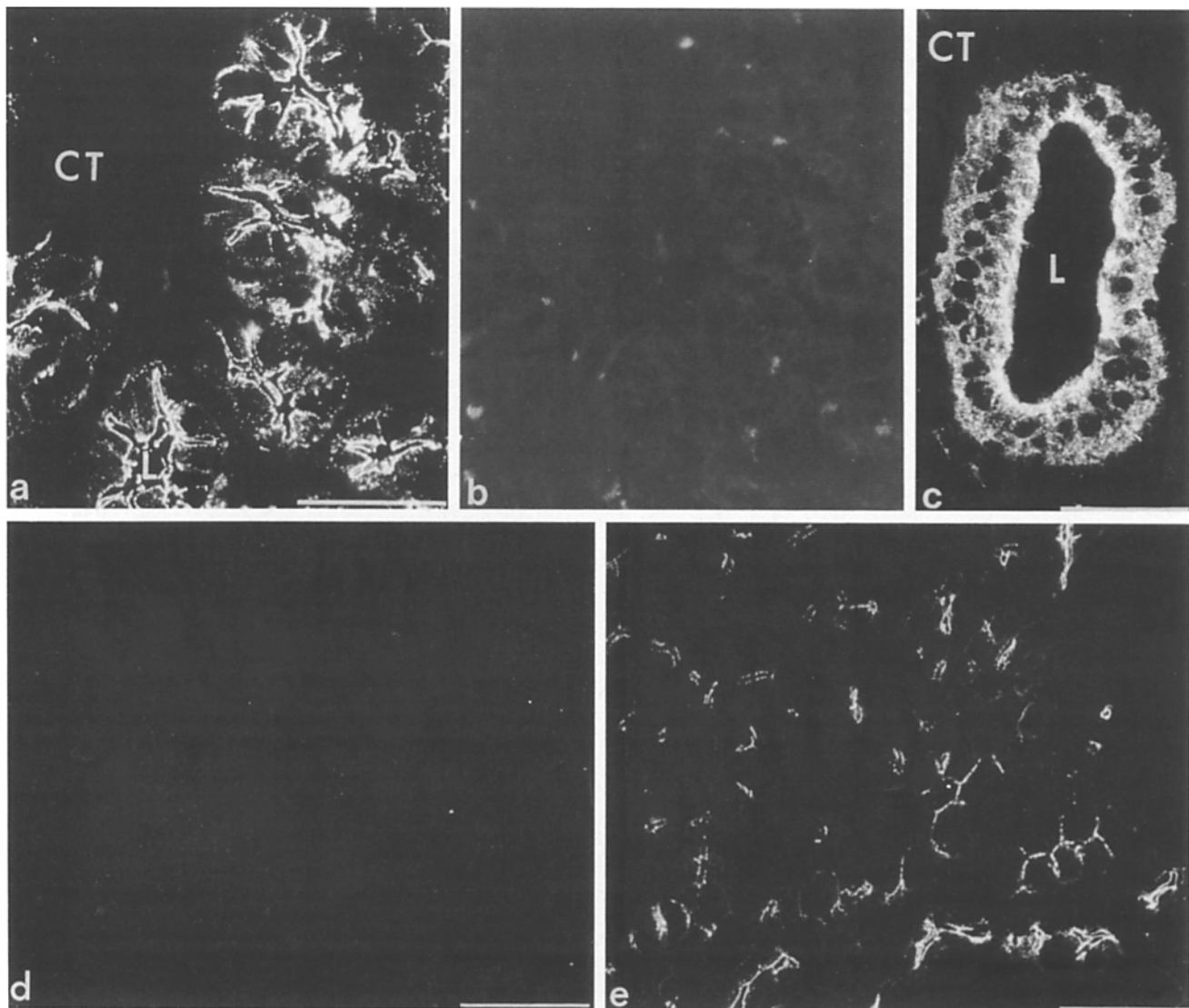
Figure 7. Immunofluorescence micrographs of cryostat sections of bovine muzzle glandular structures and liver tissue, showing the different reactions of antibodies to desmoplakin (*a* and *e*) and B6P (*b, c,* and *d*). (*a* and *b*) Same field shown by double label immunofluorescence of glandular epithelium in bovine muzzle epidermis with murine monoclonal antibody DP1 and 2-2.15 to desmoplakins (*a*) and affinity-purified guinea pig antibodies to B6P (*b*). Note that the glands are negative for B6P but positive for desmoplakin. FITC- (*a*) and Texas Red- (*b*) labeled secondary antibodies were used. *CT,* connective tissue. (*c*) Positive B6P immunostaining of the complex epithelium of a glandular duct (same tissue block as in *a* and *b*). (*d* and *e*) Double immunofluorescence microscopy of bovine liver with affinity purified antibodies to B6P (*d*) and monoclonal antibody DP1 and 2-2.15 to desmoplakin (*e*). Note the typical linear punctate arrays of desmoplakin along the bile canalicular front (*e*) and the absence of reaction of B6P antibodies (*d*). *L,* lumen. Bars, 50 μm.

zen tissues. The typical appearance of the protein is shown in Figs. 6 and 7. In the species used for the isolation of the antigen, i.e. cow, the typical stratified epithelia showed an intense immunostaining along intercellular plasma membranes (Fig. 6, *a* and *b* present results for epidermis and tongue mucosa). At higher magnification and in particularly suitable sections this fluorescence was often resolved into individual "dots" (Fig. 6 *b*), probably representing individual desmosomes, i.e., in a staining pattern similar to other desmosomal antigens (8-10, 24, 25, 28, 69). Intense reactions in linear punctate arrays were also seen on other stratified bovine epithelia such as esophagus and vagina. However, when the B6P immunostaining was compared with the reactions of other desmosomal proteins such as desmoplakin, plakoglobin,

desmoglein and "D1 antigen," the B6P immunostaining was usually less distinct; the reasons for this difference are not known. The plasma membrane bordering the basal lamina, which contains "hemi-desmosomes," was negative (Fig. 6 *b*).

