

Distinct Functions For Integrins $\alpha 3\beta 1$ in Focal Adhesions and $\alpha 6\beta 4$ /Bullous Pemphigoid Antigen in a New Stable Anchoring Contact (SAC) of Keratinocytes: Relation to Hemidesmosomes

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Abstract. Basal cells of stratified epidermis are anchored to the basement membrane zone (BMZ) of skin via hemidesmosomes. We previously identified integrin $\alpha 3\beta 1$, in focal adhesions (FAs), of cultured human keratinocytes (HFKs) as a mediator of HFK adhesion to secreted BMZ-like extracellular matrix (ECM; Carter, W. G., E. A. Wayner, T. S. Bouchard, and P. Kaur. 1990. *J. Cell Biol.* 110: 1387-1404). Here, we have examined the relation of integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$, to bullous pemphigoid antigen (BPA), a component of hemidesmosomes. We conclude that $\alpha 6\beta 4$ in HFKs localizes in a new stable anchoring contact (SAC) that cooperates with $\alpha 3\beta 1$ -FAs to mediate adhesion to ECM, based on the following. (a) Comparison of secreted ECM, with exogenous laminin, fibronectin and collagen identified ECM as the preferred ligand for HFK adhesion and spreading and for formation of both $\alpha 6\beta 4$ -SACs and $\alpha 3\beta 1$ -FAs. (b) Inhibition of HFK adhesion with combined anti- $\alpha 3\beta 1$ (PIB5) and anti- $\alpha 6\beta 4$ (GoH3) antibodies indicated that

both receptors were functional in adhesion to ECM while $\alpha 3\beta 1$ played a dominant role in spreading. (c) $\alpha 6\beta 4$ colocalized with BPA in SACs that were proximal to but excluded from FAs. Both $\alpha 6\beta 4$ -SACs and $\alpha 3\beta 1$ -FAs were in contact with the adhesion surface as indicated by antibody exclusion and interference reflection microscopy. (d) In contrast to $\alpha 3\beta 1$ -FAs, $\alpha 6\beta 4$ -SACs were present only in nonmotile cells, not associated with stress fibers, and were relatively stable to detergents and urea, suggesting a nonmotile, or anchoring function for SACs and motility functions for $\alpha 3\beta 1$ -FAs. (e) $\alpha 6\beta 4$ formed a detergent-insoluble complex with exogenous ECM in an affinity isolation procedure, confirming the ability of an unidentified ECM ligand to interact with $\alpha 6\beta 4$. (f) We suggest that $\alpha 6\beta 4$ /BPA-SACs in culture restrict migration of HFKs on ECM while $\alpha 3\beta 1$ -FAs form dynamic adhesions in spreading and migrating cells. $\alpha 6\beta 4$ /BPA-SACs in culture bear functional and compositional similarities to hemidesmosomes in skin.

BASAL cell of the human epidermis may adhere to the basement membrane zone (BMZ)¹ through hemidesmosomes (Staehelin, 1974). Hemidesmosomes are identifiable by electron microscopy as electron dense plaques localized to the cytoplasmic side of the basal membrane of keratinocytes attached to the BMZ (Selby, 1955; Odland, 1958; Shienvold and Kelly, 1976). Although little is known about the composition of hemidesmosomes, they are distinct from desmosomes (Jones et al., 1986), and are sites for keratin filament insertion at the basal membrane (Staehelin, 1974; Jones et al., 1989).

Krawczyk and Wilgram (1973) and Gipson et al. (1983) suggested that sites for insertion of anchoring fibrils into the

BMZ may be nucleation points for new hemidesmosomes. Anchoring fibrils of the BMZ contain type VII collagen (Sakai et al., 1986; Keene et al., 1987; Smith and Sybert, 1990). Additional components of the BMZ include laminin, entactin/nidogen, heparan sulfate proteoglycan, and type IV as well as type VII collagens synthesized by the basal cells (Katz, 1984; Martin and Timpl, 1987; Yurchenco and Schittny, 1990; Beck et al., 1990). Laminin and type IV collagen are structural and adhesive components localized to the lamina rara and lamina densa of the BMZ, respectively.

A correlation between the ultrastructure of hemidesmosomes and possible hemidesmosome function in basal cell adhesion to the BMZ is suggested from investigations of the autoimmune disorder, bullous pemphigoid (for review see Stanley, 1983, 1989). Bullous pemphigoid is characterized by detachment of epidermis from the BMZ. These patients also produce polyclonal autoantibodies against a 230-kD protein and a distinct 180-kD protein component of the hemidesmosome plaque. The circulating autoantibodies

1. *Abbreviations used in this paper:* BMZ, basement membrane zone; BPA, bullous pemphigoid antigen; ECM, extracellular matrix; FA, focal adhesion; HD-BSA, heat-denatured bovine serum albumin; HFK, neonatal human keratinocytes; KGM, keratinocyte growth medium; SAC, stable anchoring contact.

against these bullous pemphigoid antigens (BPAs) are not known to be causal in the disease. BPAs in keratinocytes from epidermis and cultured cells are incorporated in two pools: one insoluble and associated with the basal cell cytoskeleton (Mutasim et al., 1985, 1989) and the other soluble in isotonic buffers (Stanley et al., 1981). It has been reported that hemidesmosomes are endocytosed after trypsinization (Takahashi et al., 1985). It is not known whether these endocytosed BPAs are degraded or reused in formation of "hemidesmosome-like structures" in culture. The 230-kD BPA has been localized to the cytoplasmic plaque of hemidesmosomes (Westgate et al., 1985; Tanaka et al., 1990; Ikeda et al., 1990). The implications are that BPAs in hemidesmosomes may contribute to a physiologically significant adhesion structure in skin.

In contrast to basal cell attachment in skin, cultured cells adhere to the substrate via multiple types of adhesion structures including focal adhesions or focal contacts (FAs; Burridge et al., 1988) that contain integrin adhesion receptors (Hynes, 1987; Buck and Horwitz, 1987; Carter et al., 1990; Chen and Singer, 1982; Ruoslahti, 1988). FAs are the closest contacts of the cells with the extracellular adhesive ligands (Burridge et al., 1988) and can be identified by interference reflection microscopy (Singer et al., 1988) or antibody exclusion techniques (Neyfakh et al., 1983). The integrin $\beta 1$ subunits in FAs are the origins of actin containing stress fibers (Burridge et al., 1988). This complex of stress fibers, integrin and ligand play a major role in dynamic processes including adhesion, spreading, and migration (Straus et al., 1989; Bretscher, 1989; Zieske et al., 1989).

Adhesion of cultured neonatal foreskin keratinocytes (HFKs) to exogenous ligands, fibronectin, collagen, and secreted extracellular matrix (ECM) localizes $\alpha 5\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$, to the FAs, respectively (Carter et al., 1990). An uncharacterized component(s) of secreted ECM was the preferred ligand for localization of $\alpha 3\beta 1$ in FAs when compared to exogenous laminin, collagen, and fibronectin (Carter et al., 1990). In addition to the $\beta 1$ integrins, HFKs and other squamous epithelium also express integrin $\alpha 6\beta 4$ (or $\alpha E\beta 4$; Kajiji, et al., 1989; Hemler et al., 1989; Carter et al., 1990), a proposed laminin receptor (Cheresh et al., 1989; Lotz et al., 1990).

Both FAs and hemidesmosomes are suggested as physiologically significant structures in epithelial cell adhesion to the BMZ (Griep and Robbins, 1988). We have examined the distribution, function and interactions of BPA, integrins $\alpha 3\beta 1$, and $\alpha 6\beta 4$ in cell culture as components of FAs and a new anchoring adhesion (SAC). The relation of SACs in culture to hemidesmosomes in skin is discussed.

Materials and Methods

Materials

PMSF, *N*-ethylmaleimide, 2-mercaptoethanol, BSA, Triton X-100, protein A-Agarose, and soybean trypsin inhibitor, were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyldichlorosilane was from Pierce Chemical Co. (Rockford, IL). Peroxidase- and fluorescein-conjugated goat anti-mouse and anti-rat IgG and IgM (H and L chains) or peroxidase and rhodamine-conjugated goat anti-rabbit IgG and IgM (H and L chains) were obtained from Tago, Inc. (Burlingame, CA). [^{51}Cr]sodium chromate was from New England Nuclear (Boston, MA). Rhodamine-conjugated phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR).

Cells and Cell Culture

Normal newborn HFKs were prepared as described by Boyce and Ham (1985) and maintained in serum free keratinocyte growth medium (KGM; Clonetics Corp., San Diego, CA) containing insulin, epidermal growth factor (10 ng/ml), hydrocortisone, and bovine pituitary extract ($\sim 50 \mu\text{g}$ protein/ml).

Preparation of Extracellular Matrix Adhesion Ligands

Mouse laminin (derived from Engelbreth-Holm-Swarm (EHS) sarcoma, grown in mice) was purchased from Collaborative Research, Inc. (Bedford, MA) or prepared in this lab. The EHS sarcoma laminin migrated as two bands of ~ 200 and 400 kD on SDS-PAGE and that reacted with antilaminin antibodies by immunoblotting. No other bands reacted with antifibronectin or anti-type IV collagen antibodies or stained with Coomassie blue. Plasma fibronectin and collagen types I, and IV, were prepared as described (Wayner et al., 1988).

Antibodies

mAbs to the integrin receptors $\alpha 3\beta 1$ (PIB5, P1F2), $\alpha 2\beta 1$ (PIH5), $\alpha 5\beta 1$ (PID6), and $\beta 1$ (P4C10) have been described (Wayner and Carter, 1987; Wayner et al., 1988, 1989; Carter et al., 1990). PIH5 and PID6 inhibit fibroblast, keratinocyte, and platelet adhesion to collagen and fibronectin-coated substrates, respectively (Wayner et al., 1987; Wayner et al., 1988, Carter et al., 1990). mAb P4C10 reacts with all $\beta 1$ -containing integrins and inhibits cell adhesion to laminin, collagen, and fibronectin (Carter et al., 1990). SP2 is a control conditioned culture medium from the SP2 mouse melanoma. Monoclonal anti- $\alpha 6$ (GoH3) was from Dr. Arnold Sonnenberg (Central Lab. of Netherlands Red Cross, Amsterdam, Holland) and has been reported to inhibit platelet adhesion to laminin via $\alpha 6\beta 1$ and carcinoma adhesion to laminin via $\alpha 6\beta 4$ (Lotz et al., 1990). Rabbit anti-mouse EHS sarcoma laminin (R5922) was prepared as previously described (Wayner and Carter, 1987). Mouse mAb 3E1 against integrin $\beta 4$ was a gift from Dr. Eva Engvall (La Jolla Cancer Research Center, La Jolla, CA). Mouse mAb K8.13 anti-cytokeratin (Gigi et al., 1982) was from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antiserum against the carboxy terminus of the BPA (R1086) was prepared as described (Tanaka et al., 1990) and localizes to hemidesmosome on the cytoplasmic side of the basal membrane of keratinocytes in skin.

