The Role of Integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in Cell–Cell and Cell–Substrate Adhesion of Human Epidermal Cells

William G. Carter, Elizabeth A. Wayner, Todd S. Bouchard, and Pritinder Kaur

Fred Hutchinson Cancer Research Center and Department of Pathobiology, The University of Washington, Seattle, Washington 98104

Abstract. We have examined cultures of neonatal human foreskin keratinocytes (HFKs) to determine the ligands and functions of integrins $\alpha 2\beta 1$, and $\alpha 3\beta 1$ in normal epidermal stratification and adhesion to the basement membrane zone (BMZ) in skin. We used three assay systems, HFK adhesion to purified extracellular matrix (ECM) ligands and endogenous secreted ECM, localization of integrins in focal adhesions (FAs), and inhibition of HFK adhesion with mAbs to conclude: (*a*) A new anti-$\alpha 3\beta 1$ mAb, PIF2, localized $\alpha 3\beta 1$ in FAs on purified laminin > fibronectin/collagen, indicating that laminin was the best exogenous ligand for $\alpha 3\beta 1$. However, in long term culture, $\alpha 3\beta 1$ preferentially codistributed in and around FAs with secreted laminin-containing ECM, in preference to exogenous laminin. Anti-$\alpha 3\beta 1$, mAb PIB5, detached prolonged cultures of HFKs from culture plates or from partially purified HFK ECM indicating that interaction of $\alpha 3\beta 1$ with the secreted laminin-containing ECM was primarily responsible for HFK adhesion in long term culture. (*b*) In FA assays, $\alpha 2\beta 1$ localized in FAs coincident with initial HFK adhesion to exogenous collagen, but not laminin or fibronectin. However, in inhibition assays, anti-$\alpha 2\beta 1$ inhibited initial HFK adhesion to both laminin and collagen. Thus, $\alpha 2\beta 1$ contributes to initial HFK adhesion to laminin but $\alpha 3\beta 1$ is primarily responsible for long-term HFK adhesion to secreted laminin-containing ECM. (*c*) Serum or Ca^{2+}-induced aggregation of HFKs resulted in relocation of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ from FAs to cell–cell contacts. Further, cell–cell adhesion was inhibited by anti-$\alpha 3\beta 1$ (PIB5) and a new anti-$\beta 1$ mAb (P4C10). Thus, interaction of $\alpha 3\beta 1$ with either ECM or membrane coreceptors at cell–cell contacts may facilitate Ca^{2+}-induced HFK aggregation. (*d*) It is suggested that interaction of $\alpha 3\beta 1$ with a secreted, laminin-containing ECM in cultured HFKs, duplicates the role of $\alpha 3\beta 1$ in basal cell adhesion to the BMZ in skin. Further, relocation of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ to cell–cell contacts may result in detachment of cells from the BMZ and increased cell–cell adhesion in the suprabasal cells contributing to stratification of the skin.

THE human epidermis is a regenerating tissue composed of four stratified cell layers, stratum germinativum (basal), stratum spinosum (spinous, including suprabasal immediately above the basal), stratum granulosum (granular), and stratum corneum (cornified) in order of increasing degree of cell differentiation (Green, 1980; Sun et al., 1983; Potten and Morris, 1988; Stoler et al., 1988). The basal cells adhere to a basement membrane zone (BMZ)[1] that separates them from the dermis. The composition of the BMZ includes laminin, type IV collagen, and proteoglycans as well as other components and is synthesized by the basal cells (Katz, 1984; Martin and Timple, 1987). Differentiation of epidermis is accompanied by decreased basal cell–substratum adhesion to the BMZ (Watt, 1984; Stanley et al., 1980) followed by increased cell–cell adhesion and desmosome assembly as the cells move into the suprabasal and spinous cell layers (Klein-Szanto, 1977). Basal keratinocytes can be established in culture (Green, 1977; Rheinwald, 1980; Boyce and Ham, 1985; Fusenig, 1986; Pittelkow and Scott, 1986) and induced to aggregate and stratify by addition of Ca^{2+}, or serum. These cultures serve as models for skin regeneration and wound healing (Asselineau et al., 1985; Kopan et al., 1987).

Cell–cell and cell–substratum interactions play a major role in regulating the morphogenesis of the epidermis (Edelman, 1984; Ekblom et al., 1986). In culture, cell substratum adhesion is mediated by at least two adhesion structures, weak interactions via close contacts and strong interactions via focal adhesions (FAs; for review see Burridge et al., 1988). FAs are the points at which cells make their closest contacts with the substratum and provide the primary stabilizing force for attachment of cultured cells and the sites of initiation for actin containing stress fibers. Integrin receptors $\alpha 5\beta 1$ and $\alpha v\beta 3$ will selectively localize to FAs at sites of cell

Address reprint requests to Dr. William G. Carter, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

1. *Abbreviations used in this paper*: BMZ, basement membrane zone; ECM, extracellular matrix; FA, focal adhesion; HFK, human foreskin keratinocytes; IRM, interference reflection microscopy.

A
10 uM
B
C
D
E
F
G
10 uM
H
I

Table I. Characteristics of Anti-Integrin Antibodies

Antigen*	Antibody‡	Epitope§	Distribution‖ in skin	Inhibition of adhesion		
				Cell-Cell¶	Cell-ECM**	Focal adhesions‡‡
$\alpha1\beta1$	TS2/7		Dermis	ND	ND	ND
$\alpha2\beta1$	P1H5	*a*	B,SB,S	−	+ C,L	−
	P4B4	*b*	B,SB	−	−	+ C
	P1E6	*c*	B,SB	ND	± C,L	+ C
	P1H6	*d*	B,SB	−	ND	+ C
$\alpha3\beta1$	P1B5	*a*	B	+	+ L,F,CS	−
	P1F2	*b*	B	−	± L,F,CS	+ L,CS
$\alpha4\beta1$	P4G9		Hematopoietic	(+)	+ F	+
$\alpha5\beta1$	P1D6	*a*	−	−	+ F	−
	AB33	*b*	Muscle	ND	ND	+ F
$\alpha6\beta4/\beta1$	GoH3		B	ND	ND	± L
$\beta1$	P4C10		B,SB	+	+ C,L,F,CS	+ C,L,F,CS
	A1A5		ND	ND	ND	+ C,L,F,CS

* The indicated antibodies all react with specific epitopes located on the α subunit or α-β subunit combinations.
‡ All the indicated antibodies are monoclonal except AB33, which is a rabbit polyclonal.
§ Differences in epitopes recognized by the antibodies (indicated by *a*, *b*, *c*, etc.) have been identified by competitive binding studies on a flow cytometer.
‖ The primary distribution of the specific epitope for each antibody in cryostat sections of neonatal human foreskin: B = basal; SB = suprabasal; S = spinous layers of the epidermis. mAb P4C2 is expressed primarily in hematopoietic cells and will inhibit lymphoid cell adhesion to endothelium (Wayner, 1989, manuscript in preparation).
¶ ** Inhibition of epidermal cell adhesion to confluent monolayers of cells (cell-cell) or various ECM ligands coated on a surface (cell-ECM; L = laminin; F = fibronectin; C = collagen types I and IV; CS = a partially purified ECM complex that is secreted by basal keratinocytes and contains at least laminin).
‡‡ The ability of each antibody to detect integrins in focal adhesions on the indicated ligands is indicated.

adhesion to fibronectin and vitronectin, respectively, as observed by interference reflection microscopy (IRM, Singer et al., 1988) or by the antibody exclusion technique (Neyfakh et al., 1983), indicating that these receptors are directly involved in mediating cell adhesion to these ligands.

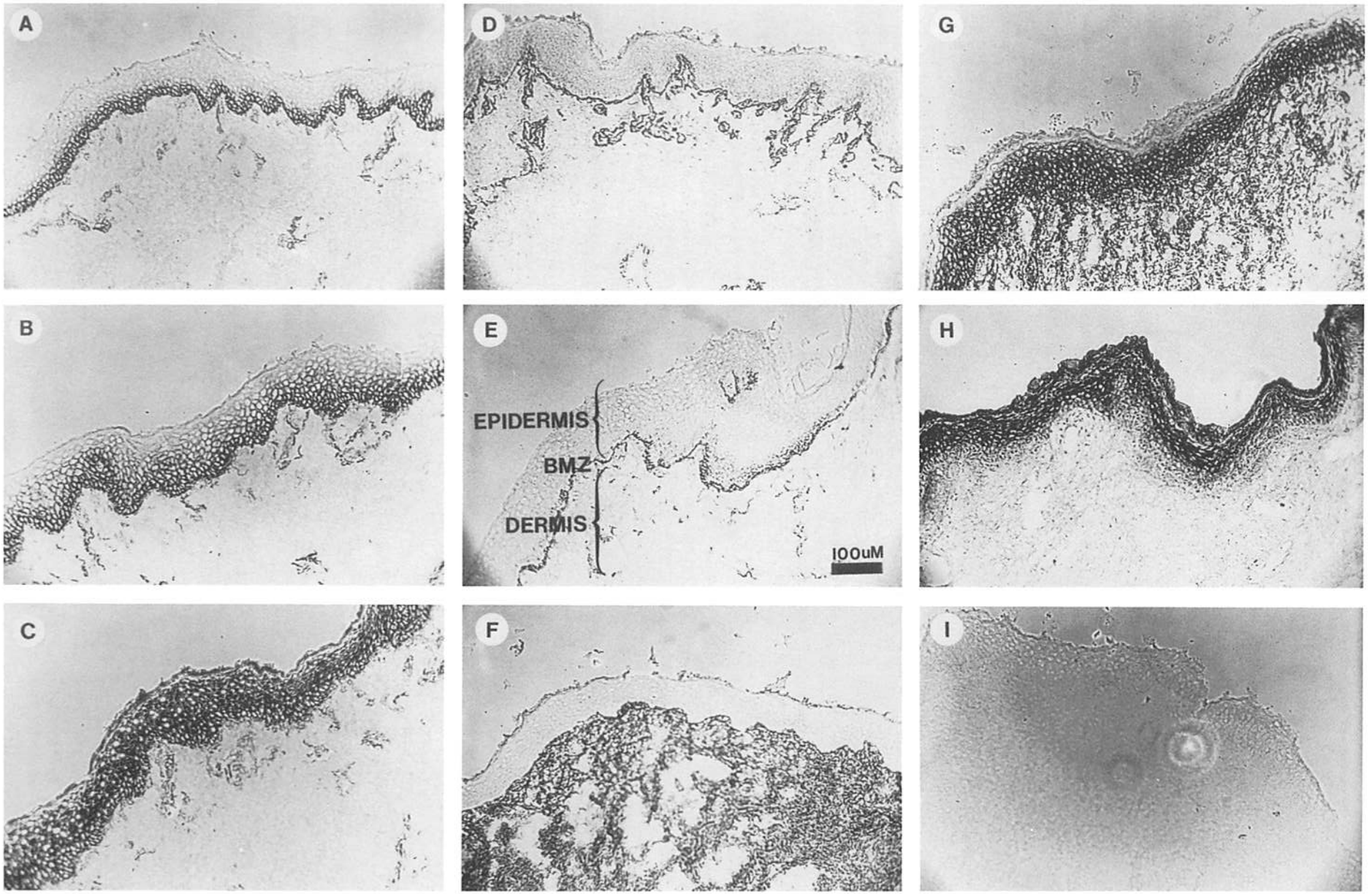
We have examined cultured human keratinocytes in order to determine the role of integrin receptor (Hynes, 1987; Buck and Horwitz, 1987; Ruoslahti, 1988; Hemler, 1988) interactions with the extracellular matrix (ECM) in regulating skin morphogenesis. In preliminary experiments we made three pertinent observations: (*a*) expression of $\alpha2\beta1$, a collagen receptor (Wayner and Carter, 1987; Wayner et al., 1988), and $\alpha3\beta1$, a promiscuous receptor with affinity for laminin (Wayner and Carter, 1987; Gehlsen et al., 1988), was restricted primarily to the suprabasal/basal and basal layers of the epidermis in skin, respectively; (*b*) monoclonal antibodies (mAbs) to $\alpha2$ inhibited epithelial cell adhesion to laminin as well as collagen; and (*c*) $\alpha3\beta1$ failed to redistribute into focal adhesions during the initial stages of epithelial cell adhesion to various purified ECM ligands. These findings suggested that $\alpha2\beta1$ and $\alpha3\beta1$ may contribute to basal cell adhesion to the BMZ and in stratification to the suprabasal region of the spinous layer. We selected human neonatal foreskin keratinocytes (HFKs) to investigate the function and ligands for integrins $\alpha2\beta1$ and $\alpha3\beta1$ in epidermis. We addressed three issues: (*a*) analysis of $\alpha2\beta1$ and $\alpha3\beta1$ interactions with purified ECM ligands and secreted BMZ components synthesized by HFKs in culture; (*b*) the effect of serum or Ca^{2+} induction on the expression and function of $\alpha2\beta1$ and $\alpha3\beta1$ in cell-cell and cell-substrate adhesion of HFKs; and (*c*) the relation between differential expression of $\alpha2\beta1$ and $\alpha3\beta1$ and keratinocyte stratification and differentiation.

