The Role of Integrins α2β1 and α3β1 in Cell–Cell and Cell–Substrate Adhesion of Human Epidermal Cells

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Abstract. We have examined cultures of neonatal human foreskin keratinocytes (HFKs) to determine the ligands and functions of integrins α2β1, and α3β1 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-α3β1 mAb, P1F2, localized α3β1 in FAs on purified laminin > fibronectin/collagen, indicating that laminin was the best exogenous ligand for α3β1. However, in long term culture, α3β1 preferentially codistributed in and around FAs with secreted laminin-containing ECM, in preference to exogenous laminin. Anti-α3β1, mAb P1B5, detached prolonged cultures of HFKs from culture plates or from partially purified HFK ECM indicating that interaction of α3β1 with the secreted laminin-containing ECM was primarily responsible for HFK adhesion in long term culture. (b) In FA assays, α2β1 localized in FAs coincident with initial HFK adhesion to exogenous collagen, but not laminin or fibronectin. However, in inhibition assays, anti-α2β1 inhibited initial HFK adhesion to both laminin and collagen. Thus, α2β1 contributes to initial HFK adhesion to laminin but α3β1 is primarily responsible for long-term HFK adhesion to secreted laminin-containing ECM. (c) Serum or Ca2+-induced aggregation of HFKs resulted in relocation of α2β1 and α3β1 from FAs to cell–cell contacts. Further, cell–cell adhesion was inhibited by anti-α3β1 (P1B5) and a new anti-β1 mAb (P4C10). Thus, interaction of α3β1 with either ECM or membrane coreceptors at cell–cell contacts may facilitate Ca2+-induced HFK aggregation. (d) It is suggested that interaction of α3β1 with a secreted, laminin-containing ECM in cultured HFKs, duplicates the role of α3β1 in basal cell adhesion to the BMZ in skin. Further, relocation of α2β1 and α3β1 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) 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 Ca2+ 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 α5β1 and αvβ3 will selectively localize to FAs at sites of cell...
Table I. Characteristics of Anti-Integrin Antibodies

| Antigen* | Antibody† | Epitope‡ | Distribution§ in skin | Cell-Cell¶ | Cell-ECM** | Focal adhesions†† |
|----------|-----------|----------|------------------------|------------|------------|------------------|
| αβ1      | TS2/7     |          | Dermis                 | ND         | ND         | ND               |
| αβ1      | 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              |
| αβ3      | P1B5      | a        | B                      | +          | + L,F,CS   | –                |
|          | P1F2      | b        | B                      | –          | ± L,F,CS   | + L,CS           |
| αβ4      | P4G9      |          | Hematopoietic          | (+)        | + F        | +                |
| αβ5      | P1D6      | a        | –                      | –          | + F        | –                |
|          | AB33      | b        | Muscle                 | ND         | ND         | + F              |
| αβ6/β1   | GoH3      |          | B                      | ND         | ND         | ± L              |
| β1       | 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 αβ2, a collagen receptor (Wayner and Carter, 1987; Wayner et al., 1988), and αβ3, 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 αβ2 inhibited epithelial cell adhesion to laminin as well as collagen; and (c) αβ3 failed to redistribute into focal adhesions during the initial stages of epithelial cell adhesion to various purified ECM ligands. These findings suggested that αβ2 and αβ3 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 αβ2 and αβ3 in epidermis. We addressed three issues: (a) analysis of αβ2 and αβ3 interactions with purified ECM ligands and secreted BMZ components synthesized by HFKs in culture; (b) the effect of serum or Ca²⁺ induction on the expression and function of αβ2 and αβ3 in cell–cell and cell–substrate adhesion of HFKs; and (c) the relation between differential expression of αβ2 and αβ3 and keratinocyte stratification and differentiation.

Materials and Methods

Materials

PMSE, 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 αβ2 and αβ3 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-αβ2; (B) anti-type I collagen; (C) IRM, (D) AB33 anti-α5β1; (E) PIIH11 anti-fibronectin; (F) IRM; (G) P4B4, anti-αβ1; (H) rhodamine-phalloidin; (I) IRM.
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; Clontech, 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 Ca²⁺, PMA, or serum to express filgrerin 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 Ca²⁺-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). Vimentin 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 A1A5, against the common integrin β1 subunit of the VLA family of receptors (Hemler, 1988) and anti-α5β1 (TS 2/7) were obtained from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). mAbs to the integrin receptors α5β1 (P3/22), α2β1 (5H5, P4B4, PIIH, PII6), α6β1 (Sp2, control). Anti-ECMR III/CD44, an intrinsic membrane proteoglycan; (H) anti-involucrin; (I) SP2, control.