Adhesion of Cells to Coverslips

Acid-washed glass coverslips (25 mm diam) were derivatized with dimethyldichlorosilane, then coated with purified ligands ($5\text{--}20 \mu\text{g}$ protein/ml) then blocked with 1% (wt/vol) heat-denatured bovine serum albumin (HD-BSA) in PBS as previously described (Carter et al., 1990). Cells were suspended and adhered to the coverslips in KGM medium for periods of 1 h to 3 d (Carter et al., 1990).

Keratinocyte Extracellular Matrix

For this study, the extracellular matrix synthesized and secreted by HFKs shall be referred to as ECM. Endogenous ECM is secreted during the time course of an assay. Exogenous ECM was purified from cultures of HFKs and used as a ligand for adhesion of other cells. Exogenous ECM was prepared by growing HFKs for 3 d in KGM on glass coverslips or 48-well virgin styrene plates coated with various ligands. The adherent cells were removed by sequential extraction with (a) 1% (vol/vol) Triton X-100 detergent in PBS, (b) 2 M urea in 1 M NaCl, and (c) 8 M urea. All extraction buffers contained 1 mM phenylmethyl sulfonyl fluoride and 2 mM *N*-ethylmaleimide as protease inhibitors. The ECM was digested with $10 \mu\text{g}/\text{ml}$ DNase I for 30 min in 1% HD-BSA/PBS. The resulting coverslips were washed with PBS, blocked with HD-BSA, and used for cell adhesion studies.

Immunofluorescence, Interference Reflection, and Antibody Exclusion Microscopy

Coverslips and cells prepared as described above were incubated with combinations of mouse or rat monoclonal antibodies and rabbit polyclonal primary antibodies diluted in 1% HD-BSA overnight as previously described (Carter et al., 1990). The slips were washed with PBS, incubated with dilutions of affinity-purified, species-specific, FITC-conjugated goat anti-mouse/rat IgG and rhodamine-conjugated goat anti-rabbit IgG secondary antibody-

ies for 1 h, and washed with PBS. Interference reflection microscopy was performed basically as described (Izzard and Lochner, 1976) and was used to identify FAs in the same field as the two-color fluorescence. FAs were also localized by the antibody exclusion technique (Neyfakh et al., 1983).

Inhibition of Cell-Substrate Adhesion

Inhibition of cell adhesion to various ligands was performed as previously described (Wayner and Carter, 1987; Carter et al., 1990). The cells were labeled with $\text{Na}_2^{51}\text{CrO}_4$ (50 $\mu\text{Ci}/\text{ml}$ for 2–4 h) and were allowed to adhere to the protein-coated surfaces in the presence of the hybridoma supernatants for 1–2 h for short-term adhesion assays (Fig. 2 A and B or Fig. 3) or 18–24 h for long term adhesion assays (Fig. 4) at 37°C. Previous studies (Carter et al., 1990) have indicated that long-term adhesion results in secretion of endogenous ECM and localization of $\alpha 3\beta 1$ in FAs regardless of the exogenous ligand that was coated on the glass surface and used to initiate the adhesion. Over a 24-h period, the endogenous ECM is deposited even when HD-BSA is used as a ligand or blocking agent. Therefore, long-term adhesion to HD-BSA-coated surfaces is due to secretion of endogenous ECM.

Immune Precipitation, and PAGE

Adherent HFKs were metabolically labeled with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$ for 18 h) followed by sequential extraction with: (a) 1% (vol/vol) Triton X-100 detergent in PBS, (b) 2 M urea in 1 M NaCl, (c) 8 M urea as described for preparation of ECM for adhesion studies; and (d) the adherent ECM was solubilized in 0.5% SDS with scraping with a rubber policeman. Each extraction buffer was added to the adherent cell layer, incubated for

15 min then removed leaving the adherent cell residue. All extraction buffer contained 1 mM PMSF, and 2 mM *N*-ethylmaleimide as protease inhibitors. Immune precipitation was performed as previously described (Wayner and Carter, 1987). SDS-PAGE gels were prepared following the basic stacking gel system of Laemmli (1970).

Tissue Staining

The distribution of receptors and ligands in tissue were determined by immunoperoxidase microscopy of cryostat sections. Cryostat sections (6 μm) were prepared from human neonatal foreskin embedded in OCT medium after snap freezing in isopentane/liquid nitrogen. Where indicated (Fig. 1), sections were incubated in PBS with or without Triton X-100 detergent (1% (vol/vol) in PBS for 10 min) before fixation in 4% paraformaldehyde in PBS. This extraction was performed to identify solubility differences in tissue components. All sections were subsequently permeabilized with 1% Triton X-100 after fixation then incubated with primary antibodies and peroxidase-conjugated secondary antibodies.

Results

$\alpha 6\beta 4$ and $\alpha 3\beta 1$ Have Distinct Organizations/Distributions in Skin

Integrin subunits $\alpha 3$, $\beta 1$, $\alpha 6$, and $\beta 4$ localize in the basal and to a lesser degree in the suprabasal cell populations adjacent

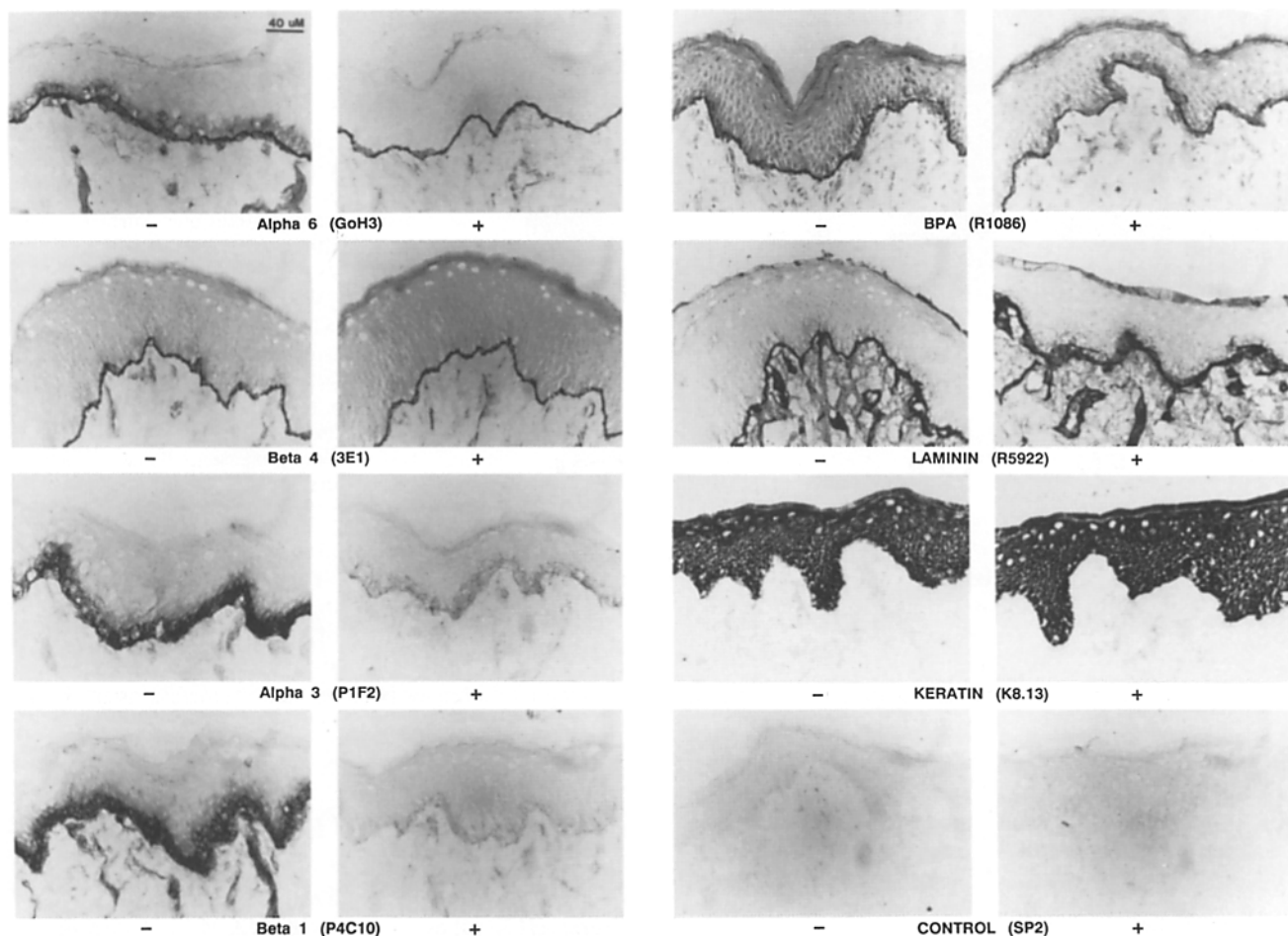


Figure 1. Localization of $\alpha 3\beta 1$, $\alpha 6\beta 4$, and BPA in cryostat sections of skin: effects of extraction with Triton X-100. Cryostat sections (6 μm) of human neonatal foreskin were treated (+) or untreated (-) with Triton X-100 detergent (1.0% [vol/vol] in PBS for 10 min), then stained with the indicated primary antibodies anti-alpha 6 (GoH3), anti-Beta 4 (3E1), etc., and peroxidase-conjugated secondary antibodies. Triton X-100 solubilized $\alpha 3\beta 1$ (PIB5), and $\beta 1$ (P4C10)-containing integrins but not $\alpha 6$ (GoH3), $\beta 4$ (3E1), BPA (R1086), laminin, or keratin from epidermis.

