Polarized Expression of Integrin Receptors ($\alpha_6\beta_4$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_5$) and their Relationship with the Cytoskeleton and Basement Membrane Matrix in Cultured Human Keratinocytes

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Abstract. In human keratinocytes cultured in conditions which allow differentiation and stratification and are suitable to reconstitute a fully functional epidermis, $\alpha_6\beta_4$ and two members of the β_1 integrin family ($\alpha_2\beta_1$ and $\alpha_3\beta_1$) were respectively polarized to the basal and lateral domains of the plasmamembrane both in growing colonies and in the reconstituted epidermis. Conversely, the α_v integrin subunit, presumably in association with β_5, was expressed at the basal surface in growing and migrating but not in stationary keratinocytes. The integrin $\alpha_6\beta_4$: (*a*) was organized in typical patches which often showed a "leopard skin" pattern where spots corresponded to microfilament-free areas; (*b*) was not associated with focal contacts containing vinculin and talin but rather corresponded to relatively removed contact areas of the basal membrane as shown by interference reflection microscopy; and (*c*) was coherent to patches of laminin secreted and deposited underneath the ventral membrane of individual cells. The two β_1 integrins ($\alpha_2\beta_1$ and $\alpha_3\beta_1$), both endowed with laminin receptor properties, were not associated with focal adhesions under experimental conditions allowing full epidermal maturation but matched the lateral position of vinculin (but not talin), cingulin, and desmoplakin, all makers of intercellular junctions. Often thin strips of laminin were observed in between the lateral aspects of individual basal keratinocytes. The integrin complex $\alpha_v\beta_5$ had a topography similar to that of talin- and vinculin-containing focal adhesions mostly in the peripheral cells of expanding keratinocyte colonies and in coincidence with fibronectin strands. The discrete topography of β_1 and β_4 integrins has a functional role in the maintenance of the state of aggregation of cultured keratinocytes since lateral aggregation was impaired by antibodies to β_1 whereas antibodies to β_4 prevented cell–matrix adhesion (De Luca, M., R. N. Tamura, S. Kajiji, S. Bondanza, P. Rossino, R. Cancedda, P. C. Marchisio, and V. Quaranta. *Proc. Natl. Acad. Sci. USA.* 87:6888–6892). Moreover, the surface polarization of integrins followed attachment and depended both on the presence of Ca^{2+} in the medium and on the integrity of the cytoskeleton. We conclude that our in vitro functional tests and structural data suggest a correlation between the pattern of integrin expression on defined plasmamembrane domains and the mechanism of epidermal assembly.

CELL adhesion is a fundamental process in the organization of multicellular organisms (reviewed by Ekblom et al., 1986; Fleming and Johnson, 1988). The regulation of cell adhesive properties is a complex process that plays a major role in morphogenetic events and in the maintenance of tissue integrity (Edelman, 1986). Recently, major advances in our understanding of the molecular mechanisms of cell adhesion have occurred, with the identification of several intercellular and cell–substratum membrane adhesion molecules (reviewed by Albelda and Buck, 1990; Buck and Horwitz, 1987; Edelman, 1986; Ekblom et al., 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987) and extracellular matrix molecules which may assemble in the basement membrane (Martin and Timpl, 1987; Yurchenko and Schittny, 1990).

The integrins are an important class of transmembrane surface receptors involved in cell–matrix and cell–cell adhesion (Buck and Horwitz,1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). They are heterodimers composed of noncovalently associated α and β subunits. So far, β subunits and at least 11 α subunits have been recognized (Ruoslahti and Giancotti, 1989), primarily on the basis of amino acid sequence homologies (Sheppard et al., 1990; Ramaswamy and Hemler, 1990). The α subunits tend to associate exclu-

sively with one of the β chains, although exceptions to this rule exist (Cheresh et al., 1989; Holzmann and Weissman, 1989; Dedhar and Gray, 1990). Integrins are then divided into subfamilies, according to their β chains (Hynes, 1987). The various integrin heterodimers share many features. For instance, all α chains are homologous to each other, and so are the β chains. Many integrins bind to a common ligand motif, centered around the tripeptide Arg-Gly-Asp (Ruoslahti and Pierschbacher, 1986, 1987). Distinguishing features are the pattern of tissue-specific expression and the spectrum of ligand specificities.

One of the most recently discovered integrins, the complex $\alpha_6\beta_4$ (Sonnenberg et al., 1988; Kajiji et al., 1989; Kennel et al., 1988), has been sequenced (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990) and found to be highly expressed by epithelial cells. Moreover, two members of the β_1 integrin family (i.e., $\alpha_2\beta_1$ and $\alpha_3\beta_1$), have been located to cell–cell contact domains in epidermal cells (Konter et al., 1989; Carter et al., 1990; De Luca et al., 1990*b*; Larjava et al., 1990; Staquet et al., 1990) while $\alpha_6\beta_4$ has been located to their basal domain in a typical pattern which suggested a polarity-dependent organization of integrins and a functional role of $\alpha_6\beta_4$ in the recognition of and in the attachment to the basement membrane both in the native and in vitro–reconstituted epidermis (De Luca et al., 1990*b*). A further epithelial integrin heterodimer, $\alpha_v\beta_5$, has been identified (Cheresh et al., 1989), and the β_5 chain sequenced (Ramaswamy and Hemler, 1990).

In this paper we studied the distribution and, when appropriate reagents were available, the functional role of integrins as a function of the cytoskeleton organization of epithelial cells. To study the role of integrins in the organization of epithelia, we used, as a model system, normal human epidermal keratinocytes cultured in vitro in conditions that allowed full epidermal differentiation (Rheinwald and Green, 1975). This system has been extensively characterized (Green, 1980; Barrandon and Green, 1987*a*,*b*; De Luca et al., 1988) and has many advantageous features for investigating tissue organizing determinants, such as: (*a*) the cultured keratinocyte is a normal cell, i.e., nontransformed; (*b*) it forms epithelial colonies and sheets closely resembling normal human epidermis (Green et al., 1979) and maintains virtually the same differentiation features and gene expression patterns of its in vivo counterpart (Green, 1980) such as to be used as routine grafting for large skin and mucosal defects (Gallico et al., 1984; De Luca et al., 1989, 1990*a*; Romagnoli et al., 1990).

In this paper we report that $\alpha_6\beta_4$: (*a*) has a novel type of organization in the basal plasmamembrane domain of epithelial cells; (*b*) a relationship with the cytoskeleton different from that displayed by any other integrins; and (*c*) a correspondence with organized laminin patches. The organization of $\alpha_6\beta_4$ is different from that shown by $\alpha_v\beta_5$ and $\alpha_2\beta_1/\alpha_3\beta_1$ integrins and suggests that it represents the prototype of a new integrin family provided with a peculiar relationship both with the extracellular matrix and the cytoskeleton. We also propose that three different laminin receptors located to discrete surface domains may interact with their physiological ligand to support both adhesion to the basement membrane and collaborate with other molecules in the maintenance of intercellular bonds.

Materials and Methods

Cell Culture

Human epidermal keratinocytes were cultured according to the methods described by Rheinwald and Green (1975). Briefly, 2-cm^2 skin biopsies from healthy volunteers were minced and trypsinized (0.05%/0.01% EDTA) by gently stirring at 37°C for 3 h. A single cell suspension was collected every 30 min. Cells were then plated (2×10^6/75 cm^2 flask) on feeder layers of lethally irradiated 3T3-J2 mouse fibroblasts (a gift from H. Green, Harvard Medical School, Boston, MA) and cultured in keratinocyte growth medium (KGM) at 37°C in a water-saturated atmosphere of 5% CO_2. KGM composition was: Dulbecco-Vogt Eagle's and Ham's F12 media (3:1 mixture) containing 10% fetal calf serum, glutamine (4 mM), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (20 pM), epidermal growth factor (10 ng/ml; a gift from C. Nascimento, Chiron Corp., Emeryville, CA), penicillin-streptomycin (50 IU/ml). Confluent primary cultures were trypsinized and passaged at a density of 4×10^3 to 1.3×10^4 cells/cm^2. Under these culture conditions, keratinocytes can be serially propagated in vitro for several passages (Green et al., 1979).

In some experiments, adherent keratinocyte colonies were squirted with several jets of buffer from the narrowed tip of a Pasteur pipette before fixation. The aim was to detach some of the cells, expose the underlying matrix, and loosen the bonds among cells.

For control purposes, a line of human keratinocytes was obtained from Clonetics Co., San Diego, CA and cultured according to manufacturer's instructions in growth medium with low Ca^{2+}.

