

Novel Function for β_1 Integrins in Keratinocyte Cell-Cell Interactions

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Abstract. We have examined the expression, localization, and function of β_1 integrins on cultured human epidermal keratinocytes using polyclonal and monoclonal antibodies against the β_1 , α_2 , α_3 , and α_5 integrin subunits. The β_1 polypeptide, common to all class 1 integrins, was localized primarily in areas of cell-cell contacts of cultured keratinocytes, as were α_2 and α_3 polypeptides, suggesting a possible role in cell-cell adhesion for these integrin polypeptides. In contrast, the fibronectin receptor α_5 subunit showed no such accumulations in regions of cell-cell contact but was more diffusely distributed in the keratinocyte plasma membrane, consistent with the absence of fibronectin at cell-cell contact sites. Colonies of cultured keratinocytes could be dissociated by treatment with monoclonal antibody specific to the β_1 polypeptide. Such dissociation of cell-cell contacts also occurred under

conditions where the monoclonal antibody had no effect on cell-substrate adhesion. Therefore, β_1 integrin-dependent cell-cell adhesion can be inhibited without affecting other cell-adhesive interactions. Antibody treatment of keratinocytes maintained in either low (0.15 mM) or high (1.2 mM) CaCl_2 also resulted in the loss of organization of intracellular F-actin filaments and β_1 integrins, even when the anti- β_1 monoclonal antibody had no dissociating effect on keratinocyte colonies at the higher calcium concentration. Our results indicate that β_1 integrins play roles in the maintenance of cell-cell contacts between keratinocytes and in the organization of intracellular microfilaments. They suggest that in epithelial cells integrins can function in cell-cell interactions as well as in cell-substrate adhesion.

IT has become increasingly clear that cell-cell and cell-substrate interactions are mediated through specific cell surface molecules. Cell-substrate adhesion occurs through the binding of extracellular molecules by their cell surface receptors. The best characterized of the cell adhesion receptors comprise a family of integral membrane glycoprotein dimers called integrins or cytoadhesins (reviewed by Buck and Horwitz, 1987; Hynes, 1987; Juliano, 1987; Ginsberg et al., 1988; Ruoslahti, 1988; Akiyama et al., 1989a). The integrins consist of an α subunit of 140,000–180,000 D noncovalently bound to a smaller β subunit of 105,000–125,000 D. The various integrin heterodimers are divided into classes or subfamilies based on the different β polypeptides (Hynes, 1987). The β_1 integrins are associated with one of at least six different α subunits. β_1 integrins include receptors for fibronectin, collagen, and laminin and also the human T cell very late antigen (VLA)¹ heterodimers (Hem-

ler et al., 1987; Hemler, 1988; Takada et al., 1988). The high-affinity fibronectin receptor is $\alpha_5\beta_1$ (or VLA-5).

Keratinocytes are specialized epithelial cells of the epidermis that function as the covering and protecting layer of the skin. The epidermis consists of multiple layers of keratinocytes that undergo terminal differentiation when they move from the mitotic basal layer. Polarized basal cells rest on the basement membrane, and their apical and lateral surfaces are in tight contact with other keratinocytes (Donaldson and Mahan, 1988). Cultured keratinocytes attach to the adhesive proteins fibronectin, collagen, laminin, and vitronectin in vitro (Clark et al., 1985; O'Keefe et al., 1985; Stenn et al., 1983; Takashima and Grinnell, 1984; Takashima et al., 1986; Toda and Grinnell, 1987; Toda et al., 1987; Woodley et al., 1988) and can migrate on fibronectin and collagens but not on laminin (Nickoloff et al., 1988; Woodley et al., 1988). Such results suggest that keratinocytes could use one or more of the integrin receptors for these adhesive proteins.

Human keratinocytes can be maintained in a highly proliferative state in culture by keeping the concentration of cal-

1. *Abbreviations used in this paper:* DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; PBS+, Dulbecco's PBS with calcium and magnesium; VLA, very late antigen.

cium in the growth medium low. Under these conditions, keratinocytes grow in colonies and do not form desmosomes or adherens-type junctions (Duden and Franke, 1988; Hennings et al., 1980; Pillai et al., 1988; Watt et al., 1984). There is, however, some membrane overlapping and formation of loose contacts between adjacent cells (Pillai et al., 1988). Under culture conditions of higher calcium concentration, the formation of adherens-type cell-cell junctions and desmosomes is promoted (Hennings et al., 1980; Pillai et al., 1988; Watt et al., 1984). Thus, keratinocytes are a particularly convenient model system for the examination of cell-cell contacts.

We have used polyclonal and monoclonal antibodies to examine the localization and function of β_1 integrins in keratinocytes. We find evidence that integrins play important roles in the formation and maintenance of cell-cell contacts and organized microfilament cytoskeleton.

Materials and Methods

Cell Culture

Normal human keratinocytes were purchased from Clonetics Corp. (San Diego, CA) after isolation from adult human surgical breast samples and culturing as described by Boyce and Ham (1985) and were cultured as described in serum-free low calcium keratinocyte growth medium supplemented with epidermal growth factor, hydrocortisone, insulin, bovine pituitary extract, antibiotics, and antimycotic (Clonetics Corp.). Low calcium (0.15 mM) medium was converted to high calcium medium (1.2 mM) where indicated by the addition of 200 mM calcium chloride solution. Cells from passages 2-6 were cultured on either standard tissue culture plastic or on glass coverslips.

Normal human foreskin fibroblasts were a generous gift from Dr. Steve Alexander (Bethesda Research Laboratories, Bethesda, MD) and were cultured in DME supplemented with 1 mM glutamine, 1 mM sodium pyruvate, antibiotics (50 μ g/ml streptomycin, 50 U/ml penicillin), and 10% heat-inactivated FCS. Cell passages 9-14 were used.

Immunological Reagents

Two rat monoclonal antibodies to either the α_5 subunit of the human fibronectin receptor (mAb 16) or the shared β_1 subunit (mAb 13) of the β_1 integrin subfamily have been described previously (Akiyama et al., 1989b; Matsuyama et al., 1989). Fab fragments were produced as described (Akiyama et al., 1989b). A polyclonal anti- β_1 antibody (4080) was produced after immunization of rabbits with a full-length β_1 intracellular domain peptide with the sequence C K L L M I I H D R R E F A K F E K E K M N A K W D T G E N P I Y K S A V T T V V N P K Y E G K, synthesized using t-Boc amino acids with a Dupont Co. (Wilmington, DE) Coupler 2100 automated peptide synthesizer. This peptide was conjugated to keyhole limpet haemocyanin (Pierce Chemical Co., Rockford, IL) with the heterobifunctional reagent m-maleimidobenzoic acid-N-hydroxysuccinimide ester essentially as described earlier (Walker et al., 1985). Antisera against the conjugate were raised in rabbits as follows. The conjugate (0.6 mg peptide) in 0.5 ml of PBS was mixed with 0.5 ml of Freund's complete adjuvant and injected subcutaneously with booster injections at 4-wk intervals. This anti-peptide antibody immunoprecipitated fibroblast integrins and Western immunoblotted β_1 and pre- β_1 subunits in fibroblast extracts. Furthermore, the localization pattern of this antibody by immunofluorescence microscopy of fibroblasts was equivalent to that of mAb 13 (not shown). Polyclonal anti-peptide antibodies (4058; with the sequence C F D S K G S R L L E S S L S S S E G E E P V E) against the α_5 subunit of the fibronectin receptor were also produced in rabbits as above and were shown to recognize the fibronectin receptor in ELISA assays and the α_5 chain in Western immunoblotting (not shown).

Polyclonal anti-human placental fibronectin receptor antibody was used for some of the immunoprecipitations and was characterized previously (Roberts et al., 1988). mAb J143 against the α_3 integrin polypeptide (Fradet et al., 1984; Rettig et al., 1986) was kindly supplied by Dr. Lloyd J. Old (Memorial Sloan-Kettering Cancer Center, New York). Monoclonal antibody recognizing α_2 was produced after immunization of mice using

platelets as the immunogen; this F17-6D7 monoclonal antibody, an IgG₁, recognized the platelet glycoprotein Ia-IIa complex (VLA-2) according to immunoprecipitation analyses of surface-iodinated human platelets. This antibody and its Fab fragment inhibit platelet aggregation by collagen and platelet adhesion to type I and III collagen-coated surfaces (Grainick et al., 1988). It also inhibited fibroblast and keratinocyte adhesion to collagen, though not to fibronectin or laminin (not shown). Anti-VLA-1 monoclonal antibody (TS2/7) for identifying the α_1 band in SDS gels was the kind gift of Dr. Martin Hemler (Dana-Farber Cancer Center, Boston, MA). Antibodies to the following molecules were also used in this study: epithelial keratin AE 1 (ICN Biomedicals, Lisle, IL), desmoplakin I and II (ICN Biomedicals), type IV collagen (ICN Biomedicals), laminin (ICN Biomedicals), human epithelial membrane antigen (Accurate Chemical & Scientific Corp., Westbury, NY), human transferrin receptor (Accurate Chemical & Scientific Corp.), human epidermal growth factor receptor (ICN Biomedicals), and human plasma fibronectin (Chen et al., 1985a, 1986b).