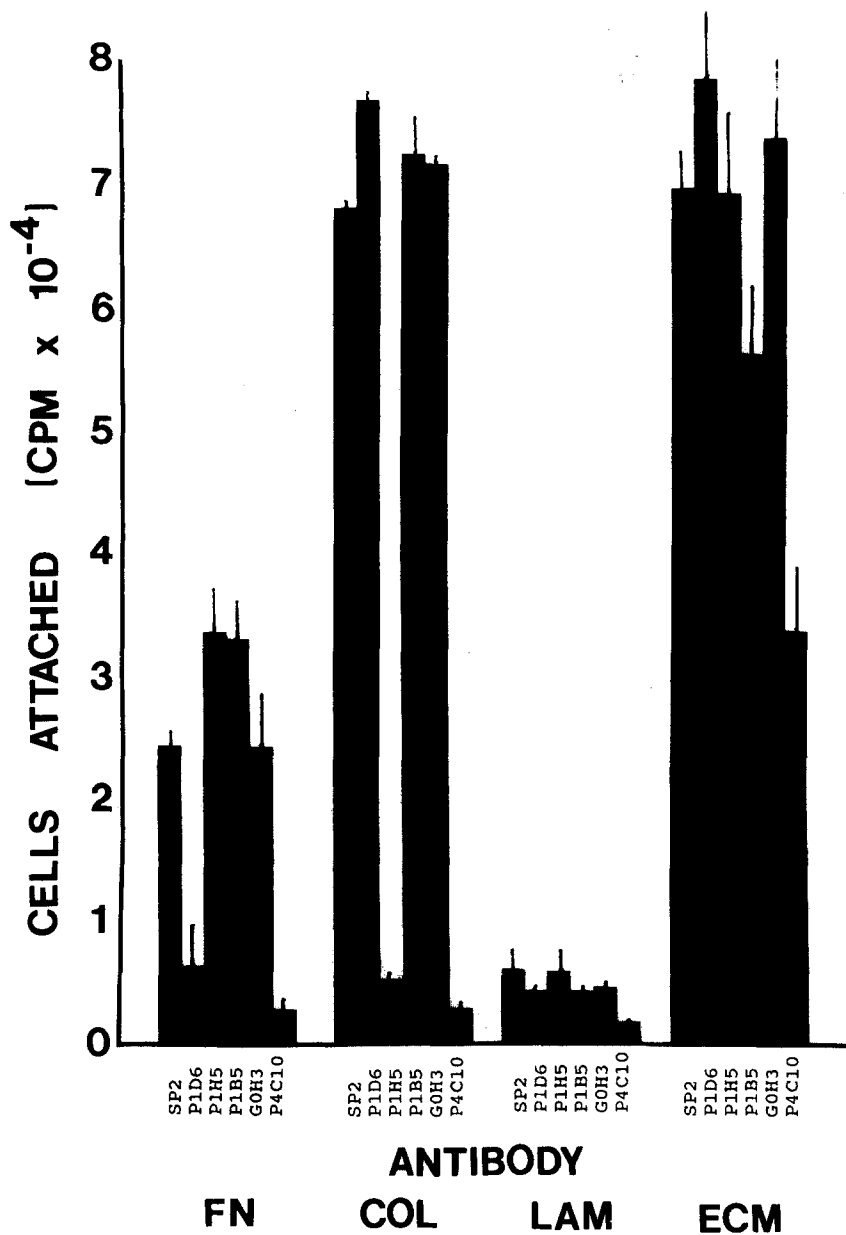


Figure 2. Anti- $\alpha 3\beta 1$ (PIB5) and anti- $\beta 1$ (P4C10) partially inhibit HFK adhesion on ECM in short-term assays: implications for additional non- $\beta 1$ receptor involvement in adhesion. Adhesion surfaces were coated with fibronectin (FN), type I collagen (COL), laminin (LAM), or HFK ECM (ECM). ^{51}Cr -labeled HFKs were incubated with the adhesion surface in the presence of inhibitory antibodies (PID6, PIH5, etc.) for 2 h, then washed to remove nonadherent cells. The adherent cells were dissolved and counted in a gamma counter. Adhesion to BSA surfaces was essentially zero. Each bar is the average of three assays.

to laminin in the BMZ of neonatal foreskin (Fig. 1). Previous results have shown that $\alpha 3$ and $\alpha 6$ combine with $\beta 1$ and $\beta 4$, respectively, in cultures of primary HFKs (Carter et al., 1990). $\alpha 3\beta 1$ was localized to the basal, lateral, and apical surfaces of the basal cells. In contrast, $\alpha 6\beta 4$ concentrated in the basal region of the basal cells with small quantities of $\alpha 6$, but not $\beta 4$, also detected in lateral and apical surfaces of these cells. BPA localized specifically to the dermal-epidermal junction near to laminin in the BMZ. This localization is consistent with the reported presence of BPA in the cytoplasmic plaque of hemidesmosomes (Tanaka et al., 1990). For comparison, keratin localized in all stratified layers of the epidermis but not the dermis.

We further distinguished $\alpha 3\beta 1$ from $\alpha 6\beta 4$ in skin. Detergent extraction of the cryostat sections before staining solubilized virtually all of the $\alpha 3$ and $\beta 1$ subunits, with much less effect on the distribution of $\alpha 6$, $\beta 4$, laminin, BPA or keratin (Fig. 1). The Triton X-100-insoluble $\alpha 6$, $\beta 4$ and BPA were

concentrated near the BMZ, consistent with their possible roles in adhesion of basal cells to the BMZ. After detergent extraction the colocalization of $\alpha 6\beta 4$ and BPA was more evident at the dermal-epidermal junction. These results indicate major differences between the organizations of $\beta 1$ and $\beta 4$ integrins in basal cells of the skin. $\alpha 6\beta 4$ constitutes a relatively stable structure in skin compared to $\beta 1$ -integrins.

Adhesion of HFKs to ECM Involves Both $\alpha 6\beta 4$ and $\alpha 3\beta 1$

We have previously identified $\alpha 3\beta 1$ in FAs as a mediator of cultured HFK adhesion to secreted ECM, and used this as a model system for basal cell adhesion to the BMZ in skin (Carter et al., 1990). ECM is a better inducer of HFK adhesion than purified laminin, fibronectin, and as good or better than collagen in short-term adhesion assays (1-2 h; Fig. 2). Although laminin is a component of the ECM, purified lami-

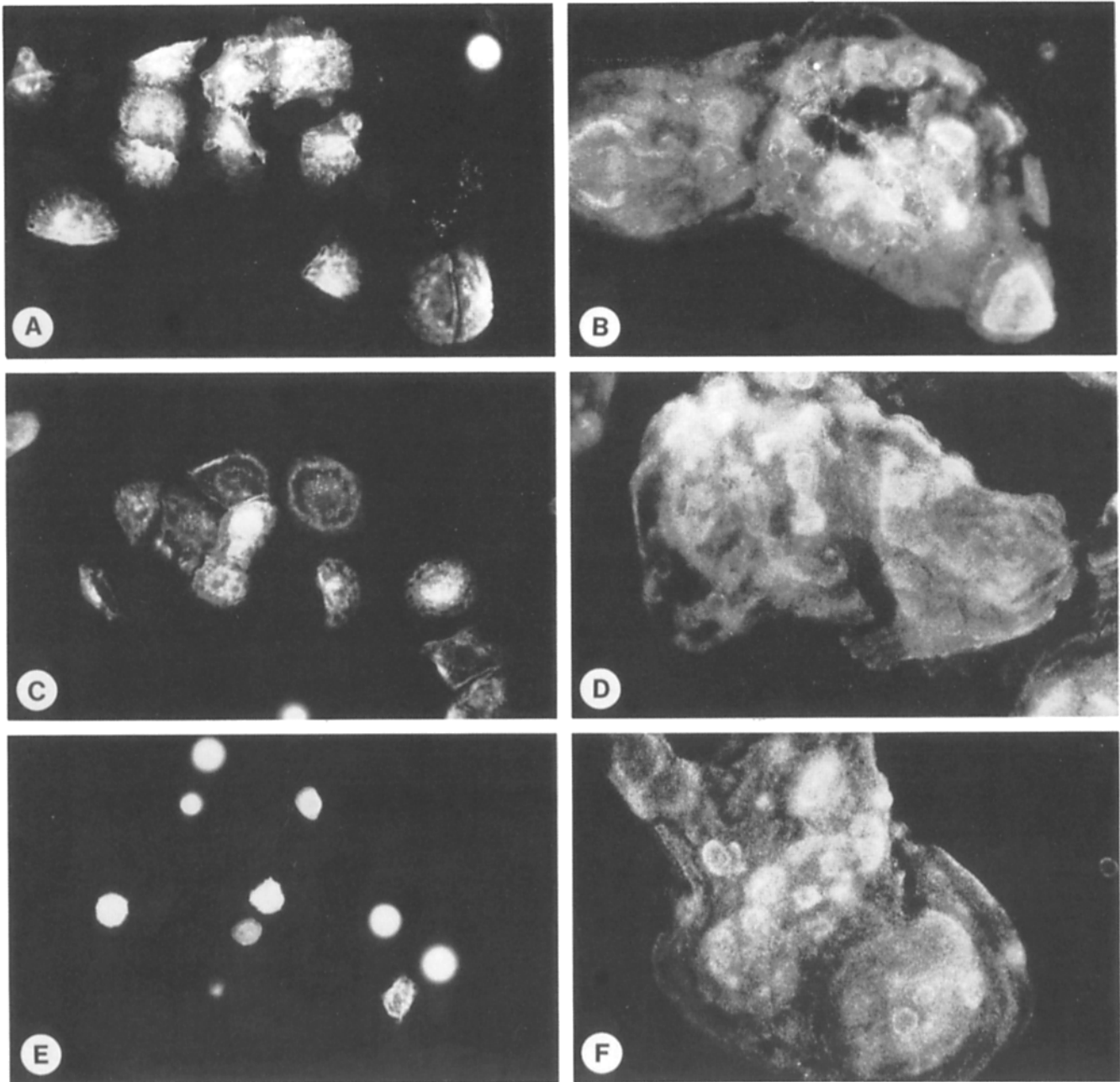


Figure 3. Anti- $\alpha 3\beta 1$ (PIB5), but not anti- $\alpha 6$ (GoH3) inhibits HFK spreading on ECM. HFK cells were grown on glass coverslips for 3 d then differentially extracted with 8 M urea to prepare adherent ECM. HFKs were allowed to adhere to the ECM for 2 h in the presence of control SP2 (A and B), anti- $\alpha 6$ (GoH3, C and D), and anti- $\alpha 3\beta 1$ (PIB5, E and F), washed to remove unbound cells then fixed and permeabilized. The adherent HFKs were stained with anti- $\alpha 6$ (GoH3; A, C, and E) and the ECM with antilaminin (R5922, B, D, and F). Anti- $\alpha 3\beta 1$, but not anti- $\alpha 6$, inhibited cell spreading on the exogenous laminin-containing ECM, but only partially inhibited cell adhesion.