In some of the B6P-positive epithelia the reaction was not homogeneous. For example, in rat cornea (Fig. 6 *c*) strong immunostaining appeared restricted to the upper layers whereas the desmosomes of the basal layer, which were intensely decorated by antibodies to desmoplakin (Fig. 6 *d*) and desmoglein (not shown), did not display significant immunofluorescence with B6P antibodies. In bovine and rat trachea, on the other hand, the small basal cells were intensely stained with B6P antibodies whereas the mucous and ciliated cells showed only a weak reaction, the significance of this was

difficult to assess (data not shown). At present, we do not know whether the selectively negative reaction on basal cells of cornea or on mucinous and ciliated cells of trachea reflects the absence of B6P in these cells or is due to a cell type-specific inaccessibility of the antigen.

No reactions of B6P antibodies were seen in the non-epithelial tissues, including connective tissue, muscles and nerves. However, an absence of B6P staining was also found in the simple epithelium lining the acini of the bovine nasal glands, which were positive for desmoplakin, as shown by double label immunofluorescence microscopy (Fig. 7, a and b). However, the complex-to-stratified epithelia of the larger glandular ducts were positively stained with B6P antibodies (Fig. 7, c).

Positive immunostaining was also observed in complex (pseudostratified) epithelia such as trachea, in the transitional epithelium (urothelium) of the bladder and in the Hassall bodies of the thymus (data not shown here; see reference 43). However, no immunoreaction was observed in all simple epithelia studied such as hepatocytes and bile duct epithelium of liver (Fig. 7, d and e), small intestine and mammary gland (not shown), and calf thymic reticulum epithelial cells. Likewise, myocardium, meninges and dendritic reticulum cells of lymph nodes were all negative (not shown). All these negative tissues, however, contain true desmosomes as demonstrated by positive reactions for other desmosomal marker proteins such as desmoplakin and desmoglein (7-11, 25, 28, 47. 52, 57. 79, 80)

Essentially identical immunofluorescence results were obtained with tissues of other species, specifically human, rat and *Xenopus laevis* (not shown here; some examples are presented in reference 43). Here again positive reactions were only obtained in stratified, complex and transitional epithelia and in the tumors derived from these tissues, including benign ones such as warts. In contrast, simple epithelia and adenocarcinomas, myocardium, and lymph node follicles were negative.

Of the diverse cell culture lines tested by immunofluorescence microscopy, the only cell line revealing a significant, albeit weak punctate reaction at cell boundaries was the human carcinoma line A-431 (not shown; data presented in reference 46).

Immunoelectron Microscopy of B6P

Immunoelectron microscopic studies using affinity-purified antibodies to B6P on ultrathin sections of Lowicryl-embedded bovine muzzle epidermis and tongue mucosa showed specific immunogold labeling in association with desmosomal plaques (Fig. 8). Other cell components, including the interdesmosomal plasma membrane regions and tonofilaments, did not show significant label.

Absence of B6P in Supernatant Fractions

As both methods used, i.e., immunoblotting of cytoskeletal preparations and immunofluorescence microscopy, might tend to select for structure-bound forms of a given protein, we also examined the possibility whether B6P may occur in soluble forms. Therefore, "soluble" proteins as obtained in supernatant fractions and in total cell lysates were analyzed. To this end we fractionated proteins from epithelial tissues and cells, such as bovine snout epidermis (Fig. 9, a and b) and cultured bovine mammary gland cells of line BMGE-H

(Fig. 9, a' and b', and c-e), obtained after homogenization and extraction with buffer of "near-physiological," low or high ionic strengths, followed by centrifugation of 100,000 g × 2 h, and analyzed the extracted and the residual pellet proteins for the presence of B6P by SDS-PAGE and immunoblotting. As shown in Fig. 9, b, b', and e, signals for B6P could not be detected in supernatant fractions. Moreover, after fractionation of the proteins from supernatant fractions by sucrose gradient centrifugation, no B6P reaction was detected by immunoblotting of the fractions (Fig. 9, a and b presents a result obtained from muzzle epidermis). Plakoglobin, a desmosomal protein known to occur in considerable soluble pools (10, 44), was used as a positive control.

Discussion

The antibodies specific for the basic M_r ~75,000 polypeptide found as B6P in desmosome-enriched fractions from bovine muzzle epidermis have allowed us to identify this protein as a desmosome-specific protein and a major component of the desmosomal plaque. This assignment of location has not been possible before because the only antisera reported to react with this protein were "mixed antigen" sera that contained primarily plakoglobin antibodies (7, 11, 15). Our present immunological data also support our previous conclusion (26, 54) that B6P is a genuine protein in its own right that is not derived from, or closely related to, any of the other desmosomal proteins, in contrast to an earlier suggestion of Cowin and Garrod (7).

The observed broad interspecies reactivity of B6P and its near identical relative molecular weight and isoelectric values in different tissues and species suggest that this cytoskeletal protein does not occur in a number of different isoforms and also indicate that it has been highly conserved during vertebrate evolution.