The specificity of anti-integrin immunolocalization was evaluated using the above monoclonal antibodies against epithelial membrane antigen, transferrin receptor, and epidermal growth factor receptor, and nonimmune IgG from rat and mouse and by comparisons with the more general localization of glycoconjugates stained by FITC-labeled wheat germ agglutinin (a lectin chosen because it binds to a variety of membrane glycoproteins) and of a carbocyanine lipid analogue. For lectin labeling, keratinocytes were cultured in either low or high calcium medium and then fixed and processed as for immunofluorescence but then stained instead with 50 μ g/ml FITC-wheat germ agglutinin (Reactifs IBF, Villeneuve La Garenne, France) for 1 h. For localization of a nonspecific lipid probe, keratinocytes were incubated 1 h in the carbocyanine dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes Inc., Eugene, OR) used as described by Honig and Hume (1986) after filtration of the dye-medium suspension through an 0.22- μ m filter; after an additional 18 h of culturing, the cells were fixed with formaldehyde and examined using a rhodamine filter combination.

Immunofluorescence Microscopy

Keratinocytes were cultured on glass coverslips in low calcium (0.15 mM) medium. When the cell layer reached half-confluence, the cells were fixed immediately or switched to high calcium (1.2 mM) medium for 15 min to 24 h. The cells were then washed briefly twice with Dulbecco's PBS with calcium and magnesium (PBS+) and fixed for 30 min with 4% formaldehyde and 5% sucrose in PBS+. After washing, the cells were permeabilized in 0.5% Triton X-100 in PBS+ for 4 min. The cells were then washed five times with PBS+, and nonspecific binding sites were blocked for 30 min with 3% BSA (Sigma Chemical Co., St. Louis, MO) in PBS+ containing 0.1% glycine. After one wash, the cells were incubated for 1 h with the indicated concentrations of antibodies in PBS containing 1 mg/ml BSA at room temperature. After extensive washing for 1 h with PBS, the first antibodies were localized using rhodamine-conjugated second antibodies (1 h at 1:100 dilution; Miles Laboratories Inc., Elkhart, IN) or using 25 or 50 μ g/ml affinity-purified IgG (Rockland, Inc., Gilbertsville, PA) in 1 mg/ml BSA in PBS. In some experiments, 10% goat or rabbit serum was used to supplement blocking solutions to reduce background staining further. In double immunolabeling experiments to localize integrin β_1 and fibronectin simultaneously, keratinocytes were stained with mAb 13 against the β_1 subunit, followed by rhodamine-labeled goat anti-rat IgG, and then with 1:100 goat 1213 anti-human plasma fibronectin IgG directly labeled with FITC. After rinsing, the coverslips were mounted in PBS containing 1 mg/ml *p*-phenylenediamine (Sigma Chemical Co.) to inhibit photobleaching (Johnson and Nogueira Araujo, 1981). Immunofluorescence was documented with a Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence and Tri-X Pan 400 film (Eastman Kodak Co., Rochester, NY) with film speed increased to 1,600 ASA by development in Diafine (Acufine, Inc., Chicago, IL).

Frozen sections (5 μ m) of adult human epidermis were rinsed with and preincubated with 0.1% BSA for 30 min. The sections were then immunostained with mAb 13 (50 μ g/ml) as described above.

Treatment of Keratinocytes with Antibodies against β_1 Polypeptides

Keratinocytes grown in plastic multiwells or on glass coverslips in low calcium (0.15 mM) medium were washed once with the medium and transferred to either low or high calcium (1.2 mM) medium supplemented with 0-500 μ g/ml monoclonal antibodies against the integrin β_1 subunit (mAb 13) or 500 μ g/ml of its Fab fragments. Control cultures received equal or

higher amounts of rat nonimmune IgG. Effects of the antibody treatment were monitored for 15 min to 24 h using phase-contrast microscopy. The treatments were terminated by fixing the cells as described for immunological staining. Parallel cultures were stained with 1% crystal violet in methanol for 3 min. Control and mAb 13-treated cultures were stained for the integrin β_1 subunit using anti-peptide antibody 4080 against the intracellular domain of β_1 .

The organization of F-actin microfilament bundles was monitored using rhodamine-labeled phalloidin (Molecular Probes Inc.), as described (Barak et al., 1980). The spreading of keratinocytes in treated vs. control cultures was measured from photographic prints by digitizing cell perimeters and calculating cell areas using Sigma-Scan software (Jandel Scientific Corp., Sausalito, CA). Statistical analyses were performed using *t* test. The reversibility of mAb 13 treatment was examined by allowing the cells to recover in normal growth medium for 24 h after a 24-h pretreatment with mAb 13 and rinsing in medium.

Cell Spreading Assays

Fibronectin and vitronectin were purified as described from fresh frozen human plasma obtained from the Department of Transfusion Medicine (National Institutes of Health, Bethesda, MD) (Akiyama and Yamada, 1985; Yatohgo et al., 1988). Type I collagen (Vitrogen 100; 3 mg/ml) was obtained from Collagen Corp. (Palo Alto, CA).

Cell spreading assays were performed essentially as described (Akiyama et al., 1986) with the following modifications. For collagen-coated wells, 25 μ l of Vitrogen 100 was added per well of 96-well multiwell plates. After drying overnight, the wells were rinsed several times with PBS before adding cells. Fibronectin and vitronectin were used at concentrations of 25 μ g/ml. Keratinocytes (10,000/well) were added to the wells in the presence or absence of mAb 13 or nonimmune rat IgG (500 μ g/ml). After 18 h, the cells were fixed and stained with crystal violet as described above. The spreading of keratinocytes was expressed as the percentage of spread cells per microscopic field. Ten random fields from three replicate wells were counted for each point, determining a spread morphology according to phase-contrast microscopy.

Immunoprecipitation and Western Immunoblotting

Human keratinocytes or fibroblasts were cultured in their normal growth media to early confluency. The cell monolayers were washed twice with methionine-free growth medium, and the cells were labeled with 100 μ Ci/ml Trans³⁵S label (specific activity >1,000 Ci/mmol; ICN Radiochemicals, Irvine, CA) in keratinocyte or fibroblast growth medium lacking methionine for 24 h. The β_1 integrins were then immunoprecipitated from cell extracts as described in detail elsewhere (Roberts et al., 1988) using polyclonal anti-fibronectin receptor antiserum (Roberts et al., 1988) and monoclonal (mAb 13) or polyclonal anti-peptide antibodies (4080) against the β_1 subunit. Specific integrin α polypeptides were immunoprecipitated using monoclonal antibodies indicated in the figure legends with protein G-agarose (Genex Corp., Gaithersburg, MD) as described (Akiyama et al., 1989b).

Western immunoblotting was performed essentially as described (Burnette, 1981) with the following modifications. Nonfat milk solids (5% wt/vol) were substituted for the BSA for initial blocking. Blots were then incubated with 1:100 or 1:200 dilutions of anti-peptide antisera against α_5 (4058) or β_1 (4080) in 5% milk solids followed by 0.5 mCi/ml ¹²⁵I-labeled protein A (specific activity 2–10 μ Ci/ μ g; New England Nuclear, Boston, MA).

Results

Immunolocalization of Epidermal Keratinocyte and Fibroblast Integrins

To examine possible roles of integrins in adhesive interactions of keratinocytes, frozen sections of normal adult human epidermis were stained with mAb 13, which recognizes the human integrin β_1 polypeptide. As shown in Fig. 1, integrin β_1 was localized primarily in the basal and suprabasal cell layers, gradually disappearing in the lower parts of the stratum spinosum. There was little or no staining of the stratum granulosum and the corneum. Surprisingly, staining for inte-

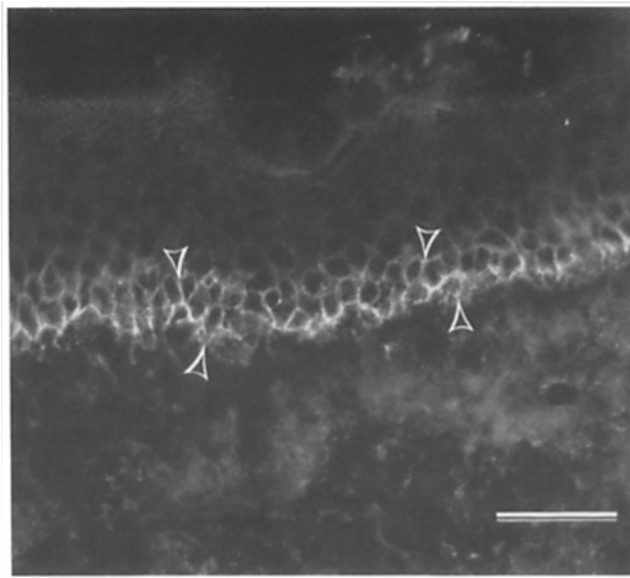


Figure 1. Localization of integrin β_1 subunit in adult human skin with mAb 13. Integrin β_1 polypeptides were visualized in frozen 5- μ m sections of adult human skin by incubating with 50 μ g/ml mAb 13 followed by rhodamine-labeled anti-rat IgG. The most intense staining was observed throughout the basal and suprabasal layers of the epidermis, while stratum granulosum and corneum were always negative. Staining of the basal cell layer closest to the basement membrane (*upward arrowheads*) is discontinuous and spot-like in some areas, while staining of cell-cell junctions (*downward arrowheads*) is continuous. Bar, 50 μ m.

grin β_1 was discontinuous adjacent to the basement membrane but was intense and mostly continuous in the regions of cell-cell contact in the suprabasal and lower stratum spinosum layers.