nin was relatively slow in inducing HFK adhesion. Anti- $\alpha 3\beta 1$ (PIB5) or anti- $\beta 1$ (P4C10) only partially inhibited cell adhesion to ECM (Fig. 2). In contrast, anti- $\alpha 2\beta 1$ (PIH5), anti- $\alpha 5\beta 1$ (PID6), and anti- $\beta 1$ (P4C10) completely blocked HFK adhesion to collagen, fibronectin, and collagen/fibronectin, respectively. An explanation for the partial inhibition was seen in Fig. 3. HFK adhesion and spreading on deposits of ECM was always confined to the ECM spots indicating the dependency of the adhesion on the ECM. The spreading of HFKs on the ECM was completely inhibited by anti- $\alpha 3\beta 1$

(PIB5; Fig. 3) while attachment was only partially inhibited (Fig. 2, ECM). For the studies in Fig. 3, we have prepared the ECM adhesion surface from long-term HFK cultures grown on BSA-coated glass. The BSA coating retards migration of HFKs and confines the secreted ECM to localized cell colonies. In the absence of the BSA treatment, HFKs deposited ECM over the entire adhesion surface, making it difficult to evaluate the selectivity of HFK adhesion to ECM.

The results from Fig. 2 and 3 suggest that short term HFK adhesion to ECM involves $\alpha 3\beta 1$ and an additional uncharac-

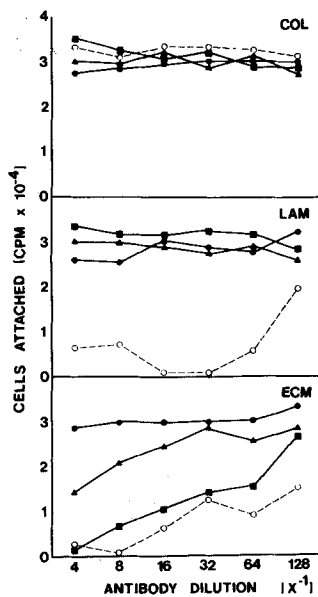


Figure 4. Combined anti- $\alpha3\beta1$ and anti- $\alpha6\beta4$ selectively inhibit HFK adhesion to laminin and ECM in long-term assays. ^{51}Cr -labeled HFKs were adhered to surfaces coated with type I collagen (COL), laminin (LAM), or HFK ECM (ECM) for 18 h in the presence of dilutions of control SP2 (●), anti- $\alpha6$ (GoH3, ▲), anti- $\alpha3\beta1$ (PIB5, ■) and combined anti- $\alpha6$ + anti- $\alpha3\beta1$ (○). The nonadherent cells were removed by washing and the adherent cells counted.

terized adhesion system that does not use a $\beta1$ -containing integrin. We examined the possible role of $\alpha6\beta4$ as this non- $\beta1$ integrin adhesion receptor. In short term adhesion studies, anti- $\alpha6$ (GoH3), had no inhibitory effect on HFK spreading (Fig. 3, compare A, C, and E) or attachment (Fig. 2) on exogenous ECM, laminin, collagen, or fibronectin. As an alternate approach, we examined the possibility that $\alpha6\beta4$ may play an increased role, relative to $\alpha3\beta1$, in long-term

HFK adhesion studies (18–24 h). In addition, we examined the possibility that $\alpha3\beta1$ might mask the function of $\alpha6\beta4$. In long-term adhesion studies, HFKs secrete their own endogenous ECM (Figs. 3 and 4) and accumulate $\alpha3\beta1$ in FAs as a result of the deposition of a preferred ligand. As exogenous ligands for the long-term adhesion studies, we used collagen (control)-, laminin-, and BSA-coated surfaces. As shown in Fig. 3, HFKs in long-term culture on BSA-coated surfaces, secrete endogenous ECM without the presence of exogenous ligands that can complicate the adhesion studies. Combined PIB5 and GoH3 inhibited HFK adhesion to laminin and ECM (Fig. 4) while adhesion to ECM was partially inhibited by either antibody alone. In control experiments, combined or single antibodies had no effect on cell adhesion (Fig. 4) or spreading (results not shown) on collagen. These results indicate that HFK adhesion to purified exogenous ECM in short-term adhesion and to endogenous ECM in long-term adhesion involves both $\alpha3\beta1$ and $\alpha6\beta4$.

Localization of $\alpha6\beta4$ and $\alpha3\beta1$ in Distinct Adhesion Structures

The distinction between the organizations of $\alpha6\beta4$ and $\beta1$ -containing integrins in skin (Fig. 1) was also observed in cultures of HFKs. $\alpha3\beta1$ localizes to FAs (Fig. 5, A–C) in long-term cultures on exogenous laminin coincident with the deposition of an ECM (Carter et al., 1990). As previously reported, $\alpha5\beta1$ and $\alpha2\beta1$ also localized to FAs when HFKs adhered to fibronectin and collagen, respectively (Carter et al., 1990). The $\alpha3\beta1$ -containing FAs ($\alpha3\beta1$ -FAs) were identifiable as black attachment sites in the focal plane of the

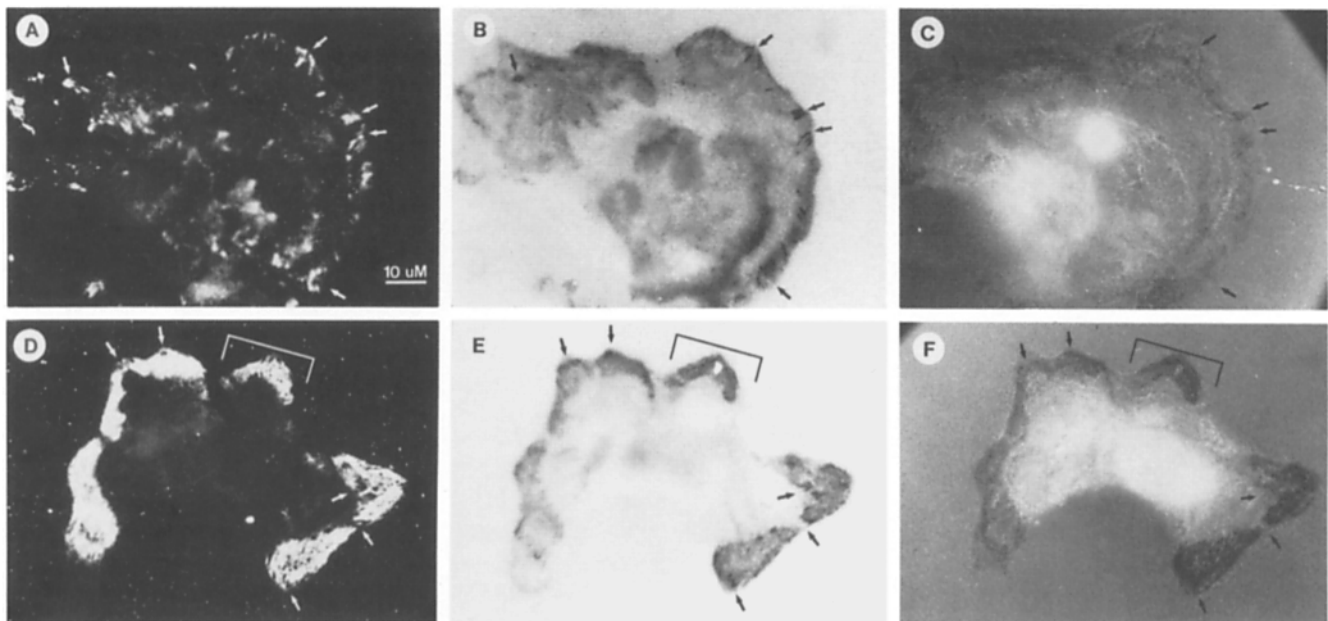


Figure 5. $\alpha6\beta4$ localizes in a new cell–substrate contact and $\alpha3\beta1$ in FAs of HFKs. HFKs were cultured for 2 d in KGM on glass coverslips coated with laminin then washed, fixed, permeabilized, and blocked. Each slip was stained with anti- $\alpha3\beta1$ (PIF2) and antilaminin (R5922) or anti- $\alpha6$ (GoH3) and antilaminin antibodies in order to localize receptors and laminin in the same field. Colocalization of the receptors in FAs and basal plaques was determined by the antibody exclusion techniques and by interference reflection microscopy. Bound primary antibodies were detected with species specific FITC- and rhodamine-conjugated secondary antibodies. A (anti- $\alpha3\beta1$), B (anti-laminin), and C (interference reflection) are all the same field. D (anti- $\alpha6$), E (antilaminin), and F (interference reflection) are all the same field. Arrows indicate $\alpha3\beta1$ -FAs in contact with the surface in the same position in each field. Brackets indicate $\alpha6\beta4$ in contact with the surface.

