

Integrin $\alpha 6/\beta 4$ Complex Is Located in Hemidesmosomes, Suggesting a Major Role in Epidermal Cell–Basement Membrane Adhesion

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Abstract. The $\alpha 6/\beta 4$ complex is a member of the integrin family of adhesion receptors. It is found on a variety of epithelial cell types, but is most strongly expressed on stratified squamous epithelia. Fluorescent antibody staining of human epidermis suggests that the $\beta 4$ subunit is strongly localized to the basal region showing a similar distribution to that of the 230-kD bullous pemphigoid antigen. The $\alpha 6$ subunit is also strongly localized to the basal region but in addition is present over the entire surfaces of basal cells and some cells in the immediate suprabasal region. By contrast staining for $\beta 1$, $\alpha 2$, and $\alpha 3$ subunits was very weak basally, but strong on all other surfaces of basal epidermal cells. These results suggest that different integrin complexes play differing roles in cell–cell

and cell–matrix adhesion in the epidermis.

Immunoelectron microscopy showed that the $\alpha 6/\beta 4$ complex at the basal epidermal surface is strongly localized to hemidesmosomes. This result provides the first well-characterized monoclonal antibody markers for hemidesmosomes and suggests that the $\alpha 6/\beta 4$ complex plays a major role in epidermal cell–basement membrane adhesion. We suggest that the cytoplasmic domains of these transmembrane glycoproteins may contribute to the structure of hemidesmosomal plaques. Immunoultrastructural localization of the BP antigen suggests that it may be involved in bridging between hemidesmosomal plaques and keratin intermediate filaments of the cytoskeleton.

THE $\alpha 6/\beta 1$ and $\alpha 6/\beta 4$ integrins are cell surface glycoprotein complexes each containing a presumably identical α subunit and unique β subunits (Sonnenberg et al., 1987, 1988a; Hemler et al., 1988, 1989; Kajiji et al., 1989). Both complexes are members of the superfamily of integrins which mediate cell–cell and cell–matrix interactions (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987). In addition, the $\alpha 6/\beta 1$ complex is part of the very late activation antigen subfamily of integrins, the members of which share a common $\beta 1$ subunit but have different α subunits (Hemler et al., 1987a; Hemler, 1990). The integrin $\alpha 6/\beta 1$ was first identified on platelets (Sonnenberg et al., 1987; Hemler et al., 1988) and since then has been found on a variety of other cell types, including epithelial (tumor) cells (Hemler et al., 1989; Sonnenberg et al., 1990a), macrophages (Shaw et al., 1990), and lymphocytes (Shimizu et al., 1990). Inhibition studies using $\alpha 6$ -specific antibodies have shown that the $\alpha 6/\beta 1$ complex functions as a specific receptor for laminin (Sonnenberg et al.,

1988b). The binding site for $\alpha 6/\beta 1$ has been located on the long arm of laminin in the elastase-derived fragment E8 (Aumailley et al., 1990; Sonnenberg et al., 1990b; Hall et al., 1990). On macrophages and lymphocytes, the $\alpha 6/\beta 1$ integrin appears to mediate cell adhesion to laminin in an activation-dependent manner (Shaw et al., 1990; Shimizu et al., 1990). Cytoskeletal association and phosphorylation of the $\alpha 6$ subunit have been suggested as possible molecular events in this activation (Shaw et al., 1990). The role of the $\alpha 6/\beta 1$ complex as an adhesion receptor is further indicated by the observation that invasion of transformed cells through reconstituted basement membranes could be strongly inhibited by an $\alpha 6$ -specific monoclonal antibody (Dedhar and Saulnier, 1990). That the $\alpha 6/\beta 1$ complex is also important in tissue morphogenesis is shown in a recent report, in which inhibition of kidney development by antibodies against $\alpha 6$ was described (Sorokin et al., 1990).

Complexes of $\alpha 6/\beta 4$ have been found on carcinoma cell lines of diverse type (Sonnenberg et al., 1988a, 1990a; Hem-

ler et al., 1989; Kennel et al., 1989; Kajiji et al., 1989). Some evidence exists that on colon carcinoma cell lines the $\alpha 6/\beta 4$ protein acts as a laminin receptor (Lotz et al., 1990). However, although $\alpha 6/\beta 4$ has been shown to be present on mammary tumor cells, its function as a laminin receptor on these cells has not been demonstrated (Sonnenberg et al., 1990b). Expression of $\alpha 6/\beta 4$ in mouse (Falcioni et al., 1986) and human tumor cells (Kimmel and Carey, 1986) has been associated with metastasis.

Recently, the cDNA for $\beta 4$ has been cloned and sequenced (Suzuki and Naitoh, 1990; Hogervorst et al., 1990). Comparison of the primary structure of this β subunit with other β subunits indicated some common structural features such as homologous cysteine-rich domains and a putative transmembrane segment, but also a unique feature: the presence of an exceptionally long cytoplasmic domain of $\sim 1,000$ amino acids.

In a former study, we reported on the distribution of $\alpha 6$ and $\beta 4$ subunits in adult and neonatal mice (Sonnenberg et al., 1990a). We used immunoperoxidase reactions to demonstrate that nearly all epithelial tissues express both $\alpha 6$ and $\beta 4$ subunits. Expression of the $\alpha 6$ subunit was seen mostly on the basal surface of epithelial cells, but basolateral distributions were also observed. By contrast, the expression of the $\beta 4$ subunit always appears to be confined to the basal surface. Expression of $\alpha 6/\beta 4$, however, is not restricted to epithelial cells, since staining for $\alpha 6$ and $\beta 4$ has also been demonstrated in peripheral nerves. There is also evidence for expression of $\beta 4$ in certain subsets of endothelial cells (Kennel, S. J., V. Godfrey, L. Y. Ch'ang, T. K. Lankford, L. J. Foote, and A. Makkinje, manuscript in preparation).

An interesting discrepancy between previous studies of the distribution of $\alpha 6/\beta 4$ complex is that the complex was not detected in skin by two-site monoclonal antibody assay (Kennel et al., 1986; Falcioni et al., 1988), but by immunoperoxidase staining skin was found to be strongly positive (Sonnenberg et al., 1990a). This suggests that $\alpha 6/\beta 4$ may be present in an insoluble form perhaps as part of a junctional structure associated with the cytoskeleton. In this study we show by immunoelectron microscopy that the $\alpha 6/\beta 4$ complex in basal epidermal cells is localized in hemidesmosomes, suggesting that it plays an important role in epidermal cell adhesion to the basement membrane.

Materials and Methods

Cell Cultures

HBL-100 human normal mammary cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Sonnenberg et al., 1990a). Cells were maintained at 37°C in an atmosphere of 5% CO₂, 95% air, and were subcultured after removal from tissue culture dishes with 0.25% trypsin, 0.1% EDTA in PBS. Suspensions of single keratinocytes were obtained from human foreskin. The upper dermal and epidermal part of the foreskin, cleared of fibrous tissue and fat, was stretched on a sterile filter paper in a petri dish with thermolysin (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO) and incubated overnight at 4°C. The epidermis was gently stripped from the dermis, collected in a tube with 0.25% trypsin (Gibco Laboratories, Grand Island, NY) and incubated for 15 min at 37°C. The cell suspension was filtered through nylon gauze. Cells were seeded in collagen-coated (Vitrogen 100; Collagen Corp., Palo Alto, CA) tissue culture flasks and grown in Modified MCDB 153 (Clonetics Corp., Boulder, CO) supplemented with bovine pituitary extract (70 μ g/ml), epidermal growth factor (10 mg/ml), insulin (5 μ g/ml), hydrocortisone (0.5 μ g/ml; Sigma Chemical

Co.), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (Flow Laboratories Inc., McLean, VA).