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). Lactoperoxidase and glucose oxidase were from Calbiochem-Behring Corp. (La Jolla, CA). Peroxidase-, and fluores-

Figure 1. Localization of $\alpha2\beta1$ and $\alpha5\beta1$ to focal adhesions and origins of stress fibers in WI-38 fibroblasts. WI-38 fibroblasts were adhered (1 h) to glass cover slips coated with collagen (type I, *A–C*, same field and *G* and *H* same field) and fibronectin (*D–F*, same field), fixed, and permeabilized. The adherent cells were incubated simultaneously with the following primary mouse and rabbit antibody combinations, followed by FITC- and rhodamine-conjugated secondary antibodies to detect bound primary antibodies or rhodamine conjugated phalloidin to detect actin containing stress fibers. Photomicrographs were prepared using FITC, rhodamine, or IRM filters. (*A*) P4B4 anti-$\alpha2\beta1$; (*B*) anti-type I collagen; (*C*) IRM, (*D*) AB33 anti-$\alpha5\beta1$; (*E*) P1H11 anti-fibronectin; (*F*) IRM; (*G*) P4B4, anti-$\alpha2\beta1$; (*H*) rhodamine-phalloidin; (*I*) IRM.


A
D
G
B
E
EPIDERMIS
BMZ
DERMIS
100uM
H
C
F
I

cein-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). Rabbit anti-mouse and anti-rat IgG (H+L) antiserum was obtained from Cappel (Cooper Laboratories Biomedical, Malvern, PA). ^{51}Cr-sodium chromate was from New England Nuclear (Boston, MA). ^{125}I was from Amersham Chemical Co. (Arlington Heights, IL).

Cells and Cell Culture

Normal HFKs were prepared as described by Boyce and Ham (1985) after sequential digestion of the tissue with dispase (grade II; Boehringer Mannheim, Indianapolis, IN) to separate the dermis from the epidermis and digestion of the epidermis with trypsin to release cells. Cultures were maintained in serum-free keratinocyte basal medium (KBM; Clonetics, San Diego, CA) containing insulin, epidermal growth factor, hydrocortisone, and bovine pituitary extract (~50 μg protein/ml) and referred to as keratinocyte growth medium (KGM) through two passages then used for experiments through passage five, while being maintained in KGM and fed every 3 d. These cells could be induced with Ca^{2+}, PMA, or serum to express filaggrin and involucrin markers. FEA (Kaur and McDougall, 1988) and EIL8 established keratinocyte cell lines (Kaur et al., 1989) were generated by transfection of HFKs with genomes from human papilloma virus 18 and 16, respectively. EIL8 cells were used for cell-cell adhesion studies (Fig. 8) because they respond to Ca^{2+}-induction similar to HFKs. FEA cells were used for adhesion to HFK ECM because they do not synthesize their own ligand for α3β1 (Fig. 12). OVCAR-4 cells (human ovarian carcinoma) were obtained as a gift from Dr. Arnoud Sonnenberg (Amsterdam, Holland).

Preparation of Extracellular Matrix Adhesive Ligands

Mouse laminin (derived from Engelbreth-Holm-Swarm sarcoma, grown in mice) was purchased from Collaborative Research, Inc. (Bedford, MA) or prepared in our laboratory. The EHS sarcoma laminin migrated as two bands of ~200 and 400 kD on SDS-PAGE and that reacted with anti-laminin antibodies by immunoblotting. No other bands reacted with anti-fibronectin or anti-type IV collagen antibodies or stained with Coomassie blue. Plasma fibronectin and collagen types I, III, IV, V, and VI were prepared as described (Wayner et al., 1988). Vitronectin and pepsinized human placental laminin were purchased from Telios Pharmaceuticals, Inc. (San Diego, CA). In some experiments, pepsinized human laminin was used instead of mouse laminin but always produced similar results.

Keratinocyte Extracellular Matrix and Conditioned Culture Medium

HFKs were maintained in serum-free KGM. Conditioned KGM was pooled, adjusted to 1 mM with PMSF and *N*-ethylmaleimide, precipitated with ammonium sulfate (50% saturation), and the pellet was dissolved and dialyzed in PBS. Fibronectin was removed from the solution by chromatography on gelatin-Sepharose. This solution could induce cell adhesion and will be referred to as culture supernatant (CS). Glass cover slips, prepared as described below, were coated with CS at a concentration of 25 μg protein/ml. Alternatively, HFK ECM was prepared by growing HFK cells to confluence on glass cover slips coated with CS. The adherent cells were removed by sequential extraction with (*a*) 1% vol/vol Triton X-100 detergent in PBS for 15 min; (*b*) 2 M urea in 1 M NaCl. The resulting cover slips were washed with PBS and used for cell adhesion studies.

Antibodies

Table I contains a summary of the characteristics of the various monoclonal and polyclonal anti-integrin antibodies used in this study. A rabbit polyclonal antibody, AB33, prepared against the cytoplasmic domain of the fibronectin receptor, integrin α5β1, (Roman et al., 1988) was used to detect α5β1 in focal adhesions. mAbs AIA5, against the common integrin β1 subunit of the VLA family of receptors (Hemler, 1988) and anti-α1β1 (TS 2/7) were obtained from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). mAbs to the integrin receptors α3β1 (PIB5), α2β1 (PIH5, P4B4, PIH6, PIE6), α4β1 (P4G9) and α5β1 (PID6) have been described (Wayner and Carter, 1987; Wayner et al., 1988, 1989). PIH5 and PID6 inhibit fibroblast and platelet adhesion to collagen and fibronectin-coated substrates, respectively (Wayner et al., 1987; Kunicki et al., 1988; Wayner et al., 1988). mAb P4C10 reacts with all β1-containing integrins and was prepared in this lab by selection of an antibody that inhibited cell adhesion to laminin, collagen, and fibronectin. mAb PIG12 anti-ECMR III/CD44 is directed against a 90–250-kD intrinsic membrane proteoglycan (Carter and Wayner, 1988) that is homologous to the lymphocyte homing receptor (Gallatin et al., 1989). Monoclonal anti-α6β1/β4 (GoH3) was from Dr. Arnoud Sonnenberg (Central Laboratory of the Netherlands, Amsterdam, Holland). mAb F9A5 reacts with tenascin and was prepared in this lab. Rabbit polyclonal anti-human fibronectin (R790), and mouse EHS sarcoma laminin (R5922) were prepared as previously described (Wayner and Carter, 1987). Rabbit polyclonal anti-type I and IV collagens were from Chemicon (El Segundo, CA). Rabbit polyclonal antibody to the E8 domain of laminin was a gift from Dr. Eva Engvall (La Jolla Cancer Research Center, La Jolla, CA). Rabbit polyclonal anti-involucrin was obtained from Dr. Robert Rice (Davis, CA).

Localization of Receptors in Focal Adhesions

Acid-washed glass coverslips (25-mm-diam) were derivatized with dimethyldichlorosilane (the slips were dipped in 10% solution in chloroform and then air dried) and then washed in chloroform and methanol to reduce nonspecific cell interaction with the glass surface. 30–60-μl aliquots of purified ligands (5–20 μg protein/ml) were spotted on the cover slips and incubated for 2–4 h. Excess ligand solution was removed with a suction apparatus to maintain a sharp boundary for each ligand spot. The surface was immediately washed and blocked with 1% heat denatured BSA in PBS for 1 h. Four to five ligand spots could easily be applied to single cover slips with no cell adhesion between the different ligand spots. The cover slips were placed in 3.5-cm petri dishes for the cell adhesion.

Cells were suspended by digestion with trypsin (0.05% wt/vol)/ethylenediamine tetraacetic acid (0.02%) in PBS, washed in RPMI 1640 containing 1 mg/ml BSA and 100 μg/ml soybean trypsin inhibitor. HFKs, FEA, and EIL8 cells were allowed to adhere to the prepared coverslips in either KGM or KBM medium, with comparable results, for periods of 1 h to 5 d. OVCAR-4 cells and fibroblasts were adhered in RPMI containing 1 mg BSA/ml.

Nonadherent cells were washed from the cover slips with warm PBS and the adherent cells fixed in warm 100 mM Na cacodylate buffer pH 7.2 containing 100 mM sucrose, 4.5 mM $CaCl_2$, and 2% formaldehyde for 20 min. The adherent and fixed cells were permeabilized with 0.5% vol/vol Triton X-100 detergent in PBS for 5 min, washed in PBS, and blocked with 10% vol/vol goat serum (fibronectin was removed from the goat serum by passage over gelatin-Sepharose).

Immunofluorescence, Interference Reflection, and Antibody Exclusion Microscopy

Cover slips and cells prepared as described above were incubated with combinations of mouse or rat mAbs and rabbit polyclonal primary antibodies diluted in 10% goat serum (fibronectin free) in PBS for 2–4 h. The use of two color fluorescence allowed us to identify both the receptors and the ligand spots in the same field and focal plane. 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 antibodies for 1 h, and washed with PBS. The cover slips were mounted with 80% glycerol containing 50 mM Tris HCl, pH 8.5, and 1 mg/ml phenylenediamine. The cells were examined with a Zeiss fluorescence microscope equipped with narrow band FITC and rhodamine filters using a 63× oil Neofluor anti-flex objective. IRM was performed basically as described (Izzard and Lochner, 1976) and was used to identify FAs

Figure 2. Localization of integrins in cryostat sections of neonatal human foreskin. Cryostat sections (6 μm) of fresh neonatal human foreskin were reacted with the indicated antibodies followed by reaction with peroxidase conjugated secondary antibodies. (*A*) PIF2, anti-α3β1; (*B*) P4B4, anti-α2β1; (*C*) PIH5, anti-α2β1; (*D*) R5922, anti-laminin; (*E*) F9A5, anti-tenascin; (*F*) PIH11, anti-fibronectin; (*G*) PIG12, anti-ECMR III/CD44, an intrinsic membrane proteoglycan; (*H*) anti-involucrin; (*I*) SP2, control.

in the same field as the two color fluorescence. FAs were also localized by the antibody exclusion technique (Neyfakh et al., 1983).

Characteristics of Integrin Localization in Focal Adhesions

In preliminary studies, we have examined the characteristics of integrin localization in FAs to develop a FA assay suitable for identifying preferential receptor-ligand interactions. Anti-$\alpha 2\beta 1$ mAbs, P1H5, P4B4, P1H6 and P1E6 react with nonoverlapping epitopes on the $\alpha 2$ subunit of the collagen receptor during initial adhesion of fibroblasts to collagen. (See Table I for a summary of these mAbs.) A summary of the characteristics for the assay is presented in Fig. 1 and is as follows: (*a*) mAbs P1H6, P1E6, and P4B4 that were noninhibitory or weakly inhibitory for cell adhesion to collagen, localized $\alpha 2$ into focal adhesions, as identified by IRM and antibody exclusion, on the basal surface of cells adhered to cover slips coated with collagen types I, III, IV, V, and VI (Fig. 1, *A–C*) but not laminin or fibronectin (Fig. 6, *C* and *D*). In contrast, the inhibitory anti-$\alpha 2$ mAb P1H5 was not detectable in FAs on collagen (Fig. 6 *A*). When not localized into FAs, $\alpha 2\beta 1$ was detected in a uniform cell surface localization or at intercellular junctions (see later results for details). Similarly, $\alpha 5\beta 1$, specifically localized in FAs, as detected with the noninhibitory Ab33, only when WI-38 fibroblasts adhered to fibronectin-coated surfaces (Fig. 1, *D–F*). (*b*) Localization of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ to FAs occurred simultaneously with the first detectable adhesion of WI-38 cells to the collagen and fibronectin surfaces (~60 min). (*c*) $\alpha 3\beta 1$ was not detected in FAs of WI-38 cells attached to any ligand, including laminin, despite the high cell surface expression of $\alpha 3\beta 1$, as seen in Fig. 3. (*d*) mAb A1A5 and P4C10 that react with the common integrin $\beta 1$ subunit, also localized to the FAs on fibronectin, collagen, and laminin, as well as a diffuse cell surface staining. (*e*) $\alpha 2\beta 1$ and $\alpha 5\beta 1$ localize to FAs at the origins of actin containing stress fibers (Fig. 1, *G* and *H*).