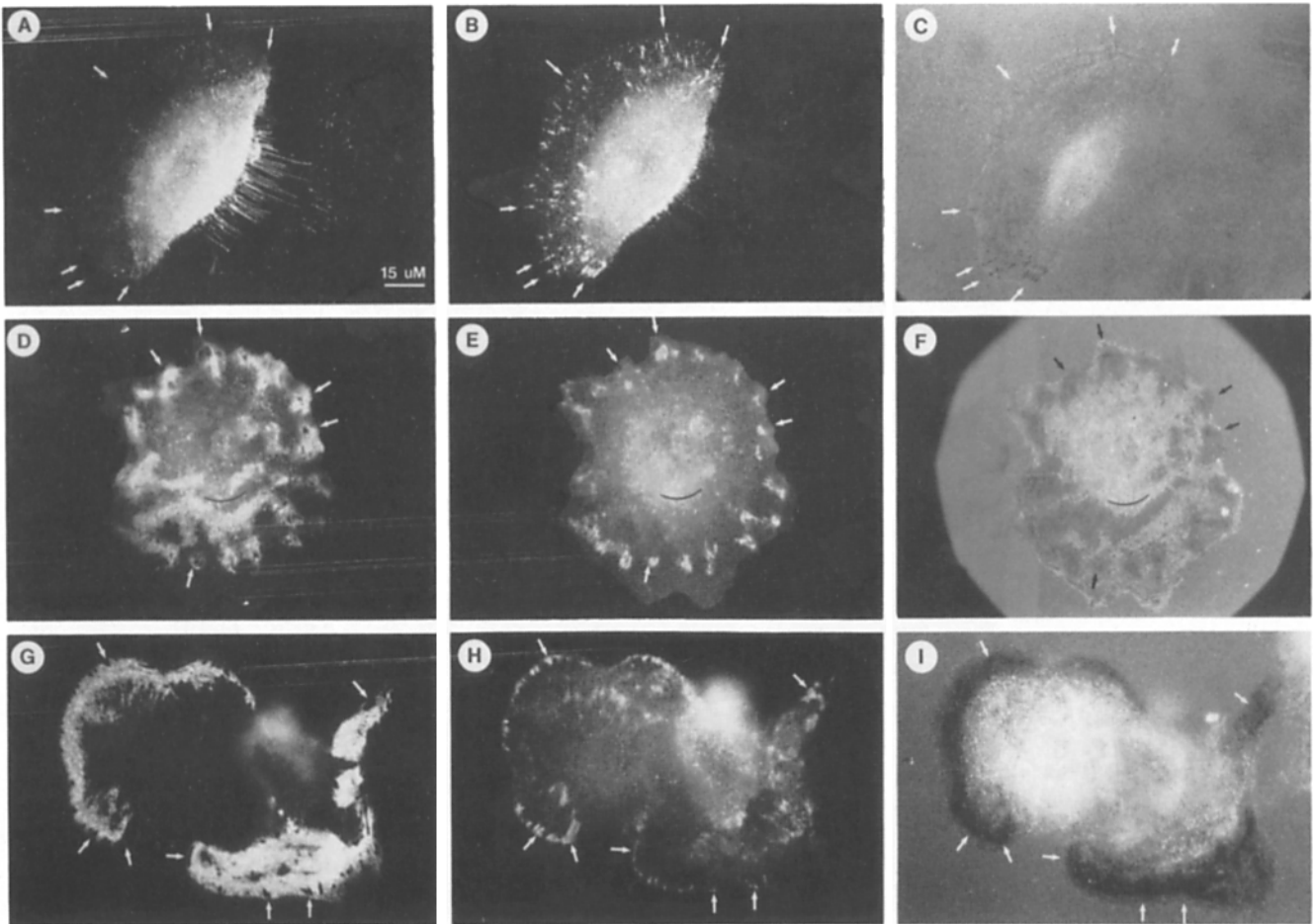


Figure 6. $\alpha 6 \beta 4$ contacts and $\alpha 3 \beta 1$ -FAs localize in three distinct patterns in stationary and motile cells. HFKs were allowed to adhere to glass coverslips coated with exogenous ECM for 2 h (A–C) or cultured on laminin for 3 d to generate endogenous ECM (D–F and G–I). $\alpha 3 \beta 1$ (B, E, and H) and $\alpha 6 \beta 4$ (A, D, and G) were identified by incubation with mAb PIF2 and GoH3 and were localized in relation to the ligand surface by interference reflection microscopy (C, F, and I). A–C, D–F, and G–I each represent a single field. Arrows indicate the absence of $\alpha 6$ and the presence of $\alpha 3 \beta 1$ in FAs at the leading edge of a migrating cell. Arrows indicate $\alpha 3 \beta 1$ FAs surrounded by $\alpha 6 \beta 4$. The arc identifies a cell–substrate contact that contains $\alpha 6$ but not $\alpha 3 \beta 1$ -FAs. Arrows identify $\alpha 3 \beta 1$ -FAs at the periphery of $\alpha 6 \beta 4$ -contacts. $\alpha 6 \beta 4$ is excluded from the FAs.

ligand by the antibody exclusion technique (see Materials and Methods). For these experiments, antibodies against laminin were used as a probe of the adhesion surface (Fig. 5 B). Although poorly resolved, the FAs for $\alpha 3 \beta 1$ were identifiable by interference reflection microscopy (Fig. 5 C).

In contrast, $\alpha 6 \beta 4$ localized in relatively large adhesion contacts that were distinct from the $\beta 1$ -integrin containing FAs (Fig. 5, D–F). The $\alpha 6 \beta 4$ -contacts localized to the same focal plane as the FAs and in proximity to the ligand-coated surfaces. Consistently, the $\alpha 6 \beta 4$ -contacts were detectable by antibody exclusion as relatively large grey areas of partial antibody exclusion in contrast to the small black foci of the FAs (Fig. 5 E). By interference reflection microscopy (Fig. 5 F), the $\alpha 6 \beta 4$ contacts were poorly resolved but detectable as large grey areas that corresponded to the grey areas of antibody exclusion (compare Fig. 5, D–F). The $\alpha 6 \beta 4$ contacts were usually in proximity to $\alpha 3 \beta 1$ -FAs but were clearly excluded from FAs (Fig. 5, D–F) as detected with anti- $\alpha 6$ mAb (GoH3).

$\alpha 6 \beta 4$ distributed in three characteristic patterns depending on the time in culture and motility of the HFKs. (a) When

HFKs were allowed to adhere to exogenous ECM for a short period (2 h), $\alpha 3 \beta 1$ was localized in FAs at the leading edge of migrating or spreading cells as detected by interference reflection microscopy (Fig. 6, B and C). In contrast, $\alpha 6 \beta 4$ polarized to the trailing edge in the same migrating cell (Fig. 6 A), with no accumulation in contacts or FAs. (b) In stationary spread cells $\alpha 6 \beta 4$ localized in relatively large polymorphic $\alpha 6 \beta 4$ contacts (Fig. 6, G–I). These $\alpha 6 \beta 4$ -contacts were characteristic of cells in culture for multiple days where they secreted ECM. In many cases, the relatively small $\alpha 3 \beta 1$ -FAs were localized around the periphery of the $\alpha 6 \beta 4$ -contacts, forming a complex adhesion structure. (c) An intermediate organization was also observed with relatively small quantities of $\alpha 6 \beta 4$ surrounding the $\alpha 3 \beta 1$ -containing FAs (Fig. 6, D–F). This organization was characteristic of cells spreading on ECM.

BPA Codistributes with $\alpha 6 \beta 4$ in a New SAC

The possibility that the $\alpha 6 \beta 4$ contacts observed in cultured HFKs may correspond to hemidesmosomes of skin was ex-

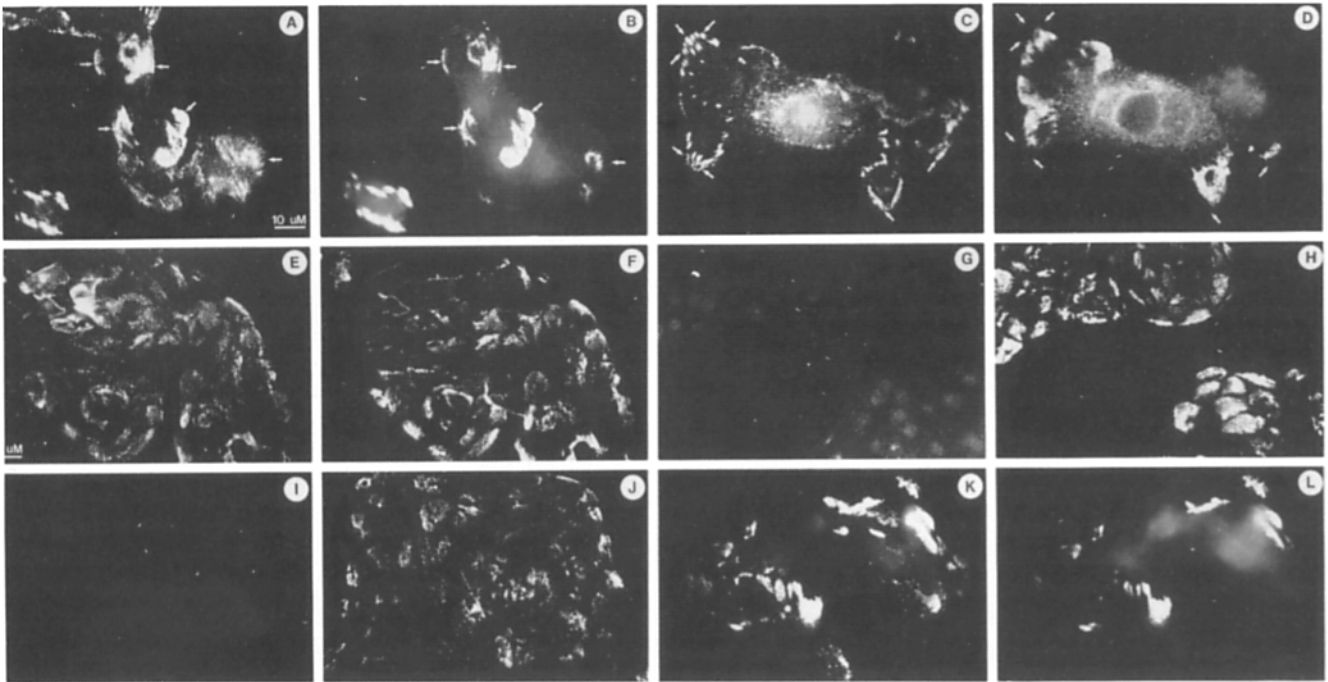


Figure 7. Codistribution of $\alpha 6\beta 4$ with BPA in SACs: relative stability of SACs and FAs to extraction with Triton X-100 detergent and urea. HFKs were cultured for 2 d on glass coverslips coated with laminin. The adherent HFKs were either fixed and permeabilized (A–D) or extracted sequentially with the following before fixation: (a) G and H, Triton X-100 (1% [vol/vol] in PBS); (b) E, F, K, and L, Triton \rightarrow 2 M urea + 1 M NaCl, and (c) I and J, Triton \rightarrow urea/NaCl \rightarrow 8 M urea. The adherent cells and cell residue were stained for BPA (R1086, B, D, F, H, J, and L) and $\alpha 6$ (GoH3, A, E, and I), $\beta 4$ (3E1, K), $\alpha 3\beta 1$ (PIF2, C) or $\beta 1$ (P4C10, G). $\alpha 6\beta 4$ codistributes with BPA in SACs. BPA is excluded from $\alpha 3\beta 1$ -FAs. Triton X-100 solubilizes all $\beta 1$ -containing integrins while 8 M urea but not 2 M urea removes $\alpha 6$ and $\beta 4$. BPA is partially resistant to 8 M urea.