All tissues in which we have detected B6P are stratified epithelia, including the urothelium and the Hassall bodies of thymus, or epithelia of high cell type complexity and architecture in which certain cells are in contact with the basal lamina but do not reach the lumen, such as tracheal epithelium. Remarkably, B6P is missing in a large number of cells that possess true desmosomes with plaques containing plakoglobin, desmoplakin, and desmoglein, including all simple epithelial cells examined, mammary gland alveoli, myocardium, and Purkinje fiber cells of heart, arachnoidal cells of meninges and dendritic reticulum cells of lymphoid follicles and thymus. Correspondingly, B6P has also been found, by immunoblotting assays and immunolocalization techniques, in several tumors derived from stratified epithelia. In contrast, the protein has not been found in a wide range of adenocarcinomas, hepatocellular carcinomas, and breast carcinomas. This suggests that the cell-type-specific expression of B6P is maintained during transformation and malignant growth.

Of the various cell culture lines examined in our study, only one, the human carcinoma cell line A-431, has been found to contain amounts of B6P that are detectable by immunoblotting and, though less clearly, immunofluorescence microscopy. It may be significant that this cell line is derived from a squamous cell carcinoma, i.e., ultimately from a stratified epithelium, whereas all the negative cell lines have originated from simple epithelia. If in future studies the
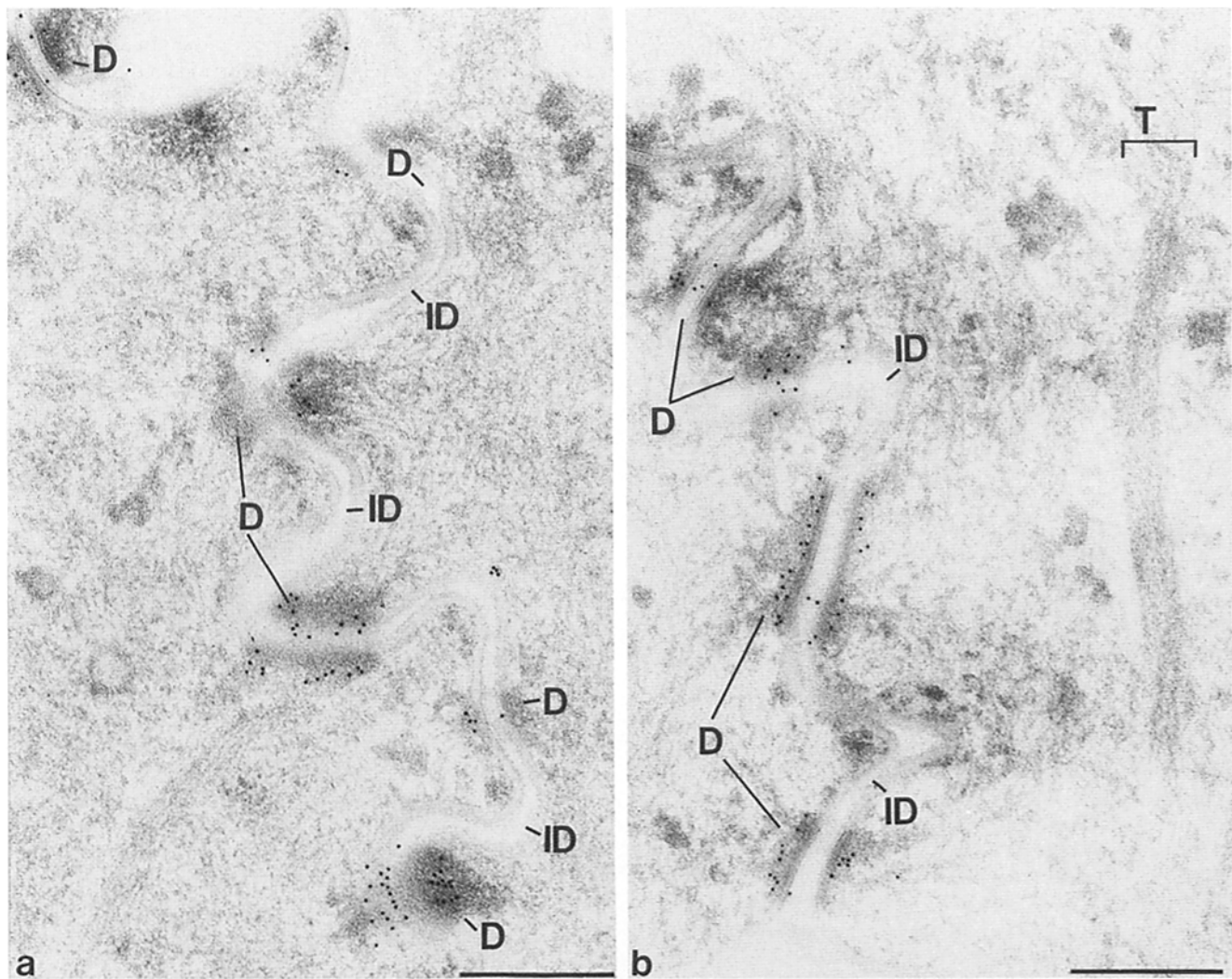
Figure 8. Immunoelectron micrographs showing the reaction of affinity-purified guinea pig antibodies to B6P on ultrathin sections of Lowicryl-embedded bovine tongue mucosa. Goat antibodies to anti–guinea pig immunoglobulins conjugated to collodial gold of 10 nm were used as secondary reagents. Note specificity of the reaction on desmosomes (*D*; oblique and tangential sections are shown in *a*, cross-sections in *b*). Ideal cross-sections show that most gold particles are located on the cytoplasmic plaques of the desmosomes (*b*). Tonofilaments (*T*) and interdesmosomal plasma membrane regions (*ID*) do not show significant labels. Bars, 0.5 μm.

selective presence of B6P could be shown in other stratified epithelia-derived lines, one might be able to conclude that the epithelial subtype specificity of B6P expression can be maintained during cell culturing in vitro.

