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Polarized integrin mediates human keratinocyte adhesion to basal lamina

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ABSTRACT **Epithelial cell interactions with matrices are critical to tissue organization. Indirect immunofluorescence and immunoprecipitations of cell lysates prepared from stratified cultures of human epidermal cells showed that the major integrins expressed by keratinocytes are $\alpha_E\beta_4$ (also called $\alpha_6\beta_4$) and $\alpha_2\beta_1/\alpha_3\beta_1$. The $\alpha_E\beta_4$ integrin is localized at the surface of basal cells in contact with the basement membrane, whereas $\alpha_2\beta_1/\alpha_3\beta_1$ integrins are absent from the basal surface and are localized only on the lateral surface of basal and spinous keratinocytes. Anti-β_4 antibodies potently inhibited keratinocyte adhesion to matrigel or purified laminin, whereas anti-β_1 antibodies were ineffective. Only anti-β_4 antibodies were able to detach established keratinocyte colonies. These data suggest that $\alpha_E\beta_4$ mediates keratinocyte adhesion to basal lamina, whereas the β_1 subfamily is involved in cell–cell adhesion of keratinocytes.**

The contact of epithelial cells with underlying basement membranes is thought to regulate several functions pertaining to formation, maintenance, and proper operation of epithelial sheets (1–3). At the molecular level, some of these functions may be sustained by interactions of cell surface receptors with extracellular matrix components, which in turn may affect cell attachment and mobility, cell division, and cell differentiation. Identification and characterization of such receptors are therefore of great interest.

Several cell surface receptors for components of basement membranes, including laminin and collagen, have been described; these receptors mostly belong to the integrin family (4–6). Integrins are particularly interesting receptors (6, 7), because they are able to transduce matrix binding into intracellular signals affecting cell organization. Integrins comprise a large family of transmembrane heterodimers, which are homologous in structure and display distinctive patterns of tissue-specific expression. These receptors mediate cell adhesion to extracellular substrates such as fibronectin, laminin, and collagen as well as cell surface ligands such as ICAM-1 (8). A common motif often found in integrin ligands is the peptide sequence Arg-Gly-Asp (RGD) (7). Recently an integrin-type receptor, $\alpha_E\beta_4$ (also called $\alpha_6\beta_4$) with preferential epithelial distribution was described (9–11). We have investigated the possible functional role of this epithelial integrin in normal human keratinocytes, cultured *in vitro* as originally described by Rheinwald and Green (12, 13). Under these conditions (14, 15), keratinocyte colonies eventually fuse, giving rise to a tridimensional tissue (stratified squamous epithelium) that can be successfully and permanently transplanted onto patients presenting large skin defects (16–21) and that maintains characteristics of the original donor site (22, 23). Thus, physiological interactions regulating cell adhesion, growth, and differentiation in a complex tissue are potentially accessible for experimental investigations.

The data we present here support the idea that the epithelial integrin $\alpha_E\beta_4$ (9), to which no clear adhesive receptor function has been thus far ascribed, mediates interactions between the basal cell of a stratified epithelium and the basement membrane.

MATERIALS AND METHODS

Cell Culture and Immunostaining. Human epidermal keratinocytes were cultured as described (14, 15). Briefly, single cell suspensions from skin biopsy specimens of healthy volunteers were grown to confluence on lethally irradiated mouse fibroblasts (3T3-J2, a gift from Howard Green, Harvard Medical School, Boston) in KGM medium (15) and passaged at a density of 4×10^3 to 1.3×10^4 cells per cm^2. These conditions result in the formation of stratified epidermis, as illustrated in detail by Green and co-workers (13, 14). For cross-sectioning, confluent epidermis was briefly treated with dispase (14) and then detached with a sharp razor blade. These sheets, or 0.5-cm^2 skin biopsy specimen from healthy volunteers, were briefly fixed in paraformaldehyde, sectioned, immunostained as described (24), and inspected on a Zeiss Axiophot microscope with epifluorescence and planapochromatic lenses.