aminated by localizing $\alpha 6\beta 4$ relative to BPA, a component of the cytoplasmic plaque of hemidesmosomes in skin. $\alpha 6$ (Fig. 7, A and B) was found to codistribute with BPA present in cultures of HFKs as components of the adhesion contacts. BPA was usually in proximity to but excluded from $\alpha 3\beta 1$ -FAs (Fig. 7, C and D) as well as $\alpha 2\beta 1$ -FAs and $\alpha 5\beta 1$ -FAs (results not shown). Like $\alpha 6\beta 4$, BPA localized to the focal plane of the adhesion surface. The distribution of $\alpha 6\beta 4$ in contacts was frequently more extensive than BPA; however, when BPA was detected it consistently codistributed with $\alpha 6\beta 4$.

Further confirmation of the unique character of the $\alpha 6\beta 4$ /BPA contacts compared to $\alpha 3\beta 1$ -FAs was apparent in the relative stabilities of the two adhesion structures. Extraction of adherent HFKs with Triton X-100 detergent removed detectable $\beta 1$ (Fig. 7 G) and $\alpha 3$ (results not shown) from FAs but had little effect on BPA in the same field (Fig. 7 H). Extraction with Triton X-100, even when followed by extraction with 2 M urea containing 1 M NaCl, failed to remove all $\alpha 6$ (Fig. 7 E), $\beta 4$ (Fig. 7 K), or codistributing BPA (Fig. 7, F and L), from contacts or from the adhesion surface. Further extraction with 8 M urea removed or denatured detectable $\alpha 6$ (Fig. 7 I) and $\beta 4$ (results not shown) on the adhesion surface but left detectable patterns of BPA in the same field (Fig. 7 J). Thus, $\alpha 6\beta 4$ /BPA contacts and $\alpha 3\beta 1$ -FAs exhibit distinct stabilities in both cell culture and skin.

Based on the unique characteristics of the $\alpha 6\beta 4$ contacts, we shall refer to the contacts as stable anchoring contacts or SACs to distinguish them from FAs. SACs shall refer to all $\alpha 6\beta 4$ -containing contacts whether BPA is present or not.

The presence of BPA in the ECM associated with the adhesion surface was confirmed by immune precipitation (Fig. 8). Adherent HFKs were metabolically labeled with [35 S]-methionine then sequentially extracted as described in Fig. 7 in order to prepare the detergent/urea-insoluble ECM. The adherent matrix was removed from the adhesion surface by scraping in SDS. BPA was enriched in the SDS extract (Fig. 8, EXTRACT, lane 4) as were other uncharacterized proteins that represent potential ligands for $\alpha 3\beta 1$ and $\alpha 6\beta 4$. BPA was immune precipitated from the SDS extract with rabbit polyclonal anti-BPA as a 230-kD protein (Fig. 8, RIP, lane 1) consistent with the previously described BPA (Stanley et al., 1981).

$\alpha 3\beta 1$ -FAs and $\alpha 6\beta 4$ -SACs Mediate Distinct Adhesion Functions

We further compared the subcellular organizations of $\alpha 3\beta 1$ -FAs and $\alpha 6\beta 4$ -SACs in order to identify possible functional distinctions between the two adhesion complexes. $\alpha 3\beta 1$ -FAs were origins for actin-containing stress fibers (Fig. 9, A–C) while the $\alpha 6\beta 4$ -SACs (Fig. 9, D–F) that surrounded the FAs were absent from the origins of the stress fibers. The failure of $\alpha 6\beta 4$ to associate with actin-containing stress fibers is consistent with the failure of $\alpha 6\beta 4$ to localize to the leading edge of cells migrating on ECM (Fig. 6, A–C). In contrast, the association of $\alpha 3\beta 1$ -FAs with stress fibers and their polarized distribution at the leading edge of migrating cells is consistent with their role in dynamic processes such as migration.

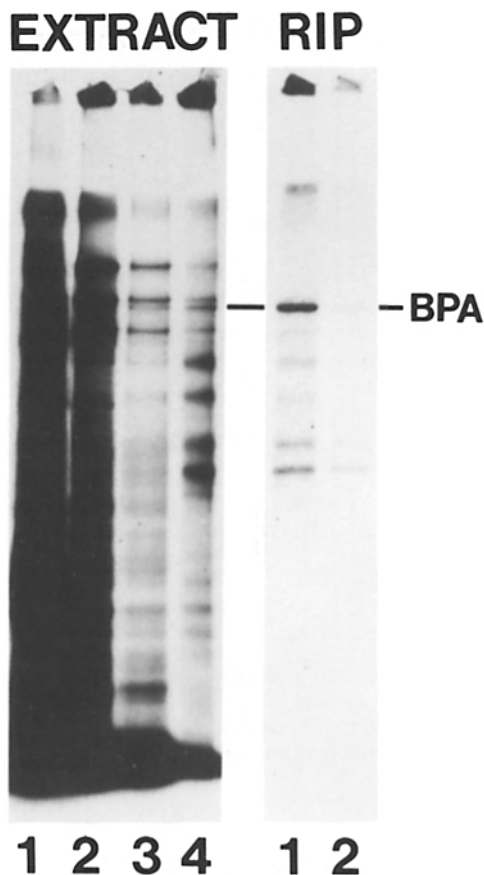


Figure 8. Immune precipitation of BPA from the detergent-insoluble matrix of HFKs. (**EXTRACT**) Confluent cultures of adherent HFKs were metabolically labeled with [³⁵S]methionine, washed and extracted sequentially with PBS containing the following in order to prepare an adherent, detergent-insoluble matrix; (**Extract 1**) 1% Triton X-100, (**Extract 2**) 2 M urea + 1 M NaCl, and (**Extract 3**) 8 M urea. The ECM was then removed from the adhesion surface by addition of 0.5% SDS followed by scraping (**Extract 4**). Each extract was fractionated by SDS-PAGE (8% gel) under reducing conditions and detected by fluorography. Radioimmune precipitation (**RIP**). The SDS extract was immune precipitated with anti-BPA antibody (R1086, lane 1) or control nonimmune antibody (lane 2) then fractionated by SDS-PAGE (8% acrylamide gel) and fluorography.

Interaction of $\alpha 6\beta 4$ -SACs and $\alpha 3\beta 1$ -FAs with ECM

Anti- $\alpha 6\beta 1$ partially inhibits HFK adhesion to ECM and $\alpha 6\beta 4$ /BPA-SACs are resistant to extraction and detachment from the ECM surface with detergent/urea. This suggests that the SACs interact with an uncharacterized ligand in the ECM. For the studies in Fig. 10, we have used antilaminin as a marker of deposited ECM. However, antilaminin does not inhibit HFK adhesion to ECM nor localization of $\alpha 3\beta 1$ into FAs (Carter et al., 1990). This suggests that laminin may not be the ligand for $\alpha 3\beta 1$ in the complex ECM. The adhesive ECM contains multiple protein bands that are potential ligands (see Fig. 8, **EXTRACT**, lane 4; Carter, W. G., and P. J. Gahr, manuscript in preparation).

The interaction of $\alpha 6\beta 4$ with ECM was investigated further. We wished to determine if HFK adhesion to an exogenous ECM surface would result in stable binding of $\alpha 6\beta 4$

to the exogenous ECM. ECM was prepared from HFKs grown on coverslips by sequential extraction, generating an adherent ECM that was insoluble in 8 M urea. The ECM was labeled with antilaminin antibodies. In control figures, the ECM was positive for laminin (Fig. 10 D) and BPA (Fig. 7 J), but lacked any detectable $\alpha 6\beta 4$ (Fig. 7 I). HFKs were added to the ECM-coated coverslips that had been labeled with antilaminin. The HFKs adhered to the deposits of ECM within 1 h and the adherent HFKs organized $\alpha 6\beta 4$ into SACs (Fig. 10 E) that contacted the exogenous ECM. During the adhesion process, $\alpha 6\beta 4$ formed a stable interaction with the exogenous ECM and this interaction was not dissociated with Triton X-100 (Fig. 10 G). This result indicates that $\alpha 6\beta 4$ in adhering HFKs rapidly binds to an uncharacterized ligand in the ECM.

Discussion

$\alpha 6\beta 4$ /BPA-SACs in Culture Are Similar to Hemidesmosomes in Skin

Hemidesmosomes have been suggested to mediate basal cell adhesion to the BMZ in skin (Staehelein, 1974). Our results indicate that $\alpha 6\beta 4$ in cultured HFKs codistributes with BPA in a new SAC that is distinct from $\beta 1$ -containing FAs, but similar to hemidesmosomes in skin. SACs are distinct from FAs based on their unique characteristics as follows (also see Table I and Fig. 11): (a) antibodies against $\alpha 6\beta 4$, in the presence of anti- $\alpha 3\beta 1$, inhibit HFK adhesion to ECM (Fig. 4) and localize $\alpha 6\beta 4$ in cell-substrate contact sites (Fig. 5); (b) the formation of $\alpha 6\beta 4$ -SACs occurs only in nonmigrating cells (Fig. 6); (c) BPA codistributes with $\alpha 6\beta 4$ -SACs but not $\alpha 3\beta 1$ -FAs (Fig. 7); (d) $\alpha 6\beta 4$ /BPA-SACs are relatively stable to detergent/urea extraction (Figs. 1, 7, and 10); and (e) $\alpha 6\beta 4$ -SACs are excluded from the origins of actin-containing stress fibers in FAs (Fig. 9). The similarities in composition, stability, cytoskeletal association, and function all suggest that SACs in culture may be related to hemidesmosomes in skin. Further, this is the first demonstration of a cell adhesion function by a hemidesmosome-like structure.