The expression of B6P is not only different between stratified and simple epithelia but differences have also been observed between various cell layers and cell types in the same epithelium as, for example, in the cornea and the trachea. The reasons for these differences are not yet understood but it is interesting to note that the basal cells intensely immunostained for B6P in the trachea are the same cells which selectively express cytokeratins usually typical for stratified epithelia (3).

Perhaps the most important conclusion from our findings is that the basic B6P is not an obligatory constituent of the desmosomal plaque and of desmosome-forming cells but an accessory plaque component expressed only in stratified epithelia or cells derived there from. Therefore, the function of this protein cannot be a fundamentally desmosomal one but

seems to be related to the special forms of cell–cell interaction and tissue architecture in stratified epithelia or to the plaque-anchorage of the specific cytokeratin IFs that are expressed in stratified and complex epithelia but are usually not found in one-layered, i.e., simple epithelia (27, 53, 61, 77). Indeed, the results of our cytokeratin binding experiments would be consistent with the hypothesis of an involvement of B6P in cytokeratin IF-anchorage at the plaque. However, as cytokeratin 18, i.e., a cytokeratin usually characteristic of simple epithelium (53), also binds to B6P, the interaction of this plaque protein does not seem to be selective for those cytokeratins expressed only in stratified cell systems, and therefore this molecular relationship cannot explain the tissue specificity of B6P expression.

In its restricted pattern of occurrence in stratified epithelia, B6P resembles desmoplakin II which so far has also been unequivocally detected only in stratified epithelia as well as in tumors and cell culture lines derived therefrom (9). However, while desmoplakin II has not yet been shown to be a

genuine, native polypeptide, our identification of B6P as translation product in vitro (26) makes it clear that the expression of B6P in stratified tissues represents a case of the synthesis of a tissue-type junction plaque protein. Interestingly, the association of B6P expression with stratification is also similar to, but not coincident with, the patterns of synthesis of involucrin, a polypeptide of molecular weight 68,000 which is characteristically found in stratified tissues and contributes to the formation of the "cornified envelope" of epidermis (17, 64, 65). Therefore, it will be important to find out how stringently the appearance of B6P, and also of desmoplakin II and involucrin, is linked to the process of stratification, for example, during fetal development of stratified and complex epithelia, during squamous metaplasia in pathologically altered simple epithelia such as obtained after vitamin A-depletion, and in cell cultures changing from monolayer growth to multilayered formations.

Our results show that not all desmosomes are identical in composition but that obligatory (basal) and accessory (cell type-specific) plaque constituents can be distinguished. Clearly, B6P belongs to this latter category. However, cell type-specific desmosomal components may not be confined to plaque proteins as certain cell surface-associated proteins ("cell adhesion molecules") that may occur in association with desmosomes also show cell type-related differences (41).

We and others have previously shown that antibodies against desmosome-specific proteins such as desmoplakin and desmoglein are valuable tools for cell typing in tumor diagnoses, as they provide differentiation markers for certain groups of tumors such as carcinomas and meningiomas (24, 52, 57, 69). The restricted distribution of B6P to stratified and transitional epithelia as well as to certain complex epithelia makes this protein an interesting candidate for a marker allowing a subtyping of carcinomas (for the value of involucrin as a marker for squamous cell carcinomas see, e.g., 66, 84). Future systematic studies on B6P expression in tumors will have to show its diagnostic potential.
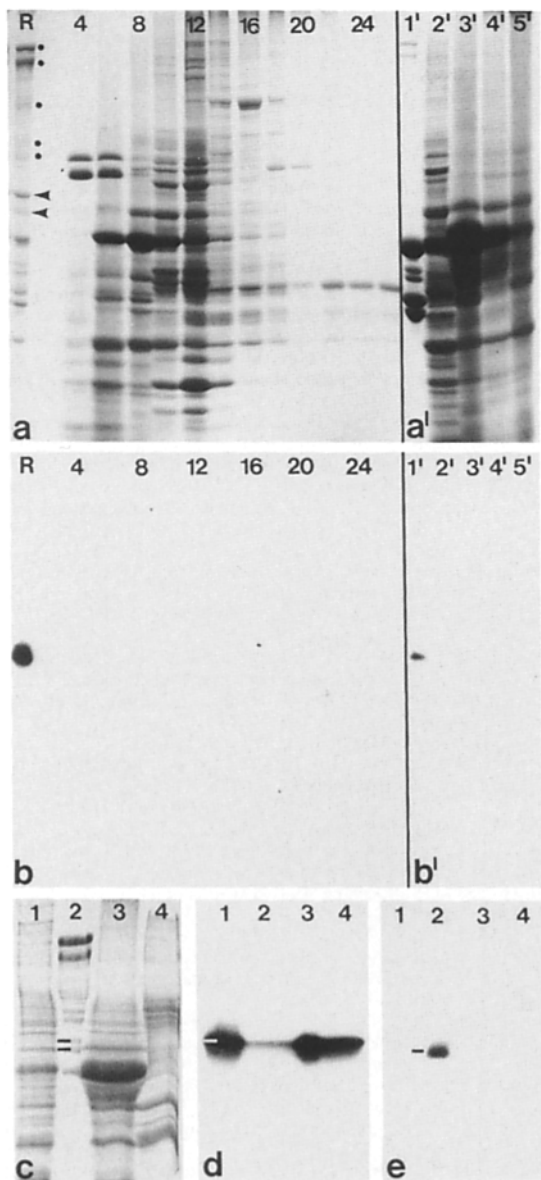
Figure 9. Demonstration of the absence of B6P in soluble protein fractions from various tissues and cell lines, as detectable by immunoblotting. (a) Coomassie Blue staining of SDS-PAGE separated polypeptides present in the 100,000 g × 2 h supernatant fraction obtained after homogenization of bovine muzzle epidermis in buffer of "near-physiological" pH and ionic strength, followed by centrifugation on a 5-30% (sucrose gradient, [wt/vol], fractionation, SDS-PAGE of fractions, and immunoblotting with B6P antibodies. Fractions 2-24 are shown (only some even number fractions are indicated at the top). R, reference lane containing desmosome-enriched fraction (as in Fig. 3 a; symbols as in Fig. 2 d, lane 1). (a') Coomassie Blue staining of SDS-PAGE (same gel as in a)-separated proteins of fractions obtained under different conditions: Lane 1', desmosome-tonofilament-enriched fraction from bovine muzzle epidermis prepared by the "pH 9 method" (54) in comparison with the 100,000 g × 2 h supernatant fraction obtained after homogenization of bovine muzzle epidermis in the same buffer (lane 2'), proteins of the pelletable fraction (100,000 g × 2 h) obtained after homogenization of cultured bovine BMGE-H cells in PBS (lane 3'), proteins of a 100,000 g × 2 h supernatant fraction obtained after homogenization of cultured BMGE-H cells in PBS (lane 4') and proteins of a 100,000 g × 2 h pellet obtained after homogenization of BMGE-H cells in high salt buffer containing Triton X-100 (lane 5'). (b and b')