Antibodies. The monoclonal antibody (mAb) S3-41, to $\alpha_E\beta_4$ complex, and the polyclonal antiserum 5710, to β_4, have been described (9). Other mAbs, and the investigators who kindly provided them, are as follows: TS2/7 (25), to α_1, and A-1A5 (25), to β_1, M. Hemler (Dana–Farber Cancer Institute, Boston); 12F1 (26), to α_2, V. Woods (University of California, San Diego); J143 (27), to α_3, A. Albino (Memorial Sloan–Kettering Cancer Center, New York); CLB-54, to β_2, R. van Lier (Netherlands Red Cross, Amsterdam); GOH3 (28), to α_E, A. Sonnenberg (Netherlands Red Cross). Goat antiserum (29) to β_1, used in adhesion assays, gave immunoprecipitation patterns identical to A-1A5.

Cell Labeling and Immunoprecipitation. Either confluent secondary cultures or third-passage cultures from breast reductive surgery (Clonetics, San Diego) were used, with identical results. Detergent lysates of [^{35}S]methionine-labeled cells were immunoprecipitated and analyzed by SDS/PAGE as described (9, 30).

Adhesion Inhibition and Detachment Assays. Ninety-six-well plates were coated for 1 hr at 37°C with mouse laminin (10 μg/ml) or matrigel (2 μg/ml) (gift from G. Allavena, IST). Keratinocytes from confluent secondary cultures were plated (3×10^4 cells per well) in KGM medium without serum, unless indicated otherwise. For inhibition assays (Fig. 4), rabbit antiserum 5710 to $\alpha_E\beta_4$, GOH3 mAb to α_E, goat antiserum to β_1 (31) (1:50 or 1:100 dilutions), control normal

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Abbreviation: mAb, monoclonal antibody.

serum, or unrelated antibodies were added at plating. In some experiments a synthetic GRGDSP peptide (gift from Guido Tarone, University of Turin, Turin, Italy) was added at plating at a concentration ranging from 0.0078 mg/ml to 1 mg/ml. For detachment assays (Fig. 3) cells were left to adhere 12 hr at 37°C prior to addition of serum. After 12 hr at 37°C with serum, cells were washed, fixed in 3% formaldehyde/2% sucrose/phosphate-buffered saline for 5 min, stained with 0.5% crystal violet/20% methanol for 15 min, washed, and dried. The dye was eluted with 50% EtOH/0.1 M sodium citrate, pH 4.2. Optical density was read in a Titertek (Flow Laboratories) Multiscan at 540 nm.

RESULTS

Immunoprecipitation. Immunoprecipitations of cell lysates prepared with the aid of detergent from metabolically labeled cells indicated that cultured human epidermal keratinocytes express the integrin $\alpha_E\beta_4$ (Fig. 1). This integrin is formed by the noncovalent association of the subunit α_E with β_4, which is generally present in three forms with slightly different apparent molecular weights. Antibodies reactive with epitopes on the α_E chain, on the β_4 chain, or on the complex of the two chains precipitated virtually identical patterns comprising the α_E band (M_r 150,000 under nonreducing and M_r 125,000 under reducing conditions) and the three β_4 bands (M_r 190,000, M_r 170,000, and M_r 120,000 under nonreducing and M_r 200,000, M_r 180,000, and M_r 150,000 under reducing conditions).

Additional immunoprecipitations showed (Fig. 1) that anti-β_1 antibodies bring down bands corresponding to β_1 (M_r 125,000 under reducing and M_r 110,000 under nonreducing conditions), β_1 precursor (M_r 110,000 under reducing and M_r 95,000 under nonreducing conditions), and other bands presumably representing α chains associating with β_1. Many heterodimers of the β_1 family have been described as laminin receptors: $\alpha_1\beta_1$ (38), $\alpha_2\beta_1$ (4), $\alpha_3\beta_1$ (5), and $\alpha_E\beta_1$ (28). Therefore, antibodies specific for these heterodimers were used. Fig. 1 shows that anti-$\alpha_2\beta_1$ precipitated bands corresponding to β_1 and to α_2 (M_r 170,000 under reducing and M_r 160,000 under nonreducing), whereas anti-$\alpha_3\beta_1$ mAb brought down β_1 complexed with two faint bands likely corresponding to α_3 and α_3 precursor, as evidenced by their molecular weights (M_r 125,000 under reducing and M_r 160,000 and M_r 130,000, respectively, under nonreducing conditions; the faintness of these bands is probably due to the low synthetic rate of this protein). No signal was detectable with anti-$\alpha_1\beta_1$ antibodies, whereas antibodies to α_E only recognized this chain in association with β_4.