Antibodies

The murine monoclonal antibodies (mAb) S3-41 and AA3 and the polyclonal antiserum 5710 to $\alpha_6\beta_4$ have been described (Kajiji et al., 1989) and generously provided by V. Quaranta, Research Institute of Scripps Clinic, La Jolla, CA. Other murine mAb, with the investigators that kindly provided them, are as follows: TS2/7, to α_1 (Hemler et al., 1983), B-5G10, to α_4 (Hemler et al., 1987*b*) from M. Hemler, Dana Farber Cancer Institute, Boston, MA; P1D6 and P1F8, to α_5 (Wayner et al., 1988) from W. Carter, Hutchinson Cancer Research Center, Seattle, WA; LM142, to α_v, from D. Cheresh, Research Institute of Scripps Clinic; A-1A5, to β_1 (Hemler et al., 1983, 1987*a*), from M. Hemler; 12F1, to α_2, (Pischel et al., 1987) from V. Woods, University of California, San Diego, CA; J143, to α_3 (Fradet et al., 1984), from L. Old, Sloan-Kettering Institute, New York; VIPI-2, to β_3, from W. Knapp, University of Vienna, Austria; CLB-54, to β_2, from R. van Lier, Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands. The rat mAb GOH3 to α_6 (Sonnenberg et al., 1987) was a gift from A. Sonnenberg, Central Laboratory of the Netherlands Red Cross. Of two rabbit antisera to β_3, one was donated by R. Pytela, Department of Medicine, University of California, San Francisco, CA (Pytela et al., 1985), the other was raised in P. C. Marchisio's laboratory (Dejana et al., 1988*b*). Goat antiserum to β_1 has been described (Conforti et al., 1989). An mAb to vinculin was purchased from Bio Makor, Rehovot, Israel (VIN 11-5, cat, No. 6501) and an mAb cross-reacting with human talin (clone 8D4) was obtained from K. Burridge, University of North Carolina at Chapel Hill, Chapel Hill, NC. Rabbit antisera to laminin and to collagen type IV were respectively from Gibco Laboratories, Grand Island, NY (cat. No. 680-3019) and from Heyl GmBH, Berlin, Federal Republic of Germany. In some experiments the laminin antibodies were preabsorbed with an excess of laminin purified from EHS mouse tumor (a kind gift of G. Taraboletti, Istituto Mario Negri Bergamo, Bergamo, Italy). A rabbit antiserum to cingulin (Citi et al., 1988, 1989) was obtained from S. Citi, Columbia University, New York, and an mAb to desmoplakin 1 and 2 (clone DP 2.15, code 695421) was purchased from ICN ImmunoBiologicals, Lisle, IL. Finally, an mAb to cellular fibronectin (IST 9) was kindly provided by L. Zardi, IST, Genova, Italy (Borsi et al., 1987; Carmemolla et al., 1987) and affinity-purified rabbit IgGs to human vitronectin were given by K. Preissner,Max Planck Institute for Thrombosis Research, Giessen, FRG (Preissner et al., 1985).

Immunostaining

Keratinocytes from confluent primary cultures (1.3×10^4 cells/cm^2) were plated onto 24-well Costar plates containing 1.1-cm^2 round glass coverslips

which had been previously coated either with a feeder layer or the indicated substrate (10 μg/ml). After indicated time, coverslip-attached keratinocytes were fixed in 3% formaldehyde (from paraformaldehyde) in PBS pH 7.6 containing 2% sucrose for 5 min at room temperature. After rinsing in PBS, cells were permeabilized by soaking coverslips for 3–5 min at 0°C in Hepes-Triton-X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100). This procedure of fixation and permeabilization permits immunostaining of cytoskeletal and adhesion components (for example, see Marchisio et al., 1984; Dejana et al., 1988*a*, *b*). Indirect single-label immunofluorescence experiments were performed as reported (Marchisio et al., 1984). Briefly, the primary antibody (usually at an Ig concentration of 10–30 μg/ml) was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS-0.2% BSA, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (DAKOPATTS, Copenhagen, Denmark) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (F-PHD; Sigma Chemical Co., St. Louis, MO). Indirect double-label immunofluorescence experiments were performed essentially as reported (Dejana et al., 1988*b*). Coverslips were mounted either in Mowiol 4-88 (Hoechst AG, Frankfurt/Main, FRG) or in 50% glycerol-PBS. Routine observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence, plan-apochromatic lenses or Antiflex 63× lens for interference reflection microscopy (IRM). Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in T-Max Developer for 10 min at 20°C. In some experiments a laser confocal fluorescence imaging system (Lasersharp MRC-500 was used to evaluate the position of integrins in the thickness of keratinocyte colonies.

Cell Radiolabeling, Lysis, and Immunoprecipitation

These experiments were carried out as described (Kajiji et al., 1989). Briefly, cells were metabolically radiolabeled and detergent extracts immunoprecipitated with LM142 mAb to α_v or with a rabbit polyclonal anti-vitronectin receptor antibody (both antibodies were a gift of D. Cheresh). Immunoprecipitates were washed and then eluted in sample buffer (Laemmli, 1970) at 100°C with or without reduction/alkylation by 10 mM dithiothreitol and 50 mM iodoacetamide and electrophoresed on SDS-PAGE (Laemmli, 1970), followed by fluorography (Laskey and Mills, 1975) on x-ray film.

Adhesion and Cohesion Assays

Costar plates (96 wells) were coated for 1 hr at 37°C with laminin (10 μg/ml in PBS) or Matrigel (either 2 μg/ml in DMEM, a gift of A. Albini, IST, Genova, Italy). Keratinocytes obtained from confluent cultures (30,000 cells/well) were plated in KGM without serum and EGF in the presence of indicated antisera or control sera from corresponding species (1:100 dilutions) and then incubated for 12 h at 37°C. Controls of cell viability during cohesion assays were routinely carried out. Cohesion assays were repeated a minimum of six times with consistent results. The cells which had adhered after exposure to the β_1 antiserum (almost no cells adhered after treatment with the β_4 antiserum; De Luca et al., 1990*b*) were fixed and stained with R-PHD as described above to evaluate their morphology.

Results

Integrin Topography in Cultured Human Keratinocytes

We previously showed both by serological and biochemical means that normal human keratinocytes express the integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ (De Luca et al., 1990*b*). Here we show that normal human keratinocytes express α_v in association with β_5 or a β_5-like molecule by SDS-PAGE mobility as demonstrated by immunoprecipitation with anti-α_v antibodies (Fig. 1). That the β chain associated with α_v is not β_3 is indicated by the lack of reactivity of normal human keratinocytes with anti-β_3 antibodies (Fig. 1) and, on the basis of

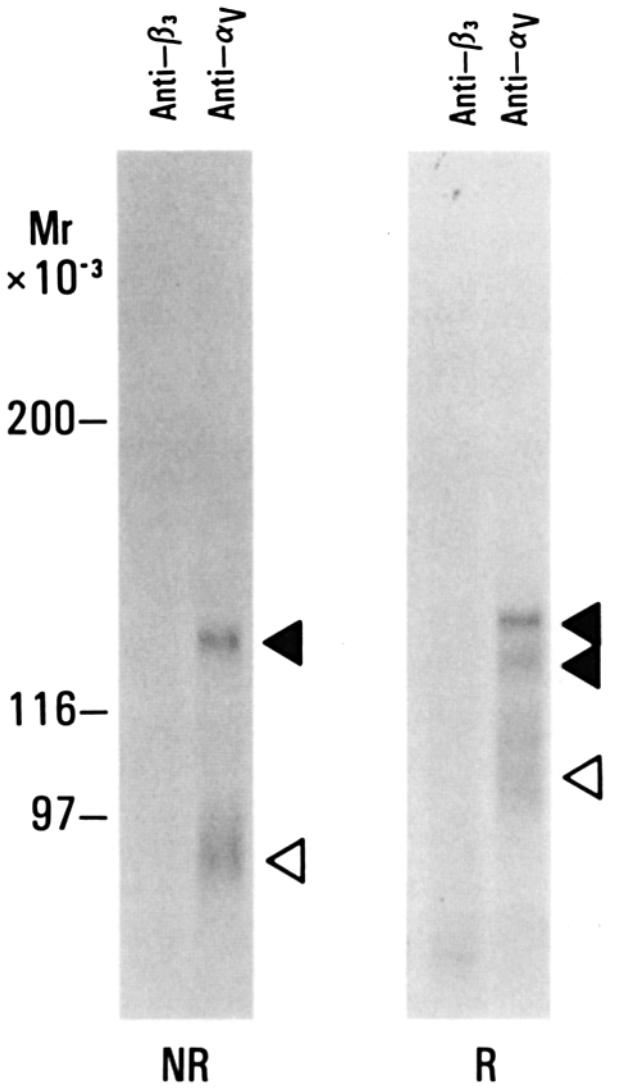

Figure 1. Immunoprecipitates from detergent lysates of metabolically radiolabeled keratinocytes with antibodies to β_3 or to α_v integrin chains (see Materials and Methods). The eluates were analyzed by SDS-PAGE under nonreducing (*NR*) or reducing (*R*) conditions, followed by autoradiography. No specific bands are detectable in the anti-β_3 lanes. In the anti-α_v lanes, the open arrowheads point at bands consistent with the mobility of a β_5 subunit, while the closed arrowheads indicate the positions of α_v bands. The higher α_v band in the reducing lane is often observed and likely represents a higher molecular mass precursor of the mature α_v chain.

mobility properties, the β chain does not correspond to any of the alternative β chains reported to associate with α_v (Holzmann and Weissman, 1989; Dedhar and Gray, 1990). Moreover, no α chain with the mobility properties of α_v could be immunoprecipitated using β_1 antibodies thus excluding the occurrence of the newly reported complex $\alpha_v\beta_1$ (Bodary and McLean, 1990; Vogel et al., 1990).