Antibodies

Rat mAbs 346-11A, against mouse $\beta 4$ and 439-9B, against human $\beta 4$ have been described (Kennel et al., 1989; Sonnenberg et al., 1990a). Mouse mAb A-1A5 is directed against the common $\beta 1$ subunit of the VLA family of receptors (Hemler et al., 1983). Mouse mAbs TS2/7 (Hemler et al., 1984), 10G11 (Giltay et al., 1989), J143 (Kantor et al., 1987), B-5G10 (Hemler et al., 1987b), and BIE5 (Hall et al., 1990) recognize the individual $\beta 1$ containing integrins, $\alpha 1/\beta 1$, $\alpha 2/\beta 1$, $\alpha 3/\beta 1$, $\alpha 4/\beta 1$, and $\alpha 5/\beta 1$, respectively. Rat mAb GoH3 (Sonnenberg et al., 1986) is directed against the $\alpha 6$ subunit and detects complexes of this subunit with either $\beta 1$ ($\alpha 6/\beta 1$) or $\beta 4$ ($\alpha 6/\beta 4$). The anti-human $\beta 4$ monoclonal antibody 5B5 was raised by immunization of mice with amnion epithelial cells freshly released from term placental membranes by trypsin treatment (Aplin et al., 1984). Screening of the resulting hybridomas was carried out initially by immunofluorescence using tissue sections. Epitopes were selected that were located exclusively at the basal surface of the epithelium. Bullous pemphigoid serum that had been shown by Western blotting to recognize the 230kd bullous pemphigoid antigen (Stanley et al., 1981) was kindly provided by Dr. F. Wojnarowski (Department of Dermatology, Slade Hospital, Oxford, UK). 5 nm gold-conjugated goat anti-rat IgG was purchased from Janssen Pharmaceutica (Beerse, Belgium). 9 nm gold-conjugated protein A was kindly provided by Dr. G. Griffith (European Molecular Biological Laboratory, Heidelberg, Germany).

Immunoelectron Microscopy

Immunoperoxidase Reaction Used in Electron Microscopy. 20- μ m cryostat sections of human skin were incubated with the integrin-specific antibodies (ascites dilution, 1:100; 1 h) and the corresponding peroxidase-conjugated second antibodies (diluted 1:50; 30 min), fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 15 min, followed by the nickel-modified DAB reaction with cobalt chloride enhancement as described by Green et al. (1989). After washing, the sections were dehydrated and embedded in a mixture of LX112 and Araldite 502. Unstained and stained sections were examined with a Philips electron microscope, model CM10.

Immunogold Labeling for Electron Microscopy. Human tongue was fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, dehydrated and embedded in LR White resin (Agar; Scientific Ltd., Essex, UK). Thin sections were incubated at room temperature with rat mAb 439-9B (ascites dilution 1:50; 2 h) and GARaG⁵ (diluted 1:40, 1 h) and stained with uranyl acetate and lead citrate. Mouse skin was fixed as described above. Ultrathin frozen sections were incubated with rat mAb 346-11A (ascites dilution 1:100; 1 h), followed by incubation with GARaG⁵ (1 h). After immunolabeling the sections were stained with uranyl acetate.

Ultrathin Frozen Section Technique. Human breast skin excised at mastectomy was dissected into 1 mm³ and impregnated with 2.3 M sucrose for 20 min before mounting on a copper stub and freezing with liquid nitrogen. Sections were cut at 100–200 nm using a RMCFS100/MT-6000 ultracyrotome (Research & Manufacturing Co., Inc., Tucson, AZ) and then transferred to Formvar film on carbon-coated 100 mesh copper grids. Blocking was achieved by incubating grids on drops of HBSS containing 5% FBS (HBSS/FBS) for 30 min at 4°C. This was followed by incubation for 30 min at 4°C in 5- μ l drops of monoclonal antibody at dilutions in the range neat to 1:20 with HBSS/FBS. Grids were then washed three times for 5 min each in HBSS containing 1% BSA (HBSS/BSA), and fixed in 4% paraformaldehyde in HBSS for 20 min at room temperature. After three 5-min washes in HBSS/BSA, grids were incubated for 30 min at room temperature in either anti-mouse IgG (Sigma Chemical Co.) or anti-rat IgG (DAKO Corp., Santa Barbara, CA) as appropriate and diluted 1:50 with HBSS/FBS. After further washing three times for 5 min in HBSS/BSA the grids were incubated with 9-nm gold-conjugated protein A (kindly supplied by Dr. G. Griffith) diluted 1:50 in HBSS/FBS for 30 min at room temperature. Alternatively sections were treated with bullous pemphigoid serum at a dilution of 1:10 in HBSS/FBS and then incubated with protein A-gold after paraformaldehyde fixation. A final washing in HBSS/BSA for three times for 5 min and four times for 1 min in distilled water was followed by an embedding/contrast step using 2% methyl cellulose/3% uranyl acetate 1:9 for 10 min on ice. Each grid was then filter paper drained of excess liquid and allowed to air dry for 20 min before sections were viewed and photographed in a Phillips EM 201.

Immunogold Labeling of Epidermal Cell Sheets. Human breast skin excised at mastectomy was rinsed in cold HBSS and dissected to obtain the epidermis with a small amount of underlying dermis. The epidermis was then detached as a sheet by collagenase treatment as follows. Strips of dissected skin (1 cm × 1 mm) were incubated in collagenase (Clostridiopeptidase A; EC 3.4.24.3; Sigma C-2139; Sigma Chemical Co.) to 160 U/ml in HBSS at 37°C for 30 to 60 min. After digestion the tissue fragments were transferred to HBSS containing 10% FBS at room temperature and the epidermis peeled away from the underlying stroma with fine forceps. In some experiments chromatographically purified collagenase (Sigma C-0773; Type VII; Sigma Chemical Co.) was used with similar results. Epidermal sheets were incubated in PBS (pH 7.4) containing 1% BSA (PBS/BSA) for 30 min at room temperature. This was followed by incubation for 1 h at room temperature in monoclonal antibody at dilutions of 0–1:200 with PBS plus 5% FBS (PBS/FBS). After three 5-min washes with PBS/BSA epidermal sheets were incubated with either anti-mouse IgG or anti-rat IgG as appropriate diluted 1:50 with PBS/FBS for 45 min. After further washing the sheets were incubated with 9-nm gold-conjugated protein A. Samples were then washed three times for 10 min in PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for a minimum of 2 h. Washing with cacodylate buffer was followed by post fixation in 1% osmium tetroxide, rinsing in distilled water, dehydrated through graded alcohols and dried acetone, and embedding in Spurr resin. Ultrathin sections were viewed and photographed in a Philips EM201 either unstained or after staining with uranyl acetate and lead citrate.