Inhibition of Cell–Substrate Adhesion

Inhibition of cell adhesion to various ligands was performed as previously described (Wayner and Carter, 1987). Briefly, 48-well virgin styrene plates were coated with adhesive ligands (5–10 μg/ml). The plates were blocked with PBS supplemented with 10 mg/ml heat-denatured BSA (HBSA). Cells were labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml for 2–4 h) and washed; then 5×10^4 cells/well were incubated with hybridoma culture supernatants (1:2 dilution in PBS supplemented with 1 mg/ml heat-denatured BSA) or control myeloma cell culture supernatant for 15 min at room temperature. The cells were allowed to adhere to the protein-coated surfaces in the presence of the hybridoma supernatants for 2–4 h at 37°C. Nonadherent cells were removed by washing with PBS, and the adherent cells were dissolved in SDS/NaOH and bound ^{51}Cr-counts per minute were quantitated in a gamma counter.

Inhibition of Cell–Cell Adhesion

Adhesion surfaces were prepared by growing E1L-8 cells to confluence in 48-well tissue-culture clusters (#3548; Costar Corp., Cambridge, MA) in KGM followed by activation of the cells by addition of 1 mM $CaCl_2$ 24 h before use in the assays. The Ca^{2+} induced cell–cell aggregation within 5 h of addition. Additional E1L8 cells were ^{51}Cr-labeled, Ca^{2+}-induced, suspended by digestion with trypsin (0.05% wt/vol in PBS containing 0.8 mM Ca^{2+} for 20 min), washed with soybean trypsin inhibitor, filtered through Nytex (Fairmont Fabrics, Pacifica, CA) to remove cell aggregates then added to the multiwell plates in RPMI containing 10% serum or KGM without serum with or without the presence of inhibitory antibodies. Essentially identical results were obtained using purified mAbs or hybridoma culture supernatants that had been dialyzed against fresh RPMI. Incubations were performed at room temperature with rocking for 60 min. Nonadherent labeled cells were removed from the adhesion assay by washing, whereas labeled cells that bound to the cell monolayer were dissolved in SDS-NaOH and counted in a gamma counter. All experiments were done in triplicate and the results are representative of at least three experiments.

Immune Precipitation, Sequential Immune Precipitation, and PAGE

Viable cells were surface labeled with ^{125}I as described (Wayner and Carter, 1987) followed by extraction with 1% vol/vol Triton X-100 detergent in 50 mM PBS, pH 7.2. 1 mM PMSF and 1 mM *N*-ethylmaleimide were added as protease inhibitors. Immune precipitation and sequential immune precipitations were performed as previously described (Wayner and Carter, 1987). SDS-PAGE gels were prepared following the basic stacking gel system of Laemmli (1970).

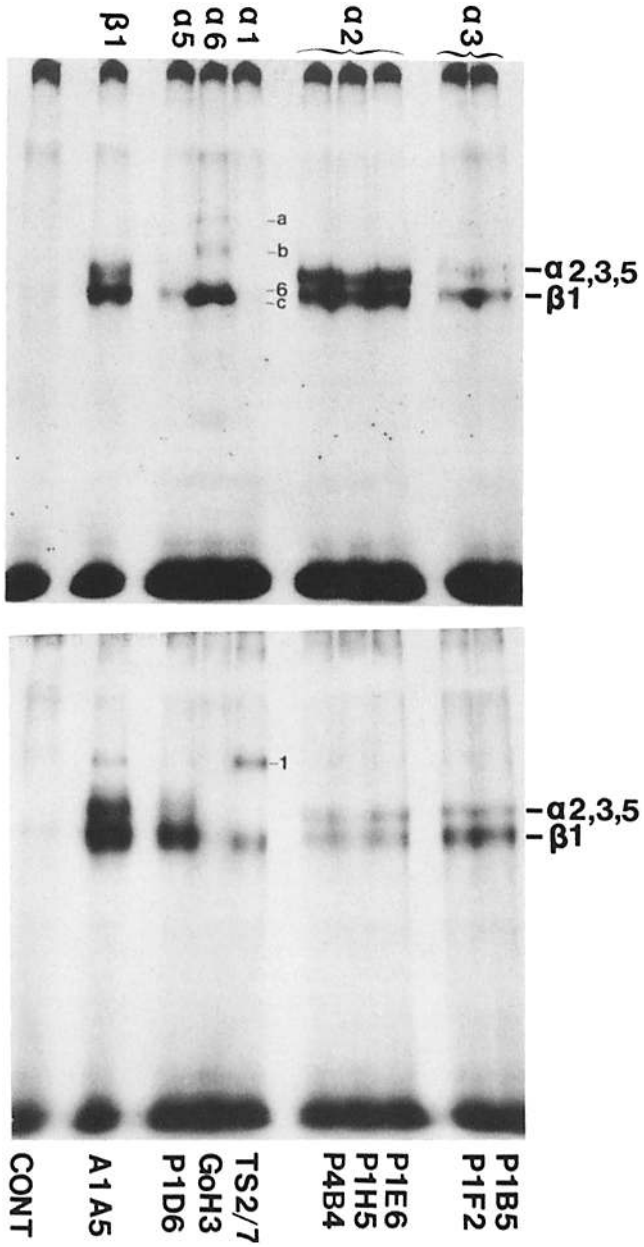


Figure 3. Immunoprecipitation of integrin receptors from neonatal human foreskin keratinocytes (HFKs) and neonatal human foreskin dermal fibroblasts (HFFs). HFKs (*top*) and HFFs (*bottom*) were surface labeled with ^{125}I-lactoperoxidase, extracted with Triton X-100 detergent and immunoprecipitated with the indicated mAbs (P1B5, P1F2, etc.). Migration of α and β subunits are indicated in the right margin. Migration of α6 and the *a*, *b*, and *c* bands of β4 (Hemler et al., 1989) are indicated in the middle lane of the top panel. Migration of α1 is indicated in the middle lane of the bottom panel.

Tissue Staining

The distribution of receptors and ligands in tissue was determined by immunoperoxidase microscopy of cryostat sections. Cryostat sections (6 μm) were prepared from human tissue samples embedded in OCT medium after snap freezing in isopentane/liquid nitrogen. All sections were fixed in 4%

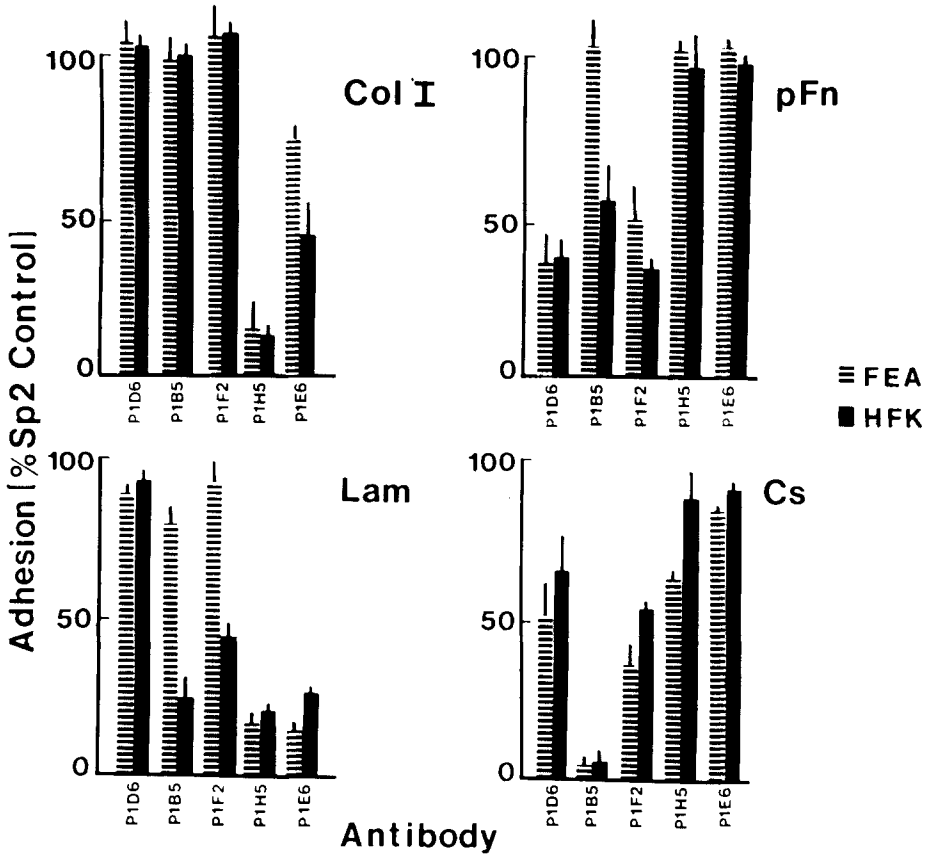


Figure 4. Inhibition of HFK and FEA adhesion to ECM components with mAbs to integrins. Nonadhesive surfaces were coated with the indicated ligands (*COL I*, type I collagen; *pFN*, human plasma fibronectin; *LAM*, mouse laminin from Engelbreth-Holm-Swarm sarcoma; *CS*, concentrated serum-free culture supernatant from HFK cells). ^{51}Cr-labeled HFK and FEA cells were allowed to adhere to the surfaces in the presence of the indicated antibodies (P1D6, P1B5, etc.) for 1 h and nonadherent cells were removed by washing. Adherent cells were dissolved and counted in a gamma counter. Adhesion is presented as the percent of cells adhering in the presence of the indicated antibodies relative to control SP2 antibodies. Each result is the average of four separate experimental events. The vertical line on each bar indicates SD. P1H5, anti-$\alpha 2\beta 1$, inhibited HFK and FEA adhesion to both collagen and laminin but not fibronectin or CS. P1B5, anti-$\alpha 3\beta 1$ partially inhibited adhesion to laminin and completely blocked adhesion to CS.

paraformaldehyde in PBS before incubation in primary antibodies and peroxidase-conjugated secondary antibodies.