Here the fine topography of integrins was investigated in its relationship with cytoskeletal components in the same cells by using indirect immunofluorescence. First of all, no above control immunofluorescence signal was detected with

1. *Abbreviations used in this paper*: CD, cytochalasin D; F-PHD, fluorescein-labeled phalloidin; IRM, interference reflection microscopy; R-PHD, rhodamine-labeled phalloidin.

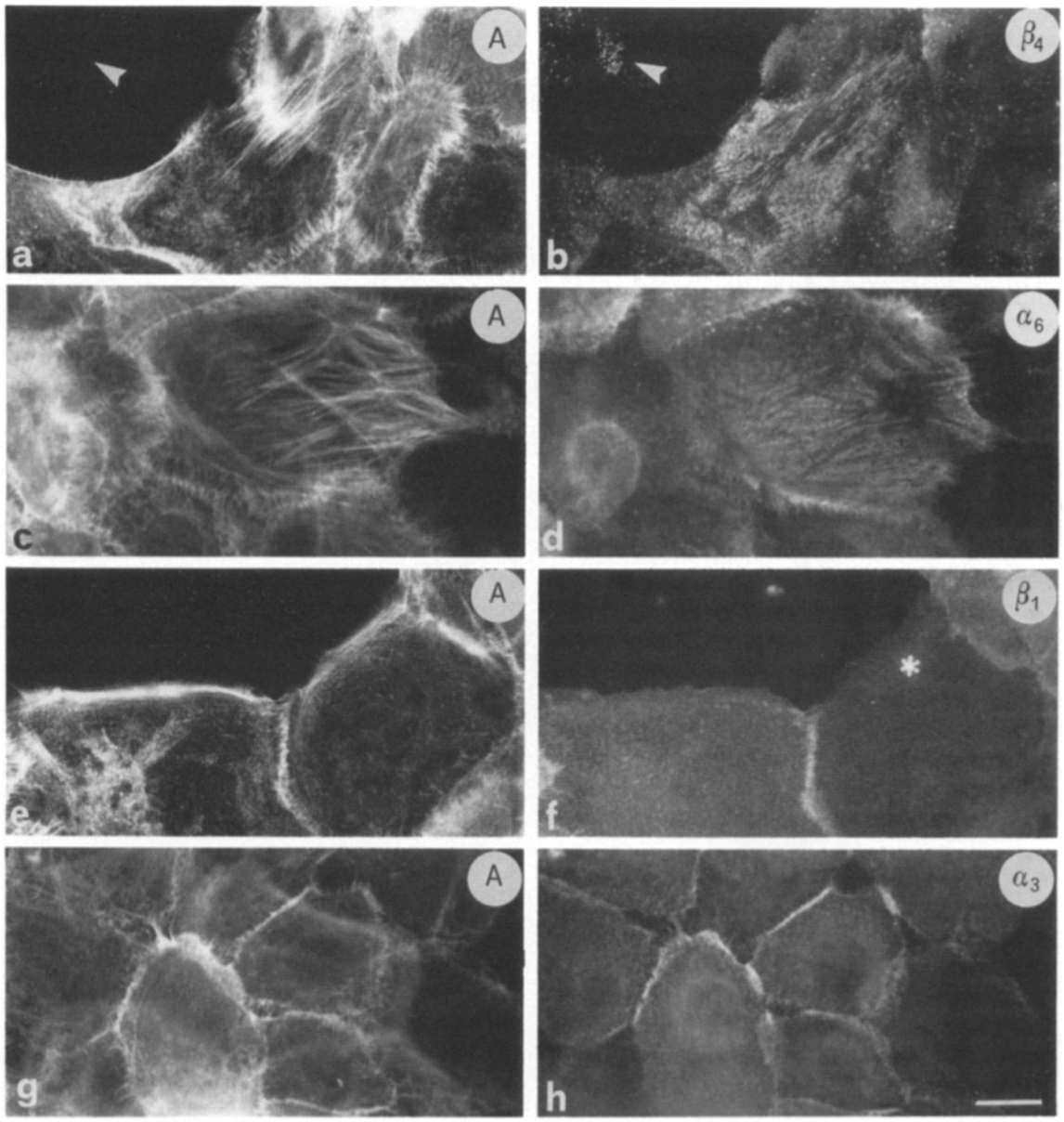

Figure 2. Distribution of β_4 (*b*), α_6 (*d*), β_1 (*f*), and α_3 (*h*) integrins in human keratinocyte colonies grown for 3 d on glass coverslips in complete medium without feeder layer. The corresponding staining for F-actin (*A*) is shown in *a*, *c*, *e*, and *g*. The cells were immunofluorescently stained with mouse mAbs and costained with fluorescein-labeled phalloidin (F-PHD). The patterns of β_4 and α_6 staining of the ventral membrane (*b* and *d*) are very similar insofar the complex is enriched in F-actin microfilament-poor areas (see also Fig. 3). Staining is also present in footprints left by cells which had been mechanically detached during the staining procedures (a and b, see arrowhead; see also Fig. 8). This suggests that the $\alpha_6\beta_4$ complex is exposed in the ventral membrane in contact with the substrate. Staining for β_1 (*f*) and α_3 (*h*, α_2 is identical to α_3) indicates that there is just a hazy staining for the β_1 complex in the ventral membrane (*f*, see *asterisk*) while fluorescence is enriched at intercellular contact rims. No F-actin staining was apparent in substrate-attached material. Bar, 5 μm.

α_1, α_4, or β_3 antibodies (not shown). Distinct localization patterns were observed with anti-β_4 compared to anti-β_1 antibodies. Both mAb S3-41 or AA3 and the rabbit serum 5710 to $\alpha_6\beta_4$ (Kajiji et al., 1989) stained keratinocytes on the basal surface with a pattern of polymorphous patches, somewhat granular in appearance (Fig. 2 *b*) and often suggesting a leopard skin pattern (Figs. 3 and 4). Cell footprints, remaining on the substratum from cells detached during fixation (Fig. 2 *b, arrowhead*; see also Fig. 8), similarly displayed granular fluorescence with β_4 antibodies. An identical pattern of reactivity (Fig. 2 *d*) was found with an mAb reactive with α_6; codistribution of α_6 and β_4 in basal patches was confirmed also in double-label immunofluorescence (Fig. 3, *a* and *b*). Almost no $\alpha_6\beta_4$ immunoreactivity was detected in the contour of the cells, in the apical zones, or in association with talin at focal adhesions (Fig. 3, *c* and *d*). Such pattern of integrin distribution was virtually identical in confluent keratinocyte colonies.

A peculiar feature of $\alpha_6\beta_4$ distribution was that it was virtually absent from areas containing submembranous bundles of F-actin microfilaments suggesting a complementary distribution of the integrin complex and the submembran-