Localization of Receptors in Focal Adhesions

Acid-washed glass coverslips (25-mm-diam) were derivatized with dimethyl dichlorosilane (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 tetracetic 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.

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 (fibriogen 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 IgG and rhodamine-conjugated goat anti-rabbit IgG secondary antibodies for 1 h, and wash 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) P1H5, anti-α3β1; (B) P4B4, anti-α2β1; (C) PIIH, anti-α2β1; (D) R5922, anti-laminin; (E) P9A5, anti-tenascin; (F) PIIH, anti-fibronectin; (G) PGI2, anti-ECMR III/CD44, an intrinsic membrane proteoglycan; (H) anti-involucrin; (J) SP2, control.
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-α2β1 mAbs, PIH5, P4B4, PIH6 and PIE6 react with nonoverlapping epitopes on the α2 subunit of the collagen receptor during initial adhesion of fibroblasts to collagen. (See Table 1 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 PIH6, PIE6, and P4B4 that were noninhibitory or weakly inhibitory for cell adhesion to collagen, localized 0~2 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-α2 mAb PIH5 was not detectable in FAs on collagen (Fig. 6 A). When not localized into FAs, α2β1 was detected in a uniform cell surface localization or at intercellular junctions (see later results for details). Similarly, α5β1, 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 α2β1 and α5β1 to FAs occurred simultaneously with the first detectable adhesion of WI-38 cells to the collagen and fibronectin surfaces (~60 min). (c) α3β1 was not detected in FAs of WI-38 cells attached to any ligand, including laminin, despite the high cell surface expression of α3β1, as seen in Fig. 3. (d) mAb AIA5 and P4C10 that react with the common integrin β1 subunit, also localized to the FAs on fibronectin, collagen, and laminin, as well as a diffuse cell surface staining. (e) α2β1 and α5β1 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 Na251CrO4 (50 μCi/ml for 2-4 h) and washed; then 5 × 10^5 cells/well were incubated with hybridoma culture 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 counted in a gamma counter. All experiments were done in triplicate and the results are representative of at least three experiments.

Inhibition of Cell-Cell Adhesion

Adhesion surfaces were prepared by growing ELL-8 cells to confluence in 48-well tissue-culture clusters (Costar Corp., Cambridge, MA) in KGM followed by activation of the cells by addition of 1 mM CaCl2 24 h before use in the assays. The Ca^{2+} induced cell-cell aggregation within 5 h of addition. Additional ELL8 cells were 3Cr-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 5 × 10^5 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 3Cr-counts per minute were quantitated 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 125I 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).

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%...
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 α2β1 and α3β1 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. α3β1 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, α2β1 detected with mAb P4B4 (or PIE6 and PIH6) was expressed on both basal and suprabasal cell layers. A second anti-α2 mAb, PIH5, also detected α2β1 in the basal/suprabasal regions but was most intensely expressed throughout the spinous cell layer. The mAbs P4B4 and PIH5 detect different epitopes on the α2 subunit that are differentially expressed in both tissue and cell culture (Wayner et al., 1989). In general, both α2β1 and α3β1 were detected on all surfaces of the cells in the basal and suprabasal strata of the epidermis. In addition, both α2β1 and α3β1 were present in the glandular epithelium of the dermis. Fibronectin was expressed throughout the dermis (Fig. 2 F). In control experiments, mAb PIG12 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 125I-surface-labeled HFKs (Fig. 3, top), with α subunit specific mAbs identified α2, α3, and α6 subunits at high levels and α5 subunit at low levels. The α2, α3, and α5 subunits were associated with the β1 subunits. As recently shown by Hemler et al. (1989) for many carcinomas, α6 was associated with the a, b, and c peptide bands of the β4 subunit of the integrin family. FEA and EIL-8 keratinocyte cell lines, expressed similar α and β subunits (data not shown). In contrast, human dermal fibroblasts isolated from the same tissue, expressed α1, α2, α3, and α5 at high levels but did not express α6 (Fig. 3, bottom).

