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 $M_r$ ~ 75,000 ("band 6 protein") which differs from all other desmosomal proteins so far identified by its positive charge (isoelectric at pH ~ 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 ~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, $\alpha$-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 ~30 nm, which often appears to be bisected...
by a midplate structure, and a pair of cytoplasmic plaques associated with intermediate-sized filaments (IFs). 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 \( M_r \sim 83,000 \)) 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 \( M_r \sim 250,000 \), termed desmoplakin I (8, 9, 24, 25, 36, 54) and a transmembrane glycoprotein of \( M_r \sim 65,000 \) 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 \( M_r \sim 215,000 \) which is immunologically and biochemically very closely related to desmoplakin I (9, 45, 54) and desmocollin, a calmodulin-binding protein of \( M_r \sim 240,000 \) (81). The cell type distribution of another group of glycopolypeptides of \( M_r \sim 130,000 \) and \( 115,000 \) ("bands 4a and 6b"); desmocollins I and II; 7, 11, 12, 35, 57, 58) is less clear.

In addition to these plaque components, a major polypeptide of \( M_r \sim 75,000 \) has been described in desmosomal preparations from bovine epidermis (band 6 polypeptide [B6P]; 26, 28, 54) which is basic (pH \( \sim 8.5 \)) 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 cyto-keratins 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 \( \sim 2.5 \) 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 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 thymus and dermis.

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 x 30 min. The resulting pellet was taken up in 20 mM phosphate buffer (pH 7.5) containing 1 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 x 20 min the pellet was washed twice by suspension in PBS and recentrifugation, and the final pellet was stored frozen at \(-20^\circ C\). 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). Origin and conditions for cell culture growth have been described (25, 28, 36, 54). From observations that this protein is reduced by high salt buffer and Triton X-100 were obtained from these monolayer cultures as previously described (9, 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 (\( \sim 20 \) 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 Al 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 \( \mu \)g/ml of each leupeptin and pepstatin (Boehringer, Mannheim, FBG), 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 x 20 min. The pellet was resuspended in 200 ml of "CASC Bi 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 x 20 min. The upper white layer was removed and resuspended in \( \sim 100 \) ml CASB Bi buffer with a motor-driven, glass-Teflon Potter-Elvehjem device. The pellet obtained after centrifugation at 13,000 g x 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 \(-20^\circ C\).

**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 (54).

**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 PMSE). 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 Separations 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 dialyzed and concentrated by vacuum dialysis against 5 mM Tris buffer (pH 7.4) and diluted 1:1 with complete Freund's 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 antibodies were emulsified with complete Freund's adjuvant for the first time and with incomplete Freund's 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 (30) have been described. Monoclonal cytokeratin antibodies K8.13 (34), K8,pan1-8.13 (from Progen Biotechnik, 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 dialyzed and concentrated by vacuum dialysis against PBS using collodion bags (model No. SM 3200; 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 1:100, 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 labeling immunofluorescence microscopy as described (for methodological refs. see 10, 25, 28, 33, 62).

Immunoelectron Microscopy

For postembedding immunolocalization on ultrathin sections conjugates with colloidal gold were used. Lowicryl K4M-embedded sections 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 colloidal-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

Polyepitopes 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% NaCl (third wash), and finally with Tween-PBS alone. Bound antibodies were detected by incubation of 125I-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 polyepitopes after transfer to nitrocellulose paper using the method of Hatzfeld et al. (39). In most experiments, 125I-labeled purified human cytokeratins 8 or 18 (~2 × 106 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 KCI, 0.5% Triton X-100, 10 mM NaCl for 3 h, with several buffer changes. Specifically bound radioidinated cytokeratins were autoradiographically detected as used for immunoblotting.

Peptide Map Analysis

Polyepitope 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 epithelium 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 PMSE, 0.5 mM E64) and "buffer B" (2 mM NaHCO3, 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 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.

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 epithelium were fraction-
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 coelectrophoresis 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 Iα-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 (DGαa and b), plakoglobin (PG) and B6P (6) are denoted (for nomenclature see reference 8). Brackets denote cytokeratins Iα-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.

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. 1a 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 Mr 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,C1), myocardial 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 reactions 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.

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 types, we separated the proteins of isolated bovine 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-
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 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.

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 (Mr, >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 Kd8,13, Kpan8,13 and antibody IFA which reacts with the B6P component (arrowhead) but Kpan1-8,136 reacts with all type II cytokeratins in the gel plus human and bovine cytokeratin 18. None of these antibodies reacts with the B6P component (arrowhead). (a) Kpan1-8,136 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) Kc 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 Kpan1-8,136 reacts with several epidermal cytokeratins as well as human and bovine cytokeratin 8 (b), IFA reacts with all IF proteins in the gel; and Kc 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 [32P]-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 7) whereas cytokeratin 8 has only bound to cytokeratins VI and VII (lane 2).
mosomonal 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 cytokeratin such as cytokeratin 18 to B6P (Fig. 5f, 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. 5f, 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-
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 epithelial 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 cleft 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).

**Immuno electron 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 x 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 Mr ~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
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 therefrom. 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
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 epithelium prepared by the "pH 9 method" (54) in comparison with the 100,000 g × 2 h supernatant fraction obtained after homogenization of bovine muzzle epithelium in the same buffer (lane 2'), proteins of the pellatable 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')

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.

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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 f 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 epithelium; 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).
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