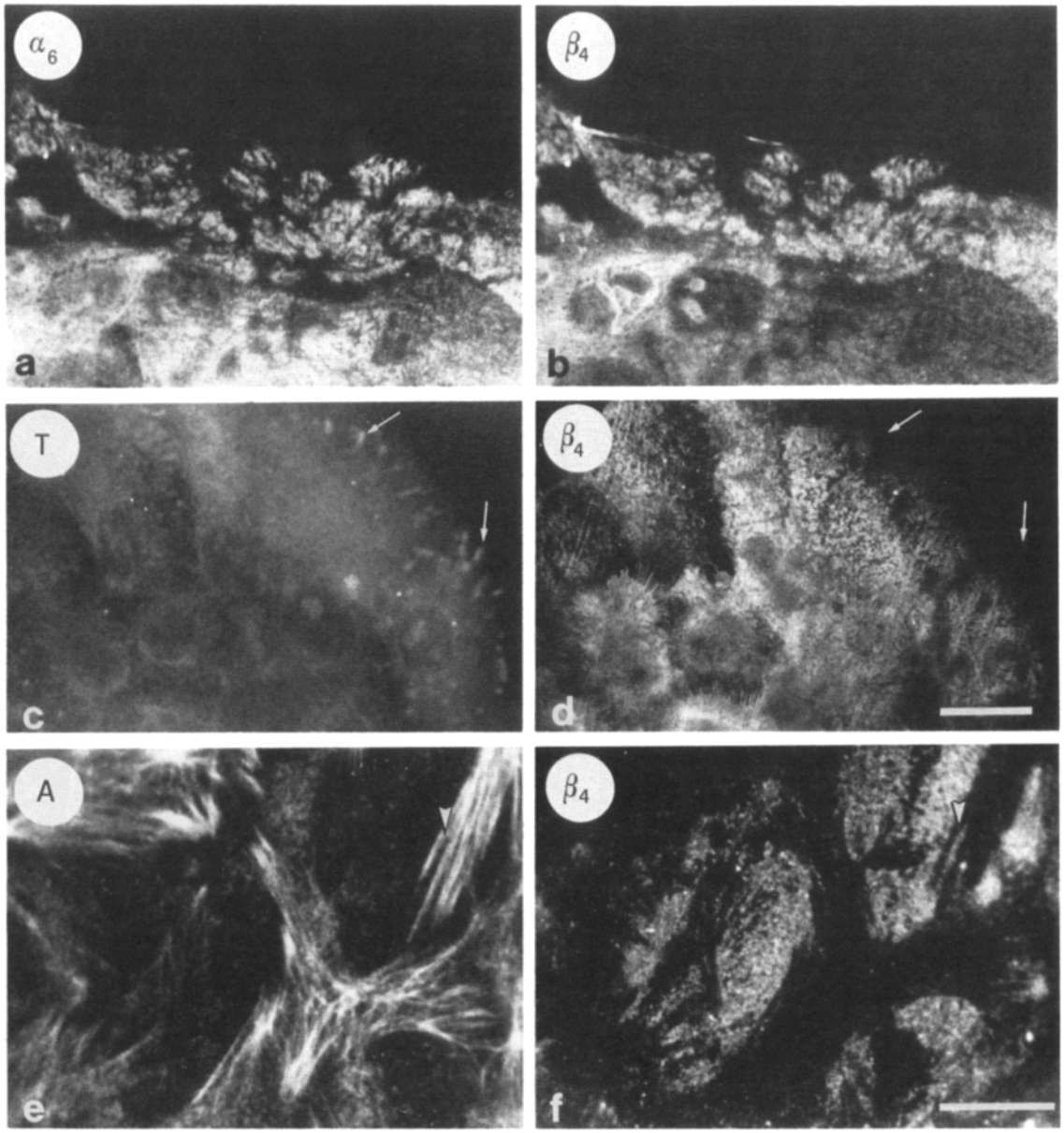

Figure 3. Double-label indirect immunofluorescence staining of human keratinocyte colonies grown for 3 d on glass coverslips in complete medium without feeder layer. Virtual codistribution of α_6 (*a*) and β_4 (*b*) indicates that immunostaining with the corresponding antibodies (rabbit Igs to the cytoplasmic domain of α_6 and mAb S3-41 to β_4) shows the basal location of the $\alpha_6\beta_4$ heterodimer in rather typical patches. No obviously similar pattern was observed by coimmunostaining for talin (*c*, rabbit antiserum to human platelet talin; *T*) and mAb S3-41 to β_4 (*d*): arrows indicate the position of some peripheral talin-positive focal adhesions (*c*) located in β_4-free areas (*d*) of the ventral attachment area. The exclusion of the $\alpha_6\beta_4$ complex from areas showing bundles of microfilaments (staining with F-PHD for F-actin; *A* is shown in *e* and *f*: almost negative imaging appears from the comparison of the two frames (e.g., at arrowheads). Bar, 5 μm.

ous microfilament meshwork (compare the paired pictures in Fig. 2*a* and *b*, *c* and *d*, and Fig. 3, *e* and *f*). When observed in interference reflection microscopy (IRM), an optical system which shows the distance between the attachment surface and the ventral membrane of cultured cells (for example, see Izzard and Lochner, 1976), $\alpha_6\beta_4$ patches never corresponded to focal adhesions but rather to areas slightly more removed from substrate than those corresponding to microfilamentous strands which, instead, were more closely apposed to the substrate (Fig. 4). Cell types other than keratinocytes, i.e., occasional human fibroblasts, dendritic cells (probably melanocytes), and the 3T3-J2 cells of the feeder layer, were consistently negative for $\alpha_6\beta_4$.

The anti-β_1, as well as the anti-α_2 and anti-α_3 mAbs predominantly stained those areas of plasmamembrane involved in cell-to-cell contact in growing colonies (Fig. 2, *e–h*) as well as in the reconstituted epidermis. Little $\alpha_{2/3}\beta_1$ staining was seen in the basal surface areas found reactive with β_4 antibodies. Similarly, cell footprints that contained $\alpha_6\beta_4$ did not contain β_1 (not shown). In our hands, no specific staining was observed with α_5 mAbs in keratinocytes but, in the same cultures, occasional human fibroblast-like cells displayed elongated streaks along stress fibers, indicating that the antibody could indeed recognize $\alpha_5\beta_1$ at focal contacts (not shown).

Further evidence for the segregation of β_1 and β_4 inte-

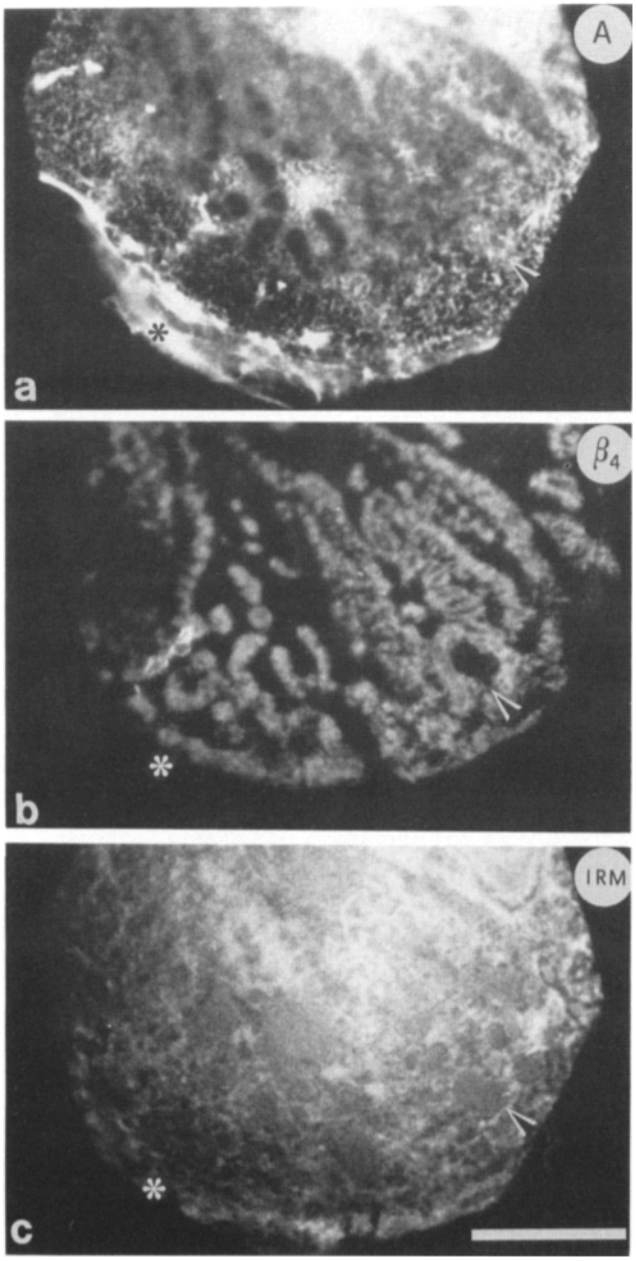

Figure 4. High power picture of the fluorescence staining for F-actin (*a*), $\alpha_6\beta_4$ (*b*), and the corresponding IRM pattern (*c*) of a large keratinocyte located at the periphery of a growing colony. Irregular patches of $\alpha_6\beta_4$ show a preferential location in F-actin poor areas and, conversely, $\alpha_6\beta_4$ is excluded from areas where microfilaments are abundant (e.g., in the areas indicated by the asterisks and by the arrowhead). The F-actin-rich and $\alpha_6\beta_4$-free areas of the ventral membrane are darker in IRM indicating a closer proximity with the substrate (see arrowhead) than those corresponding to $\alpha_6\beta_4$ patches. IRM black focal contact streaks are found only at the edges of the cells and never correspond to $\alpha_6\beta_4$ patches (see also Fig. 3). In general, the IRM pattern of cultured keratinocytes is completely different from that of a fibroblast-like cell (for review see Burridge et al., 1988). Bar, 5 μm.