To explore the distribution and function of the β_1 integrins in greater detail, we then examined cultured human keratinocytes using a series of subunit-specific monoclonal and polyclonal anti-integrin antibodies. The keratinocytes were routinely maintained in low calcium medium but in some experiments were switched to a high calcium medium as described in Materials and Methods. As shown in Fig. 2, the integrin β_1 polypeptide as localized with mAb 13 was mainly found in regions of cell-cell contacts (A). In noncontact regions (e.g., A, upper right), staining was present but with a weaker, diffuse, or sometimes spot-like distribution. Staining with monoclonal antibodies that recognize the α_2 collagen receptor subunit (Fig. 2 B) and the multiligand collagen/laminin/low-affinity fibronectin receptor α_3 subunit (Fig. 2 C) showed similar localization patterns as mAb 13. In contrast, mAb 16, which recognizes the fibronectin receptor α_5 subunit, had a weak and diffuse localization without accumulation at cell-cell boundaries (D).

When the medium was changed from low to high calcium, the localization of β_1 , α_2 , and α_3 increased at cell-cell contacts accompanied by a decrease in the amount of diffuse apical membrane staining (Fig. 2, E–G). This effect was observable within 15 min of changing the calcium concentration and was essentially complete after 2 h (not shown). Nonconfluent keratinocytes in low calcium often showed some accumulation of β_1 polypeptides at local regions of membrane immediately opposing neighboring cells (Fig. 3, A, B,

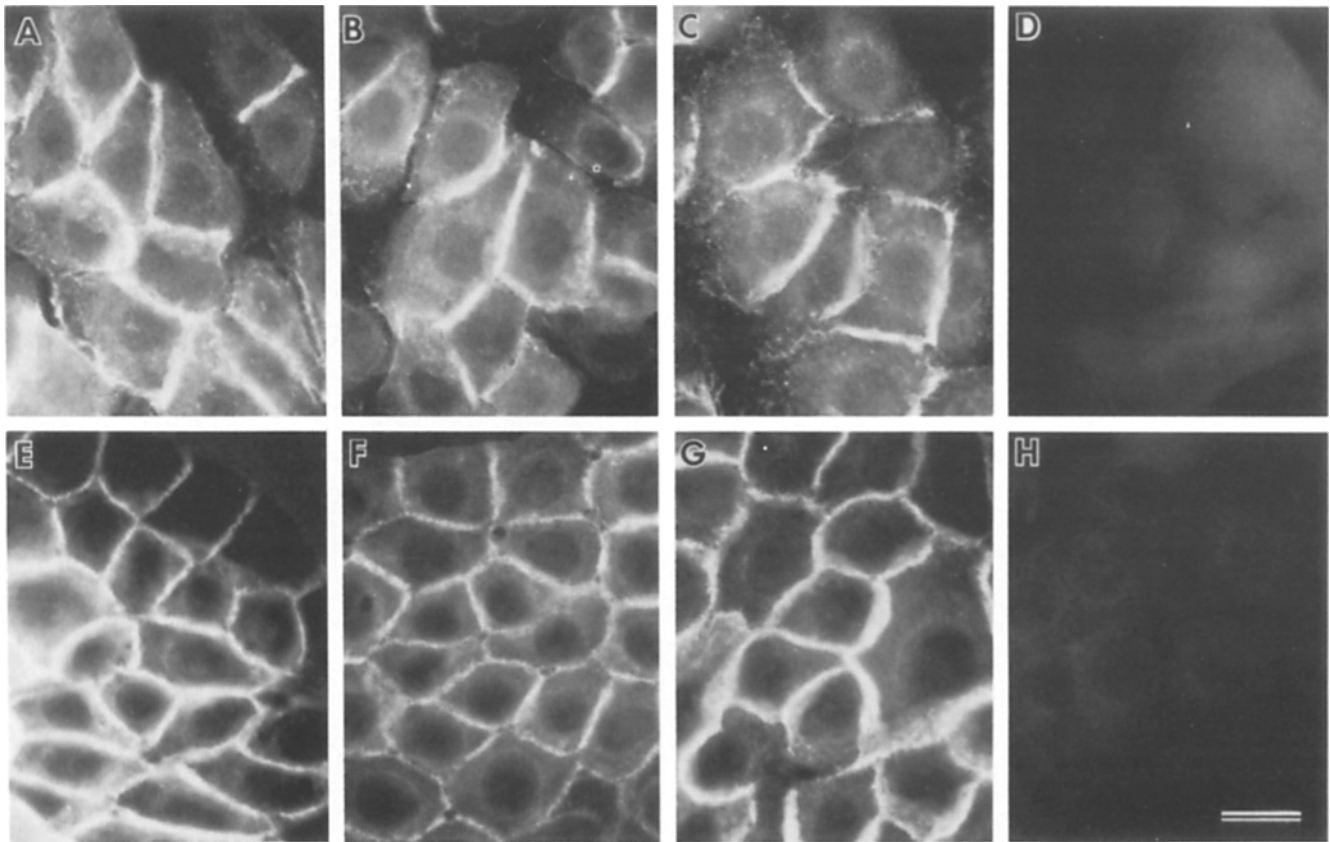


Figure 2. Localization of β_1 integrins in cultured keratinocytes. Cells were cultured for 24 h in low calcium (0.15 mM; *A-D*) or high calcium (1.2 mM; *E-H*) medium and stained with monoclonal antibodies against the (*A* and *E*) β_1 , (*B* and *F*) α_2 , (*C* and *G*) α_3 , (*D* and *H*) α_5 integrin subunits; antibody concentrations were, respectively, 50 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 1:100 mouse serum containing monoclonal J143, and 50 $\mu\text{g/ml}$. In *A-C* and *E-G*, note the extensive accumulation of integrin subunits at cell-cell contact sites. In *D* and *H*, there is no apparent α_5 accumulation at cell-cell contact sites. Bar, 20 μm .

and *F*). When cells were switched to high calcium and stained without permeabilization, this phenomenon became particularly striking with almost all β_1 polypeptides found in cell-cell contacts (Fig. 3, *C-D*).

The matrix proteins fibronectin (Fig. 3, *E* and *G*), type IV collagen, and laminin (not shown) were not found in cell-cell contact regions. Fibronectin was localized in perinuclear intracellular pools as well as along membrane processes and in strands left behind as cells migrated (Fig. 3, *E* and *G*). Double immunofluorescence localization of fibronectin and β_1 integrins in the same cells as shown in Fig. 4, *A* and *D*, directly confirmed the general lack of colocalization of fibronectin and β_1 integrins. No accumulation of fibronectin could be detected in the cell-cell contact regions that stained heavily with the mAb 13 anti-integrin monoclonal antibody. The only exception concerning colocalization was in the perinuclear region, which presumably represents fibronectin and integrins in endoplasmic reticulum and Golgi apparatus before secretion. As a control, keratinocytes grown on porous nitrocellulose filters to allow free access of antibodies to the basal surfaces of the cell also displayed the same pattern of integrin β_1 localization (not shown).

To rule out the possibility of a general accumulation of membrane glycoproteins in regions of cell-cell contacts, separate control cultures of keratinocytes were first stained with antibodies that recognize the transferrin receptor, the

epidermal growth factor receptor, or a general epithelial membrane antigen. Each of these control antibodies stained the plasma membrane in diffuse or micropunctate patterns, with no accumulation at regions of cell-cell contacts (Fig. 4). Moreover, staining of a wider selection of membrane glycoproteins with wheat germ agglutinin did not mimic the localization patterns of the α_2 , α_3 , or β_1 integrin subunits (Fig. 4 *C*). Some cells showed moderately increased labeling at regions of membrane overlap that did not match the extent of accumulation of integrin staining and that resembled the pattern of staining seen at some regions of membrane overlap stained by the carbocyanine dye DiI used as a nonspecific lipid probe (cf. Fig. 4, *C* and *F*). To summarize, none of these controls mimicked the marked accumulation of integrins in cell contact regions.

To compare the localization of the integrin β_1 polypeptide in keratinocytes with that in fibroblasts, these cells were cocultured in high calcium keratinocyte medium. As shown in Fig. 5, mAb 13 staining was concentrated in cell-cell contact regions in keratinocytes (Fig. 5, *A* and *C*). In contrast, mAb 13 stained primarily in a perinuclear distribution and in focal contacts of fibroblasts (Fig. 5, *B* and *D*), consistent with previously published reports (e.g., reviewed in Akiyama et al., 1989a). There was no observable increased staining in regions of fibroblast-fibroblast contact (Fig. 5, *B* and *D*) nor in regions of fibroblast-keratinocyte contact (Fig.