Labeling and Immunoprecipitation of Integrins

Cells were surface labeled with ^{125}I using lactoperoxidase (Sonnenberg et al., 1988a) and lysed with a buffer containing 1% NP-40, 100 mM NaCl, 4 mM EDTA, and 25 mM Tris-HCl, pH 7.5. The lysates were clarified by centrifugation at 15,000 rpm and precleared by incubation with protein A-Sepharose (Pharmacia, Uppsala, Sweden). After incubation with antibodies against integrin subunits, immunocomplexes were collected with protein A-Sepharose. Affinity-purified rabbit anti-rat IgG was included in immunoprecipitations involving the rat monoclonal antibodies BIE5, GoH3, and 439-9B. Immunoprecipitated material was analyzed by SDS-PAGE.

Results

Antibody Characterization

To characterize antibodies against integrin $\alpha 6$, $\beta 1$, and $\beta 4$ subunits, immunoprecipitations were performed from detergent extracts of ^{125}I surface-labeled HBL-100 cells. Fig. 1, lane 1 shows that antibody A-1A5 immunoprecipitated the $\beta 1$ subunit with associated α subunits, consistent with previous data that this antibody binds to the $\beta 1$ subunit (Hemler et al., 1983). Antibody GoH3 to integrin $\alpha 6$ immunoprecipitated the $\alpha 6$ subunit together with $\beta 1$ and $\beta 4$ subunits (Fig. 1, lane 2); the relative intensities suggest that this cell line expresses $\alpha 6/\beta 4$ strongly and $\alpha 6/\beta 1$ weakly (Sonnenberg et al., 1990a). Fig. 1, lane 3 shows that antibody 439-9B to the $\beta 4$ subunit (Falcioni et al., 1988) immunoprecipitates $\alpha 6/\beta 4$, and lane 4 shows that antibody 5B5 also precipitates $\alpha 6/\beta 4$. The 5B5 epitope is absent from the surface of platelets (which contain $\alpha 6\beta 1$ but not $\alpha 6\beta 4$; data not shown), indicating that 5B5 recognizes a $\beta 4$ -associated epitope.

Immunofluorescence and Immunoelectron Microscopic Analysis of Integrins in Human Skin

Immunofluorescent staining of cryostat sections of normal human breast skin with antibodies against $\beta 4$ antigen showed an apparently continuous line of reactivity along the basal surface of the basal epidermal cells (Fig. 2 a). A similar appearance of continuous linear basal reactivity was obtained by staining with bullous pemphigoid serum (Fig. 2 b) and with anti-laminin antibody (not shown). This indicates that all three of these antigens are strongly localized to the basal

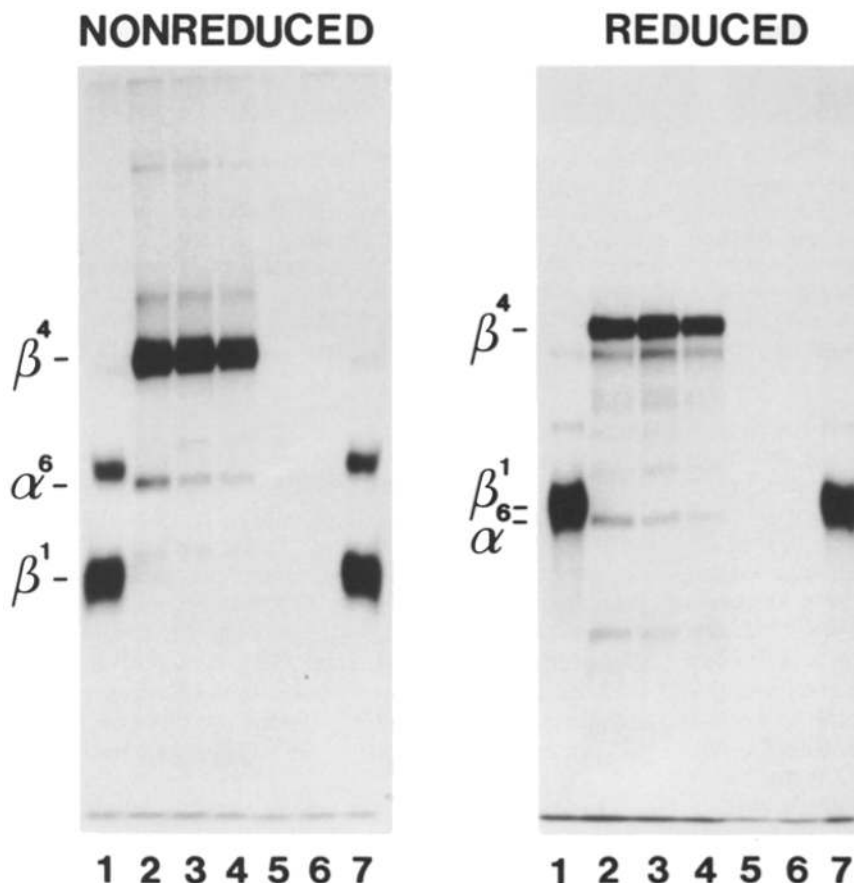


Figure 1. Immunoprecipitation of integrins from the normal human mammary cell line HBL-100. The procedure, which used detergent extracts from surface radioiodinated cells, was as described previously (Sonnenberg et al., 1990a). The following monoclonal antibodies were used: lanes 1 and 7, anti- $\beta 1$ (A-1A5); lane 2, anti- $\alpha 6$ (GoH3); lane 3, anti- $\beta 4$ (439-9B); lane 4, anti- $\beta 4$ (5B5); lanes 5 and 6, control monoclonal antibodies. Samples were run on 5% polyacrylamide gels in the presence of SDS under nonreducing (left) or reducing (right) conditions.