Autoradiographs corresponding to (a and a'), showing the immunoblot reaction with guinea pig antibodies to B6P. Note that no reaction is recognized in the various soluble fractions as well as in all fractions of cultured BMGE-H cells, in contrast to the positive reactions in the specific control samples (lane R in b and 1' in b'). (c) Coomassie Blue staining of SDS-PAGE-separated polypeptides of different fractions from cultured bovine mammary gland epithelial cells: (lane 1) total cellular protein fraction; (lane 2) desmosomal polypeptides from bovine muzzle epidermis; lane 3, polypeptides of the 100,000 g × 2 h supernatant fraction obtained after homogenization of BMGE-H cells in physiological buffer A and 0.05% Triton X-100; lane 4, polypeptides of the 100,000 g × 2 h pellet, corresponding to the supernatant fraction in lane 3. The positions of plakoglobin (upper) and B6P are denoted by bars. (d and e) Autoradiographs corresponding to c, showing the comparison of the immunoblot reactions with guinea pig antibodies to plakoglobin (d) and B6P (e). Note the negative reaction in all fractions of cultured BMGE-H cells with B6P antibodies (e), as compared with positive plakoglobin reactions in the specific control samples (d; lane 2 in e).

Kapprell et al. An Accessory Desmosomal Plaque Protein

1689

References

1. Achtstätter, T., M. Hatzfeld, R. Quinlan, D. C. Parmelee, and W. W. Franke. 1986. Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. *Meth. Enzymol.* 134:355-371.

2. Bartnik, E., M. Osborn, and K. Weber. 1985. Intermediate filaments in non-neuronal cells of invertebrates: isolation and biochemical characterization of intermediate filaments from the esophageal epithelium of the mullusc *Helix pomatia. J. Cell Biol.* 101:427-440.

3. Blobel, G. A., R. Moll, W. W. Franke, and I. Vogt-Moykopf. 1984. Cytokeratins in normal lung and lung carcinomas. I. Adenocarcinomas, squamous cell carcinomas, and cultured cell lines. *Virchows Arch. (Cell Pathol.)* 45:407-429.

4. Boller, K., D. Vestweber, and R. Kemler. 1985. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. *J. Cell Biol.* 100:327-332.

5. Cohen, S. M., G. Gorbsky, and M. S. Steinberg. 1983. Immunochemical characterization of related families of glycoproteins in desmosomes. *J. Biol. Chem.* 258:2621-2627.

6. Cooper, D., A. Schermer, R. Pruss, T.-T. Sun. 1984. The use of aIF, AE₁, and AE₃ monoclonal antibodies for the identification and classification of mammalian epithelial keratins. *Differentiation.* 28:30-35.

7. Cowin, P., and D. R. Garrod. 1983. Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. *Nature (Lond.)* 302:148-150.

8. Cowin, P., W. W. Franke, C. Gründ, H.-P. Kapprell, and J. Kartenbeck. 1985. The desmosome-intermediate filament complex. *In* The Cell in Contact. G. Edelman, and J.-P. Thiery, editors. John Wiley and Sons, New York. 427-460.

9. Cowin, P., H.-P. Kapprell, and W. W. Franke. 1985. The complement of desmosomal plaque proteins in different cell types. *J. Cell Biol.* 101:1442-1454.

10. Cowin, P., H.-P. Kapprell, W. W. Franke, J. Tamkun, and R. O. Hynes. 1986. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell.* 46:1063-1073.

11. Cowin, P., D. Mattey, and D. Garrod. 1984. Distribution of desmosomal components in the tissues of vertebrates, studied by fluorescence antibody staining. *J. Cell Sci.* 66:119-132.

12. Cowin, P., D. Mattey, and D. Garrod. 1984. Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab'. *J. Cell Sci.* 70:41-60.