Inhibition of Keratinocyte Adhesion with mAbs

Anti-α2β1 (PIH5), and anti-α5β1 (PID6) 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
Figure 6. HFK α2β1 localizes in focal adhesions on collagen but not laminin or fibronectin as detected with noninhibitory mAbs (PIE6, P4B4, P1H6) but not inhibitory mAbs (PIH5). HFKs were adhered to surfaces coated with collagen (Type I; labeled CN I), fibronectin (FN), or laminin (LN) for 5 h, and then reacted with the indicated mAbs. (A) mAb PIH5 that inhibits cell adhesion to collagen failed to detect α2β1 in FAs on collagen. (B) Noninhibitory mAb PIE6 identified α2β1 in FAs on collagen. (C) PIE6 did not detect α2β1 in FAs on fibronectin. (D) PIE6 did not detect α2β1 in FAs on laminin. Noninhibitory mAbs P4B4 and P1H6 gave results similar to PIE6.

Ligand specificities for α2β1 and α3β1 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 α2β1 and α5β1 (Fig. 5, PIE6 and AB33, respectively) into FAs. Despite the observed inhibition of cell adhesion to both collagen and laminin by anti-α2β1 (Fig. 4, Col and Lam, PIH5), the anti-α2β1 mAbs (P4B4, PIE6, and P1H6) only localized to HFK FAs on collagen (Fig. 5, PIE6 and Fig. 6 B) but not laminin (Fig. 6 D) or fibronectin (Fig. 6 C). In general, anti-α2, -α3, or -α5 mAbs that could inhibit cell adhesion to ligands did
not detect α subunits in FAs while noninhibitory anti-α mAbs effectively stained FAs (Fig. 6 A, inhibitory P1H5 and 6 B, noninhibitory P1E6). 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 αβ1 (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 αβ1 in FAs of HFKs and the best exogenous ligand was laminin (Fig. 5, P1F2).

αβ1 and αβ1 in Cell–Cell Adhesion

We investigated the role of αβ1 and αβ1 in the stratification process in skin. Our work focused on two aspects of this question: (a) evaluation of the possible roles for αβ1 and αβ1 in cell–cell adhesion and (b) identification of physiologically relevant ligands for αβ1 and αβ1 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 αβ1 (Fig. 7 G) and αβ4 in prolonged cultures of HFKs (over 24 h). After the localization of αβ1 in FAs, αβ1 polarized to the opposite end of the same cell (Fig. 7, G and H).

The distribution of αβ1, αβ1, and αβ1 in these three locations was affected by the exogenous ECM ligand. For example, αβ1 localized to putative cell–cell contacts above the focal plane of the adhesion surface when the cells were adherent on fibronectin (Fig. 6 C and 7 B) or laminin. In contrast, αβ1 concentrated in 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, αβ1 preferentially localized at the cell–cell contacts. The relocation of αβ1 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 Ca2+-induced HFK aggregation may decrease αβ1 and αβ1 in FAs and increase localization into cell–cell contacts. Addition of 1 mM CaCl2 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. αβ1 localized preferentially in the putative cell–cell contact sites (Fig. 7 A). When HFKs were grown on collagen in the presence of CaCl2, αβ1 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).

The localization of αβ1 and αβ1 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-αβ1 (P1D6), anti-αβ1 (P1F2), anti-α4 (P4G9), nor 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 PIB5 mAb, reacting with a specific epitope on αβ1 successfully inhibited cell–cell adhesion.

αβ1 Colocalizes with a Secreted, Laminin-containing ECM in FAs

In contrast to the rapid localization of αβ1 and αβ1 into FAs on collagen and fibronectin, αβ1 appeared more slowly in FAs of HFKs. Within 5 h, HFKs localized αβ1 into FAs on exogenous fibronectin while αβ1 was diffuse over the cell surface (Fig. 9). However, after 24–96 h of culture on fibronectin (Fig. 9) αβ1 was displaced to one edge of the cell while αβ1 was relocated into FAs. Consistent with this observation, anti-αβ1 mAb PIB5, partially inhibited the initial stages of cell adhesion to fibronectin or laminin (Fig. 4) but efficiently detached long term cultured cells (>5 h).