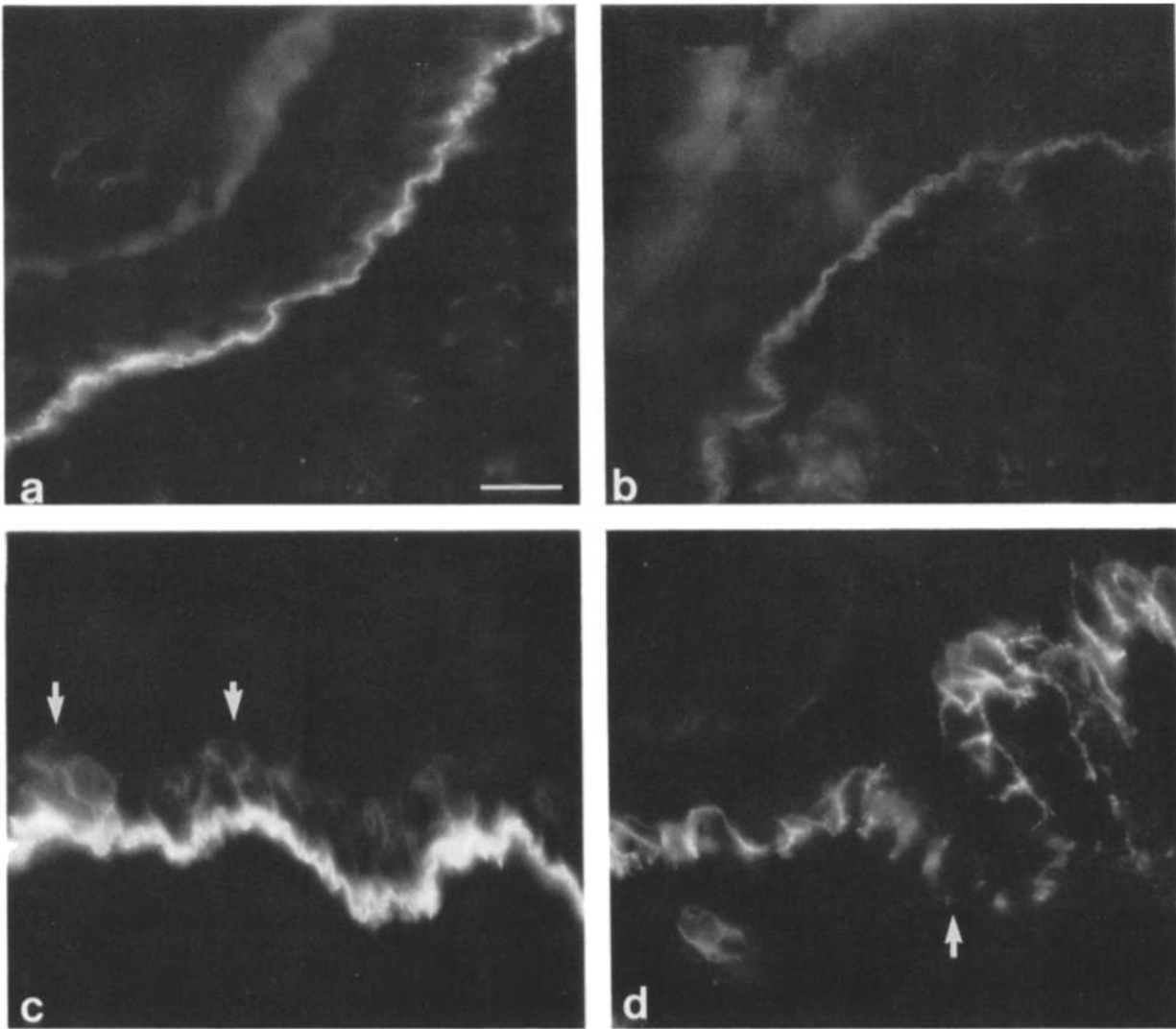


Figure 2. Fluorescent staining of cryostat sections of human breast skin with (a) anti- $\beta 4$ (5B5), (b) BP serum, (c) anti- $\alpha 6$ (GoH3), and (d) anti- $\beta 1$ (A-1A5). Anti- $\beta 4$ gives strong staining of the basal region of the basal epidermal cells (a), which at the light microscope level appears similar in distribution to the staining given by BP serum (b). Anti- $\alpha 6$ also gives strong staining of the basal region, but, in addition, gives weaker but positive staining of the lateral and apical surfaces of the basal cells and some suprabasal cells (arrows in c). It is shown below that all three of these antigens are associated with hemidesmosomes. That the staining for these antigens appears continuous in the basal regions in these fluorescence micrographs probably reflects the extremely high density of hemidesmosomes. In contrast with the other three antigens, $\beta 1$ is located principally on the apical and lateral surfaces of basal cells, sometimes with an apparently punctate distribution (d). Sparse punctate staining of the basal surfaces of basal cells can sometimes be seen (arrow in d). Bar, 20 μm .

epidermal-basement membrane region. Staining with antibody to the $\alpha 6$ integrin subunit also gave intense staining of the basal epidermal surface but, in addition, outlined the entire surfaces of basal cells as well as cells in the immediate suprabasal region (Fig. 2 c). Antibody to the $\beta 1$ subunit gave strong staining of the cell-cell contacts of basal keratinocytes and weaker positive staining of cells in the immediate suprabasal layers (Fig. 2 d). Staining of the cell-matrix contacts with this antibody was weakly positive and quite distinct from the strong continuous staining obtained for $\beta 4$, $\alpha 6$, and bullous pemphigoid antigen. The $\alpha 2$ and $\alpha 3$ subunits were found to have similar distribution to $\beta 1$, that is prominent in cell-cell contacts but scarcely found in contact with the basement membrane (not shown).

Ultrastructural localization of the integrin $\alpha 3$, $\alpha 6$, and $\beta 1$

subunits was possible by using the enhanced immunoperoxidase reaction (see Materials and Methods), whereas localization of $\beta 4$ could also be demonstrated by immunogold labeling of fixed tissue sections. The ultrastructural immunoperoxidase staining of human skin confirmed the localization of the $\alpha 6$ and $\beta 4$ along the basal surfaces of basal epidermal cells (Fig. 3, a and b). The $\alpha 3$ and $\beta 1$ subunits were detected on surfaces of basal cells that were in contact with other cells, but were scarcely found on those in contact with the basement membrane (Fig. 3, c and d). Thus, the $\beta 1$ - and $\beta 4$ -containing integrins on basal cells appeared to be quite distinct.

The basal epithelial cells are attached to the basement membrane by numerous hemidesmosomes. The ultrastructural appearance of these hemidesmosomes is shown in a