Results

Expression of Integrins in Epidermal Tissue

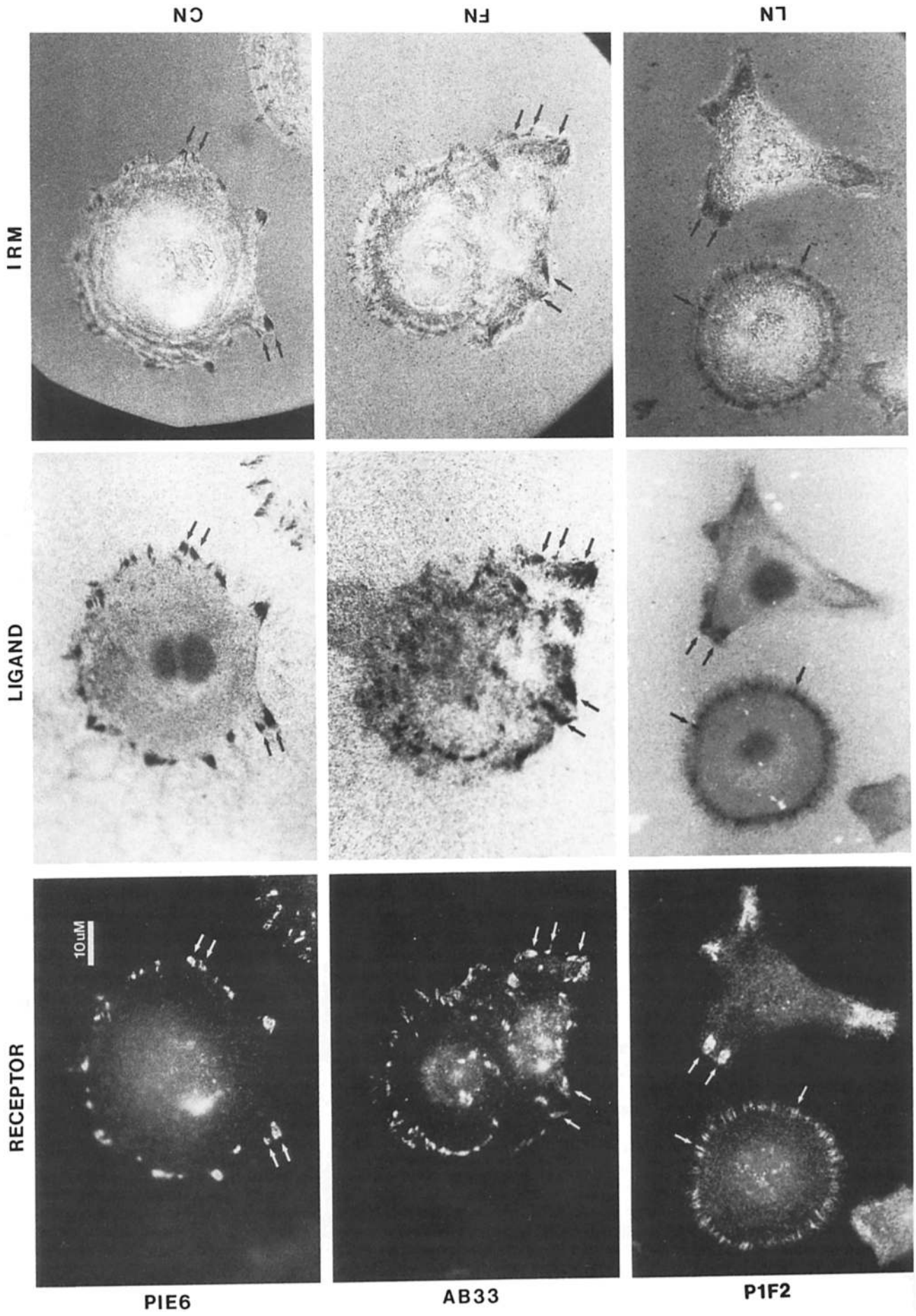
A summary of the characteristics of the various anti-integrin α subunits used in this study is presented in Table I. A differential expression of the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins by epithelial cells was seen in cryostat sections of neonatal foreskin (Fig. 2, *A–C*) as well as fetal (Wayner et al., 1988) and adult skin. $\alpha 3\beta 1$ was weakly and sporadically expressed in suprabasal cells and strongly expressed by basal epidermal cells (Fig. 2 *A*) in contact with the BMZ. This zone contains laminin, tenascin (Fig. 2, *D* and *E*), and type IV collagen. In contrast, $\alpha 2\beta 1$ detected with mAb P4B4 (or P1E6 and P1H6) was expressed on both basal and suprabasal cell layers. A second anti-$\alpha 2$ mAb, P1H5, also detected $\alpha 2\beta 1$ in the basal/suprabasal regions but was most intensely expressed throughout the spinous cell layer. The mAbs P4B4 and P1H5 detect different epitopes on the $\alpha 2$ subunit that are differentially expressed in both tissue and cell culture (Wayner et al., 1989). In general, both $\alpha 2$B1 and $\alpha 3\beta 1$ were detected on all surfaces of the cells in the basal and suprabasal strata of the epidermis. In addition, both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ were present in the glandular epithelium of the dermis. Fibronectin was expressed throughout the dermis (Fig. 2 *F*). In control experiments, mAb P1G12 localized ECMR III/CD44 in the basal and spinous layers of the epidermis and in the dermis (Fig. 2 *G*). ECMR III/CD44 is an intrinsic membrane proteoglycan (Carter and Wayner, 1988) related to the lymphocyte homing receptor/CD44 antigen (Gallatin et al., 1989). In contrast, involucrin (Fig. 2 *H*), a component of the cross-linked envelope of keratinocytes (Rice and Green, 1979) was localized to late-stage differentiated and stratified keratinocytes.

Expression of Integrins in Keratinocyte Cultures

Cultures of neonatal HFKs were established in defined (serum-free) culture medium. Immunoprecipitation of integrins from detergent extracts of ^{125}I-surface-labeled HFKs and (Fig. 3, *top*), with α subunit specific mAbs identified $\alpha 2$, $\alpha 3$, and $\alpha 6$ subunits at high levels and $\alpha 5$ subunit at low levels. The $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits were associated with the $\beta 1$ subunits. As recently shown by Hemler et al. (1989) for many carcinomas, $\alpha 6$ was associated with the a, b, and c peptide bands of the $\beta 4$ subunit of the integrin family. FEA and E1L-8 keratinocyte cell lines, expressed similar α and β subunits (data not shown). In contrast, human dermal fibroblasts isolated from the same tissue, expressed $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ at high levels but did not express $\alpha 6$ (Fig. 3, *bottom*).

Inhibition of Keratinocyte Adhesion with mAbs

Anti-$\alpha 2\beta 1$ (P1H5), and anti-$\alpha 5\beta 1$ (P1D6) were found to inhibit initial HFK and FEA cell adhesion to collagen and fibronectin, respectively (Fig. 4). This is consistent with the expression of these subunits in tissue and culture. In contrast


IRM
LIGAND
RECEPTOR
10uM
PIE6
AB33
P1F2

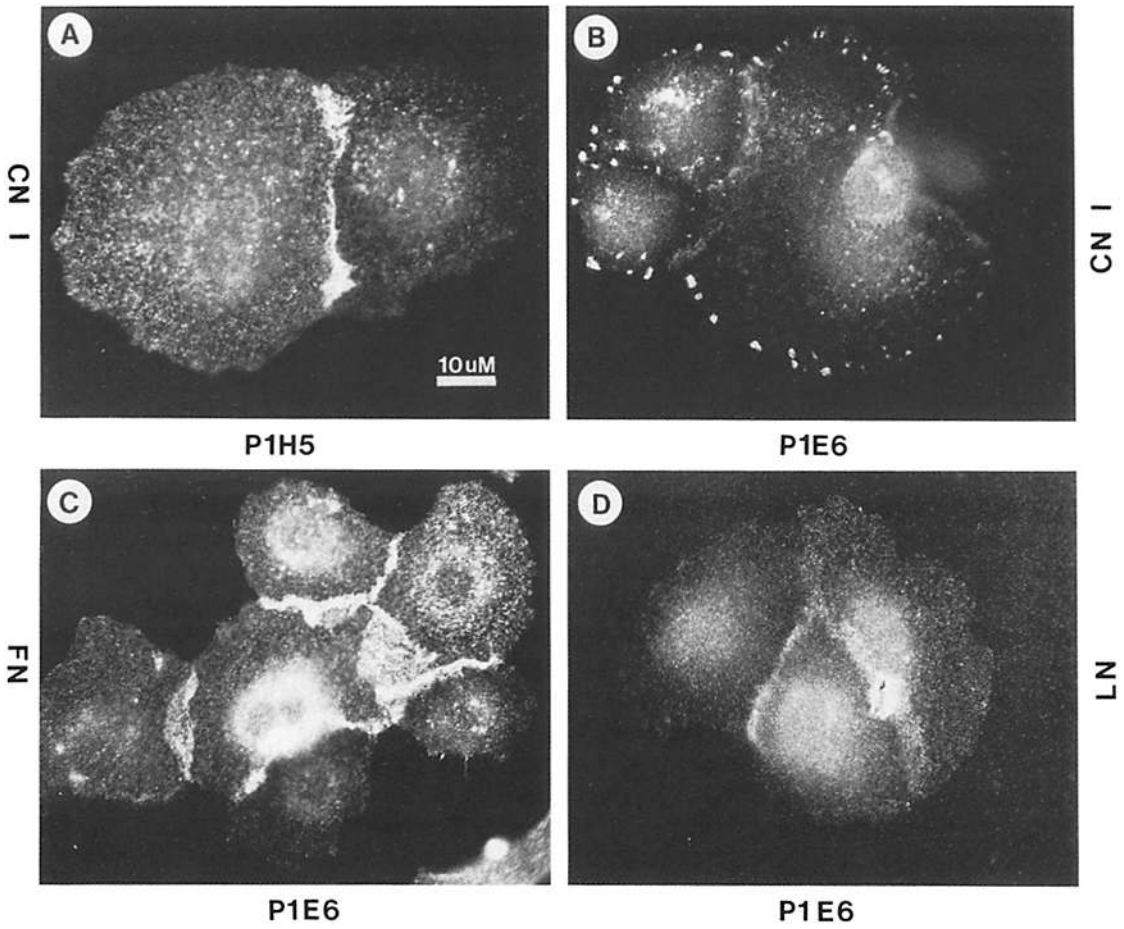


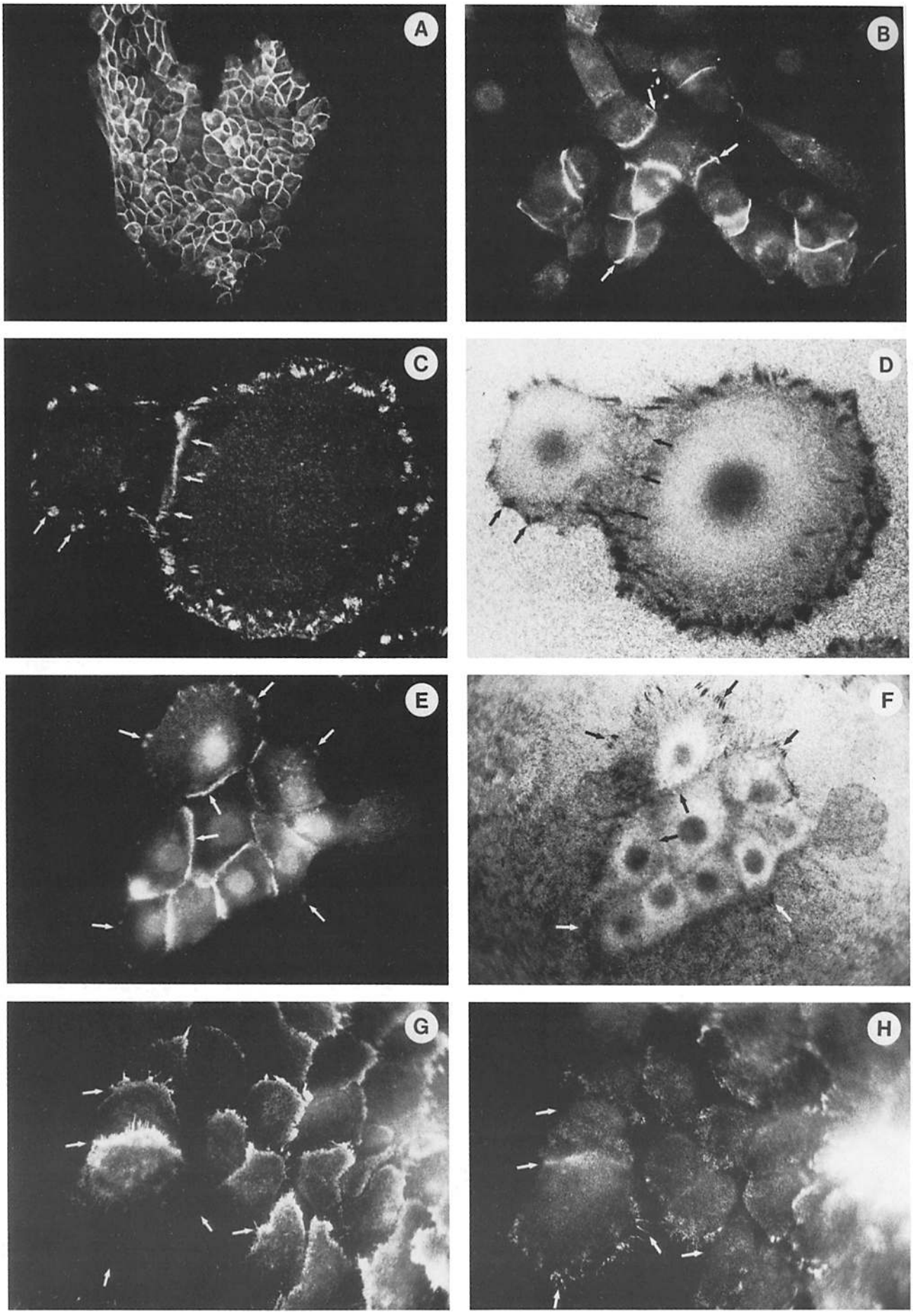
Figure 6. HFK $\alpha 2\beta 1$ localizes in focal adhesions on collagen but not laminin or fibronectin as detected with noninhibitory mAbs (P1E6, P4B4, P1H6) but not inhibitory mAbs (P1H5). HFKs were adhered to surfaces coated with collagen (Type I; labeled *CN 1*), fibronectin (*FN*), or laminin (*LN*) for 5 h, and then reacted with the indicated mAbs. (*A*) mAb P1H5 that inhibits cell adhesion to collagen failed to detect $\alpha 2\beta 1$ in FAs on collagen. (*B*) Noninhibitory mAb P1E6 identified $\alpha 2\beta 1$ in FAs on collagen. (*C*) P1E6 did not detect $\alpha 2\beta 1$ in FAs on fibronectin. (*D*) P1E6 did not detect $\alpha 2\beta 1$ in FAs on laminin. Noninhibitory mAbs P4B4 and P1H6 gave results similar to P1E6.