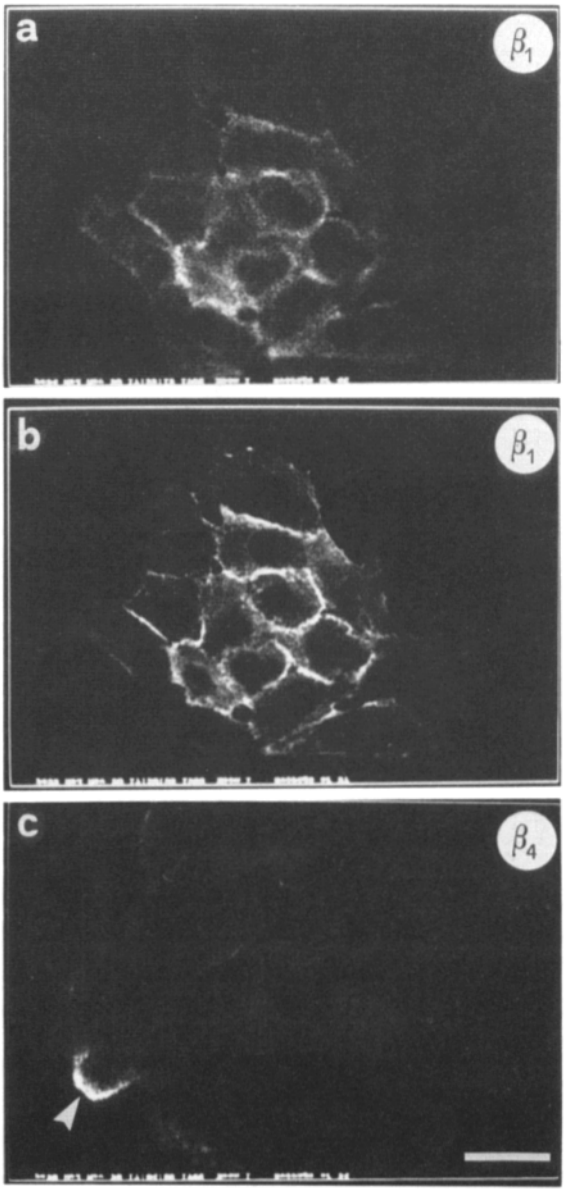

Figure 5. Confocal sectioning images obtained with a Lasersharp MRC-500 fluorescence imaging system of human keratinocyte colonies grown for 6 d on feeder layer and stained for β_1 (*a* and *b*) and β_4 (*C*). Optical sectioning was adjusted 1.5 (*b* and *c*) and 3 μm (*a*) above the plane of adhesion. The upper two pictures, obtained from the same colony, show two digitally reconstructed optical sections showing that β_1 integrins are exposed on the lateral surface at two levels 1.5 apart while β_4 is virtually absent 1.5 μm above the adhesion plane (*c*). The arrow in *c* indicates a cell at the periphery of the colony that shows positivity to $\alpha_6\beta_4$ with a slab being somewhat raised from the substrate. Bar, 10 μm.

grins in keratinocyte colonies was obtained by laser scanning confocal microscopy. Coverslips immunostained with either mAb A-1A5 for β_1 or S3-41 for β_4 were scanned on consecutive planes along the vertical axis and digitally reconstructed. Anti-β_1 immunoreactivity could be followed a few micrometers upward from the plane of attachment within colonies, indicating that β_1 integrins are enriched on lateral surfaces (Fig. 5, *a* and *b*). Anti-β_4 reactivity, instead, was absent in equivalent planes of focus removed from the attachment surface, i.e., starting from 1.5 μm above the substratum (Fig. 5 *c*).

The different topography of β_1 and β_4 integrins was consistently observed in growing and stationary keratinocytes

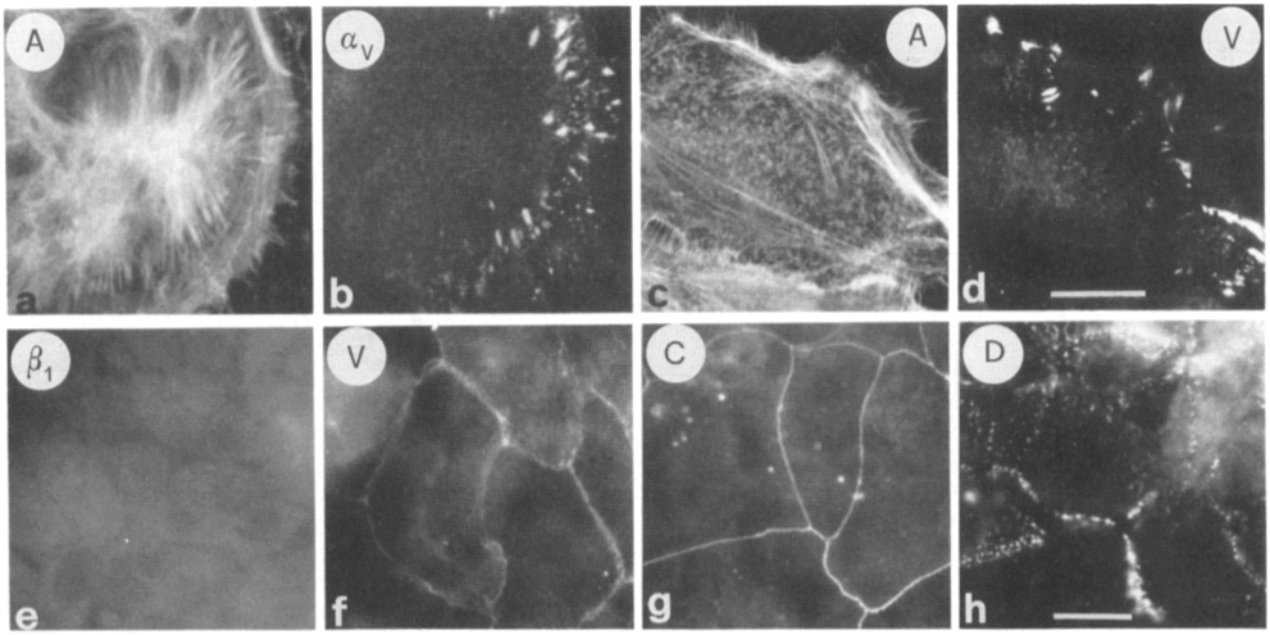

Figure 6. Distribution of α_v (*b*) and vinculin (*d*) in human keratinocyte colonies grown for 3 d on glass coverslips in complete medium without feeder layer. The corresponding staining for F-actin is shown in *a* and *c* (*A*). The localization of talin is not shown and is identical to that of vinculin (see also Fig. 3 *c*). The keratinocytes that had migrated outward (*e–h*) are devoid of integrins (e.g., β_1) associated to recognizable cellular structures (*e*) and show vinculin (*f*), cingulin (*g*), and desmoplakins in tiny dots (*h*) at cell-to-cell boundaries. No talin was found in cells of the upper layers. Bars, 5 μm.

whether they were initially seeded on fibroblast feeder layers or on artificial substrates like laminin, fibronectin, or vitronectin, suggesting that the sorting of β_1 and β_4 integrins to discrete domains of epidermal cells was an intrinsic property of adherent keratinocytes (see also below).

The localization of the integrin chain α_v was then studied using the mAb LM142. We assumed that the localization of α_v corresponded to that of the heterodimer $\alpha_v\beta_5$ in view of the reported absence of β_3 or other β chains from epithelial cells (Cheresh et al., 1989; De Luca et al., 1990*b*). It was found that α_v was located to tiny IRM dark streaks mostly at the periphery of the basal aspect of expanding keratinocytes and in association with the endings of short microfilament bundles (Fig. 6, *a* and *b*). A similar location was shown by the cytoskeletal proteins vinculin (Fig. 6, *c* and *d*) and talin (see Fig. 3 *c*). On the basis of the similar location of α_v, vinculin and talin as the endings of microfilament bundles, we suggest that α_v, most probably in association with β_5, is the epithelial integrin chain that interacts with both vinculin and talin (and, hence, with the microfilamentous cytoskeleton) and forms minute focal contacts in basal keratinocytes. Moreover, the discrete localization of vinculin and talin to the peripheral focal contacts, that are also α_v positive, highlights the absence of such cytoskeletal molecules from areas containing $\alpha_6\beta_4$ (see Fig. 3, *c* and *d*). Data awaits to be confirmed by the colocalization of α_v and β_5 when appropriate immune reagents for β_5 are available.

We then looked at the cells that had migrated outward and formed the upper layers of the colonies. We found that no integrins were associated with obvious cellular structures (e.g., β_1, Fig. 6 *e*) but gained evidence that junctions of different types had formed and that proteins like vinculin (a marker of adhesion junctions, Geiger et al., 1985), cingulin (a marker of tight junctions, Citi et al., 1988, 1989), and desmoplakins 1 and 2 (markers for desmosomes, Müller and Franke, 1982) lined the boundaries among the tile-shaped cells of upper layers (Fig. 5, *e–h*). No talin immunoreactivity was observed in keratinocytes belonging to the upper epidermal layers (not shown) in line with the reported absence of talin from adhesion junctions of epithelial cells (reviewed by Burridge et al., 1986, 1988).

In summary, these data show that $\alpha_6\beta_4$ and $\alpha_v\beta_5$ integrins are located to different adhesion structures of the basal membrane and two β_1 integrins are enriched in the lateral membrane of keratinocytes, in a rather mutually exclusive distribution and that the interaction of these different integrins with the keratinocyte cytoskeleton is different.