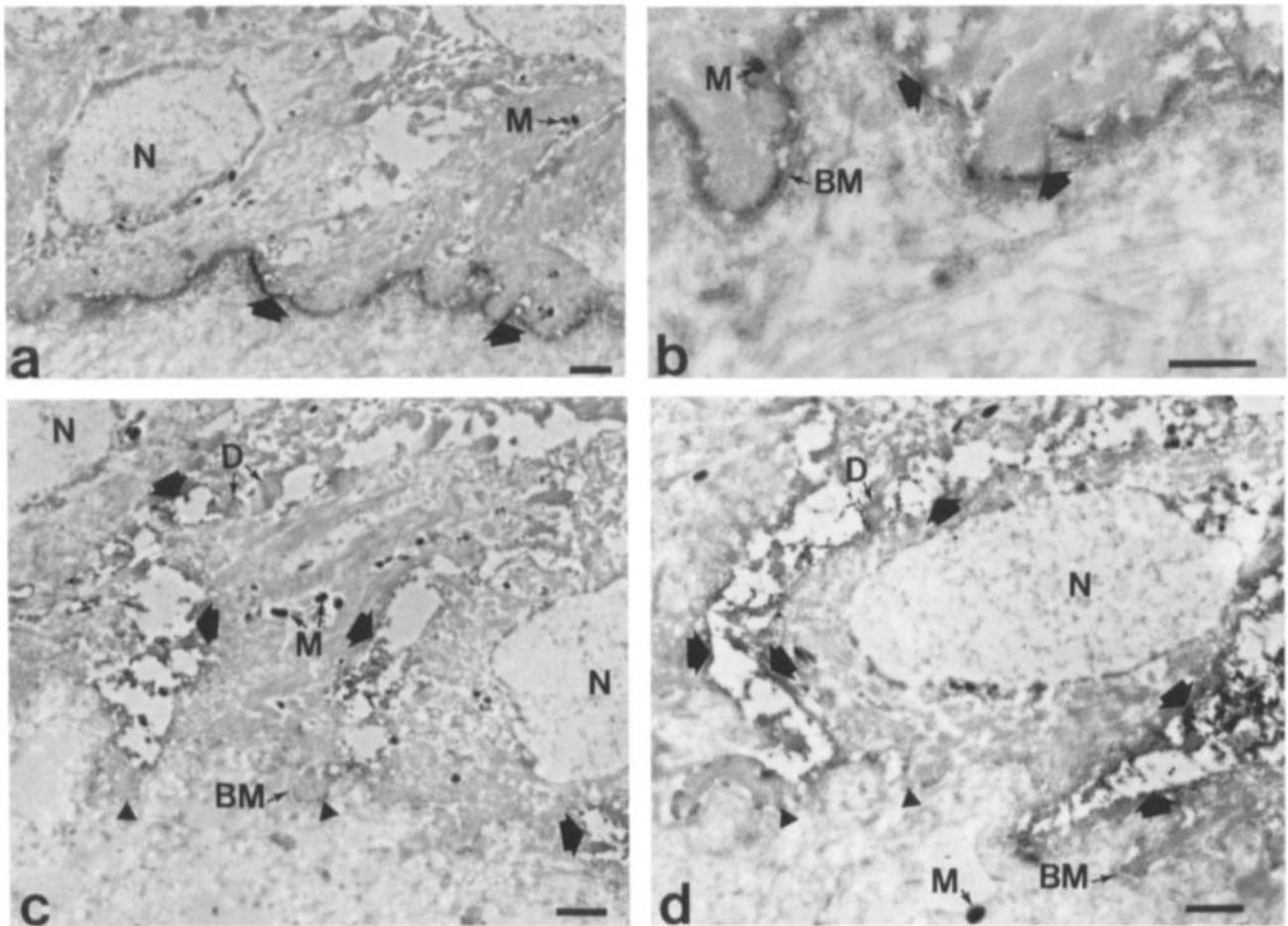


Figure 3. Ultrathin sections of human skin incubated with mAbs (a) anti- $\alpha 6$ (GoH3); (b) anti- $\beta 4$ (439-9B); (c) anti- $\alpha 3$ (J143); (d) anti- $\beta 1$ (A-1A5), and peroxidase conjugate. The electron micrographs show the basal cells of the epidermis lying on the basement membrane (BM). In a and b the peroxidase reaction product (arrows) is seen on the surface of the cells in contact with the basement membrane. In c and d reaction (arrows) is seen on the lateral cell surface. The surface in contact with the basement membrane is unstained. Hemidesmosomes (arrowheads) are clearly visible. N, nucleus; D, desmosomes; M, melanin. Bars, 1,000 nm.

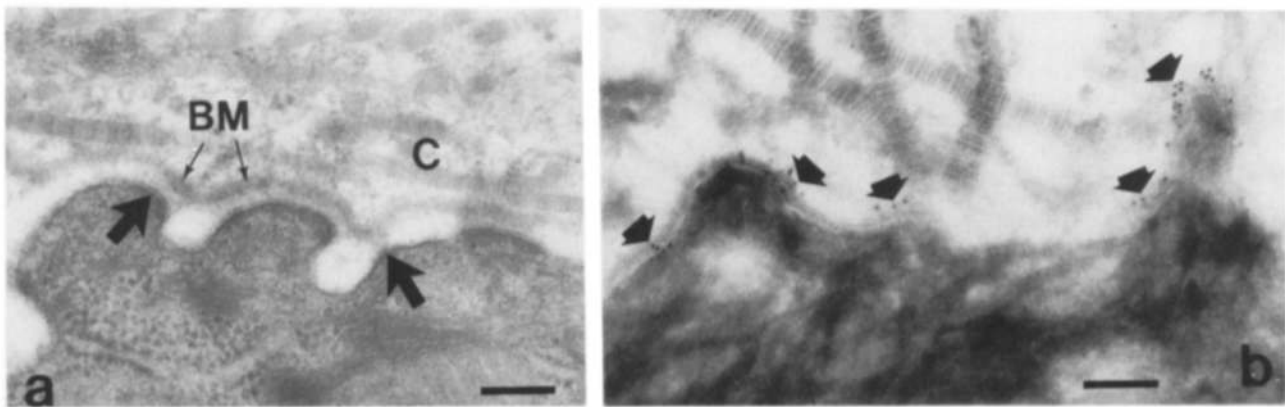


Figure 4. (a) Thin section of mouse ear skin fixed with 2.5% glutaraldehyde and 1% OsO₄ and embedded in a mixture of LX112 and Araldite. An area of a basal cell and the underlying connective tissue is shown with hemidesmosomes consisting of an electron dense plaque (arrows) underneath the plasma membrane; a basement membrane (BM) is shown immediately underlying the hemidesmosome and the connective tissue of the dermis with collagen fibrils (C). (b) Ultrathin cryosection of mouse ear skin incubated with anti- $\beta 4$ (346-11A) and gold-conjugated second antibody. All hemidesmosomes are labeled (arrows). Bars: (a) 200 nm; (b) 100 nm.