anti-$\alpha 2$ mAbs P1E6 and P4B4, that react with nonoverlapping epitopes on $\alpha 2$ had little effect on HFK and FEA adhesion to collagen. Surprisingly, anti-$\alpha 2\beta 1$ (P1H5 and P1E6) also inhibited HFK and FEA cell adhesion to laminin. As we have previously reported (Wayner and Carter, 1987), anti-$\alpha 2\beta 1$ failed to inhibit fibroblast adhesion to laminin. Anti-$\alpha 3\beta 1$ (P1B5 and P1F2) partially inhibited HFK adhesion to laminin and fibronectin but was less effective on FEA cells (see later Results and Discussion). Taken together, the tissue distribution (Fig. 2), expression of integrin subunits in cell culture (Fig. 3), and inhibition of cell adhesion (Fig. 4), suggest that basal keratinocytes express $\alpha 2\beta 1$ and $\alpha 3\beta 1$ to high levels and use both receptors in their adhesion to components present in the BMZ.

$\alpha 2\beta 1$ and $\alpha 3\beta 1$ in Focal Adhesions

Ligand specificities for $\alpha 2\beta 1$ and $\alpha 3\beta 1$ were determined by localization of integrins in FAs of cells adhered to different ECM ligands (see Materials and Methods and Fig. 1). Initial adhesion of HFKs to collagen and fibronectin, was coincident with the localization of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ (Fig. 5, P1E6 and Ab33, respectively) into FAs, respectively. Despite the observed inhibition of cell adhesion to both collagen and laminin by anti-$\alpha 2\beta 1$ (Fig. 4, *Col* and *Lam*, *P1H5*), the anti-$\alpha 2\beta 1$ mAbs (P4B4, P1E6, and P1H6) only localized to HFK FAs on collagen (Fig. 5, *P1E6* and Fig. 6 *B*) but not laminin (Fig. 6 *D*) or fibronectin (Fig. 6 *C*). In general, anti-$\alpha 2$, -$\alpha 3$, or -$\alpha 5$ mAbs that could inhibit cell adhesion to ligands did

Figure 5. Localization of integrins $\alpha 2\beta 1$, and $\alpha 3\beta 1$, and $\alpha 5\beta 1$ in focal adhesions of HFKs adherent to collagen, laminin, and fibronectin, respectively. HFKs adhered to glass cover slips coated with collagen (*CN*, type I), fibronectin (*FN*), and laminin (*LN*) for 8 h, fixed, and permeabilized then reacted with antibody combinations: mouse mAb anti-$\alpha 2\beta 1$ (P1E6) + rabbit polyclonal anti-type I collagen; rabbit polyclonal anti-$\alpha 5\beta 1$ (AB33) + mouse mAb anti-fibronectin (P1H11); and mouse mAb anti-$\alpha 3\beta 1$ (P1F2) + rabbit polyclonal anti-laminin (R5922). Localization of the receptor, ligand and focal adhesions by IRM are indicated. The same field is examined in the three panels of each row and identical representative FAs are indicated by arrows. Each receptor localizes in FAs when plated on the appropriate ligand.


A
B
C
D
E
F
G
H

not detect α subunits in FAs while noninhibitory anti-α mAbs effectively stained FAs (Fig. 6 *A*, *inhibitory PlH5* and 6 *B*, *noninhibitory PlE6*). This suggested that antibodies that compete with the ligands for binding to the receptors cannot detect the ligand-occupied receptors in FAs.

Only some anti-α subunits are useful for identifying integrins in FAs (Fig. 6, *A* and *B*). Therefore, we identified ten different mAbs that immunoprecipitate α3β1 (see Fig. 3, P1B5 and P1F2). These mAbs were further screened for their ability to localize in FAs of HFKs adherent on fibronectin, laminin, collagen (types I and IV), and vitronectin. Out of these mAbs, only one, P1F2, localized α3β1 in FAs of HFKs and the best exogenous ligand was laminin (Fig. 5, *PlF2*).

α2β1 and α3β1 in Cell-Cell Adhesion

We investigated the role of α2β1 and α3β1 in the stratification process in skin. Our work focused on two aspects of this question: (*a*) evaluation of the possible roles for α2β1 and α3β1 in cell-cell adhesion and (*b*) identification of physiologically relevant ligands for α2β1 and α3β1 in the basal cell layer adjacent to the BMZ.

Integrin subunits in cultures of HFKs localized in three distinct distribution patterns; at regions of cell-substratum contact (Figs. 5, 6 *B* and 7 *C*), at sites of putative cell-cell contact (Fig. 6, *A* and *C*; Fig. 7, *A*, *B*, and *E*), and in an unexplained polarized distribution. The polarized distribution was most prominent for α5β1 (Fig. 7 *G*) and α6β4 in prolonged cultures of HFKs (over 24 h). After the localization of α3β1 in FAs, α5β1 polarized to the opposite end of the same cell (Fig. 7, *G* and *H*).

The distribution of α2β1, α3β1, and α5β1 in these three locations was affected by the exogenous ECM ligand. For example, α2β1 localized to putative cell-cell contacts above the focal plane of the adhesion surface when the cells were adherent on fibronectin (Figs. 6 *C* and 7 *B*) or laminin. In contrast, α2β1 concentrated into FAs on the basal surface of cells attached to collagen (Figs. 6 *B* and 7 *C*). If the collagen-adherent cells were in contact with other cells, α2β1 preferentially localized at the cell-cell contacts. The relocation of α2β1 into cell-cell contacts was paralleled by a decrease in cell-substrate attachment as detected by decreased formation of FAs on the substrate below the cell-cell contacts (Fig. 6 *B* and 7, *C* and *D*).

We examined the possibility that serum or Ca^{2+}-induced HFK aggregation may decrease α2β1 and α3β1 in FAs and increase localization into cell-cell contacts. Addition of 1 mM Ca^{2+} to the defined culture medium of HFKs grown on collagen, fibronectin or laminin induced formation of adherent, multicell aggregates or colonies. These were first observable after 4–5 h of incubation. α3β1 localized preferentially in the putative cell-cell contact sites (Fig. 7 *A*). When HFKs were grown on collagen in the presence of Ca^{2+}, α2β1 remained in FAs at the periphery of the colony and increased in cell-cell contacts toward the center of the colony. The number of FAs decreased in the center of the colonies (Fig. 7, *E* and *F*).

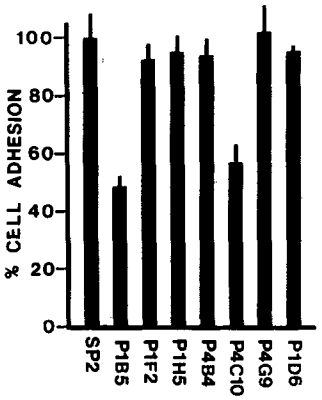


Figure 8. Inhibition of cell-cell adhesion with anti-α3β1 (P1B5) and anti-β1 (P4C10) mAbs. EIL-8 were grown in the presence of Ca^{2+} to induce cell-cell aggregates, labeled with ^{51}Cr then suspended by digesting with trypsin in the presence of Ca^{2+}. Labeled cells were added to confluent cultures of Ca^{2+}-induced EIL-8 cells in the presence of control SP2 culture supernatant or the indicated hybridoma culture supernatants and agitated for 60 min before washing to removed unattached cells. The percent cell adhesion relative to SP2 control (100%) is presented based on the quantity of labeled cells that attached to the unlabeled monolayer. The average of triplicate assays is presented. Essentially identical results were also obtained using purified mAbs in KGM in the absence of serum. The vertical line on each bar represents SD.

The localization of α2β1 and α3β1 into cell-cell contacts suggested that these receptors may play a role in both cell-substrate and cell-cell adhesion. As seen in Fig. 8, a new anti-β1 mAb (P4C10), and anti-α3 mAb (P1B5) partially inhibited adhesion of labeled cells in suspension to confluent cultures of unlabeled cells. In contrast, neither anti-α5β1 (P1D6), anti-α3β1 (P1F2), anti-α4 (P4G9), or anti-α2 (P1H5, P4B4) inhibited cell-cell adhesion. The possibility that the antibodies might indirectly affect cell-cell adhesion by crosslinking of the cell surface is unlikely because only the P1B5 mAb, reacting with a specific epitope on α3β1 successfully inhibited cell-cell adhesion.

α3β1 Colocalizes with a Secreted, Laminin-containing ECM in FAs

In contrast to the rapid localization of α2β1 and α5β1 into FAs on collagen and fibronectin, α3β1 appeared more slowly in FAs of HFKs. Within 5 h, HFKs localized α5β1 into FAs on exogenous fibronectin while α3β1 was diffuse over the cell surface (Fig. 9). However, after 24–96 h of culture on fibronectin (Fig. 9) α5β1 was displaced to one edge of the cell while α3β1 was relocated into FAs. Consistent with this observation, anti-α3β1 mAb P1B5, partially inhibited the initial stages of cell adhesion to fibronectin or laminin (Fig. 4) but efficiently detached long term cultured cells (>5 h)

Figure 7. Ca^{2+}-induced aggregation of HFKs relocates α2β1 from focal adhesions to cell-cell contacts. Integrins were found to distribute in three different patterns in HFKs, at cell-substratum contacts, cell-cell contacts and in a polarized pattern as follows. (*A*) P1B5 localized α3β1 to cell-cell junctions on collagen (type IV) after Ca^{2+}-induced cell-cell aggregation and (*B*) P4B4 localized α2β1 at cell-cell contacts on fibronectin. (*C* and *D*, same field) P1E6 localized α2β1 on collagen type IV to both focal adhesions and cell-cell contacts as determined by antibody exclusion with anti-type IV collagen. (*E* and *F*, same field) Ca^{2+}-induced aggregation relocated α2β1 from focal adhesions to cell-cell contacts. (*G* and *H*, same field) Ab33 localized α5β1 in a polarized pattern at the opposite end of cells from the α3β1 (P1F2)-containing focal adhesions at the opposite side of cells.