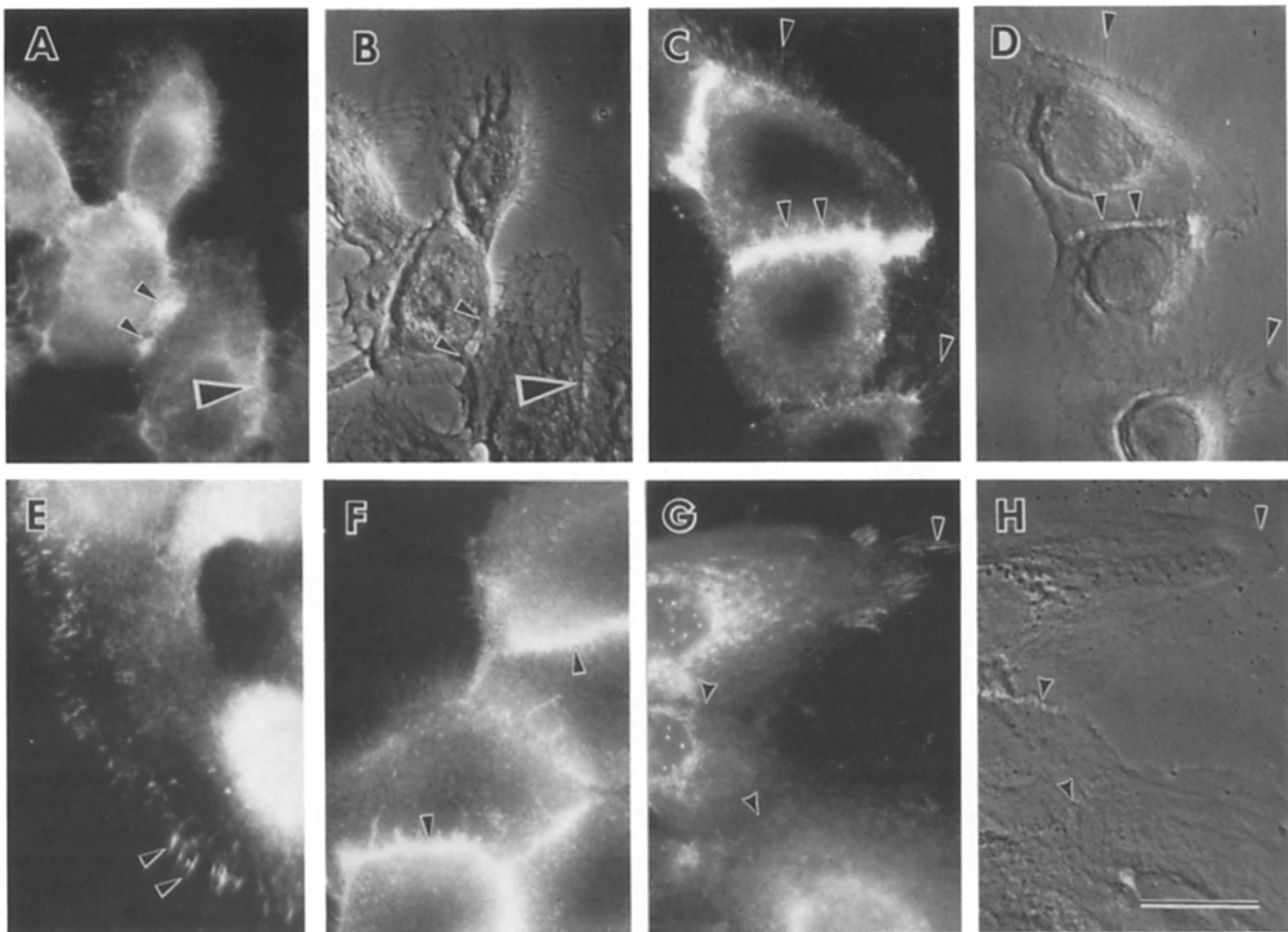


Figure 3. Localization of integrin β_1 and fibronectin in keratinocytes cultured either in low calcium (A–F) or high calcium (G–H) medium. Cells were permeabilized with 0.5% Triton for 4 min in A, B, E, G, and H or left untreated in C, D, and F. (A) Localization of β_1 integrins with mAb 13 (50 $\mu\text{m}/\text{ml}$). (B) Phase-contrast image of the field in A. In A and B, note the accumulation of integrins in regions of cell–cell contact indicated by small arrowheads. In cells with loose contacts, the staining is concentrated in plasma membrane at multiple sites of cell–cell contacts (large arrowhead). (C) Localization of integrins with mAb 13 (50 $\mu\text{g}/\text{ml}$) on nonpermeabilized keratinocytes. (D) Phase-contrast image corresponding to C. Integrins are visualized at sites of cell–cell contact and less in retraction fibers indicated by arrowheads. (E) Localization of fibronectin on keratinocytes cultured in low calcium medium; note fibronectin along membrane processes (arrowheads). (F) Localization of β_1 integrins by mAb 13 in the same experiment set as shown in E; note that unlike fibronectin integrins are present at cell–cell contact sites (arrowheads). (G) Localization of fibronectin in keratinocyte colonies maintained in high calcium medium. (H) Phase-contrast image of G; note the presence of fibronectin in perinuclear regions and along plasma membrane projections (arrowhead) but not in cell–cell contact sites (curve-sided arrowheads). Bar, 20 μm .

5 A). The same localization results were obtained in cocultures in regular fibroblast medium (not shown).

Biochemical Comparison of Keratinocyte and Fibroblast Integrins

Two polyclonal antibodies were used to compare keratinocyte and fibroblast integrins. Antibody 3847 was raised against highly purified human placental fibronectin receptor and has already been characterized (Roberts et al., 1988). A second polyclonal antibody, designated antibody 4080, was raised against a 48-residue synthetic peptide representing the cytoplasmic domain of the β_1 subunit. Both antibodies gave identical results when used in immunoprecipitations (Fig. 6). As shown in Fig. 6, lanes 1 and 2, metabolically labeled β_1 receptors immunoprecipitated from keratinocytes grown

in low calcium medium appear as two major components of 160 and 130 kD under nonreducing conditions. A 110-kD β_1 precursor is only faintly visible. In contrast, as shown in Fig. 6, lanes 3 and 4, immunoprecipitated, metabolically labeled fibroblast receptors consist of three major bands with a relatively large amount of β_1 precursor, as previously described (Akiyama and Yamada, 1987; Akiyama et al., 1989b). Close inspection of Fig. 6 shows also that the mature keratinocyte β_1 polypeptide has a slightly larger apparent size (~ 5 kD) in keratinocytes. This difference is probably due to differences in cell type–specific intracellular processing since the β_1 precursors from both cell types comigrate. These differences are most clearly shown in the densitometric scan of the autoradiograms shown in Fig. 6. After chemical reduction, several minor bands appear, apparently due to resolution of different α polypeptides. These bands have