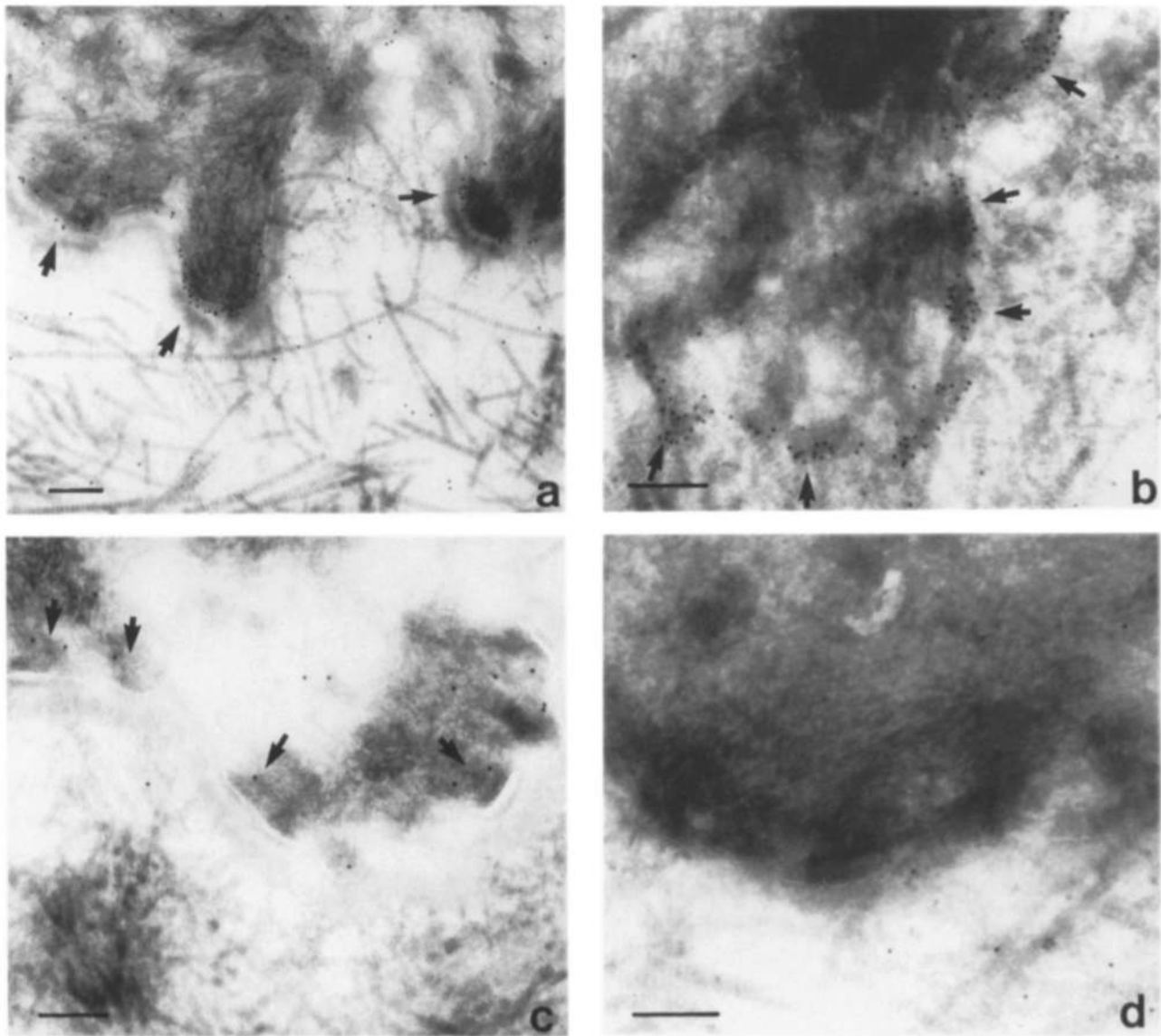


Figure 5. Protein A-gold staining of ultrathin cryostat sections of human breast skin with (a) anti- $\alpha 6$ (GoH3), (b) anti- $\beta 4$ (5B5), and (c) BP serum. The skin sample was not fixed before freezing, sectioning and staining because the epitopes recognized by the monoclonal antibodies are sensitive to paraformaldehyde treatment. We always experience some nonspecific background staining with this technique as indicated in the control (d), which was incubated with complete hybridoma culture medium, followed by anti-mouse Ig and protein A-gold. (a) Concentration of labeling for $\alpha 6$ at the extracellular regions of hemidesmosomes in the region of the lamina lucida (arrows). (b) Partly tangential section showing strong association of $\beta 4$ staining with hemidesmosomes (arrows). (c) Staining with BP serum with the inner plaque region of hemidesmosomes (arrows). The staining is not intense but is comparable both in intensity and location with that found in previous studies (see text). Photographs are at different magnifications; Bars, 200 nm.

thin section of mouse ear skin (Fig. 4 a) where the hemidesmosomal plaques, close to the basal plasma membrane, are indicated. Immunogold staining, using ultrathin cryostat sections of mouse ear skin showed strong staining of the hemidesmosomes with anti- $\beta 4$, the cell surface in between remaining unstained (Fig. 4 b). The basement membrane cannot be distinguished in this section.

Localization of $\alpha 6$ and $\beta 4$ as well as bullous pemphigoid antigen in the basal region of keratinocytes was also carried out on ultrathin frozen sections of human breast skin. The $\alpha 6$ subunit was strongly localized to hemidesmosomes by this technique. The majority of gold particles were located

at the external face of the hemidesmosomal plasma membrane in the lamina lucida region of the basement membrane (Fig. 5 a). $\beta 4$ subunit also showed hemidesmosomal localization and was especially intensely labeled in sections where the hemidesmosomes were cut obliquely (Fig. 5 b), presumably because this allowed easier access to the labeling reagents. Bullous pemphigoid antigen was strongly localized to the basal cytoplasm of the basal keratinocytes (Fig. 5 c). Rather than being directly associated with hemidesmosomal plaques that are close to the plasma membrane, the labeling appeared to be between the plaques and the so-called inner plaques where the intermediate filaments insert (see Tanaka

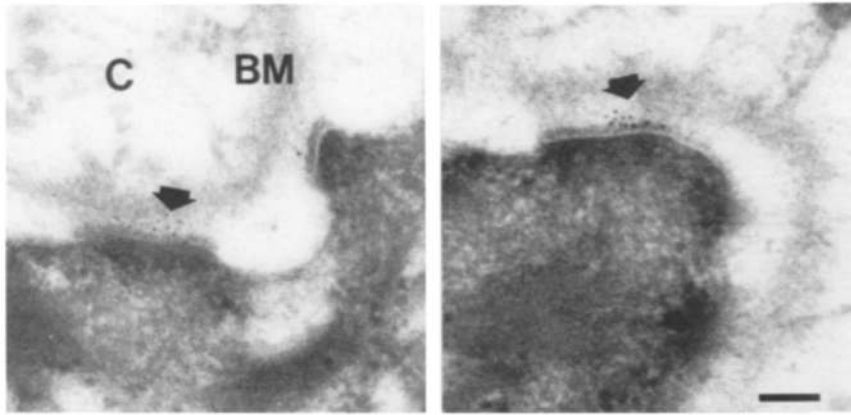


Figure 6. Ultrathin sections of human tongue embedded in LR white and incubated with anti- $\beta 4$ (439-9B) and gold-conjugated second antibody. The majority of the hemidesmosomes are labeled (arrows), whereas the rest of the cell surface, basement membrane (BM) and collagen (C) is unlabeled. Bar, 100 nm.

et al., 1990). No consistent labeling of the basal surface for $\beta 1$ integrin was obtained by this technique.

In thin sections of human tongue embedded in LR White resin, the morphology of the basement membrane and the hemidesmosomes proved to be best preserved (Fig. 6). Although the degree of labeling in this experiment was not as high as in the ones shown in Figs. 4 *b* and 5 *b*, the localization of the $\beta 4$ subunit in hemidesmosomes was clearly visible.

To analyze further the localization of integrins on the basal epidermal surface, collagenase-separated human breast epidermis was labeled with subunit-specific antibodies. This technique allows better access of antibodies and gold-labeled reagents to the basal cell surface and, because the cells are not permeabilized before labeling, provides unequivocal evidence for the extracellular localization of antibody binding sites. Fig. 7, *a* and *b* shows that $\alpha 6$ - and $\beta 4$ -specific antibodies reacted with the exposed extracellular faces of hemidesmosomes. In a sample of 141 hemidesmosomes for anti- $\beta 4$ and 113 hemidesmosomes for anti- $\alpha 6$, all showed labeling. Areas of membrane between hemidesmosomes were largely but not entirely devoid of label. Counts of gold particles associated with the exposed basal surface showed that with $\beta 4$ -specific antibodies 89.7% of gold particles were located on hemidesmosome and with $\alpha 6$ -specific antibodies 87.3% of gold particles were associated with hemidesmosomes. The $\beta 1$ -specific antibody gave positive but much less intense labeling of the exposed basal surface, but showed no obvious association with hemidesmosomes (Fig. 7 *c*). Controls in which the first antibodies were omitted showed complete absence of attached gold particles (Fig. 7 *d*). No labeling for cytoplasmic antigens such as bullous pemphigoid and cytokeratin was obtained by this technique. These results confirm that the $\alpha 6$ and $\beta 4$ integrin subunits are associated with hemidesmosomes on the surface of basal keratinocytes. Further, the binding sites for the A-1A5 and 5B5 antibodies are confirmed to be extracellular. The labeling of the basal surface for $\beta 1$ subunit is weak but positive.

Expression of Integrins in Primary Keratinocyte Cultures

To support the immunohistochemical and immunoelectron microscopical observations, the expression of integrins on keratinocytes was examined by immunoprecipitation. Primary cultures of keratinocytes were ^{125}I surface labeled and lysates of these cells were treated with monoclonal antibodies

against integrin α and β subunits. As shown in Fig. 8, keratinocytes express the $\alpha 2$ and $\alpha 3$ subunits in association with $\beta 1$, whereas the $\alpha 6$ subunit was found primarily associated with $\beta 4$. Furthermore, primary keratinocytes expressed $\alpha 5\beta 1$ at low levels, but $\alpha 1\beta 1$ or $\alpha 4\beta 1$ were not detectable.