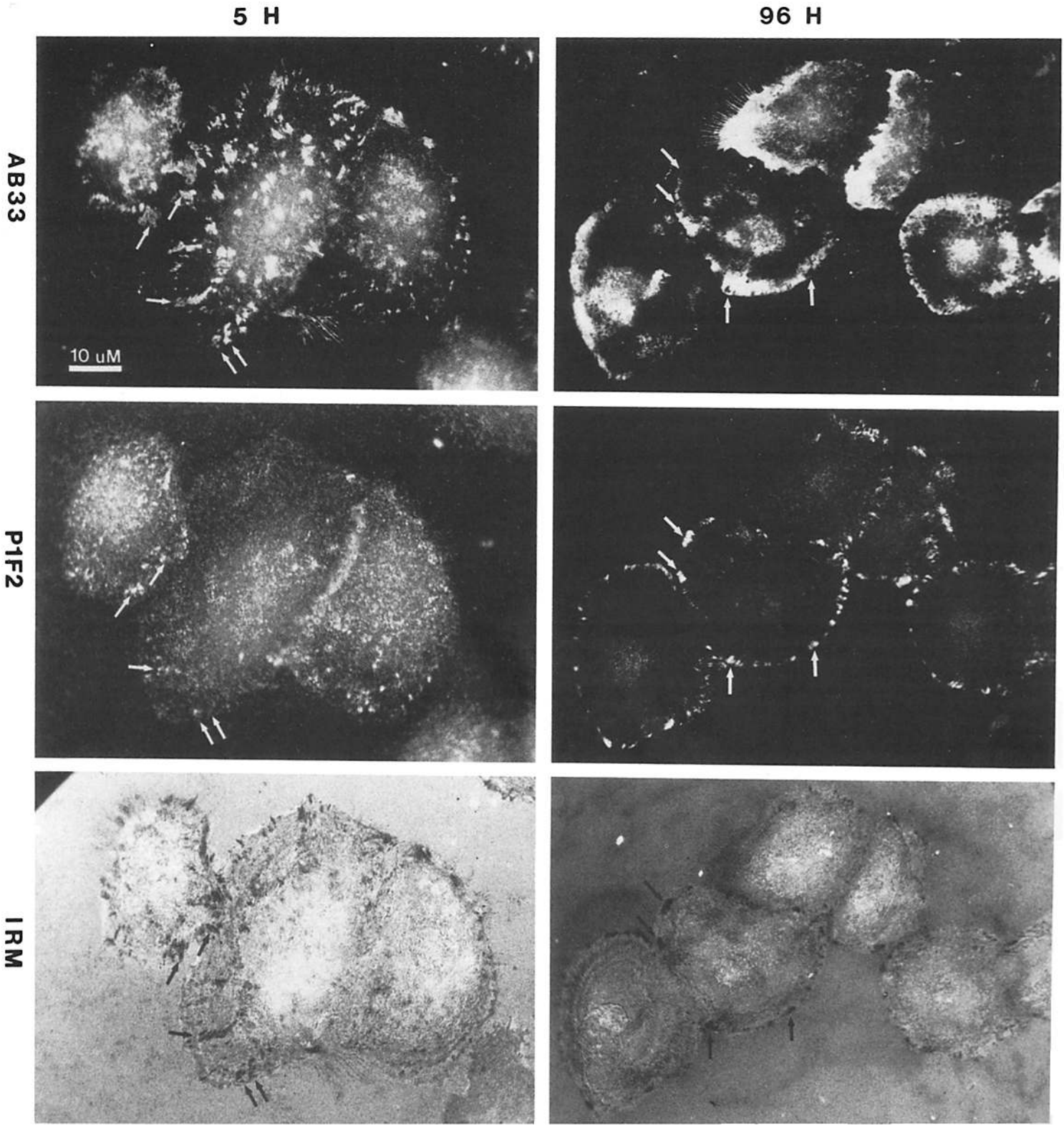


Figure 9. $\alpha 3\beta 1$ localizes in focal adhesion on laminin, fibronectin, and collagen after cell adhesion has occurred. HFKs were adhered to glass slides coated with fibronectin for 5 and 96 h, then fixed, permeabilized, and reacted simultaneously with anti-$\alpha 3\beta 1$ (P1F2) and anti-$\alpha 5\beta 1$ (AB33) antibodies. The distribution of the receptors and focal adhesions was determined by fluorescence microscopy and IRM. The three panels under *5 H* and *96 H* are the same field.

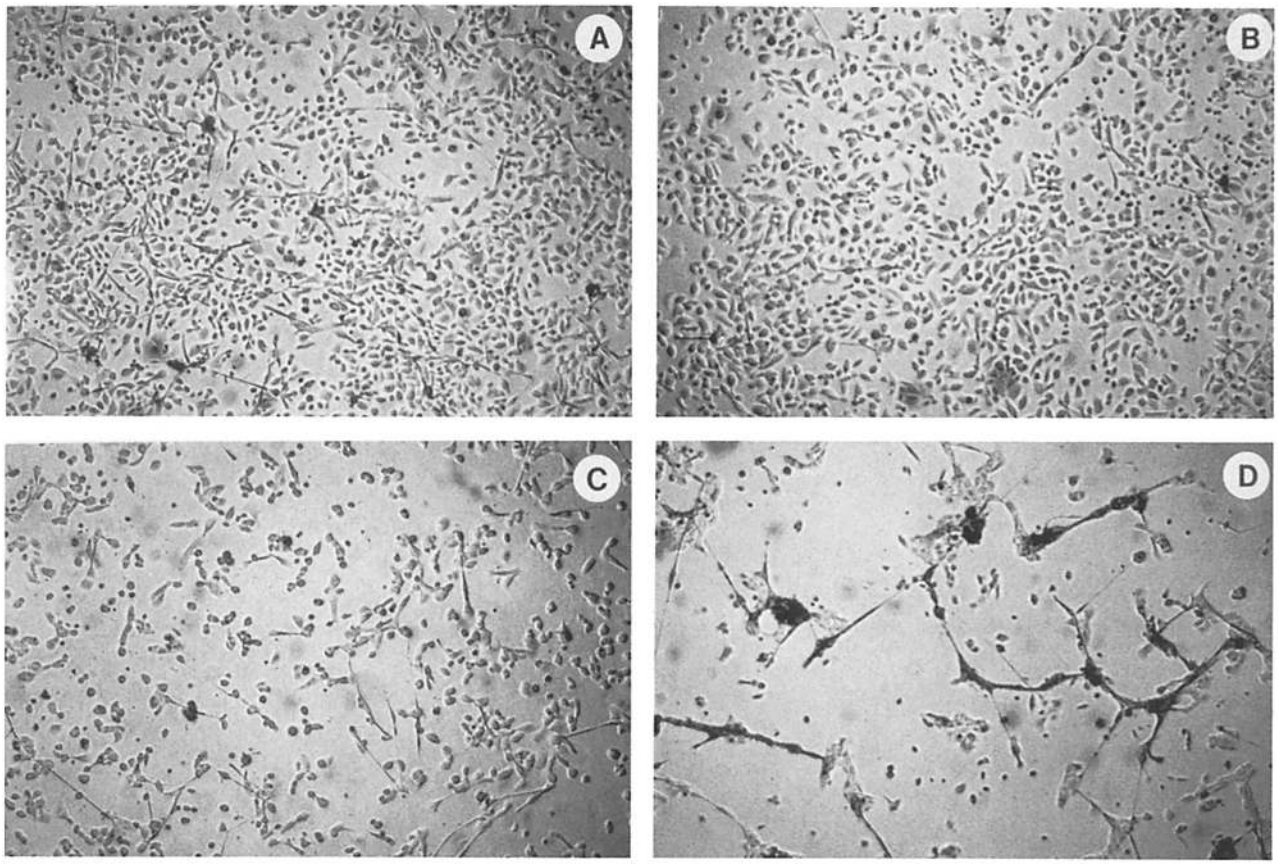


Figure 10. Anti-$\alpha 3\beta 1$ (PlB5) detaches HFK cells from long term cultures. HFK cells were attached and spread on glass cover slips coated with fibronectin (shown), collagen, and laminin (not shown) for 1 d; then the indicated antibodies were added to the culture supernatants (25 μg antibody/ml) and grown for an additional 24 h and photographed. Similar results were obtained with all ligands tested. (*A*) Mouse IgG control; (*B*) PlG12 anti-ECMR III/CD44, control; (*C*) PlD6 anti-$\alpha 5\beta 1$; (*D*) PlB5 anti-$\alpha 3\beta 1$.

from both laminin and fibronectin surfaces (Fig. 10 *D*). In contrast, anti-$\alpha 5\beta 1$ (PlD6) inhibited initial HFK adhesion to exogenous fibronectin (Fig. 4, pFN) but was only slightly better than control antibodies (Fig. 10, *A* and *B*) in detaching prolonged cultures from exogenous fibronectin (Fig. 10 *C*).

The time-dependent accumulation of $\alpha 3\beta 1$ into FAs (Fig. 9) suggested that HFKs might be secreting ECM during long-term culture that interacts with $\alpha 3\beta 1$ in preference to exogenous laminin, collagen or fibronectin. To investigate this possibility, we examined the distribution of $\alpha 3\beta 1$ into FAs of OVCAR-4 cells, a human ovarian carcinoma, that forms distinct FAs on laminin (Fig. 11). 18 hrs after addition of OVCAR-4 cells to exogenous laminin, $\alpha 3\beta 1$ (Fig. 11, *A* and *B*) localized into and on either side of FAs (Fig. 11, *B* and *C*). At higher magnification, the focal concentration of $\alpha 3\beta 1$ (Fig. 11 *D*) colocalized with focal concentrations of secreted laminin-containing ECM (Fig. 11 *E*) in and adjacent to FAs as identified by IRM (Fig. 11 *F*) and antibody exclusion (Fig. 11 *E*). Colocalization of the focal concentrations of $\alpha 3\beta 1$ and secreted laminin (Fig. 11, *G* and *H*) also occurred during cell growth on fibronectin surfaces.

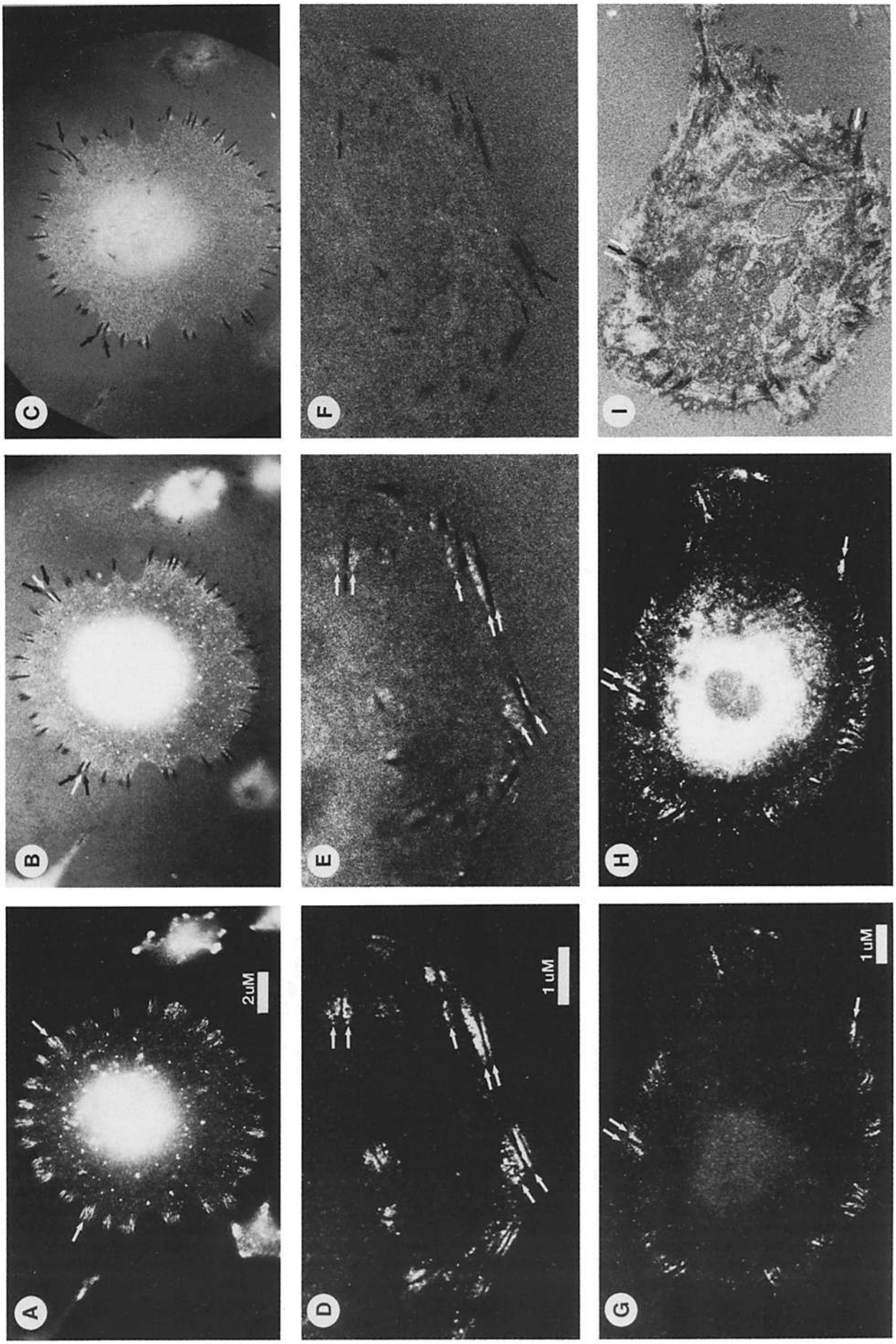
Secreted, insoluble ECM was prepared by differential extraction of HFKs (see Materials and Methods) and provided an optimal ligand for localization of $\alpha 3\beta 1$ in FAs (Fig. 12). FEA cells either in long or short term culture on laminin (Fig. 12, *A* and *B*) accumulated $\alpha 3\beta 1$ into belts at the cell periphery but failed to form distinct FAs. However, adhesion of FEA cells to ECM prepared from prolonged cultures of HFK cells (3-d cultures) caused FEA cells to localize $\alpha 3\beta 1$ into FAs that functioned as the origins for actin containing stress fibers (Fig. 12, *C–F*). The colocalization occurred coincident with initial FEA adhesion to the HFK ECM (3 h).