Distribution of Matrix Proteins in Cultured Keratinocytes

Deposition and organization of fibronectin, collagen type IV, vitronectin, and laminin were studied with specific antibodies in human keratinocytes cultured with or without feeder layer of 3T3-J2 cells. It was found that fibronectin was organized around the peripheral keratinocytes of exponentially growing colonies and by feeder cells (Fig. 7) and formed a circumferential meshwork around each colony. Fibronectin was missing from underneath both growing and confluent keratinocytes (Fig. 7).

Laminin was deposited under both expanding and confluent keratinocytes and was also intensely secreted by feeder cells and by occasional dendritic cells that could represent melanocytes. Double-label immunofluorescence staining was performed on cells that had been lightly squirted by a stream of buffer before fixation to optimize antibody access. Under these conditions $\alpha_6\beta_4$ and laminin ap-

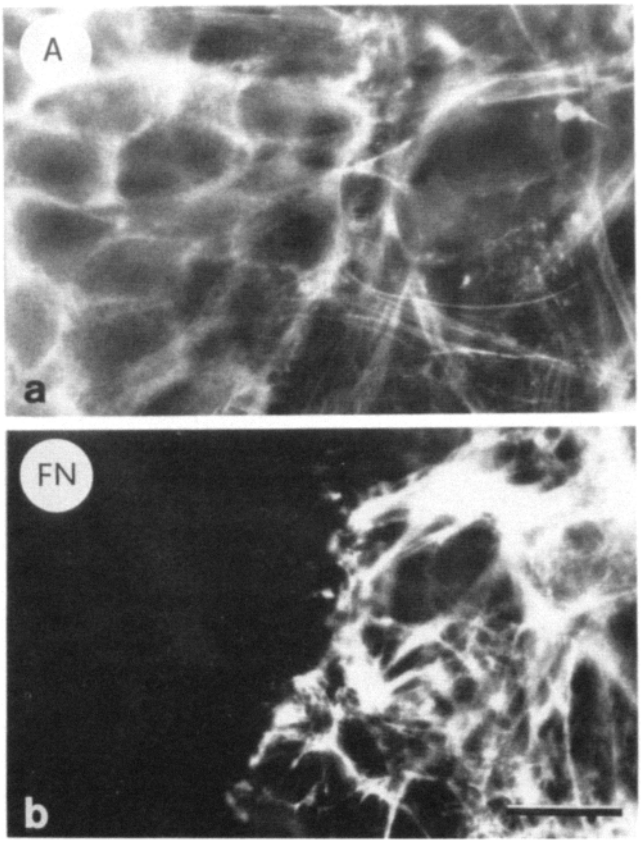

Figure 7. Distribution of fibronectin (*b*) and F-actin (*a*) in keratinocyte colonies cultured in the presence of fibroblast feeder layer. A fibronectin network (*b, right*) lines the border of a keratinocyte colony (*a, left*) costained for F-actin (*A*) with F-PHD. No fibronectin immunoreactivity (determined by the cellular fibronectin-specific mAb IST-9) was found underneath keratinocytes. Bar, 5 μm.

peared to codistribute with a similar leopard skin pattern (Fig. 8, *a* and *b*). The same pattern was found in cell footprints left by removed cells (Fig. 8, *c* and *d*). Absorption of the laminin antibodies with Engelbreth-Holm-Swarm tumor laminin abolished laminin staining (not shown). Under the same experimental conditions (i.e., when cells had been squirted with a jet of buffer and presumably slightly dissociated to allow access to antibodies), laminin was occasionally enriched in thin discrete strips corresponding to cell–cell boundaries (Fig. 3, *e* and *f*). The latter data awaits support by immunocytochemistry at the electron microscope level.

Collagen type IV was distributed in a rather homogeneous meshwork (not shown) and vitronectin was negative except for a faint background signal presumably due to the presence of serum in the culture medium (not shown).

In summary, a meshwork of fibronectin was found in apposition with the peripheral row of keratinocytes in exponentially growing colonies but was missing from confluent colonies. Conversely, laminin and collagen type IV were found underneath keratinocytes that actively synthesized, secreted, and organized these basement membrane proteins. Moreover, the laminin organized by cultured keratinocytes displayed a deposition pattern similar to that of integrin $\alpha_6\beta_4$ on the basal membrane of cells and was also occasionally found between cells.

Effects of Substrates, Ca^{2+} Deprivation and of Cytoskeleton-disrupting Drugs on Integrin Distribution in Cultured Keratinocytes

We seeded keratinocytes on substrates composed of purified matrix proteins like laminin, fibronectin, or vitronectin (components either of the basement membrane or of the provisional matrix of regenerating epidermis) or on Matrigel which represents an artificial basement membrane and is composed of laminin, collagen type IV, nidogen, and heparan sulfate proteoglycan (Kleinman et al., 1983). The aim was to detect possible fine differences in integrin distribution as a function of different substrates of attachment. No obvious difference was shown in β_1, β_4, and $\alpha_v\beta_5$ patterns at times ranging from 12 to 72 h, indicating that the typical distribution of these integrins did not depend on the initial recognition of a well-defined substrate but rather on the interaction with the matrix that keratinocytes themselves autonomously produced and organized.

Keratinocyte stratification into epidermis is known to depend on the presence of Ca^{2+} in the culture medium (Watt and Green, 1982; Watt, 1984; Magee et al., 1987). In the absence of added Ca^{2+} or with low Ca^{2+}, keratinocytes failed to form colonies and to differentiate in cells that soon moved to outward layers. We found that the sorting out of β_1 and β_4 integrins to lateral and basal domains, respectively, was impaired by Ca^{2+} deficiency but rather integrins remained diffusely distributed on the cell surface (Fig. 9, *b* and *d*). A very similar pattern was shown by a secondary line of keratinocytes (Clonetics Co., San Diego, CA) that grow with low Ca^{2+} and are often used as an in vitro model for keratinocyte differentiation (Fig. 9, *e–h*). It must be noted that, without Ca^{2+}, a fair amount of β_4, but not β_1, cross-reactive material is shed and found attached to the substratum (e.g., Fig. 9, *b* and *f*).

We then tested the effects of the microfilament-disrupting drug cytochalasin D (CD; Carter, 1967) and of colcemid, a microtubule-depolymerizing drug, on the integrin pattern of keratinocytes both during the spreading process and on established colonies. Attachment was fully inhibited by colcemid (1 μg/ml), supporting the concept that functional microtubules are required for cell adhesion (Osborn and Weber, 1976) and epithelial polarization (Eilers et al., 1989).

In contrast, CD treatment (2 μg/ml) allowed attachment and spreading of keratinocytes but prevented colony formation in a way similar to that produced by exposure to β_1 antibodies (see below). The pattern of β_4 distribution at the basal aspect of cells was not coarsely altered (Fig. 9 *j*) but rather followed the typical rearrangement of microfilament organization induced by CD treatment (Weber et al., 1976). Instead, CD prevented the sorting of β_1 integrins to the lateral membrane. The β_1 integrins were in part diffuse on the whole membrane without being enriched at lateral aspects or retained within cells (Fig. 9 *l*). No major changes in β_1 and β_4 distribution were produced by either drugs in established keratinocyte cultures.

The above experiments suggest that the sorting of integrins to be appropriate membrane domains require the presence of Ca^{2+} and a functional cytoskeleton, conditions that are both prerequisites for epidermal maturation.