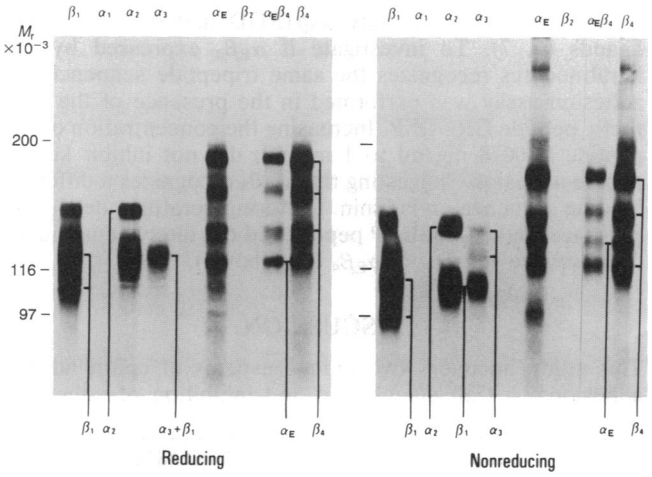

FIG. 1. Identification of integrins in lysates of radiolabeled keratinocytes. See text for the names and sources of antibodies. The antigenic determinants recognized by each antibody are either located on one of the two integrin subunits or are formed by their association. In either case, α and β chains coprecipitate because they are noncovalently associated. Antibody to β_1 (slot labeled β_1 on the upper side) precipitates bands corresponding to β_1 and β_1 precursor together with other bands corresponding to β_1-associated α chains. Antibody to α_1 (upper label) is negative. Antibodies to α_2 and α_3 (upper label) precipitate bands corresponding to α_2 and α_3, respectively, together with β_1 bands. The double band for α_3 (nonreducing lane) may be due to incomplete protein processing, and its faintness with respect to the β_1 band is probably due to a lower turnover rate. Antibody to α_E (upper label) precipitates α_E together with three bands representing the three molecular forms of β_4. The M_r 95,000 band in the nonreducing lane of anti-α_E is an often observed breakdown product of β_4. Antibody to β_2 is negative. Antibodies to determinants either formed by association of α_E and β_4 or located on β_4 precipitate the $\alpha_E\beta_4$ complex. Reducing and nonreducing lanes are shown, since the changes in molecular weights of the various proteins help in their identification.

Immunofluorescence. By immunofluorescence, $\alpha_E\beta_4$ was detected in cells of the basal layer of the confluent stratified epithelium formed by keratinocytes in culture. Within the basal cells, expression of $\alpha_E\beta_4$ was restricted to those regions of the cell membrane in close contact with substrate (Fig. 2). On sections of skin biopsies, an identical distribution of $\alpha_E\beta_4$ on basal cells was observed (Fig. 2). In contrast, basal cells stained with anti-β_1 and anti-α_3 antibodies showed staining only on their lateral surfaces (Fig. 2), confirming very recent results (32). Anti-α_2 gave an identical pattern (not shown). There was no significant staining of the basal surface of the basal cells. In sections of epidermis, anti-β_1 and anti-α_3 produced a similar staining pattern, on both basal and spinous cells (Fig. 2).

The time required for $\alpha_E\beta_4$ and $\alpha_2/\alpha_3\beta_1$ integrins to become polarized was monitored on keratinocytes plated on different substrates. Up to 4 hr after plating, $\alpha_E\beta_4$ and $\alpha_2/\alpha_3\beta_1$ integrins were distributed homogeneously on the cell surface (not shown). Polarization of both integrins was complete about 12