HFKs also secreted a soluble form of the endogenous $\alpha 3\beta 1$ ligand. The soluble ligand was partially purified from serum-free HFK conditioned culture medium. This crude secreted soluble material induced HFK and FEA cell adhesion within 1 h. This adhesion was specifically inhibited by anti-$\alpha 3\beta 1$ but not by the other antibodies (*PlB5*, Fig. 4, *CS*) further differentiating the adhesion-promoting activity of secreted ECM from purified laminin (Fig. 4, *Lam*). In control experiments, metabolic labeling of HFK and OVCAR-4 cells with [^{35}S]methionine followed by immune precipitation, confirmed the presence of laminin, fibronectin, and a released form of ECMR III/CD44 antigen, a heparan sulfate/chondroitin sulfate proteoglycan as well as other components, secreted by the cells (results not shown).

Discussion

$\alpha 3\beta 1$ Preferentially Interacts with Secreted, Laminin-containing ECM

We have used two approaches to investigate the ligand-binding specificity of integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$. First, we examined the distribution of integrins in tissues to identify cell populations that expressed high levels of both $\alpha 2\beta 1$ and


A
2uM
B
C
D
1uM
E
F
G
1uM
H
I

$\alpha 3\beta 1$. This resulted in the selection of basal cell keratinocytes (HFKs) and other epithelial cells for further study. Second, cultures of epithelial cells were utilized for cell adhesion studies and for colocalization of integrins into FAs with various exogenous purified and endogenous secreted ECM ligands. Based on these studies we conclude that the best purified exogenous ligand for localization of $\alpha 3\beta 1$ in FAs is laminin. However, HFKs and OVCAR-4 cells, in long-term culture, secrete ECM that contains laminin and other components, that codistribute with $\alpha 3\beta 1$ in and around FAs in preference to purified exogenous laminin. OVCAR-4 cells were found to deposit type IV collagen as part of the ECM ligand for α3B1 (Carter et al., unpublished observations). Thus, the composition of this secreted ECM is similar to BMZ in tissue and both are assembled by basal keratinocytes. These results suggest that interaction of $\alpha 3\beta 1$ with the secreted BMZ-like ECM is instrumental in maintenance of cell–substratum adhesion in long term culture and may be causal in basal cell adhesion to the BMZ in skin. The failure of anti-$\alpha 3\beta 1$ mAbs to inhibit FEA cell adhesion to laminin and the inability of FEA cells to localize $\alpha 3\beta 1$ in FAs may be due to the absence of a secreted BMZ-like ECM in FEA cultures. Previous studies based on inhibition of cell adhesion with mAb anti-$\alpha 3\beta 1$ (Wayner and Carter, 1987; Wayner et al., 1988) and affinity chromatography (Wayner and Carter, 1987; Gehlsen et al., 1988, 1989)[2], have described $\alpha 3\beta 1$ as a promiscuous receptor with affinity for multiple purified ECM components, including laminin, fibronectin, and collagen. The minimal component of the secreted laminin-containing ECM responsible for interaction with $\alpha 3\beta 1$ in FAs is laminin. This conclusion is based on localization in FAs, inhibition of cell adhesion with mAb PIB5 and direct binding assays (Gehlsen et al., 1988, 1989). However, type IV collagen, nidogen/entactin, glycosaminoglycan, or other components may also be involved. Local high densities of laminin or an optimal interaction/organization of laminin with other components of the ECM may result in a preferred interaction of $\alpha 3\beta 1$ with laminin. Recently, studies have identified a laminin homologue (Hunter et al., 1989) and laminin-containing complexes (Chiu et al., 1986) that facilitate adhesion. The report by Terranova et al. (1980) that laminin secreted by PAM 212 epithelial cells facilitates the adhesion of those cells to type IV collagen may also reflect the binding specificity of $\alpha 3\beta 1$ for a secreted BMZ-like complex of laminin and type IV collagen.

Our results suggest that HFK adhesion to laminin, particularly in long-term culture, probably involves multiple integrin receptors including $\alpha 2\beta 1$ and $\alpha 3\beta 1$. In addition, our results have not excluded $\alpha 6\beta 4$ as a potential laminin receptor involved in initial HFK adhesion. $\alpha 6\beta 1$ has been defined as a laminin adhesion receptor in platelets (Sonnenberg et al., 1988) and anti-$\alpha 6\beta 4$ (or $\alpha E\beta 4$, Kajiji et al., 1989) inhibited cell adhesion to laminin (Cheresh et al., 1989). Collagen is an optimal ligand for $\alpha 2\beta 1$ expressed by fibroblasts and platelets (Wayner and Carter, 1987). In HFKs, $\alpha 2\beta 1$ was also shown to contribute to initial HFK adhesion to laminin as well. Anti-$\alpha 2\beta 1$ mAbs inhibits HFK and FEA adhesion to both collagen and laminin. However, $\alpha 2\beta 1$ localizes in FAs only on collagen. Apparently, $\alpha 2\beta 1$ interacts weakly with laminin and contributes to initial epidermal cell adhesion to laminin but fails to form the stable attachments characterized by $\alpha 2\beta 1$ interaction with collagen in FAs of both epithelial and mesenchymal cells. This suggests that only certain receptor–ligand combinations may be able to induce FA formation. It also suggests that fibroblasts/platelets and epithelial cells regulate binding of $\alpha 2\beta 1$ to collagen or collagen/laminin via posttranslational modification or alternate splicing of the receptor. The role of $\alpha 2\beta 1$ in cell adhesion to collagen and laminin has been confirmed by affinity purification of $\alpha 2\beta 1$ from detergent extracts of endothelial cells by chromatography on immobilized laminin (Languino et al., 1989). The recent identification of a new collagen, type XIII, localized in the epidermis, must also be considered as a potential ligand for $\alpha 2\beta 1$ (Sandberg et al., 1989).

A Role for $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in Cell–Cell Adhesion

We localized integrins in three distinct distributions in cultures of HFKs including (*a*) cell–substratum contacts, characterized by $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 5\beta 1$ concentration in FAs; (*b*) $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in cell–cell contacts above the focal plane of the FAs; and (*c*) $\alpha 5\beta 1$ and $\alpha 6\beta 4$ concentration in filapodia that were usually polarized to ends of cells and in opposition to the $\alpha 3\beta 1$-containing FAs. In most cases, concentration of an integrin in any of these sites was reversible and subject to control by at least two factors, the available exogenous ligand and/or the aggregation/differentiation status of the cells. During the initial stages of cell adhesion to collagen, $\alpha 2\beta 1$ concentrated in FAs on the basal surface of the cells. Prolonged culturing resulted in the deposition of the BMZ-like ECM, movement of $\alpha 2\beta 1$ into cell–cell contacts and the movement of $\alpha 3\beta 1$ into FAs. Although, $\alpha 2\beta 1$ initially localized in FAs when HFKs were adherent to collagen, Ca^{2+}-induction of HFK aggregation and differentiation, also caused $\alpha 2\beta 1$ to relocate from FAs into cell–cell contacts at the center of colonies with a general decrease in cell–substratum contacts wherever cell–cell contacts predominated.

The ability of Ca^{2+}-induction to relocate $\alpha 2\beta 1$ and $\alpha 3\beta 1$ into cell–cell contacts suggests possible dual functions for

2. After the submission of this manuscript, Gehlsen et al. (1989) published that $\alpha 3\beta 1$ interacts with multiple ligands (laminin > fibronectin > type IV collagen) in affinity binding assays. Kaufmann et al. (1989) also localized $\alpha 3\beta 1$ to cell–cell contacts.

Figure 11. OVCAR-4 cells colocalize $\alpha 3\beta 1$ with a secreted laminin/type IV-containing complex. OVCAR-4 cells were adhered to cover slips coated with laminin (*A–C* and *D–F*) and fibronectin (*G–I*) for 24 h, fixed, permeabilized, and reacted with mouse mAb anti-$\alpha 3\beta 1$ (*PIF2, A, D,* and *G*) and rabbit polyclonal anti-laminin (*R5922, B, E,* and *H*). $\alpha 3\beta 1$ and laminin were localized by fluorescence microscopy and focal adhesions by IRM (*C, F,* and *I*). *A–C, D–F,* and *G–I* are the same field. *White arrows* indicate representative focal concentrations of $\alpha 3\beta 1$ that colocalize with concentration of secreted laminin. *Black arrows* indicate representative focal adhesions. $\alpha 3\beta 1$ was found to colocalize with bright concentrations of laminin in proximity to identifiable focal adhesions in *A–C*. At higher magnification in *D–F*, the bright concentrations of laminin and $\alpha 3\beta 1$ were localized to regions between and around the focal adhesions. Cells adherent to fibronectin (*G–I*) secreted laminin that colocalized with $\alpha 3\beta 1$.

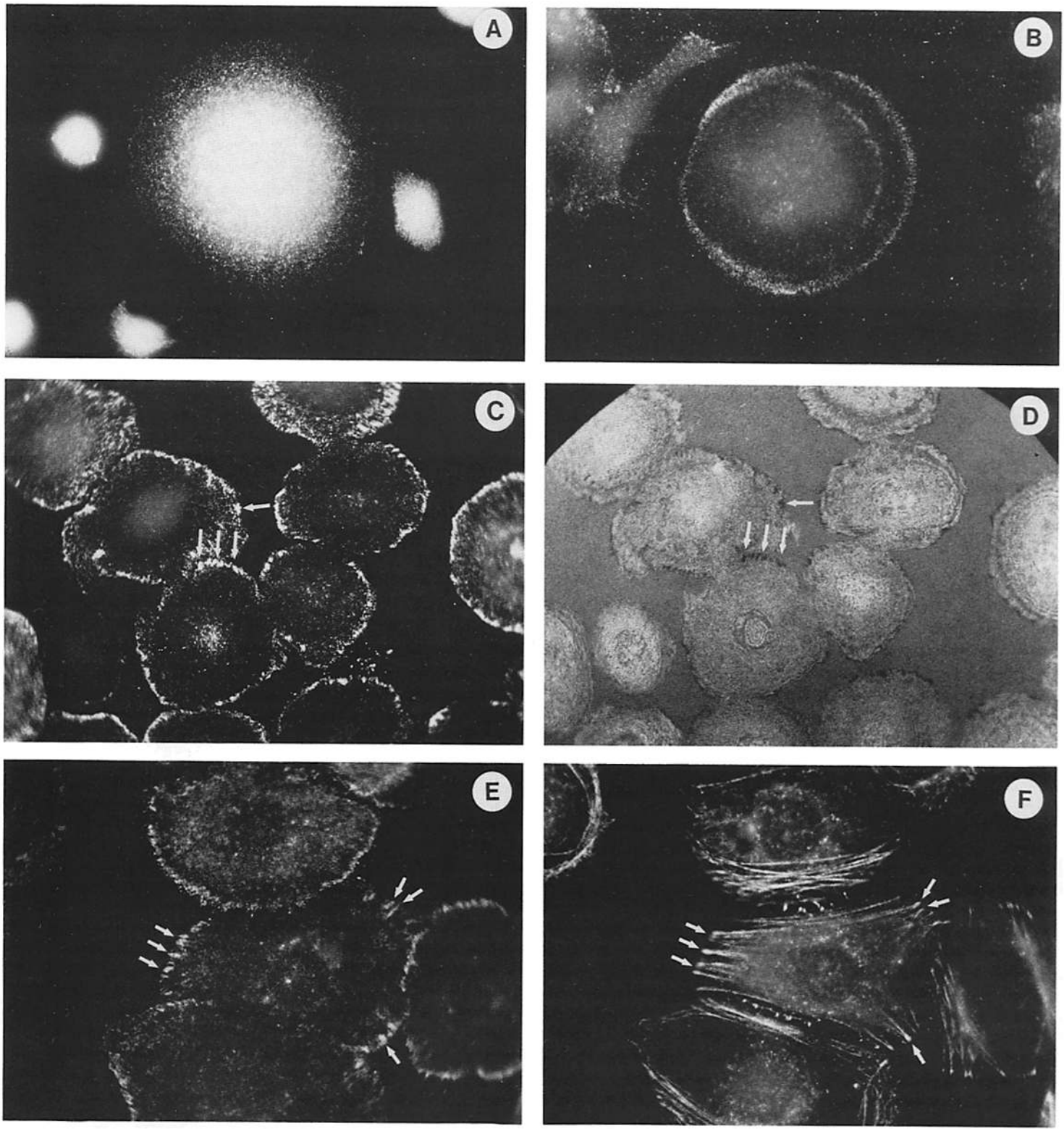


Figure 12. FEA $\alpha 3\beta 1$ localizes in focal adhesion at stress fiber origins during initial FEA adhesion to HFK ECM but not laminin. FEA cells were adhered to surfaces coated with (*A* and *B*) laminin and (*C–F*) HFK ECM for various times and reacted with various antibodies. (*A*) PIF2 localized $\alpha 3\beta 1$ in a diffuse cell surface pattern but not focal adhesions in 3 h and (*B*) in a continuous ring in 72 h on laminin (*C* and *D*, same field). PIF2 localized $\alpha 3\beta 1$ in focal adhesions in 3 h as determined by interference reflection microscopy and (*E* and *F*, same field) at the origins of stress fibers.