13. Damsky, C. H., J. Richa, D. Solter, K. Knudsen, and C. A. Buck. 1983. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell.* 34:455-466.

14. Denk, H., R. Krepler, E. Lackinger, U. Artlieb, and W. W. Franke. 1982. Biochemical and immunocytochemical analysis of the intermediate filament cytoskeleton in human hepatocellular carcinomas and in hepatic enoplastic nodules of mice. *Lab. Invest.* 46:584-596.

15. Docherty, R. J., J. G. Edwards, D. R. Garrod, and D. L. Mattey. 1984. Chick embryonic pigmented retina is one of the group of epithelioid tissues that lack cytokeratins and desmosomes and have intermediate filaments composed of vimentin. *J. Cell Sci.* 71:61-74.

16. Drochmans, P., C. Freudenstein, J.-C. Wanson, L. Laurent, T. W. Keenan, J. Stadler, R. Leloup, and W. W. Franke. 1978. Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis. *J. Cell Biol.* 79:427-443.

17. Eckert, R. L., and H. Green. 1986. Structure and evolution of the human involucrin gene. *Cell.* 46:583-589.

18. Elder, J. H., R. A. Pickett, H. Hampton, and R. A. Lerner. 1977. Radioiodination of proteins in single polyacrylamide gel slices. *J. Biol. Chem.* 252:6510-6515.

19. Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375-412.

20. Franke, W. W., P. Cowin, M. Schmelz, and H.-P. Kapprell. 1987. The desmosomal plaque and the cytoskeleton. *In* Junctional Complexes of Epithelial Cells. Ciba Foundation Symposium 125. G. Bock and S. Clark, editors. John Wiley and Sons, Chichester. 26-48.

21. Franke, W. W., H.-P. Kapprell, and P. Cowin. 1987. Immunolocalization of plakoglobin in endothelial junctions: identification as a special type of zonulae adhaerentes. *Biol. Cell* 59:205-218.

22. Franke, W. W., H.-P. Kapprell, and H. Müller. 1983. Isolation and symmetrical splitting of desmosomal structures in 9 M urea. *Eur. J. Cell Biol.* 32:117-130.

23. Franke, W. W., D. Mayer, E. Schmid, H. Denk, and E. Borenfreund. 1981. Differences of expression of cytoskeletal proteins in cultured rat hepatocytes and hepatoma cells. *Exp. Cell Res.* 134:345-365.

24. Franke, W. W., R. Moll, H. Müller, E. Schmid, C. Kuhn, R. Krepler, U. Artlieb, and H. Denk. 1983. Immunocytochemical identification of epithelium-derived human tumors with antibodies to desmosomal plaque proteins. *Proc. Natl. Acad. Sci. USA.* 80:543-547.

25. Franke, W. W., R. Moll, D. L. Schiller, E. Schmid, J. Kartenbeck, and H. Müller. 1982. Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related. *Differentiation.* 23:115-127.

26. Franke, W. W., H. Müller, S. Mittnacht, H.-P. Kapprell, and J. L. Jorcano. 1983. Significance of two dimensional plaque-associated polypeptides of molecular weights 75000 and 83000. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:2211-2215.

27. Franke, W. W., D. L. Schiller, R. Moll, S. Winter, E. Schmid, I. Engelbrecht, H. Denk, R. Krepler, and B. Platzer. 1981a. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J. Mol. Biol.* 153:933-959.

28. Franke, W. W., E. Schmid, C. Grund, H. Mueller, I. Engelbrecht, R. Moll, J. Stadler, and E.-D. Jarasch. 1981b. Antibodies to high molecular weight polypeptides of desmosomes: specific localization of a class of junctional proteins in cells and tissues. *Differentiation.* 20:217-241.

29. Franke, W. W., K. Weber, M. Osborn, E. Schmid, and C. Freudenstein. 1978. Antibody to prekeratin. Decoration of tonofilament-like arrays in various cells of epithelial character. *Exp. Cell Res.* 116:429-445.

30. Franke, W. W., S. Winter, C. Grund, E. Schmid, D. L. Schiller, and E. D. Jarasch. 1981. Isolation and characterization of desmosome-associated tonofilaments from rat intestinal brush border. *J. Cell Biol.* 90:116-127.

31. Geiger, B., Z. Avnur, T. Volberg, and T. Volk. 1985. Molecular domains of adherens junctions. *In* The Cell in Contact. G. Edelman and J.-P. Thiery, editors. John Wiley and Sons, New York. 461-489.

32. Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscope studies of membrane-microfilament interactions: distribution of α-actinin, tropomyosin and vinculin in intestinal epithelia brush border and in chicken gizzard smooth muscle cells. *J. Cell Biol.* 91: 614-628.

33. Geiger, B., E. Schmid, and W. W. Franke. 1983. Spatial distribution of proteins specific for desmosomes and adhaerens junctions in epithelial cells demonstrated by double immunofluorescence microscopy. *Differentiation.* 23:189-205.

34. Gigi, O., B. Geiger, Z. Eshhar, R. Moll, E. Schmid, S. Winter, D. L. Schiller, and W. W. Franke. 1982. Detection of a cytokeratin determinant common to diverse epithelial cells by a broadly cross-reacting monoclonal antibody. *EMBO (Eur. Mol. Biol. Organ) J.* 1:1429-1437.

35. Giudice, G. J., S. M. Cohen, N. H. Patel, and M. S. Steinberg. 1984. Immunological comparison of desmosomal components from several bovine tissue. *J. Cell Biochem.* 26:35-45.