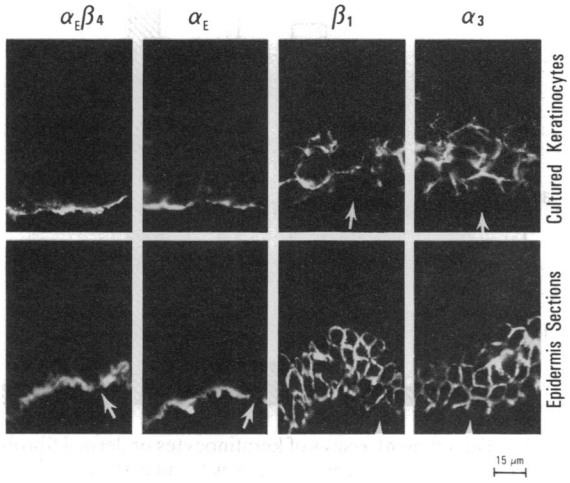

FIG. 2. Distribution of $\alpha_E\beta_4$, α_E, β_1, and $\alpha_3\beta_1$ integrins in confluent keratinocyte cultures (upper row) and in sections of skin biopsies obtained from healthy volunteers (lower row), detected by immunofluorescence. Sections are all oriented with the outermost layer of the stratified epithelium on the upper side. Arrows point at the basal aspects of cells of the basal layer. The anti-β_4 ($\alpha_E\beta_4$ panels) and anti-α_E (α_E panels) antibodies show overlapping patterns, narrowly localized at the basal surface of cells. The arrowheads in the lower row ($\alpha_E\beta_4$ and α_E panels) point at negative cells, which may represent melanocytes (not expressing $\alpha_E\beta_4$). Antibodies to β_1 (β_1 panels) and α_3 (α_3 panels) show staining concentrated on the lateral-apical aspect of cells in the Malpighian layer, whereas the basal aspect of basal cells (arrowheads) is not stained. Anti-α_2 antibodies gave similar results (not shown).

hr after plating (Fig. 3 *a* and *b*). For comparison, Fig. 3 shows the presence of β_1 (Fig. 3*c*) and the absence of $\alpha_E\beta_4$ (Fig. 3*d*) on dermal fibroblasts.

Localization of $\alpha_E\beta_4$ to the basal surface occurred whether the keratinocytes were plated on 3T3-J2 (Fig. 3 *a* and *b*), on purified laminin (the dominant basal lamina glycoprotein) (33), or on purified fibronectin or uncoated glass (not shown). Independent of the substrate, a homogeneous layer of laminin was detected underneath growing colonies (3–5 days after plating), suggesting autonomous extracellular matrix formation by keratinocytes (M.D.L. and P.C.M., unpublished data). In summary, these data demonstrate a polarized expression of integrins on the cell membrane of normal human epidermal cells.

Detachment and Adhesion Assays. The localization of $\alpha_E\beta_4$ to the substrate-contacting region of epidermal basal cells suggested experiments aimed at probing a possible role for the receptor in adhesion. Keratinocytes obtained from confluent secondary cultures were plated on either laminin, matrigel (an extract of basal lamina containing laminin, collagen type IV, nidogen/entactin, and heparan-sulfate proteoglycan) (34), or fetal calf serum (a source of vitronectin and fibronectin). After 12 hr, when integrins were presumably localized (Fig. 3 *a* and *b*), anti-β_4 and anti-β_1 antibodies were added for an additional 12 hr (detachment assay). In the presence of anti-β_4, keratinocytes adhering to laminin or matrigel were mostly detached (Fig. 3). No effect was found on keratinocytes plated on uncoated wells in the presence of serum or on dermal fibroblasts attached to any of the substrates (Fig. 3). In contrast, anti-β_1 had no effect in detaching keratinocytes from any substrate, whereas significant disruption of fibroblast adhesion was readily observed (Fig. 3).