Discussion

Hemidesmosomes are cell-matrix junctions that have been characterized and defined by their ultrastructural appearance (Weiss and Ferris, 1954; Kelly, 1966; Shienvold and Kelly, 1976; Ellison and Garrod, 1984). Cytoplasmically they consist of dense plaques that are associated with the inner face of the basal plasma membrane. Bundles of intermediate filaments run through the peripheral cytoplasm to associate with the inner aspect of the dense plaque. It has been suggested that these filaments are continuous, and that rather than terminating at the edge of the plaque they loop back into the cytoplasm. Filaments of different ultrastructure extend between the plaque and the tonofilaments (Kelly, 1966). Extracellularly, hemidesmosomes are associated with the lamina lucida of the basement membrane where anchoring filaments which traverse the basement membrane can sometimes be detected (Ellison and Garrod, 1984).

In this study we have investigated the localization of the $\alpha 6/\beta 4$ complex in epidermis. We have shown that this complex is localized in hemidesmosomes in the basal epidermal cells. This localization suggests that the $\alpha 6/\beta 4$ complex plays an important role in binding epidermal cells to the basement membrane. Presumably the basement membrane contains a component that acts as a ligand for the $\alpha 6/\beta 4$ complex though this ligand has not yet been identified. Our results provide the first well characterized monoclonal antibody markers for hemidesmosomes.

$\alpha 6$ and $\beta 4$ are transmembrane glycoproteins, the cytoplasmic domain of $\beta 4$ being unusually large and markedly different from that of any of the other known integrins (Suzuki and Naitoh, 1990; Hogervorst et al., 1990). The membrane-proximal cytoplasmic structure of the hemidesmosomes is a dense plaque and it seems likely that cytoplasmic domains of hemidesmosomal glycoproteins contribute to the structure of this plaque. The unusual cytoplasmic domain of $\beta 4$ may be specialized for participation in plaque formation. It is interesting to note that one of the major desmosomal glycoproteins, *dgl* or desmoglein, whose cytoplasmic domain is

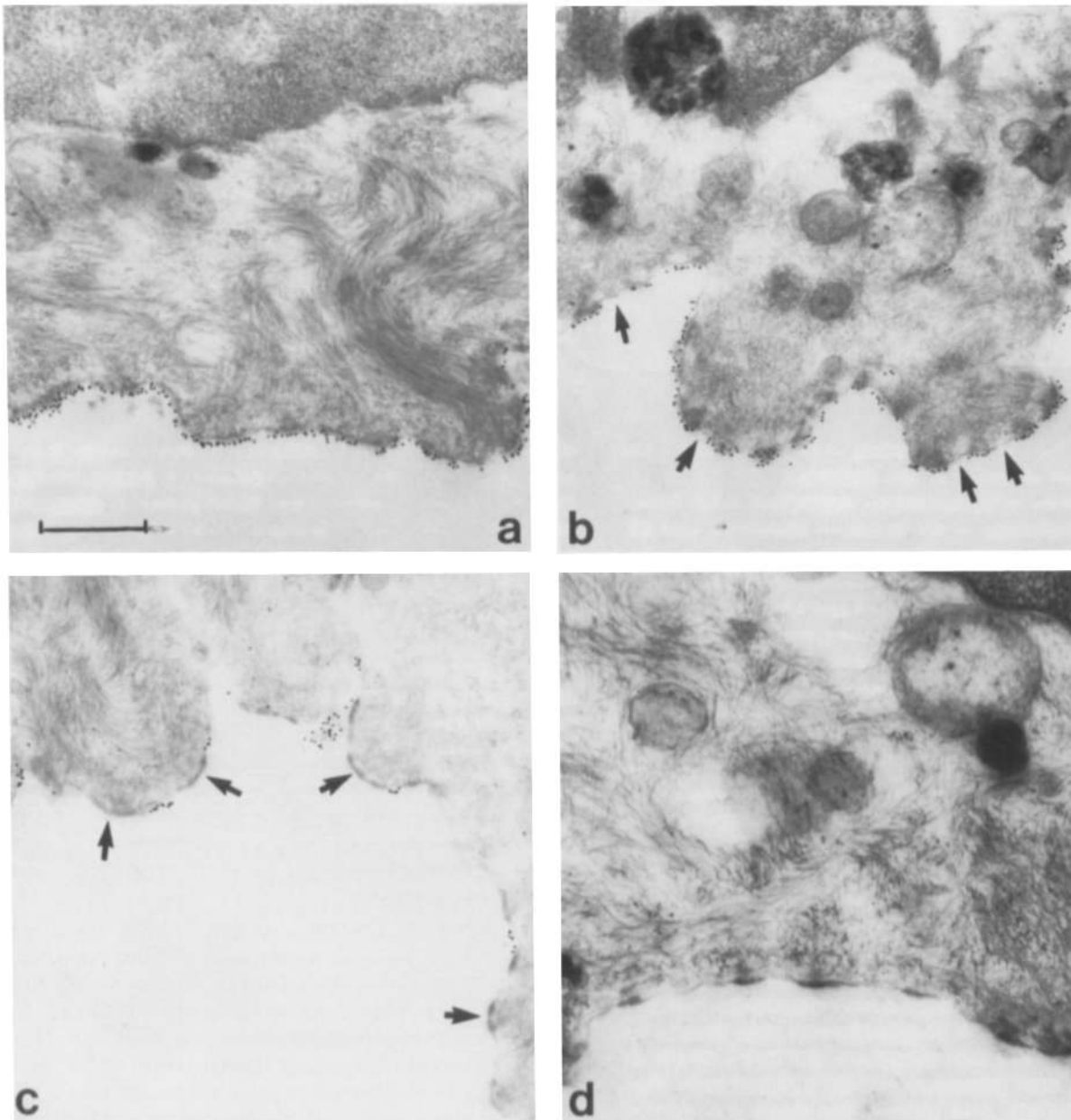


Figure 7. Electron micrographs showing protein A-gold labeling of collagenase-stripped epidermis with (a) anti- $\alpha 6$ (GoH3), (b) anti- $\beta 4$ (5B5), and (c) anti- $\beta 1$ (A-1A5). *d* is control incubated with complete hybridoma culture medium, followed by anti-mouse Ig and protein A-gold. *a* and *b* show strong association of $\alpha 6$ and $\beta 4$ with hemidesmosomes. In *a* the basal cell surface consists of an almost continuous row of densely labeled hemidesmosomes consistent with apparently continuous staining seen at the light microscope level (Fig. 2). In *b* some areas of nonhemidesmosomal membrane are present (arrows) and are almost devoid of staining. (c) Staining for $\beta 1$ is weak but positive and is not obviously associated with hemidesmosomes (arrows). The control (*d*) is devoid of staining. Bar, 500 nm.

located in the desmosomal plaque (Miller et al., 1987; Steinberg et al., 1987), also has a large and unusual cytoplasmic domain (Parrish et al., 1990; Koch et al., 1990), while being related to cadherins in its extracellular domain (Koch et al., 1990). However, there is no apparent homology between the cytoplasmic amino acid sequences of $\beta 4$ and *dgl*.