These results indicate that (a) $\alpha 6\beta 4$ may interact with BPA inside the cell in SACs and (b) $\alpha 6\beta 4$ and $\alpha 3\beta 1$ preferentially interact with an uncharacterized ligand(s) in the ECM that may be distinct from purified laminin (see Fig. 11). The carboxy terminus of the 230-kD BPA is localized to the cytoplasmic domain of hemidesmosomes in adherent HFKs (Tanaka et al., 1990) suggesting that BPA is a cytoplasmic protein. However, it is possible that the amino terminus of the 230-kD BPA or the 180 kD form of BPA may be exposed to the extracellular domain. Our results indicate that cytoplasmic BPA of SACs remains associated with the ligands in the 8 M urea-insoluble ECM used in our adhesion studies. Reasonably, $\alpha 6\beta 4$ may also be associated with the ECM as a link between intracellular BPA and extracellular ligand. However, the denaturing extraction conditions used in preparation of the ECM precluded identification of the labile epitopes in $\alpha 6\beta 4$ recognized with mAbs Goh3 and 3E1.

$\alpha 3\beta 1$ -FAs and $\alpha 6\beta 4$ -SACs in Adhesion to ECM

The proposed role for $\alpha 3\beta 1$ as a mediator of HFK adhesion to the ECM is based on multiple lines of evidence including inhibition of attachment and spreading with anti- $\alpha 3\beta 1$

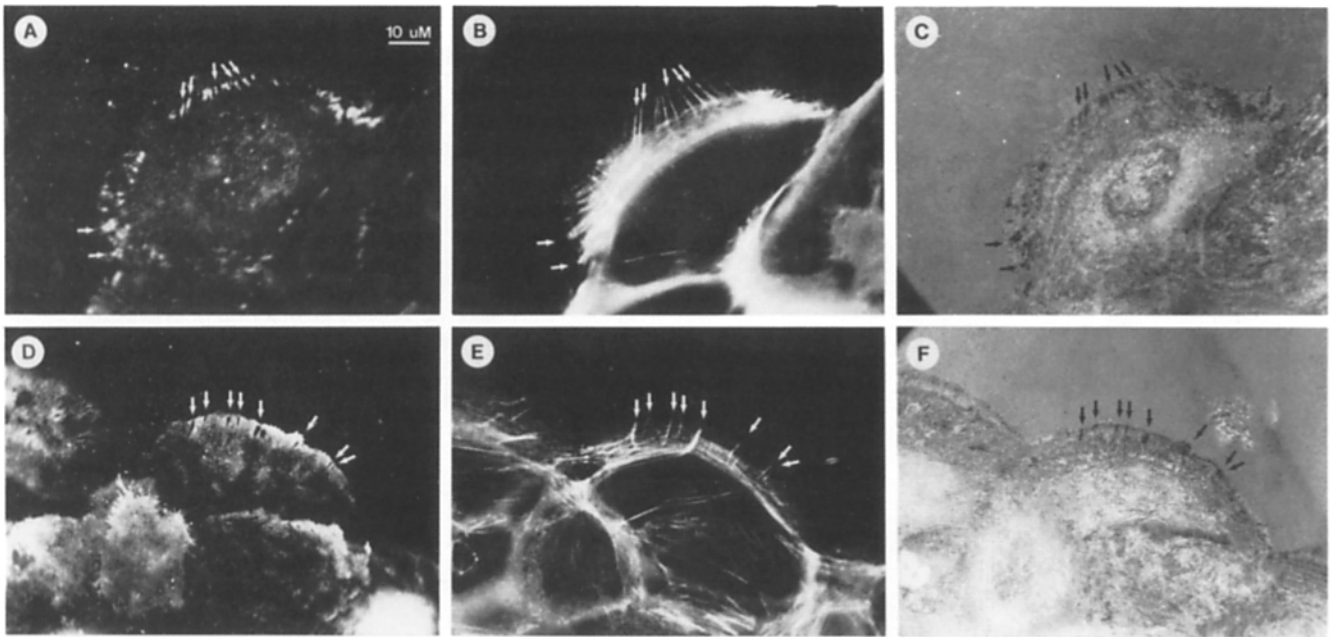


Figure 9. $\alpha 3\beta 1$ -FAs, but not $\alpha 6\beta 4$ /BPA-SACs, are the origins for actin-containing stress fibers. HFKs were cultured for 2 d on glass coverslips coated with laminin. Cells were stained with rhodamine-conjugated phalloidin (*B* and *E*) to localize actin-containing stress fibers and anti- $\alpha 6$ (GoH3, *D*) or anti- $\alpha 3\beta 1$ (PIF2, *A*) in relation to contacts with the adhesion surface detected with interference reflection microscopy (*C* and *F*). Arrows identify $\alpha 3\beta 1$ -FAs at the origin of actin-containing stress fibers (*A*–*C*, same field) or $\alpha 6\beta 4$ excluded from FAs and stress fibers (*D*–*F*, same field).

(PIB5; Figs. 2–4), and localization of $\alpha 3\beta 1$ in FAs on ECM (Carter et al., 1990). In addition, $\alpha 3\beta 1$, from other cells has been identified as a receptor for fibronectin/collagen/laminin (Wayner and Carter, 1987) and laminin (Gehlsen et al., 1988, 1989) by affinity chromatography. The data supporting the role of $\alpha 6\beta 4$ in HFK adhesion includes: (a) inhibition of HFK adhesion to ECM and laminin by combinations of anti- $\alpha 6\beta 4$ (GoH3, *D*) with anti- $\alpha 3\beta 1$ (PIB5, *A*), (b) colocalization of $\alpha 6\beta 4$ in SACs in contact with the adhesion surface, and (c) association of $\alpha 6\beta 4$ in SACs in a detergent-insoluble complex with exogenous ECM during adhesion. When $\alpha 6$ is associated with $\beta 4$, anti- $\alpha 6\beta 4$ (Cheresh et al., 1989) and anti- $\alpha 6$ (GoH3; Lotz et al., 1990) have been reported to inhibit cell adhesion to laminin. In addition, anti- $\alpha 6$ (GoH3) has been reported to inhibit platelet adhesion to laminin when $\alpha 6$ is associated with $\beta 1$ (Sonnenberg et al., 1988).

In our short-term (1–2 h) adhesion studies, the HFK preference for ligands was ECM > collagen > fibronectin > laminin. In long-term adhesion studies (18–24 h) on laminin and other ligands, HFKs synthesize their own ECM (Carter et al., 1990) and this results in the formation of $\alpha 3\beta 1$ -FAs

and $\alpha 6\beta 4$ -SACs. We have identified a possible ligand for $\alpha 6\beta 4$ -SACs present in the HFK ECM and BMZ of skin that is distinct from laminin (Carter, W. G., and P. J. Gahr, manuscript in preparation). By using our culture and assay conditions, $\alpha 3\beta 1$ -FAs plays the dominant role in adhesion and spreading on ECM and overshadows the role of $\alpha 6\beta 4$ -SACs. Conceivably, $\alpha 6\beta 4$ may play a dominant role in mediating adhesion in other culture systems or in tissue. This possibility is consistent with the localization of $\alpha 6\beta 4$ /BPA at the dermal–epidermal junction in normal skin.

Distinct Adhesion Functions for $\alpha 3\beta 1$ -FAs and $\alpha 6\beta 4$ -SACs

Differences in location, composition, and stability of $\beta 1$ -FAs and $\alpha 6\beta 4$ -SACs (Table I, Fig. 11) suggest distinct functions for these two adhesion structures. Anti- $\alpha 2\beta 1$ (PIH5) and anti- $\alpha 5\beta 1$ (PIF8/PID6) inhibit cell migration on collagen and fibronectin, respectively (Straus et al., 1989). Similarly, anti- $\alpha 3\beta 1$ (PIB5) inhibits cell migration on ECM since it inhibits spreading and partially detaches HFKs even when

Figure 10. HFKs attach $\alpha 6\beta 4$ to exogenous HFK-ECM in a Triton X-100/urea-insoluble complex. HFKs grown on glass coverslips for 3 d deposit laminin on the adhesion surface (*A* and *B* are the same field; *A*, stained with anti- $\alpha 6$, GoH3; *B*, antilaminin, R5922). ECM was prepared by sequential extraction of HFKs grown on glass coverslips as seen in *A* and *B*. The adherent ECM contained laminin (*D*, R5922) and BPA but not $\alpha 6\beta 4$ (*C*, GoH3) or $\beta 1$ -containing integrins. The ECM was incubated with antilaminin antiserum (R5922) and washed in order to label the exogenous ECM. HFKs were attached to the prelabeled ECM for 2 h then fixed and permeabilized (*E* and *F*) or extracted with 1% Triton X-100 before fixation (*G* and *H*). Adherent HFKs or detergent-insoluble matrix were incubated with anti- $\alpha 6\beta 4$ (GoH3) followed by FITC- and rhodamine-conjugated secondary antibodies to detect both the prelabeled laminin in the ECM and $\alpha 6\beta 4$ attached to the ECM as a detergent-insoluble complex. The HFKs selectively attached to the laminin-containing ECM spots. Triton extraction of the adherent HFKs left endogenous $\alpha 6\beta 4$ (*G*) attached to the exogenous ECM (*H*).