36. Gorbsky, G., and M. S. Steinberg. 1981. Isolation of the intercellular glycoproteins of desmosomes. *J. Cell Biol.* 90:243-248.

37. Gorbsky, G., S. M. Cohen, M. Shida, G. J. Giudice, and M. S. Steinberg. 1985. Isolation of the non-glycosylated proteins of desmosomes and immunolocalization of a third plaque protein: desmoplakin III. *Proc. Natl. Acad. Sci. USA.* 82:810-814.

38. Gumbiner, B., and K. Simons. 1987. The role of uvomorulin in the formation of epithelial occluding junctions. *In* Junctional Complexes of Epithelial cells, Ciba Foundation Symposium 125. G. Bock and S. Clark, editors. John Wiley and Sons Ltd., Chichester, England. 168-186.

39. Hatzfeld, M., G. Maier, and W. W. Franke. 1987. Cytokeratin domains involved in heterotypic complex formation determined by in-vitro binding assays. *J. Mol. Biol.* 197:237-255.

40. Johnstone, A., and R. Thorpe. 1982. Immunocytochemistry in Practice. Blackwell Scientific Publications, Oxford. 1-298.

41. Jones, J. C. R., K. M. Yokoo, and R. D. Goldman. A cell surface desmosome-associated component: identification of a tissue-specific cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 83:7282-7286.

42. Jorcano, J. L., J. K. Franz, and W. W. Franke. 1984. Amino acid sequence diversity between bovine epidermal cytokeratin polypeptides of the basic (type II) subfamily as determined from cDNA clones. *Differentiation.* 28: 155-163.

43. Kapprell, H.-P. 1987. Identifizierung und Charakterisierung von Plaque-Proteinen der interzellulären Verbindungsstrukturen. Ph.D. Thesis. Faculty of Biology, University of Heidelberg, Federal Republic of Germany.

44. Kapprell, H.-P., P. Cowin, and W. W. Franke. 1987. Biochemical characterization of the soluble form of the junctional plaque protein, plakoglobin, from different cell types. *Eur. J. Biochem.* 166:505-517.

45. Kapprell, H.-P., P. Cowin, W. W. Franke, H. Ponstingl, and H. J. Opferkuch. 1985. Biochemical characterization of desmosomal proteins isolated from bovine muzzle epidermis: amino acid and carbohydrate composition. *Eur. J. Cell Biol.* 36:217-229.

46. Kapprell, H.-P., R. Duden, K. Owaribe, M. Schmelz, and W. W. Franke. 1987. Subplasmalemmal plaques of intercellular junctions: common and distinguishing proteins. *In* The Cell in Contact. G. Edelman, editor. John Wiley and Sons. New York. In press.

47. Kartenbeck, J., K. Schwechheimer, R. Moll, and W. W. Franke. 1984. Attachment of vimentin filaments to desmosomal plaques in human meningiomal cells and arachnoidal tissue. *J. Cell Biol.* 98:1072-1081.

48. Krohne, G., R. Stick, J. A. Kleinschmidt, R. Moll, W. W. Franke, and P. Hausen. 1982. Immunological localization of a major karyoskeletal protein in nucleoli of oocytes and somatic cells of *Xenopus laevis. J. Cell Biol.* 94:749-754.

49. Kyhse-Andersen, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Meth.* 10:203–209.

50. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.

51. Miller, K., D. Mattey, H. Measures, C. Hopkins, and D. Garrod. 1987. Localisation of the protein and glycoprotein components of bovine nasal epithelial desmosomes by immunoelectron microscopy. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:885–889.

52. Moll, R., P. Cowin, H.-P. Kapprell, and W. W. Franke. 1986. Desmosomal proteins: new markers for identification and classification of tumors. *Lab. Invest.* 54:4–25.

53. Moll, R., W. W. Franke, D. L. Schiller, B. Geiger, and R. Krepler. 1982. The catalog of human cytokeratin polypeptides: patterns of expression of specific cytokeratins in normal epithelia, tumors and cultured cells. *Cell.* 31:11–24.

54. Müller, H., and W. W. Franke. 1983. Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. *J. Mol. Biol.* 163:647–671.

55. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133–1142.

56. Ogou, S.-I., C. Yoshida-Noro, and M. Takeichi. 1983. Calcium-dependent cell-cell adhesion molecules common to hepatocytes and teratocarcinoma stem cells. *J. Cell Biol.* 97:944–948.

57. Parrish, E. P., D. R. Garrod, D. L. Mattey, L. Hand, P. V. Steart, and R. O. Weller. 1986. Mouse antisera specific for desmosomal adhesion molecules of suprabasal skin cells, meninges, and meningioma. *Proc. Natl. Acad. Sci. USA.* 83:2657–2661.

58. Penn, E. J., C. Hobson, D. A. Rees, and A. I. Magee. 1987. Structure and assembly of desmosome junctions: biosynthesis, processing and transport of the major protein and glycoprotein components in cultured epithelial cells. *J. Cell Biol.* 105:567–68.

59. Peyriéras, N., F. Hyafil, D. Louvard, H. L. Ploegh, and F. Jacob. 1983. Uvomorulin: a nonintegral membrane protein of early mouse embryo. *Proc. Natl. Acad. Sci. USA.* 80:6274–6277.

60. Pruss, R. M., R. Mirsky, M. C. Raff, R. Thorpe, A. J. Dowding, and B. H. Anderton. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell.* 27:419–428.

61. Quinlan, R. A., D. L. Schiller, M. Hatzfeld, T. Achtstätter, R. Moll, J. L. Jorcano, T. M. Magin, and W. W. Franke. 1985. Patterns of expression and organization of cytokeratin intermediate filaments. *In* Intermediate Filaments. E. Wang, D. Fischman, R. K. H. Liem, and T.-T Sun, editors. *Ann. N. Y. Acad. Sci.* 455:282–306.