$\alpha 6$ and $\beta 4$ subunits are expressed in nearly all epithelial cells as well as peripheral nerves and certain subsets of endothelial cells (Sonnenberg et al., 1990a; Kennel, S. J., V. Godfrey, L. Y. Ch'ang, T. K. Lankford, L. J. Foote, and A. Makkinje, manuscript in preparation). Not all of these possess hemidesmosomes so the $\alpha 6/\beta 4$ complex must also reside in nonplaque locations. Different cytoplasmic interac-

tions in various locations may be mediated by alternative structures of the $\beta 4$ cytoplasmic domain generated by cell type-specific alternative mRNA splicing (Tamura et al., 1990). These authors have also proposed that the unique structure of $\beta 4$ suggests its involvement in unique cytoskeletal interactions.

We suggest that interaction between the cytoskeleton and transmembrane glycoproteins is indirect. A hemidesmosome-associated protein that has been extensively studied in recent years is the bullous pemphigoid (BP)¹ antigen (230 kD)

1. Abbreviation used in this paper: BP, bullous pemphigoid.

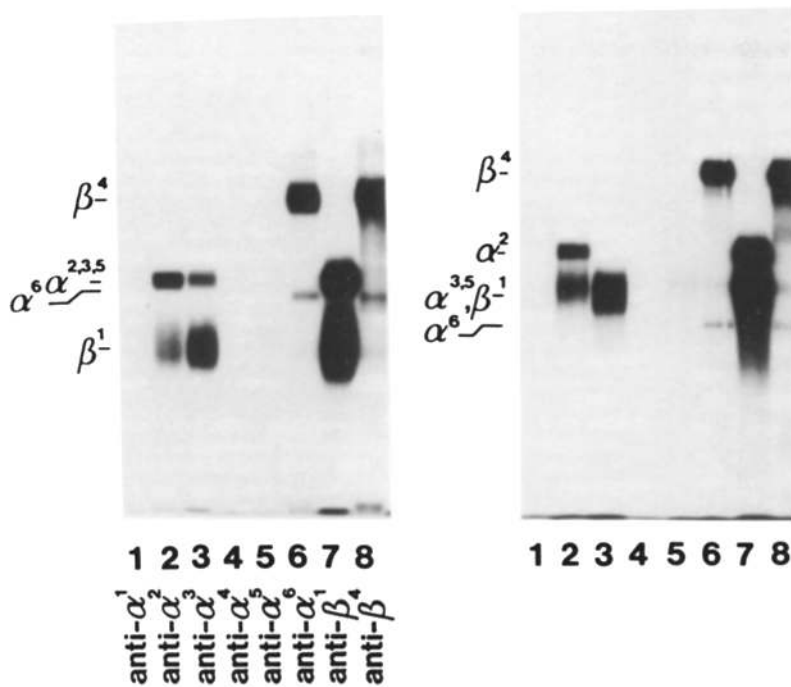


Figure 8. Analysis of integrins on human keratinocytes. Lysates of ^{125}I -labeled keratinocytes were immunoprecipitated with mAbs anti- α (TS2/7), anti- α 2 (10G11), anti- α 3 (J143), anti- α 4 (B5-G10), anti- α 5 (BIE5), anti- α 6 (GoH3), anti- β 1 (A-1A5) and anti- β 4 (439-9B). Samples were analyzed on SDS-polyacrylamide (5%) gels under nonreducing (*left*) and reducing (*right*) conditions.

(Stanley et al., 1981, 1988). This molecule is recognized by autoantibodies present in sera from patients with the bullous pemphigoid disease and has been localized intracellularly, in association with the hemidesmosomal plaque (Westgate et al., 1985). Our present immunolocalization studies, together with those of Westgate et al. (1985) and Tanaka et al. (1990) lead us to suggest that the BP antigen may occupy a location in hemidesmosomes similar to that which we have proposed for desmoplakins in desmosomes, that is between the membrane-associated plaque and the intermediate filaments (Miller et al., 1987; Garrod et al., 1990). An association between BP antigen and keratin filaments has been suggested previously (Jones et al., 1986) and is further supported by the recent finding that the carboxyl-terminal domain of BP antigen and that of two desmosomal plaque proteins, desmoplakins I and II, are very similar (Green et al., 1990). It has been suggested that the desmoplakins I and II are involved in linking the keratin filaments to the cell surface and that they may interact with the keratin network via these homologous domains. However, O'Keefe et al. (1989) were unable to demonstrate interaction between isolated desmoplakins and keratin filaments, while other desmosomal components have been shown to exhibit keratin binding activity. These are desmoccalmin (Tsukita and Tsukita, 1985), B6P (Kapprell et al., 1988), and a 140-kD protein related to lamin B (Cartaud et al., 1990). It may be that the component that binds hemidesmosomes to intermediate filaments has yet to be identified. Another candidate is the 180-kD antigen recognized by some bullous pemphigoid autoantibodies (Diaz et al., 1990), which has been localized to hemidesmosomal plaques (Klatte et al., 1989).

It has been reported that formation of new hemidesmosomes during epidermal attachment to the basement membrane is dependent upon extracellular Ca^{2+} (Trinkaus-Randall and Gipson, 1984). It is possible that this may be interpreted in

relation to the proposed involvement of the α 6/ β 4 complex in hemidesmosomal adhesion since integrin-mediated adhesion is well known to be divalent cation dependent (Ruoslahti and Pierschbacher, 1987). This is analogous to the finding that Ca^{2+} -dependent adhesion of desmosomes may be ascribed to the relationship between desmosomal glycoproteins and cadherins (Holton et al., 1990; Koch et al., 1990; Collins et al., 1991).

A protein of 125-kD that is recognized by a monoclonal antibody, prepared against a protein preparation containing hemidesmosomal components, has recently been described by Klatte et al. (1989). This protein has been localized at the lamina lucida side of the hemidesmosomes. Because of its localization and size, the 125-kD protein might be similar to the α 6 integrin subunit.

In skin, the α 2/ β 1 and α 3/ β 1 integrins were strongly expressed at the lateral borders of basal epidermal cells, but only weakly at the basal side. The presence of these two integrins at cell-cell borders was noted previously (Peltonen et al., 1989; Larjava et al., 1990; Carter et al., 1990), and it was proposed that the observed distribution of the β 1 integrins in skin indicates a role of these molecules in cell-cell interactions in epidermis (Peltonen et al., 1989). Consistent with this, antibodies against β 1 were found to dissociate colonies of cultured keratinocytes (Larjava et al., 1990). Our finding that α 6 shows weak cell-cell border localization in addition to strong basal location suggests, that it, too, may have a role in cell-cell interaction. Basolateral distribution of α 6 has been observed previously in other epithelia (Sonnenberg et al., 1990a; Sorokin et al., 1990).

Location of the α 6/ β 4 integrin complex to hemidesmosomes emphasizes the marked difference in biochemical composition between hemidesmosomes and desmosomes which has been demonstrated by several groups (Jones et al., 1986; Miller et al., 1987; Schwarz et al., 1990). Since loss of adhe-

sion to the basement membrane is an essential event in epidermal stratification, it seems that regulation of $\beta 4$ integrin expression is likely to play an important role in this process. In addition to bullous pemphigoid, loss or abnormality of hemidesmosomes has been reported in certain diseases including various types of carcinoma (Schenk, 1979; White and Gohari, 1984) and junctional epidermolysis bullosa (Tidman and Eady, 1986). Our results provide a basis for the investigation of the involvement of hemidesmosomal adhesion in these processes.

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