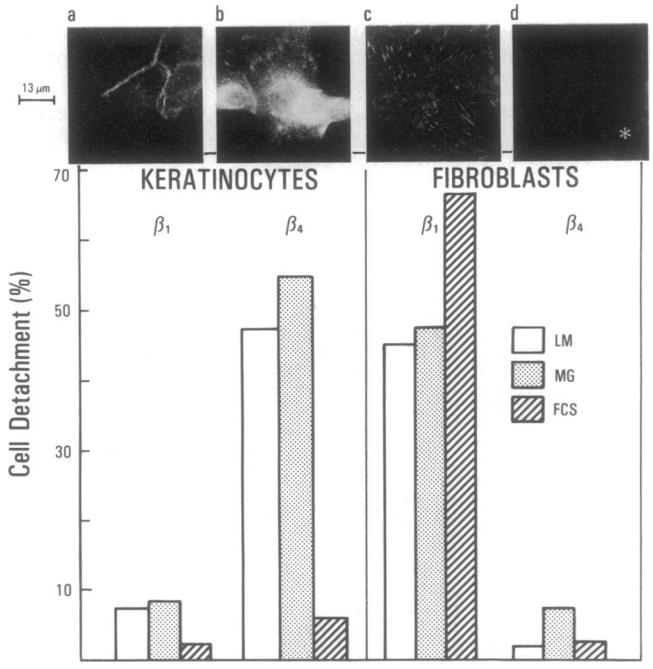

FIG. 3. Detachment assays of keratinocytes or dermal fibroblasts by antisera to specific integrins. Cells were plated on wells coated with laminin (LM) or matrigel (MG). After 12 hr cells were incubated for an additional 12 hr with anti-β_4 or anti-β_1 antibodies. In parallel, cells were plated on uncoated surfaces in the presence of 5% fetal calf serum (FCS). Each point is the average of triplicates from four experiments. Variation among triplicates was <5%. (*Inset*) Immunofluorescence staining of keratinocytes (*a* and *b*) or dermal fibroblasts (*c* and *d*) with anti-β_1 (*a* and *c*) or anti-β_4 (*b* and *d*) antibodies. The view is from the top—i.e., the apical aspect—of cells. Cells adhered to laminin-coated coverslips under the exact conditions of adhesion and detachment assays to show that in such assays intercellular (*a*) and basal (*b*) distribution of β_1 and β_4 integrins, respectively, was maintained. The asterisk indicates a $\alpha_E\beta_4$-positive keratinocyte in the dermal fibroblast culture. Cells were immunostained as in Fig. 2, except for permeabilization by a 5-min exposure to Triton X-100 (15) after fixation.

When antibodies were added to the assay at the time of cell inoculation (adhesion inhibition assay), antibodies to $\alpha_E\beta_4$ caused >80% inhibition of keratinocyte adhesion to laminin or matrigel and some inhibition of adhesion (around 40%) of cells plated in the presence of serum but without laminin or matrigel (Fig. 4). As expected, no effect on dermal fibroblasts was found (Fig. 4). In contrast to anti-β_4, anti-β_1 did not significantly inhibit keratinocyte adhesion, whereas it abolished dermal fibroblast adhesion almost completely (Fig. 4). In these assays, cells were allowed to attach for 12 hr since keratinocytes reached a plateau of adhesion at this time point (Fig. 4 *Inset*). The greater effect of anti-β_1 in preventing adhesion of keratinocytes than in detaching them may indicate a minor cooperation of the β_1 integrins in the first phase of keratinocyte adhesion.

Association of the β_4 to the α_E subunit demonstrated by the immunoprecipitation and the immunofluorescence studies was confirmed by the adhesion inhibition assay in the presence of the GOH3 mAb to α_E. This antibody inhibited keratinocyte adhesion on matrigel (49% inhibition) and on laminin (53% inhibition). Anti-α_E did not inhibit fibroblast adhesion on any substrate and unrelated mAbs were ineffective on keratinocyte adhesion. Taken together, these results indicate that $\alpha_E\beta_4$ mediates keratinocyte attachment to basal lamina, possibly by functioning as a laminin receptor.

The lack of effect on adhesion of keratinocytes by anti-$\alpha_2\beta_1$ and $\alpha_3\beta_1$ reflects the absence of these integrins from the basal surface of the basal cells. Many integrins recognize the tripeptide sequence Arg-Gly-Asp (RGD) in their extracellular ligands (6, 7). To investigate if $\alpha_E\beta_4$ expressed by the keratinocytes recognizes the same tripeptide sequence, an adhesion assay was performed in the presence of the synthetic peptide GRGDSP. Increasing the concentration of the peptide (0.0078 mg/ml to 1 mg/ml) did not inhibit keratinocyte adhesion, suggesting that $\alpha_E\beta_4$ recognizes a different binding sequence on laminin. Culturing keratinocytes in the presence of the GRGDSP peptide did not alter the immunofluorescence staining of $\alpha_E\beta_4$ (not shown).