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Figure 9. α3β1 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-α3β1 (P1F2) and anti-α5β1 (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-α3β1 (PIB5) 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) P1G12 anti-ECMR III/CD44, control; (C) P1D6 anti-α5β1; (D) PIB5 anti-α3β1.

from both laminin and fibronectin surfaces (Fig. 10 D). In contrast, anti-α5β1 (P1D6) 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 α3β1 into FAs (Fig. 9) suggested that HFKs might be secreting ECM during long-term culture that interacts with α3β1 in preference to exogenous laminin, collagen or fibronectin. To investigate this possibility, we examined the distribution of α3β1 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, α3β1 (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 α3β1 (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 α3β1 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 α3β1 in FAs (Fig. 12). FEA cells either in long or short term culture on laminin (Fig. 12, A and B) accumulated α3β1 into belts at the cell periphery but failed to form distinct FAs. However, adhesion of FEA cells to ECM prepared from prolonged cultures of

Discussion

α3β1 Preferentially Interacts with Secreted, Laminin-containing ECM

We have used two approaches to investigate the ligand-binding specificity of integrins α2β1 and α3β1. First, we examined the distribution of integrins in tissues to identify cell populations that expressed high levels of both α2β1 and
α3β1. 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 α3β1 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 α3β1 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 α3β1 (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 α3β1 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-α3β1 mAbs to inhibit FEA cell adhesion to laminin and the inability of FEA cells to localize α3β1 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-α3β1 (Wayner and Carter, 1987; Wayner et al., 1988) and affinity chromatography (Wayner and Carter, 1987; Gehlsen et al., 1988, 1989)2, have described α3β1 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 α3β1 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 α3β1 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 α3β1 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 α2β1 and α3β1. In addition, our results have not excluded α6β4 as a potential laminin receptor involved in initial HFK adhesion. α6β4 has been defined as a laminin adhesion receptor in platelets (Sonnenberg et al., 1988) and anti-α6β4 (or α6E8, Kajiji et al., 1989) inhibited cell adhesion to laminin (Cheresh et al., 1989). Collagen is an optimal ligand for α2β1 expressed by fibroblasts and platelets (Wayner and Carter, 1987). In HFKs, α2β1 was also shown to contribute to initial HFK adhesion to laminin as well. Anti-α2β1 mAbs inhibits HFK and FEA adhesion to both collagen and laminin. However, α2β1 localizes in FAs only on collagen. Apparently, α2β1 interacts weakly with laminin and contributes to initial epidermal cell adhesion to laminin but fails to form the stable attachments characterized by α3β1 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 α2β1 to collagen or collagen/laminin via posttranslational modification or alternate splicing of the receptor. The role of α3β1 in cell adhesion to collagen and laminin has been confirmed by affinity purification of α3β1 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 α2β1 (Sandberg et al., 1989).

### Figure 11.

OVCAR-4 cells colocalize α3β1 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-α3β1 (PIF2, A, D, and G) and rabbit polyclonal anti–laminin (RS922, B, E, and H). α3β1 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 α3β1 that colocalize with concentration of secreted laminin. Black arrows indicate representative focal adhesions. α3β1 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 α3β1 were localized to regions between and around the focal adhesions. Cells adherent to fibronectin (G–I) secreted laminin that colocalized with α3β1.
Figure 12. FEA α3β1 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 α3β1 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 α3β1 in focal adhesions in 3 h as determined by interference reflection microscopy and (E and F, same field) at the origins of stress fibers.
α2β1 and α3β1 in both cell-cell and cell-substratum adhesion. This possibility was supported by the partial inhibition of cell-cell adhesion by anti-α3β1 (PIB5) and anti-β1 (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 α4β1 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 (Holzmann et al., 1989) is relevant to the role of α3β1 in both cell-cell and cell-substrate adhesion. Although anti-α3β1 (PIB5) and anti-α4β1 (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 β1-containing integrins may mediate cell-cell adhesion indirectly by bridging integrin receptors located in two adhering cells. Alternatively, the direct interaction of α3β1 and α4β1 with unidentified membrane co-receptors in adhering cells, may still be inhibited by the anti-α3β1 (PIB5), -α4 (P4C2) and -β1 (P4C10) mAbs.

The Role of α2β1 and α3β1 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, α3β1 localized primarily to the basal cell layer where, presumably, it associates with the laminin-containing ECM secreted by the basal cells. Although α2β1, 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 α3β1 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 Ca2+-induction, downregulates α3β1 and/or relocates α2β1 and α3β1 from the substratum into intercellular contacts, permitting cell movement into the suprabasal strata.

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