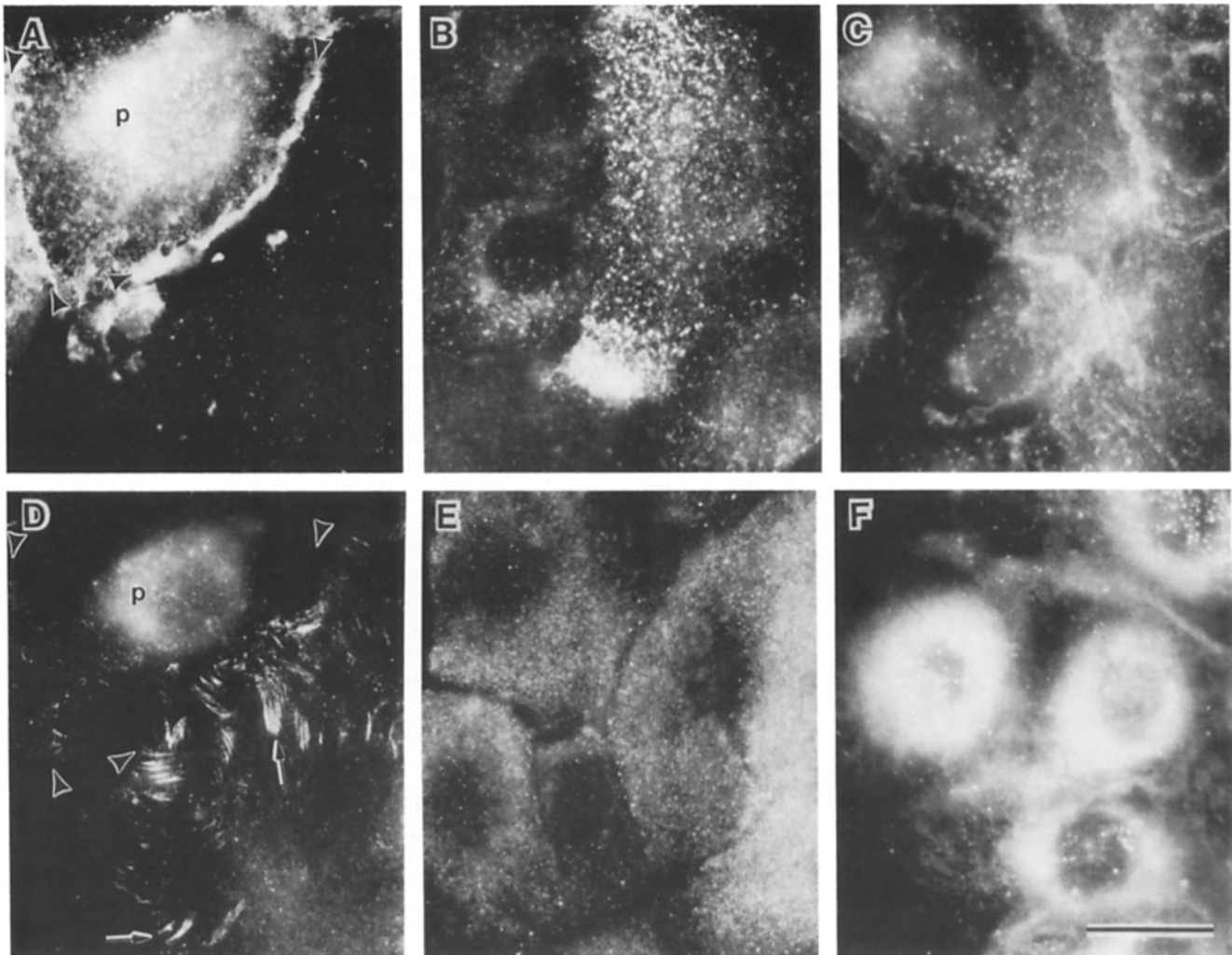


Figure 4. Comparative localization of integrins and other membrane-associated molecules. Double immunofluorescence labeling for integrin β_1 (A) and fibronectin (D) on the same cells. The arrowheads mark the ends of each of two curved zones of accumulation of labeling for integrins with mAb 13 at regions of cell-cell contact. Note that the localization for fibronectin in D fails to correlate with the cell-cell accumulation of integrins and that the only site of correspondence of labeling is in the perinuclear region (p). Also shown are localization patterns for the human transferrin receptor (B), wheat germ agglutinin (C), the epithelial membrane antigen (E), and the carbocyanine lipid probe DiI (F). None of these patterns of localization appears to mimic the accumulation of integrins in cell-cell contacts. Bar, 20 μm .

been identified as α_1 , α_2 , α_3 , and α_5 (200, 150, ~ 135 , and ~ 135 kD, respectively), based on control experiments using subunit-specific monoclonal antibodies for α_1 , α_2 , and α_3 (not shown). The presence and size of β_1 and α_5 were confirmed in Western immunoblotting using peptide antibodies 4080 and 4058 (not shown). There are no apparent changes in integrin bands immunoprecipitated from keratinocytes grown and metabolically labeled for 24 h high calcium medium (not shown).

Dissociation of Keratinocyte Colonies by Anti- β_1 Antibodies (mAb 13)

The results shown in Figs. 1-5 suggest that integrins may play an important role in cell-cell adhesion of keratinocytes. This possibility was directly examined by treating cultures of keratinocytes with mAb 13. As shown in Fig. 7, treatment of keratinocytes in low calcium medium with 0.5 mg/ml mAb 13 resulted in disruption of cell-cell contacts and dissocia-

tion of keratinocyte colonies. Cells originally in epithelioid clusters separated and assumed a polarized morphology, displaying leading lamellae that were frequently broad and characterized by numerous membrane ruffles (Fig. 7). This effect was dose dependent, and disruption of cell-cell contact could be observed with as little as 1 $\mu\text{g/ml}$ mAb 13 (not shown). To rule out apparent dissociation effects caused by artefactual aggregation of receptor by divalent intact IgG, keratinocyte colonies were also treated with monovalent Fab fragments of mAb 13. As shown in Fig. 7 D, Fab 13 fragments had the same biological effect as intact mAb 13. As further controls, nonimmune rat IgG had no effect on the keratinocyte colonies (Fig. 7 B). Thus, cell-cell adhesions in keratinocyte colonies appeared to require the integrin β_1 subunit. Treatment of keratinocytes with equal concentrations of each of the anti- α subunit monoclonal antibodies had no effect. The effect of mAb 13 was also not due to any detectable cytotoxicity or effects on protein synthesis. Keratinocytes treated with mAb 13 displayed no significant dif-

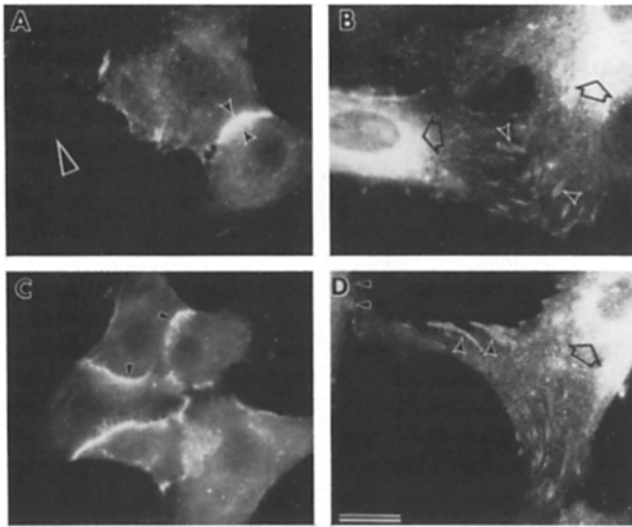


Figure 5. Localization of integrins in keratinocyte-fibroblast cocultures. Cells were cultured for 24 h in high calcium keratinocyte growth medium before fixing and visualization of integrins with mAb 13 (50 $\mu\text{g/ml}$) and rhodamine-coupled anti-rat IgG. Keratinocytes (A and C) and fibroblasts (B and D) were photographed from the same culture using different exposure times because the optimal exposure time for photographing keratinocytes was too short to visualize integrins on fibroblasts. In A, the large arrowhead indicates the location of a fibroblast that is barely visible adjacent to two keratinocytes that are in contact with each other. Integrins are localized at cell-cell contact sites between the two keratinocytes (small arrowheads) but are absent at keratinocyte-fibroblast contact sites. In C, the arrowheads indicate the accumulation of integrins at cell-cell contact sites within a colony of keratinocytes. In B and D, the open arrows indicate the perinuclear intracellular distribution of integrins in fibroblasts and the presence of integrins in focal contacts (larger arrowheads). No integrins are present at cell-cell contact sites between two fibroblasts (smaller arrowheads). Bar, 20 μm .

ference in viability as determined by trypan blue exclusion or in protein synthesis as determined by incorporation of [^{35}S]methionine. Moreover, the patterns of 29 protein bands detected by autoradiography after SDS-polyacrylamide electrophoresis of methionine-labeled cells were identical with or without mAb 13 treatment (data not shown).

Interestingly, the disruption of cell-cell contacts of keratinocytes by mAb 13 appeared to have no major effect on their substrate adhesion under the culture conditions used. As shown in Fig. 7 and also quantified separately (not shown), spread surface areas of the dissociated keratinocytes were often the same as the surface areas of keratinocytes in colonies. In previous studies, mAb 13 was shown to have a strong effect on fibroblast cell spreading on laminin and fibronectin but not on vitronectin substrates (Akiyama et al., 1989b). To rule out simultaneous effects of mAb 13 on cell-cell and cell-substratum adhesion, effects on pure vitronectin substrates were evaluated. Keratinocytes were first tested for receptor specificity by allowing them to spread on substrates prepared with purified fibronectin, collagen type I, or vitronectin in low calcium medium in the presence and absence of mAb 13. As shown in Fig. 8, mAb 13 inhibited keratinocyte spreading on collagen and fibronectin but not on vitronectin. Keratinocyte spreading on fibronectin

was substantially but never completely inhibited by mAb 13, similar to results obtained with fibroblasts (Akiyama et al., 1989b), suggesting that keratinocytes can use receptors not requiring the integrin β_1 subunit for some degree of spreading on fibronectin. Our results are consistent with previously published results showing that cells use the β_1 integrins for adhesion on collagen and fibronectin and the β_3 integrin vitronectin receptor for adhesion on vitronectin (reviewed in Akiyama et al., 1989a).

As shown in Fig. 9, treatment of keratinocytes growing on vitronectin substrates resulted in the dissociation of cell colonies, independent of any effect on cell spreading. Thus, cell surface integrin β_1 polypeptides appear to play an important role in the maintenance of cell-cell adhesions of keratinocytes in low calcium medium, independent of their possible roles in cell-substrate adhesion.

In high calcium medium, keratinocytes become more flattened, and tighter cell-cell junctions form (Hennings et al., 1980). Under these conditions, mAb 13 had only a transient effect on cell-cell adhesion. A small amount of colony disruption could be observed at the beginning of the treatment (not shown). The effect of high calcium medium on keratinocyte clusters was reversible. Keratinocytes colonies grown in high calcium and transferred into low calcium medium were dissociated by treatment with mAb 13 (not shown).