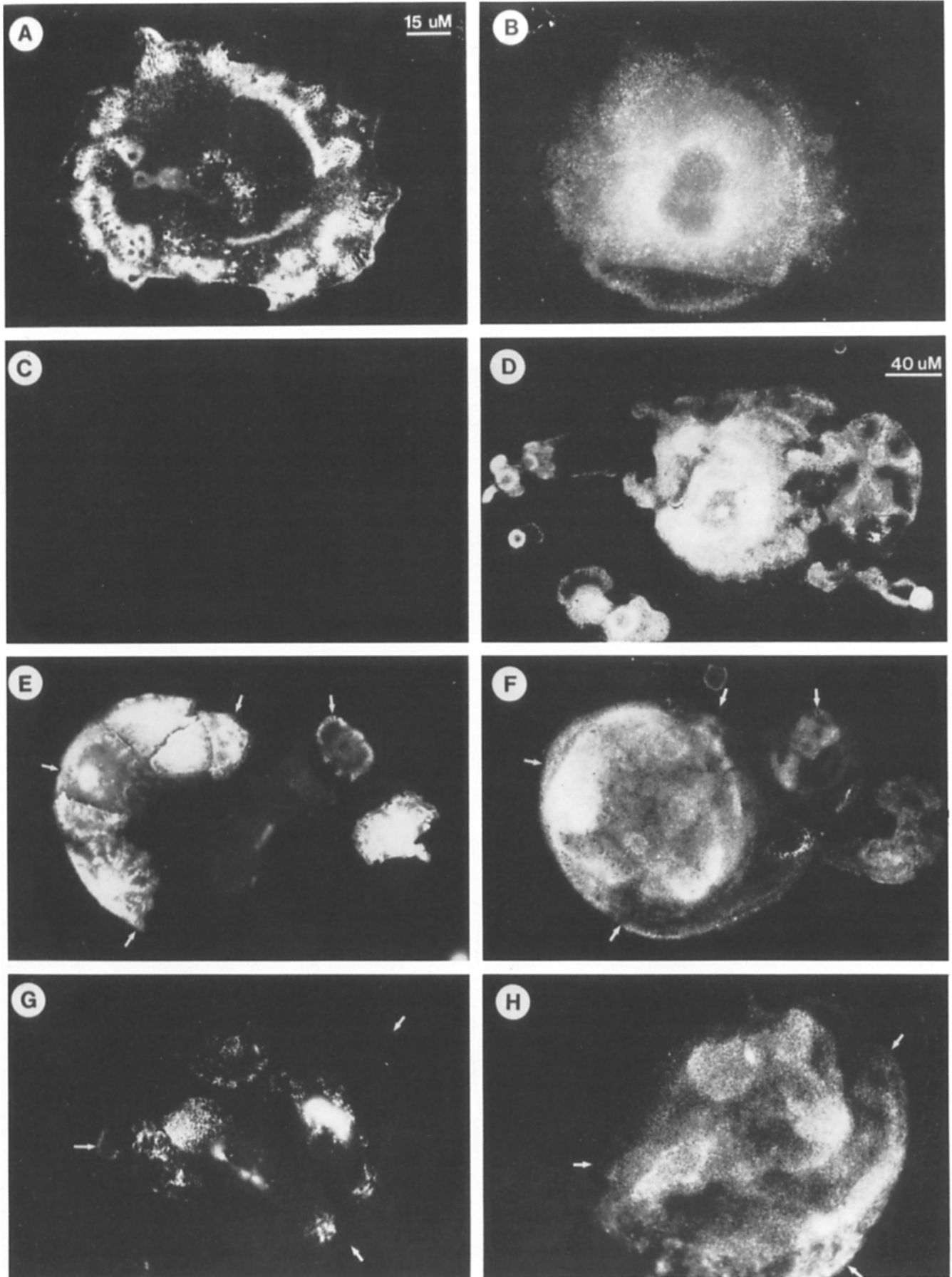


Table I. Characteristics of Focal Adhesions and Stable Anchoring Contacts (SACs) in Cultured Human Neonatal Foreskin Keratinocytes

Characteristic	FA	SAC
Receptor	$\beta 1$ integrins	$\alpha 6\beta 4$
Ligand	$\alpha 3\beta 1$: ECM, L $\alpha 2\beta 1$: C, L $\alpha 5\beta 1$: F	$\alpha 6\beta 4$: ECM
Pemphigoid antigen	Not present	Codistributes with $\alpha 6\beta 4$
Distribution of integrins	All sides of basal cell	Basal membrane of basal cell
Cytoskeletal association	Actin-containing stress fibers	No stress fibers (keratin filaments?)
Stability	Soluble in detergent	Insoluble in detergent/urea
Function	Dynamic adhesion (spreading, migration)	Stable adhesion (retention)

added after attachment (Carter et al., 1990). Additional support for the role of FAs in mediating migration of squamous epithelium was provided by Zieske et al. (1989). These results indicate a generalized dynamic role for $\beta 1$ -containing integrins in FAs as mediators of spreading, attachment, and migration through their association with actin-containing stress fibers and their specific ligands. Conversely, our results indicate that $\alpha 6\beta 4$ -SACs plays an anchoring or retention role based on the following. (a) $\alpha 6\beta 4$ /BPA-SACs in culture and in tissue are relatively stable to extraction with detergent/urea. (b) SACs form only in nonmigrating cells. (c) SACs do not associate with contractile actin-containing stress fibers important for motility.

The lack of $\alpha 6\beta 4$ codistribution with actin-containing

stress fibers is consistent with the published sequence for $\beta 4$ (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). Most of the peptide sequence of $\beta 4$ lies within the cytoplasm and has little homology to $\beta 1$. Conceivably the unique cytoplasmic domain of $\beta 4$ may preferentially interact with BPA in SACs, directly or indirectly, instead of with actin filaments.

We suggest that interaction of $\alpha 6\beta 4$ /BPA-SACs with the BMZ-like ECM in cultured HFKs may play a role in restricting cell motility on the ECM. These components may play a similar role in anchoring the basal cells to the BMZ in the form of hemidesmosomes in skin. In contrast, the relatively unstable but dynamic $\alpha 3\beta 1$ -FAs mediate motility functions in HFK-ECM interactions. $\alpha 3\beta 1$ and $\alpha 2\beta 1$ can also relocate to cell-cell junctions in differentiating keratinocytes where

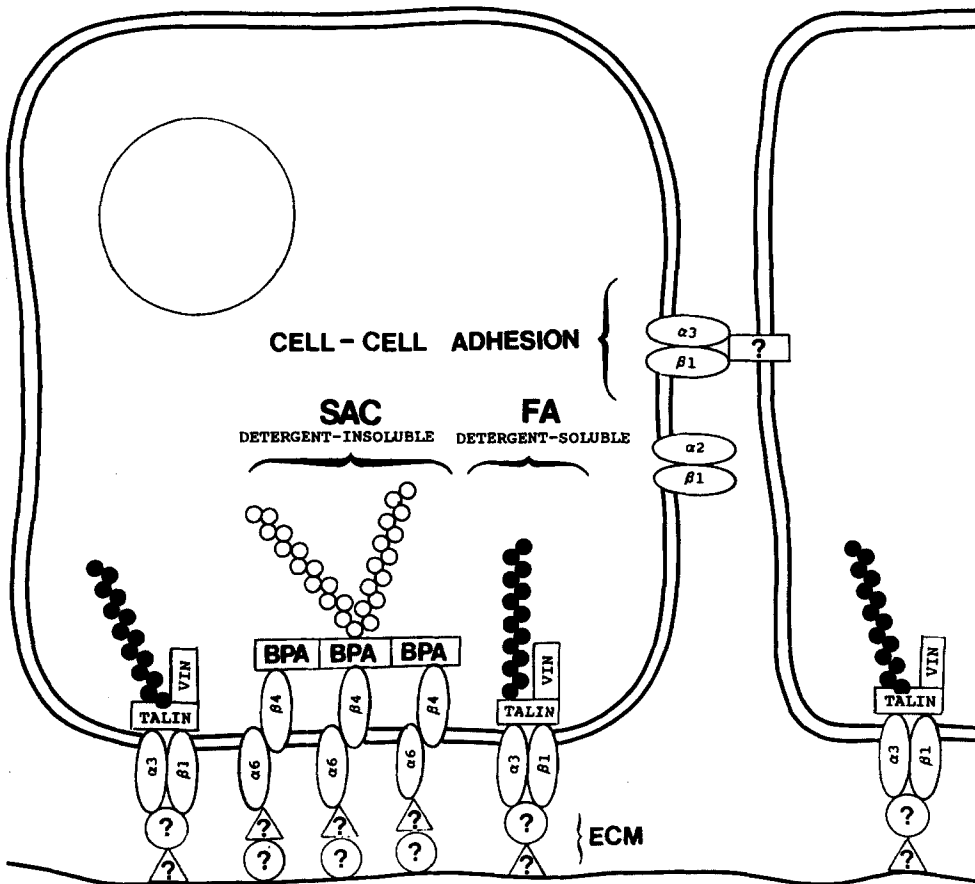


Figure 11. Diagrammatic representation of $\alpha 6\beta 4$ /BPA-SACs and $\alpha 3\beta 1$ -FAs in HFKs adherent to ECM and adjacent HFKs. Integrin $\alpha 3\beta 1$ colocalizes with actin-containing stress fibers (\bullet), talin and vinculin (VIN) in detergent-soluble FAs, and interacts with an uncharacterized ligand in the ECM (?). $\alpha 3\beta 1$ and $\alpha 2\beta 1$, the collagen receptor, can relocate from FAs to cell-cell contacts. $\alpha 3\beta 1$ may facilitate intercellular adhesion via an uncharacterized intercellular ligand or coreceptor (?). In contrast, transmembrane $\alpha 6\beta 4$ colocalizes with cytoplasmic BPA in SACs, and forms a detergent-insensitive complex with an uncharacterized ligand (?) present in the ECM. Preliminary results identify keratin (\circ), as a component of SACs. FAs and SACs may mediate motility and retention functions, respectively.

they may function in cell-cell interactions (Kaufmann et al., 1989; Larjava et al., 1990; Carter et al., 1990). The dynamic character of $\alpha 3\beta 1$ may facilitate movement of daughter cells away from the BMZ and mediate stratification of the skin (Carter et al., 1990). Proper morphogenesis would require a balance of SACs and FAs to mediate adhesive functions. Conceivably, low expression of $\alpha 6\beta 4$ or BPA would permit the function of the motile $\beta 1$ -integrins to predominate with possible cell invasion of the dermis.

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