$\alpha 2\beta 1$ and $\alpha 3\beta 1$ in both cell–cell and cell–substratum adhesion. This possibility was supported by the partial inhibition of cell–cell adhesion by anti-$\alpha 3\beta 1$ (PIB5) and anti-$\beta 1$ (P4C10) mAbs. The inability of the anti-α antibodies to completely inhibit cell–cell interactions is probably due to concurrent involvement of cell-CAM 120/80 and desmosomes (Cunningham, 1986) in the cell–cell adhesion process. The recent identification of $\alpha 4\beta 1$ as a cell adhesion receptor for the CS-1 domain of fibronectin (Wayner et al., 1989) as well as a mediator of lymphocyte-endothelial interactions (Holtzmann et al., 1989) is relevant to the role of $\alpha 3\beta 1$ in both cell–cell and cell–substrate adhesion. Although anti-$\alpha 3\beta 1$ (PIB5) and anti-$\alpha 4\beta 1$ (P4C2, P4G9) have been shown to inhibit cell–substrate adhesion, the same mAbs also interfere with cell–cell adhesion (Wayner, W. G., et al., manuscript in preparation). It is possible that the ECM ligands for these $\beta 1$-containing integrins may mediate cell–cell adhesion indirectly by bridging integrin receptors located in two adhering cells. Alternatively, the direct interaction of $\alpha 3\beta 1$ and $\alpha 4\beta 1$ with unidentified membrane co-receptors in adhering cells, may still be inhibited by the anti-$\alpha 3\beta 1$ (PIB5), -$\alpha 4$ (P4C2) and -$\beta 1$ (P4C10) mAbs.

The Role of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in Skin Stratification

Differentiation-dependent stratification in skin is accompanied by decreased adhesion of basal cells with the BMZ (Stanley et al., 1980; Watt, 1984), movement of cells into the suprabasal regions and a corresponding increase in cell–cell interactions and formation of desmosomes (Klein-Szanto, 1977). In cryostat sections of human skin, $\alpha 3\beta 1$ localized primarily to the basal cell layer where, presumably, it associates with the laminin-containing ECM secreted by the basal cells. Although $\alpha 2\beta 1$, which is also present in the basal cell layer, interacts with laminin or type IV collagen and may also contribute to the basal cell adhesion to the BMZ, our results with cell culture suggest that $\alpha 3\beta 1$ is the primary mediator of basal cell–BMZ interactions. These cell–substratum interactions may maintain cell localization at the BMZ until differentiation signals, such as Ca^{2+}-induction, downregulates $\alpha 3\beta 1$ and/or relocates $\alpha 2\beta 1$ and $\alpha 3\beta 1$ from the substratum into intercellular contacts, permitting cell movement into the suprabasal strata.

We would like to thank Dr. Rebecca Blanton for help in preparing the HFK cells and Tina Toennis for technical assistance.

Support for this research was provided by grants to W. G. Carter from the American Cancer Society (grant no. BC-419) and the National Institute of Health (grant no. RO1-CA49259).

Received for publication 14 September 1989 and in revised form 21 November 1989.

References

Asselineau, D., B. Bernhard, C. Bailly, and M. Darmon. 1985. Epidermal morphogenesis and induction of the 67kd keratin polypeptide by culture of human keratinocytes at the liquid-air interface. *Exp. Cell Res.* 159:536–539.

Boyce, S. T., and R. G. Ham. 1985. Cultivation, frozen storage, and clonal growth of normal human epidermal keratinocytes in serum free media. *J. Tissue Culture Methods.* 9:83–93.

Buck, C. F., and A. F. Horwitz. 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 3:179–205.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.

Carter, W. G., and E. A. Wayner. 1988. Characterization of a collagen-binding, phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193–4201.

Cheresh, D. A., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha v\beta x$) is responsible for distinct adhesive properties of carcinoma cells. *Cell.* 57:59–69.

Chiu, A. Y., W. D. Matthew, and P. H. Patterson. 1986. A monoclonal antibody that blocks the activity of a neurite regeneration-promoting factor: studies on the binding site and its localization in vivo. *J. Cell Biol.* 103:1383–1398.

Cunningham, B. 1986. Cell adhesion molecules: a new perspective on molecular embryology. *Trends Biochem. Sci.* 11:423–426.

Edelman, G. M. 1984. Cell adhesion and morphogenesis: the regulator hypothesis. *Proc. Natl. Acad. Sci. USA.* 81:1460–1464.

Ekblom, P., D. Vestweber, and R. Kemler. 1986. Cell-matrix interactions and cell adhesion during development. *Annu. Rev. Cell Biol.* 2:27–47.

Fusenig, N. E. 1986. Mammalian Epidermal Cells in Culture in Biology of the Integument. Vol. 2. Vertebrates. J. Bereiter-Hahn, A. G. Matoltsy, and K. S. Richards, editors. Springer-Verlag, Berlin/Heidelberg. 409–442.

Gallatin, W. M., E. A. Wayner, P. A. Hoffman, T. St. John, and W. G. Carter. 1989. Structural homology between lymphocyte receptors for high endothelium and class III extracellular matrix receptor. *Proc. Natl. Acad. Sci. USA.* 86:4654–4658.

Gehlsen, K. R., L. Dillner, E. Engvall, and E. Ruoslahti. 1988. The human laminin receptor is a member of the integrin family of cell adhesion receptors. *Science (Wash. DC).* 241:1228–1229.

Gehlsen, K. R., K. Dickerson, W. S. Argraves, E. Engvall, and E. Ruoslahti. 1989. Subunit structure of a laminin-binding integrin and localization of its binding site on laminin. *J. Biol. Chem.* 264:19034–19038.

Green, H. 1977. Terminal differentiation of cultured human epidermal cells. *Cell.* 11:405–416.

Hemler, M. E. 1988. Adhesive protein receptors on hematopoietic cells. *Immunol. Today.* 9:109–113.

Hemler, M. E., C. Crouse, and A. Sonnenberg. 1989. Association of the very late antigen (VLA) α^6 subunit with a novel protein: a possible alternative to the common VLA β_1 subunit on certain cell lines. *J. Biol. Chem.* 26:1–7.

Holzmann, B., B. W. McIntyre, and I. L. Weissman. 1989. Identification of a murine peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an α chain homologous to human VLA-4α. *Cell.* 56:37–46.

Hunter, D. D., V. Shah, J. P. Merlie, and J. R. Sanes. 1989. A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. *Nature (Lond.).* 338:229–233.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129–159.

Kajiji, S., N. Tamura, and V. Quaranta. 1989. A novel integrin $\alpha E\beta 4$ from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:673–680.

Katz, S. I. 1984. The epidermal basement membrane zone: structure, ontogeny, and role in disease. *J. Am. Acad. Dermatol.* 11:1025–1037.

Kaufmann, R., D. Frosch, C. Westphal, L. Weber, and C. E. Klein. 1989. Integrin VLA-3: ultrastructural localization at cell-cell contact sites of human cell cultures. *J. Cell Biol.* 109:1807–1815.

Kaur, P., and J. K. McDougall. 1988. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *J. Virol.* 62:1917–1924.

Kaur, P., J. K. McDougall, and R. Cone. 1989. Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. *J. Gen. Virol.* 70: 1261–1266.

Klein-Szanto, A. J. P. 1977. Sterologic baseline data of normal human epidermis. *J. Invest. Dermatol.* 68:73–75.

Kopan, R., T. Grazina, and E. Fuchs. 1987. Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. *J. Cell Biol.* 105:427–440.

Kunicki, T. J., D. J. Nugent, S. J. Staats, R. P. Orchekowski, E. A. Wayner, and W. G. Carter. 1988. The human fibroblast class II extracellular matrix receptor mediates platelet adhesion to collagen and is identical to the platelet Ia/IIa complex. *J. Biol. Chem.* 263:4516–4519.

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

Languino, L. R., K. R. Gehlsen, E. A. Wayner, W. G. Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use $\alpha 2\beta 1$ integrin as a laminin receptor. *J. Cell Biol.* 109:2455–2462.

Martin, G. R., and R. Timpl. 1987. Laminin and other basement membrane components. *Annu. Rev. Cell Biol.* 3:57–85.

Neyfakh, A. A., I. S. Tint, T. M. Svitkina, A. D. Bershadsky, and V. I. Gelfand. 1983. Visualization of cellular focal contacts using a monoclonal antibody to 80 kd serum protein adsorbed on the substratum. *Exp. Cell Res.* 149:387–396.

Pittelkow, M. R., and R. E. Scott. 1986. New techniques for the in vitro culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clin. Proc.* 61:771–777.

Potten, C. S., and R. J. Morris. 1988. Epithelial stem cells *in vivo*. *J. Cell Sci. Suppl.* 10:45–62.

Rheinwald, J. G. 1980. Serial cultivation of normal human epidermal keratinocytes. *Methods Cell Biol.* 21A:229–254.

Rheinwald, J. G., and M. A. Beckett. 1980. Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. *Cell.* 22:629–632.

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

Roman, J., R. LaChance, T. J. Broekelmann, C. J. Roberts, E. A. Wayner, W. G. Carter, and J. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implication for oncogenic transformation and for cell recognition of fibronectin matrices. *J. Cell Biol.* 108:2529–2543.

Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57: 375–413.

Sandberg, M., M. Tamminen, H. Hirvonen, E. Vuorio, and T. Pihlajeniemi. 1989. Expression of mRNAs coding for the α1 chain of type XIII collagen in human fetal tissues: comparison with expression of mRNAs for collagen types I, II, and III. *J. Cell Biol.* 109:1371–1379.

Singer, I. I., S. Scott, D. W. Kawka, D. M. Kazazis, J. Gailit, and E. Ruoslahti. 1988. Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. *J. Cell Biol.* 106:2171–2182.

Sonnenberg, A., P. W. Modderman, and F. Hogervorst. 1988. Laminin receptor on platelets is the integrin VLA-6. *Nature (Lond.).* 336:487–489.

Stanley, J. R., J. M. Foidart, J. C. Murray, G. R. Martin, and S. I. Katz. 1980. The epidermal cell which selectively adheres to a collagen substrate is the basal cell. *J. Invest. Dermatol.* 74:54–58.

Stoler, A., R. Kopan, M. Duvic, and E. Fuchs. 1988. Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. *J. Cell Biol.* 107:427–446.

Sun, T., R. Eichner, W. G. Nelson, B. A. Scheffer, C. G. Tseng, R. A. Weiss, M. Jarvinen, and J. Woodcock-Mitchell. 1983. Keratin classes: molecular markers for different types of epithelial differentiation. *J. Invest. Dermatol.* 81:109s-115s.

Terranova, V. P., D. H. Rohrback, and G. R. Martin. 1980. Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell.* 22:719–726.

Watt, F. M. 1984. Selective migration of terminally differentiating cells from the basal layer of cultured human epidermis. *J. Cell Biol.* 98:16–21.

Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for type VI collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol.* 105:1873–1884.

Wayner, E. A., W. G. Carter, R. S. Piotrowicz, and T. J. Kunicki. 1988. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.* 107:1881–1891.

Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.