Effect of Anti- β_1 Antibodies on Actin Microfilaments

As shown in Fig. 10, keratinocytes grown in low calcium medium contained a well-defined network of encircling subcortical F-actin microfilaments and bundles as reported earlier (Green et al., 1987). Treatment of cells with mAb 13 resulted in a disruption of F-actin filament organization. Although still tending to be concentrated at the cell periphery, the actin appeared much more disorganized and discontinuous, with local accumulations into spot-like structures and short bundles extending into the numerous lateral filopodia (Fig. 10 B). Treatment with mAb 13 also resulted in a more diffuse pattern of integrin localization (Fig. 11 B).

Keratinocytes in high calcium medium contain a particularly well-defined subcortical network of F-actin filaments, often closely associated with regions of cell-cell contact (Fig. 10 C). Although mAb 13 had little dissociating effect on keratinocytes in high calcium, there were quite obvious effects on the organization of F-actin microfilaments and on cell surface integrin localization. Treatment with mAb 13 resulted in the loss of microfilament organization, and integrin localization became more diffuse and discontinuous at cell-cell contact sites, even though the cells remained associated (Fig. 10 D and Fig. 11 D). Thus, the effect of mAb 13 on keratinocyte actin filament organization in high calcium conditions appears independent of its effect on cell dissociation.

Discussion

The major findings of this study indicate that β_1 integrin receptor subunits are concentrated in cell-cell contacts of keratinocytes in vivo and in culture and that these receptors play a functional role in the maintenance of cell-cell interactions of human keratinocytes.

The surprising finding that the integrin β_1 subunit was

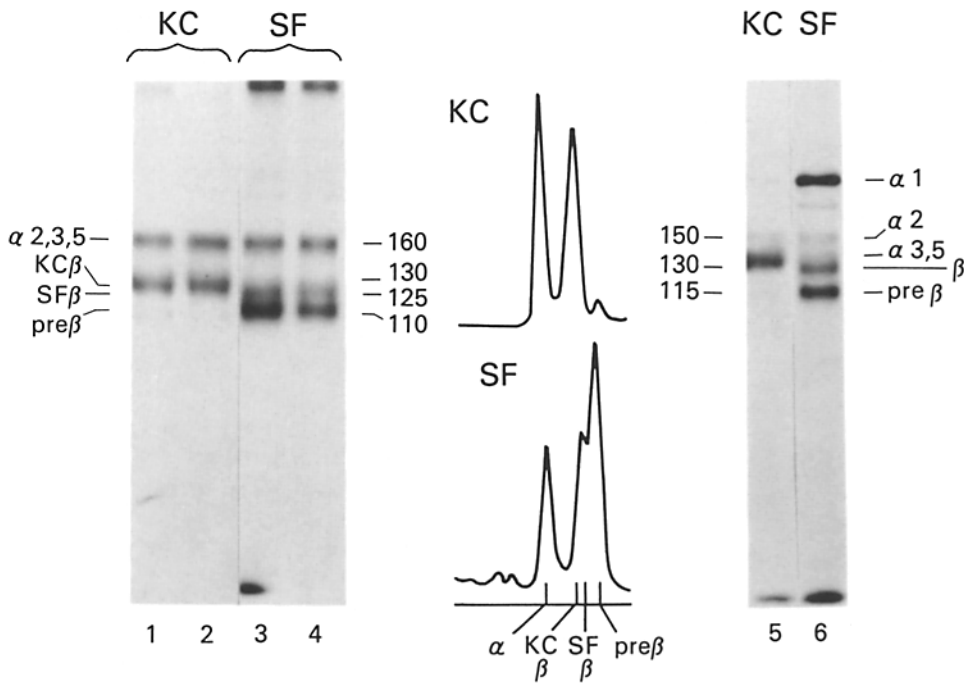


Figure 6. Biochemical analysis of keratinocyte and skin fibroblast integrins. Keratinocytes (KC) and skin fibroblasts (SF) were metabolically labeled for 24 h with [³⁵S]methionine and immunoprecipitated with either polyclonal antiserum raised against a synthetic peptide representing the cytoplasmic domain of the integrin β_1 subunit (lanes 1 and 3) or polyclonal antiserum raised against highly purified human placental fibronectin receptor (lanes 2 and 4-6). Integrin polypeptides were resolved by SDS-gel electrophoresis in the presence (lanes 5 and 6) or absence of DTT (lanes 1-4) and visualized by fluorography. The positions of integrin α_1 , α_2 , and α_3 subunits were determined by immunoprecipitating metabolically labeled keratinocyte and fibroblast cell extracts with mAbs TS2/7, F17-6D7, and J143, respectively. The position of β_1 and α_5 subunits were determined by West-

ern blotting using polyclonal peptide antibodies (4080 and 4058, respectively) as described in Materials and Methods. Note the slightly slower mobility of β_1 polypeptides from keratinocytes (KC β) and the relative lack of pre- β polypeptides. Densitometric scans of lanes 1 and 3 are also shown in the center panels.

localized in regions of cell-cell contact was the first indication of a novel cell-cell adhesive role for this molecule in epithelial cells. This possibility was confirmed by the demonstration that the anti- β_1 mAb 13 could disrupt the formation and maintenance of cell-cell contacts under conditions of low calcium concentration. To rule out that cell-matrix interactions were not involved in the dissociation of keratinocytes, we also cultured keratinocytes on vitronectin, where

the extracellular matrix contacts require different types of integrin receptors (i.e., vitronectin receptors) (Juliano, 1987; Ruoslahti, 1988; Yamada, 1988; Akiyama et al., 1989a). Anti- β_1 integrin antibodies did not affect cell spreading on vitronectin but still dissociated the cells similarly as on plastic substrates, indicating that the β_1 integrins are indeed involved more directly in cell-cell interactions.

Although integrins were present in cell-cell contact sites,

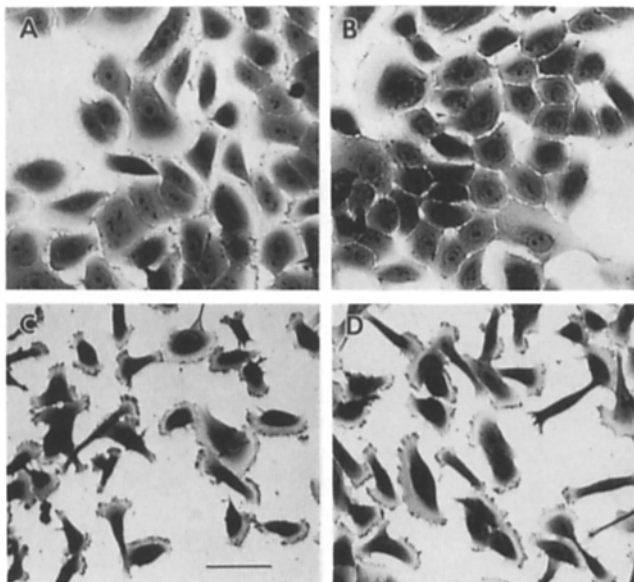


Figure 7. Dissociation of colonies of cultured keratinocytes by mAb 13. Cells in tissue culture wells were left untreated as a control (A) or treated for 24 h with 0.5 mg/ml nonimmune rat IgG (B), mAb 13 (C), or Fab fragments of mAb 13 (D). Bar, 50 μ m.

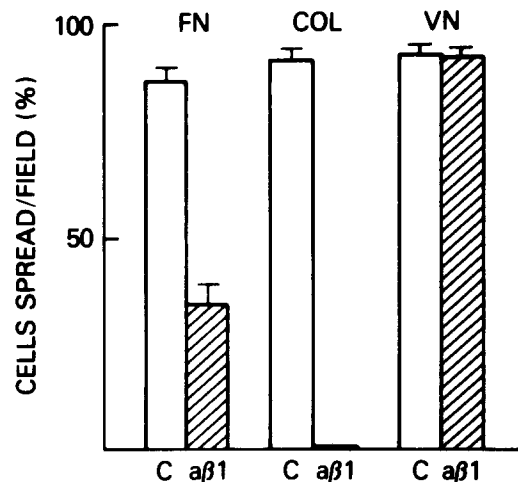


Figure 8. Inhibition of keratinocyte spreading by mAb 13. Keratinocytes were added to substrates prepared with 25 μ g/ml fibronectin (FN), 25 μ g/ml vitronectin (VN), or 3 mg/ml collagen in the form of Vitrogen (COL) either alone (bars C) or in the presence of 0.5 mg/ml mAb 13 (bars a β_1) and cultured for 18 h. The cells were then fixed and stained with crystal violet and scored for spreading. The average of 10 fields \pm SEM is shown.