Effects of Antibodies to β_1 on Cohesion of Keratinocytes In Vitro

The experiments were performed with human keratinocytes

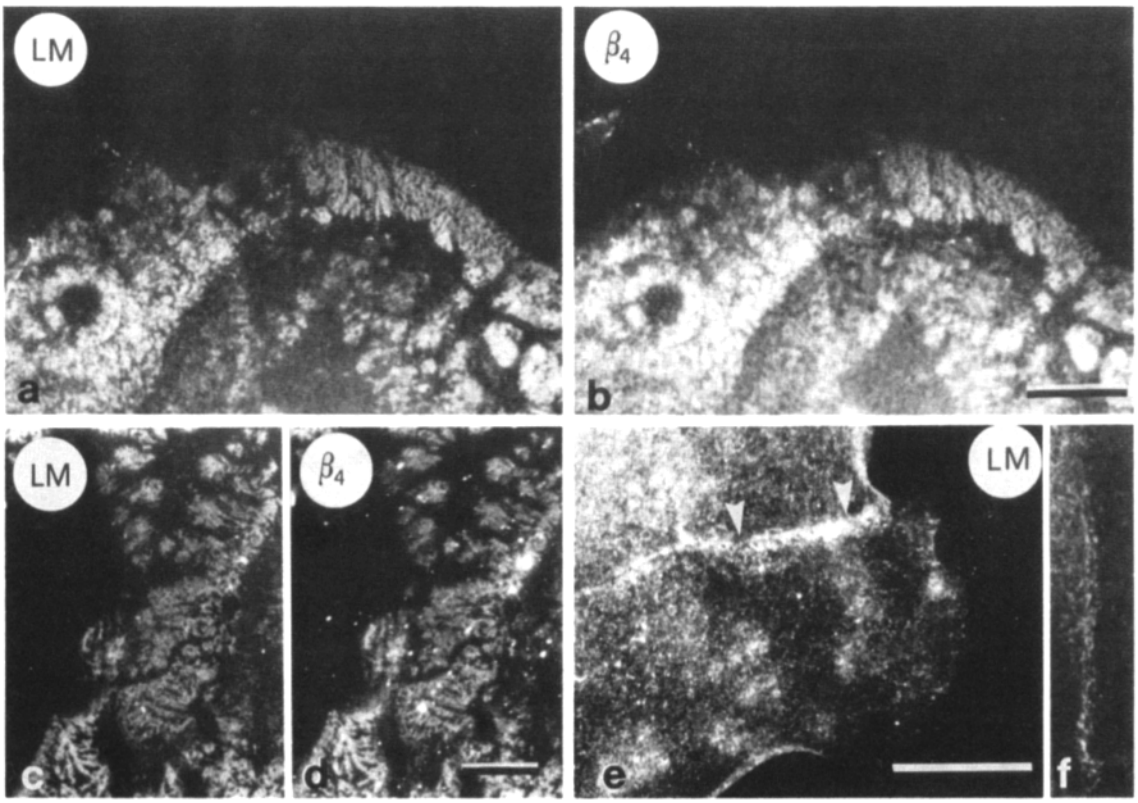

Figure 8. Double-label immunofluorescence costaining of laminin (*a* and *c*) and β_4 (*b* and *d*) in keratinocyte colonies that had been lightly squirted by a jet of buffer from a Pasteur pipette before fixation. Laminin (*LM*, detected by rabbit Igs to Englebreth-Holm-Swarm mouse tumor laminin) and β_4 (detected by mAb S3-41) are codistributed underneath keratinocyte colonies (*a* and *b*). Codistributed patches of β_4 and laminin are also seen where cells have been detached and have left their footprints (*c* and *d*). In a few residual cells that had been squirted, intercellular boundaries are intensely positive for laminin (*e*, *arrowheads*). The inset (*f*) shows a detail of the appearance of laminin at intercellular rims. Bars, 5 μm.

isolated and resuspended from cultures that had reached confluency for at least 24 h. We reported that the anti-β_1 serum had negligible effect on adhesion of cells while the anti-β_4 serum inhibited adhesion by >90% (De Luca et al., 1990). By observing the morphology of keratinocytes seeded in the presence of anti-β_1 serum, we found that colony organization was severely impaired and individual cells were less spread and almost devoid of cell-to-cell contacts (Fig. 10 *a*) while they formed regular colonies with goat preimmune serum (Fig. 10 *b*). This experiment suggests that β_1 integrins are required for keratinocyte aggregation, a condition required for in vitro epidermal maturation.

Discussion

The maturation of epidermis is a complex process which has been reproduced in vitro to obtain epidermal sheets that are used for repair of skin defects including severe burns (Green et al., 1979; Gallico et al., 1984; De Luca et al., 1989). Normal epidermal differentiation and the healing of skin wounds require cell proliferation and lateral migration (Barrandon and Green, 1987*b*) followed by outward positioning of differentiated keratinocytes that end up with the formation of a multilayered squamous epithelium. In this paper we present evidence that this complex phenomenon requires the polarized distribution of at least two integrin subfamilies that are respectively involved in the formation of bonds between cells of the basal layer with the basement membrane and in stabilizing cell-to-cell lateral recognition. To our knowledge this represents evidence for a topographically defined positioning of integrin adhesion receptors in a single cell type.

The newly described integrin $\alpha_6\beta_4$ plays a leading role in this process because (*a*) specific antibodies prevent the adhesion of keratinocytes (De Luca et al., 1990*b*) and (*b*) because $\alpha_6\beta_4$ is specifically restricted to the basal domain of keratinocytes both in vivo and in vitro and thus comes in tight contact with the basement membrane. The integrin $\alpha_6\beta_4$ is an integrin molecule that is amazingly abundant in the basal domain of most epithelial cells such as it may be considered as the natural candidate receptor for the epithelial basement membrane. Although the actual ligand of the extracellular domain of $\alpha_6\beta_4$ has not yet been biochemically identified (Sonnenberg et al., 1990), it is likely that it may be laminin itself or an association of laminin with another matrix component and, even if coherent distribution may not be considered as direct evidence, our data support this possibility. However, there is no codistribution of $\alpha_6\beta_4$ with focal contacts. Focal contacts, indeed, are hardly noted in confluent keratinocytes and mostly restricted to the peripheral cells of growing colonies. By using the IRM technique we found that the basal membrane of keratinocytes adhered via scattered spots of "close contacts" (Izzard and Lochner, 1976) alternating with spots slightly more removed from the adhesion substrate on the basis of their lighter IRM signal. The latter

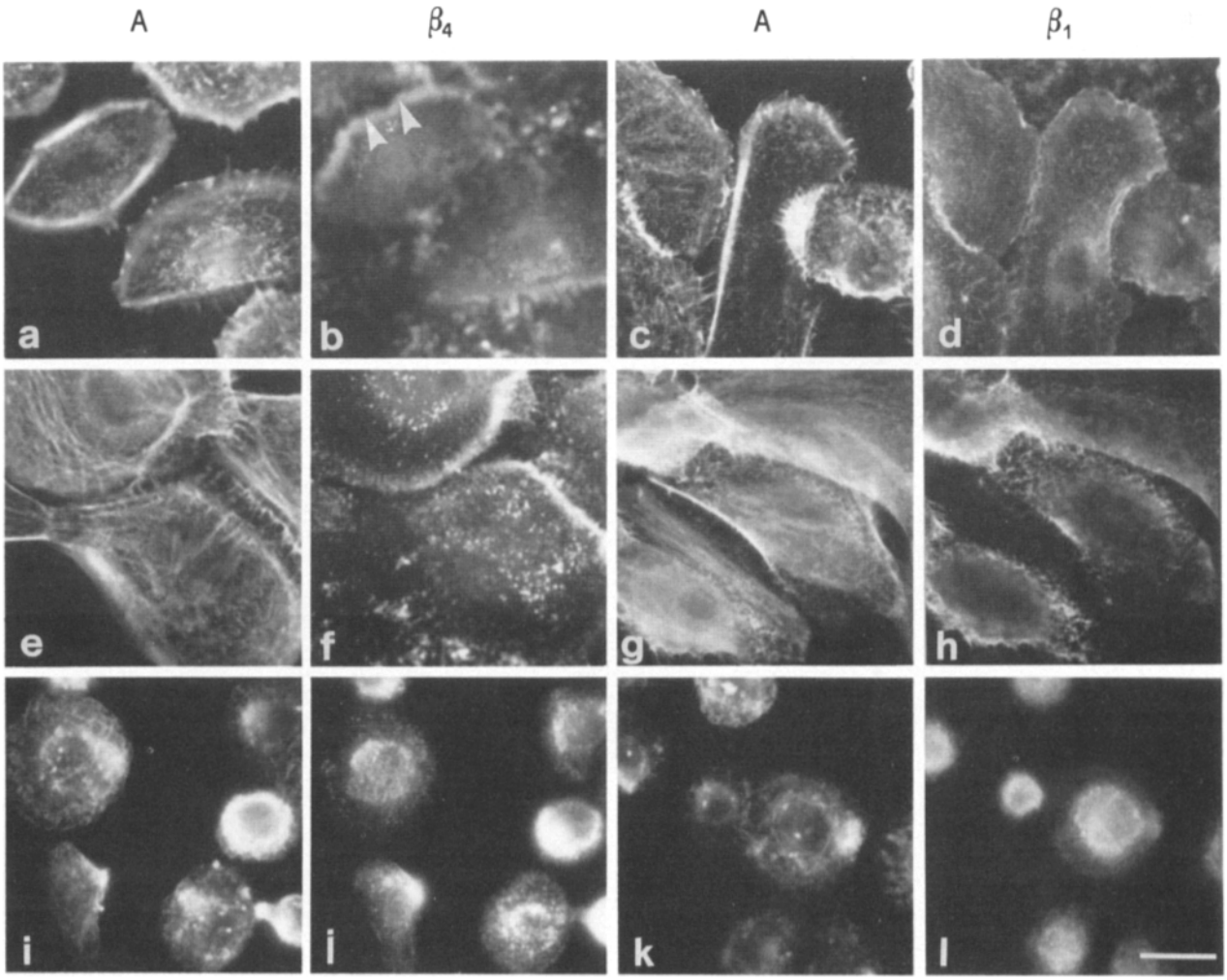

Figure 9. Distribution of β_4 (*b* and *f*) and β_1 integrins (*d* and *h*) in human keratinocytes in primary culture (*a–d*) or in secondary culture (human keratinocytes from Clonetics, *e–h*) grown for 3 d in complete medium without feeder layer and in the absence of Ca^{2+}. The corresponding staining for F-actin (*A*) is shown in *a*, *c*, *e*, and *g*. In both cell types, grown in the absence of Ca^{2+}, integrins are distributed all over the surface; in particular, β_4 (*b* and *f*) seems to be enriched at lateral borders simply because it is found also laterally. *b* was photographed slightly out of focus to maximize such information. The rounded cells (*i–l*) are primary keratinocytes exposed to cytochalasin D and immunostained for β_4 (*j*) or β_1 (*l*) and costained for F-actin (*i* and *k*). The distribution of β_4 at the attachment surface is in tiny dots and streaks that follow the rearrangement of F-actin induced by CD (*i*) while β_1 is not organized and mostly retained within cells (*l*). Bar, 10 μm.