DISCUSSION

This study uncovers two unique aspects of epithelial cell adhesion. (*i*) Cell detachment and inhibition of adhesion,

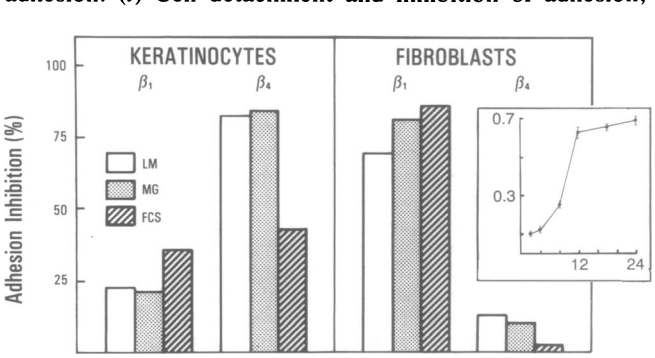

FIG. 4. Prevention of adhesion of keratinocytes or fibroblasts by antisera to specific integrins. Cells were plated on laminin (LM), matrigel (MG), or uncoated wells in medium containing 5% fetal calf serum (FCS). Anti-β_4 or anti-β_1 was added immediately. Each point was averaged from triplicates of four experiments. Variation among triplicates was <5%. (*Inset*) Time course of keratinocyte attachment to matrigel-coated plates. The SEM among triplicates was ≤6%. Time is given in hours on the *x* axis; optical absorbance is given on the *y* axis.

caused by specific anti-β_4 and anti-α_E antibodies, suggest that the adhesion of confluent keratinocyte layers to basal lamina can be largely accounted for by a single integrin, $\alpha_E\beta_4$. The role of this integrin in attachment of cells to basement membranes is of potential general importance, because of its epithelial distribution (34) and its localization on basal surfaces (e.g., in gut epithelium and in cytotrophoblast) (35). (*ii*) Immunofluorescence data show that in keratinocytes integrin expression is targeted to discrete plasma membrane domains. This phenomenon is quite distinct from the substrate-dependent clustering of integrins in focal adhesion plaques (24, 36) (e.g., dermal fibroblasts; Fig. 3 *Inset*) and appears to be correlated with epithelial polarization (3, 37). Thus, asymmetric distribution of $\alpha_E\beta_4$ to basal surfaces occurred on all substrates we tested (laminin, fibronectin, matrigel, uncoated glass), was rather patchy, and did not coincide with adhesion plaques, as assessed by actin costaining or interference reflection microscopy (unpublished data). Similarly, β_1 integrins were sorted to lateral surfaces independent of substrate and did not codistribute with stress fibers (unpublished data).

These observations suggest a relation between integrin polarization and regulation of adhesion in epithelial cells. For example, in our assays, inhibition of adhesion could only be measured reliably at 12 hr—i.e., when keratinocyte adhesion to substrate reaches a plateau (Fig. 4 *Inset*). At 2 hr (the standard time length for adhesion assays—e.g., in mesenchymal type cells), adhesion was poor (Fig. 4 *Inset*). Interestingly, by immunofluorescence maximum concentration of $\alpha_E\beta_4$ on the basal surface was seen at 12 hr after plating (Fig. 3*b*), whereas at shorter times the molecule appeared uniformly expressed on the plasma membrane (not shown). Similarly, sorting of β_1 integrins to lateral surfaces also required $\approx$12 hr (Fig. 3*a*). These times are similar to polarization times for other cell types (3). It is thus likely that integrin polarization sets the stage for proper adhesive interactions such that $\alpha_E\beta_4$ mediates adhesion to basal lamina, whereas the β_1 integrins mediate intercellular adhesion. An alternative possibility is that keratinocytes, in order to adhere properly, must modify the substrate by secreting and organizing matrix components, a process that would also require incubation times on the order of 12 hr.

A decisive advantage of the keratinocyte culture system we adopted is that it offers the opportunity to test these alternative possibilities since the molecular mechanisms underlying these phenomena can be dissected *in vitro* under essentially physiological conditions. In this context it should be stressed that transformed cultured cell lines, which are known to polarize poorly, or nonphysiological keratinocyte culture conditions may instead not be suitable for such studies.

To establish whether or not, in epithelial cells, the sorting of integrins to distinct surface domains is a primary regulator of adhesive and migratory properties, several issues need clarification. A critical point is to verify to what extent integrin polarization in these cells is substrate independent. To this end, a better knowledge of basal lamina components is necessary. In addition, the $\alpha_E\beta_4$ ligand has to be demonstrated at a molecular level. Unfortunately, biochemical approaches to this problem—e.g., column chromatography on various matrix proteins—have been thus far unsuccessful in our hands. Furthermore, the type of interactions in which the β_1 receptors are involved at intercellular sites remains to be defined. Ligands for $\alpha_2\beta_1$ and $\alpha_3\beta_1$ described to date are matrix components, but a cell surface ligand must exist for the β_1 receptor.

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