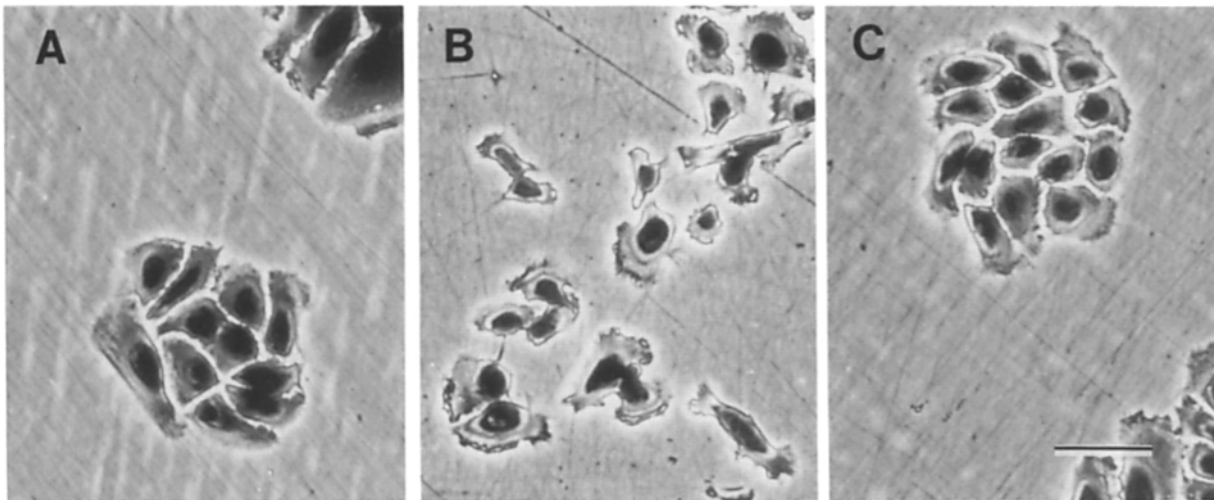


Figure 9. Dissociation of keratinocyte colonies on vitronectin substrates by mAb 13. Keratinocytes were grown on vitronectin substrates in low calcium medium and then left as a control (A) or treated for 24 h with 0.5 mg/ml mAb 13 (B) or nonimmune rat IgG (C). Bar, 50 μ m.

neither fibronectin, laminin, nor type IV collagen colocalized to these sites. These findings do not formally exclude the possibility that such ligands are actually present at sites of integrin accumulation but were not visible due to exceptionally complete epitope masking. Nevertheless, we favor the hypotheses that in keratinocytes, loose cell-cell adhesions involve either the direct association of integrin dimers on adjacent cells or, alternatively, an as yet unidentified ligand molecule that can bridge adjacent cells.

Our results also indicate that the integrin β_1 subunit is important for the organization of intracellular F-actin microfilaments in these epithelial cells. Treatment of keratinocytes with anti- β_1 mAb resulted in the disruption of organized F-actin microfilaments even when the cells were growing in the high calcium medium conditions, where the anti- β_1 mAb had no apparent effect on the dissociation of keratinocyte colonies. Treatment of keratinocytes with the anti- β_1 mAb also resulted in a more diffuse integrin distribution, with a loss of cell surface β_1 clustering at contact sites. These latter results are consistent with those obtained with cultured fibroblasts but where β_1 integrins were instead clustered in extracellular matrix contacts (Akiyama et al., 1989b). The results also show that although anti- β_1 antibodies failed to dissociate cells in high calcium, it did interfere with a cell-cell interaction system. The high calcium conditions promote the formation of adherens- and desmosome-type junctions (Hennings et al., 1980). It is likely that disruption of more than one junctional unit is needed to dissociate cells in high calcium conditions. In fact, many of the antibodies against cell-cell adhesion molecules have only short-term effects on epithelial cell-cell junctions (Gumbiner et al., 1988; Volk and Geiger, 1986b). Cell-cell adhesion molecules such as cadherins are also apparently required for actin microfilament organization in other epithelial cells (Hirano et al., 1987; Nagafuchi and Takeichi, 1988). Thus, both types of adhesion system may contribute to transmembrane cytoskeletal organization.

Previous investigations indicated that a functionally active fibronectin receptor is absent in freshly isolated keratinocytes and is only expressed after several days in culture (Takashima and Grinnell, 1985; Takashima et al., 1986;

Toda and Grinnell, 1987; Toda et al., 1987). In the present study, possibly functional fibronectin receptors $\alpha_3\beta_1$ and some $\alpha_5\beta_1$ were found in keratinocytes of passages 3–6. Fibronectin has been previously described in trails that keratinocytes leave when they move in culture (Kubo et al., 1987), which is consistent with the results of this study. In addition, most of the fibronectin is deposited between cells and substratum and not found on top of the cell layer or between cells. The high-affinity fibronectin receptor ($\alpha_5\beta_1$) was found in low amounts relatively diffusely distributed over the keratinocyte plasma membrane. These results suggest that fibronectin and its receptors function predominantly in cell-substrate adhesion, consistent with the lack of fibronectin localization at cell-cell contact sites.

Some interesting biochemical features of integrin receptors of human keratinocytes were found. Although the pattern of keratinocyte integrins resembled that of fibroblasts, little β_1 precursor was observed in either localization or metabolic labeling experiments. This result suggests that unlike in fibroblasts, the β_1 chains are processed relatively rapidly in keratinocytes. The significance of this finding is not known at present. Also, it is not known why the mature β_1 chains are slightly larger in keratinocytes, though the change appears to be posttranslational.

A reexamination of the integrin immunofluorescence micrographs published previously seems to reveal sometimes similar localization of these receptors to epithelial cell-cell boundaries as seen here in keratinocytes (Chen et al., 1985b; Chen et al., 1986a; Duband et al., 1986; Fujimoto and Singer, 1988). Moreover, previous localizations of one of the integrins, VLA-3 or J143 antigen in skin (Fradet et al., 1984; Klein et al., 1987; Wayner et al., 1989) appear similar to that of β_1 shown in Fig. 1, with general expression over the entire plasma membrane of basal cells without polarized accumulation at the interface with the basement membrane substrate. In a study published while this manuscript was under review, Kaufmann et al. (1989) describe direct electron microscopic localization of the VLA-3 molecule at sites of cell-cell contact, especially in tumor cells. They also report a similar strongly increased localization at sites of epithelial-fibroblast adhesion. We did not observe β_1 localization