correspond to areas where $\alpha_6\beta_4$ and laminin are facing each other, while closer spots correspond to $\alpha_6\beta_4$-free and F-actin-rich areas. A very similar pattern of adhesion where laminin is not coherent with focal adhesions has been described in transforming growth factor-β-treated thyroid cells in vitro (Garbi et al., 1990). Moreover, $\alpha_2\beta_1$, a basal laminin receptor of endothelial cells (Languino et al., 1989) has never been found in association with focal contacts (Lampugnani et al., 1990). Therefore, adhesion to laminin does not occur via supramolecular structures of the focal adhesion type but rather via integrin receptors that entertain a looser relationship with the substrate and never correspond directly to the microfilamentous meshwork of the cell.

Even more elusive is the potential cytoplasmic ligand of the β_4 chain which, unlike other integrin β chains, has a very large cytoplasmic domain ~1,000 amino acid residues long (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). For the reasons detailed above we feel confident in suggesting that the ligand of such long polypeptide strand exposed to the cytoplasmic environment is not any microfilament-associated molecule since β_4 is almost excluded from F-actin-rich domains of the ventral membrane. The identification of the cytoplasmic interaction of β_4 is a very challenging task.

Interesting and new is also the assigned role of β_1 integrins in the mechanics of epidermal assembly that has been previously reported by ours and other research groups (Carter et al., 1990; De Luca et al., 1990*b*; Larjava et al., 1990). These integrins are members of the largest subfamily and wide is the spectrum of ligand specificities displayed by β_1 integrins. In human keratinocytes we do not have any evidence that β_1 integrins, including the major fibronectin receptor $\alpha_4\beta_1$, are involved in basal matrix recognition but rather they are involved in cell-to-cell recognition. This goes along with recent findings that (*a*) $\alpha_4\beta_1$ is involved in intercellular recognition (Takada et al., 1989; Campanero et al.,

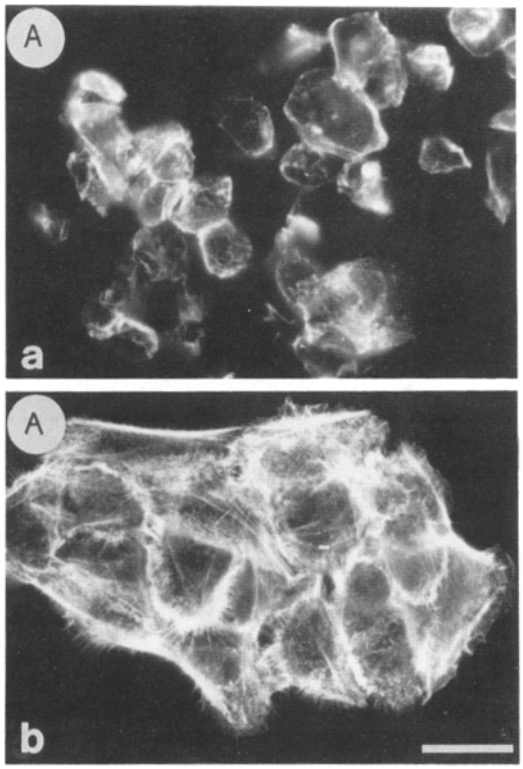

Figure 10. Inhibition of keratinocyte cohesion by goat antibodies to β_1 keratinocytes obtained from subconfluent primary cultures were passaged in secondary cultures and grown as described in Materials and Methods. They were trypsinized and immediately plated on 24-well plates in the presence of β_1 antibodies. The cohesion assay was carried for 12 h. The cells that have attached in the presence of β_1 antibodies that do not inhibit adhesion (De Luca et al., 1990*b*) are not completely spread (i.e., they have not fully organized their cytoskeleton) and are not aggregated in colonies as the cells (*b*) that had been incubated in the presence of goat preimmune serum. Cells were fixed, permeabilized, and stained with R-PHD for F-actin. Bar, 15 μm.

1990), (*b*) $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are located to intracellular spaces in different cell types (Kaufmann et al., 1989; Zutter, M. M., and S. A. Santoro, 1989; *J. Cell Biol.* 109:106a [Abstr.]), and that (*c*) $\alpha_2\beta_1$ and $\alpha_4\beta_1$ are located to cell–cell contacts in human endothelial cells (Lampugnani et al., 1990). The integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ may play roles in keeping cells of the basal layers together. Both are laminin receptors, although they bind laminin with different affinities (Gehlsen et al., 1988; Languino et al., 1989; Wayner et al., 1989; Kirchhofer et al., 1990), and some laminin is indeed found in between the lateral domains of cultured keratinocytes such as we propose that an interaction with laminin may also be involved in $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin-mediated lateral adhesion. However, we cannot rule out the possibility that they may interact with each other by hitherto undescribed homophylic bonds or with other cell adhesion molecules such as A-CAM (Geiger et al., 1985) and uvomorulin (Ekblom et al., 1986) or, finally, recognize unknown ligands.

A major point is whether integrins correspond to specific intercellular junctions in cultured keratinocytes. At the level of resolution of light microscope immunocytochemistry we cannot obtain any more detailed information but, in view of the punctate versus continuous pattern of desmoplakins and β_1 integrins we can rule out a discrete integrin location to intercellular desmosomes. Even more difficult is to assign $\alpha_6\beta_4$ to a specific junction. The only junctions that are found between the basement membrane and the ventral plasmamembrane of basal epidermal cells are hemidesmosomes (Farquhar and Palade, 1963). Indeed, at hemidesmosomes, the plasma membrane is somewhat removed from the attachment surface, a feature shared with $\alpha_6\beta_4$ spots. It is then tantalizing to speculate that $\alpha_6\beta_4$ is involved in hemidesmosome formation also in view of the fact that keratin filaments and not microfilaments converge on hemidesmosomes.

These and previous data also suggest that, while β_1 integrins do not have any detectable role in cell-to-substratum adhesion in this particular cell system, $\alpha_6\beta_4$ plays a major role in supporting the adhesion of keratinocytes firmly attached to their basement membrane and part of a highly differentiated cultured epidermis (De Luca et al., 1990*b*). As suggested by their peculiar location, β_1 integrins may play an important role during histogenesis by allowing lateral recognition of keratinocytes and in building up epidermal sheets.

A further epithelial-specific integrin, $\alpha_v\beta_5$, has been recently described in human carcinoma cells (Cheresh et al., 1989). We have indirectly located $\alpha_v\beta_5$ using an mAb to α_v since no immune reagent was available to β_5 and β_3 is absent in normal human keratinocytes. The location of $\alpha_v\beta_5$ is restricted to peripheral cells in exponentially growing colonies in coincidence with a fibrillar network of fibronectin. Since no vitronectin is apparently produced by keratinocytes, it is likely that $\alpha_v\beta_5$ binds to fibronectin at small adhesion plaques that are located at the endings of short stress fibers and contain vinculin and talin. The role of $\alpha_v\beta_5$ would then be related to colony expansion and keratinocyte locomotion that have previously been ascribed to fibronectin recognition and/or deposition during epidermal growth and regeneration processes (Takashima and Grinnell, 1985; Adams and Watt, 1989). Antibodies inhibiting $\alpha_v\beta_5$ function, when available, will clarify this point.

The keratinocyte integrin phenotype coincides with that of some malignantly transformed epithelial cell lines (Kajiji et al., 1989; Cheresh et al., 1989), suggesting that keratinocytes may also be representative of cells from other epithelial sources, e.g., from nonstratified or secretory epithelia. A distinguishing feature of this phenotype (De Luca et al., 1990) is the apparent absence from recognizable cell structures of the $\alpha_5\beta_1$ fibronectin receptor and the $\alpha_v\beta_3$ vitronectin receptor well characterized also for its multiple binding properties in many other adherent cell types such as fibroblasts, endothelial cells, osteoclasts, and tumor cells (Cheresh, 1987; Cheresh and Spiro, 1987; Dejana et al., 1988*a*, *b*, 1989, 1990; Fath et al., 1989; Pytela et al., 1985; Singer et al., 1988; Zambonin-Zallone et al., 1989). It will be interesting to see how widespread among epithelial cells the keratinocyte phenotype is.

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