62. Ramaekers, F. C. S., M. Osborn, E. Schmid, K. Weber, H. Bloemendal, and W. W. Franke. 1980. Identification of the cytoskeletal proteins in lens-forming cells, a special epithelioid cell type. *Exp. Cell Res.* 127:309–327.

63. Rentrop, M., R. Nischt, B. Knapp, J. Schweizer, and H. Winter. 1987. An unusual type-II 70-kilodalton keratin protein of mouse epidermis exhibiting postnatal body-site specificity and sensitivity to hyperproliferation. *Differentiation.* 34:189–200.

64. Rice, R. H., and H. Green. 1979. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell.* 18:681–694.

65. Rice, R. H., and S. M. Thacher. Involcrin: a constituent of cross-linked envelopes and marker of squamous maturation. 1986. *In* Biology of the Integument. 2. Vertebrates. J. Bereiter-Hahn, A. G. Matoltsy, and K. S. Richards, editors. Springer-Verlag, Heidelberg, Federal Republic of Germany. 752–761.

66. Said, J. W., G. Nash, A. F. Sassoon, I. P. Shintaku, S. Banks-Schlegel. 1983. Involucrin in lung tumors. A specific marker for squamous differentiation. *Lab. Invest.* 49:563–568.

67. Schiller, D. L., W. W. Franke, and B. Geiger. 1982. A subfamily of rela-

tively large and basic cytokeratin polypeptides as defined by peptide mapping is represented by one or several polypeptides in epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:761–769.

68. Schmelz, M., R. Duden, P. Cowin, and W. W. Franke. 1986. A constitutive transmembrane glycoprotein of M_r 165,000 (desmoglein) in epidermal and non-epidermal desmosomes. I. Biochemical identification of the polypeptide. *Eur. J. Cell Biol.* 42:177–183.

69. Schmelz, M., R. Duden, P. Cowin, and W. W. Franke. 1986. A constitutive transmembrane glycoprotein of M_r 165,000 (desmoglein) in epidermal and non-epidermal desmosomes. II. Immunolocalization and microinjection studies. *Eur. J. Cell Biol.* 42:184–199.

70. Schmid, E., D. L. Schiller, C. Grund, J. Stadler, and W. W. Franke. 1983. Tissue type specific expression of intermediate filament proteins in a cultured epithelial cell line from bovine mammary gland. *J. Cell Biol.* 96:37–50.

71. Skerrow, C. J., and A. G. Matoltsy. 1974. Isolation of epidermal desmosomes. *J. Cell Biol.* 63:515–523.

72. Skerrow, C. J., and A. G. Matoltsy. 1974. Chemical characterization of isolated epidermal desmosomes. *J. Cell Biol.* 63:524–531.

73. Staehelin, L. A. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* 39:191–283.

74. Steinberg, M. S., H. Shida, G. J. Giudice, M. Shida, N. H. Patel, and O. W. Blaschuk. 1987. On the molecular organization, diversity and functions of desmosomal proteins. *In* Junctional Complexes of Epithelial Cells. Ciba Foundation Symposium 125. G. Bock and S. Clark, editors. John Wiley and Sons, Chichester. 3–19.

75. Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.

76. Suhrbier, A., and D. Garrod. 1986. An investigation of the molecular components of desmosomes in epithelial cells of five vertebrates. *J. Cell Sci.* 81:223–242.

77. Sun, T.-T., R. Eichner, A. Schermer, D. Cooper, W. G. Nelson, and R. A. Weiss. 1984. Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. *In* Cancer Cells. 1. The Transformed Phenotype. A. Levine, W. Topp, g. Vande Woude, and J. D. Watson, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 169–176.

78. Thomas, J. O., and R. D. Kronberg. 1975. An octamer of histones in chromatin and free solution. *Proc. Natl. Acad. Sci. USA.* 72:2626–2630.

79. Thornell, L.-E., A. Eriksson, B. Johnsson, U. Kjörell, W. W. Franke, I. Virtanen, V.-P. Lehto. 1985. Intermediate filaments and associated proteins in heart Purkinje fibers: a membrane-myofibril anchored cytoskeletal system. *In* Intermediate Filament. E. Wang, D. Fischman, R. K. H. Liem, and T.-T. Sun, editors. The New York Academy of Sciences, New York. Vol. 455.213–240.

80. Tokuyasu, K. T., A. H. Dutton, and S. J. Singer. 1983. Immunoelectron microscopic studies of desmin (skeletin) localization and intermediate filament organization in chicken cardiac muscle. *J. Cell Biol.* 96:1736–1742.

81. Tsukita, S., and S. Tsukita. 1985. Desmocalmin: a calmodulin-binding high molecular weight protein isolated from desmosomes. *J. Cell Biol.* 101:2070–2080.

82. Volk, T., and B. Geiger. 1984. A 135-kd membrane protein of intercellular adherens junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2249–2260.

83. Volk, T., and B. Geiger. 1986. A-CAM: a 135 kd receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. *J. Cell Biol.* 102:1441–1450.

84. Warhol, M. J., J. Roth, J. M. Lucocq, G. S. Pinkus, and R. H. Rice. 1985. Immuno-ultrastructural localization of involucrin in squamous epithelium and cultured keratinocytes. *J. Histochem. Cytochem.* 33:141–149.

85. Wessel, E. F., and U. I. Fluegge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141–143.