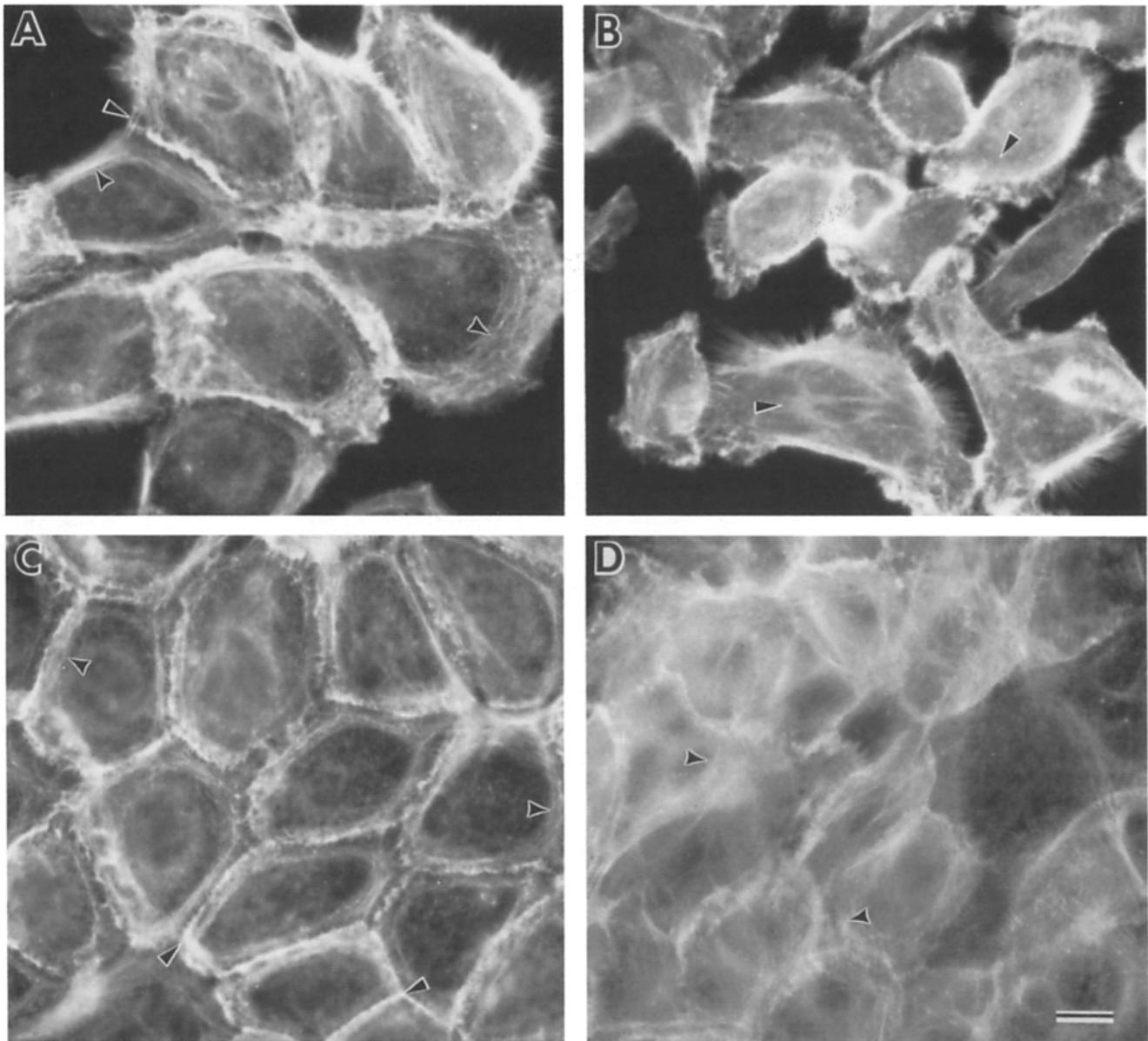


Figure 10. The effect of mAb 13 on the localization of actin in keratinocytes. Cells were cultured in either low calcium (*A* and *B*) or high calcium medium (*C* and *D*). Cells were also either left as controls (*A* and *C*) or treated with 0.5 mg/ml mAb 13 for 24 h (*B* and *D*). F-actin filaments were localized with rhodamine phalloidin. In *A*, the actin filaments in control cells in low calcium form a submembranous network of filaments and bundles (*curved arrowheads*) with some ending at cell-cell contact regions (*straight arrowhead*). After treatment with mAb 13 (*B*), the actin filaments become reorganized, with many regions becoming disorganized (*arrowheads*) or accumulating in peripheral filopodia. In *C*, the actin filaments in control cells in high calcium medium form a submembranous network as observed in low calcium medium but now more strikingly concentrated in a cortical band (*curved arrowheads*) and located immediately adjacent to cell-cell contact sites (*straight arrowheads*). After treatment with mAb 13 (*D*), the actin filaments become disorganized to a more diffuse pattern throughout the cells with discontinuous, disrupted patterns (*curved arrowheads*) and partial disappearance from cell-cell contacts. Bar, 20 μm .

at keratinocyte-fibroblast contacts, but the apparent differences may be due to differing cell types or sensitivities of detection. What, then, is the relationship of integrins to other junctional proteins found in cell-cell contacts of keratinocytes? Cell-cell adhesion molecules, especially on epithelial cells, have been characterized in detail (reviewed by Edelman, 1986; Takeichi, 1988) and include, among others, uvomorulin or L-CAM (E-cadherin), N-CAM, and A-CAM (N-cadherin) (Boller et al., 1985; Geiger et al., 1983, 1985a,b; Volk and Geiger, 1986a,b; Green et al., 1987). Uvomorulin

and A-CAM are extensively studied (Gumbiner et al., 1988; Takeichi, 1988; Volk and Geiger, 1986a,b). Uvomorulin staining is rather diffuse in low calcium conditions, which differs from the integrin pattern of staining that instead concentrates in cell-cell contacts even in low calcium. Staining of uvomorulin and A-CAM, however, closely resembles that of integrins when epithelial cells are switched to high calcium medium (compare with Gumbiner et al., 1988; Volk and Geiger, 1986b).

The dissociating effects of anti- β_1 integrin antibodies also

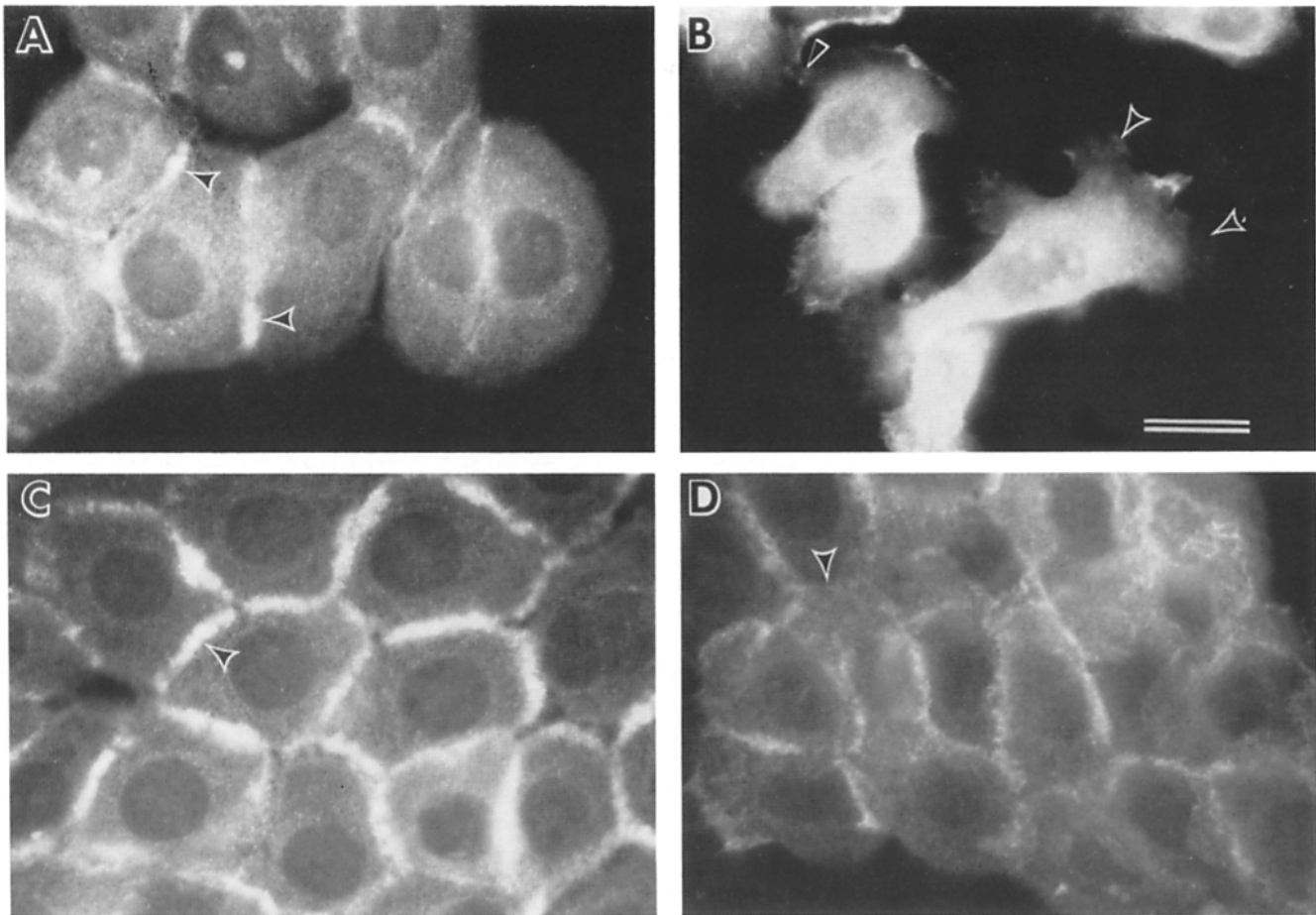


Figure 11. The effect of mAb 13 on the localization of β_1 integrins in keratinocytes. Cells were cultured in either low calcium (*A* and *B*) or high calcium medium (*C* and *D*). Cells were also either left as controls (*A* and *C*) or treated with 0.5 mg/ml mAb 13 for 24 h (*B* and *D*). Integrins were localized with rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the intracellular domain of the β_1 subunit. In untreated cells cultured in low calcium medium (*A*), integrins are localized at cell-cell contact sites (arrowheads). In mAb 13-treated cells (*B*), the distribution of integrins is more diffuse (curved arrowheads), except in regions of membrane ruffles, and they disappear from cell-cell contact sites (arrowhead). In untreated cells cultured in high calcium medium (*C*), integrins are localized at cell-cell contact sites (arrowhead). In mAb 13-treated cells (*D*), the distribution of integrins is more diffuse and is decreased at cell-cell contact sites (arrowhead). Bar, 20 μ m.

resemble those obtained with antibodies against cell CAM 120/80 (L-CAM; Damsky et al., 1981; 1986). Localization in cell-cell contacts are similar, as may be the culture morphology of cells treated with anti-cell CAM 120/80 antibodies (Damsky et al., 1981, 1986). The dimeric integrin receptors are clearly different from the above-mentioned cell-cell adhesion molecules, which are thought to be monomeric cell surface glycoproteins and to have different amino acid sequences. There is, however, some NH₂-terminal similarity in the sequence between cell adhesion molecules and integrins that might indicate some evolutionarily similar origin of these groups of molecules (Takada et al., 1987).

It is interesting to compare our results with recent reports suggesting that VLA-4 ($\alpha_4\beta_1$) plays a role in lymphocyte binding to endothelium and in cytolytic T lymphocyte lysis B lymphocytes (Holzmann et al., 1989; Takada et al., 1989). Both of these interactions involve individual lymphocytes and are heterotypic (i.e., between distinct cell types). The same VLA-4 integrin, however, has also recently been shown to be a cell-substrate adhesion receptor of lymphocytes that binds to a specific site in fibronectin (Wayner et al., 1989);

so its modes of action in heterotypic cell-cell interactions remain unclear.

In conclusion, localization and functional studies of β_1 integrins in keratinocytes suggests their novel role in the formation and maintenance of cell-cell contacts, including actin cytoskeletal organization.

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