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Cell adhesion to laminin 1 or 5 induces isoform-specific clustering of integrins and other focal adhesion components

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SUMMARY

Laminin 1 (α1β1γ1) and laminin 5 (α3β3γ2) induce cell adhesion with different involvement of integrins: both are ligands for the α6β1 integrin, while α3β1 integrin has affinity for laminin 5 only. These two laminin isoforms therefore provide good models to investigate whether α3β1 and α6β1 integrins play different roles in signal transduction and in focal adhesion formation. Laminin 1 or 5 induced adhesion of normal human skin fibroblasts to a similar extent but promoted different overall cell shapes. On laminin 1 the fibroblasts formed mainly filopodia-like structures, while on laminin 5 they developed lamellipodias. Staining of fibrillar actin with fluorescein-phalloidin revealed a similar organisation of the actin cytoskeleton on both substrates. However, integrin subunits and several cytoskeletal linker proteins, including vinculin, talin, and paxillin, showed an isoform-specific arrangement into focal adhesions. On laminin 1 they were recruited into thick and short aggregates localized at the termini of actin stress fibers, while on laminin 5 they appeared as dots or streaks clustered on a long portion of actin microfilaments. To test whether the differing affinity of laminin 1 or 5 for α3β1 integrin would explain the formation of morphologically different focal adhesions, cells were seeded on laminin 1 under conditions in which α3β1 integrins were occupied by a function-blocking antibody. This resulted in the formation of focal adhesions similar to that observed on laminin 5, where the integrin is occupied by its natural ligand. These results provide the first evidence for a cross-talk between α3β1 and α6β1 integrins and indicate that occupancy of α3β1 integrins results in a trans-dominant regulation of α6β1 integrin clustering and of focal adhesions. It suggests that recruitment of integrins and cytoskeletal linker proteins are laminin isoform-specific and that tissue specific expression of laminin isoforms might modulate cell behavior by the activation of distinct sets of integrins and by the induction of distinct molecular assemblies within the cell adhesion signaling complexes.

Key words: Laminin, Integrin, Focal adhesion

INTRODUCTION

Laminins form a family of heterotrimeric molecules ubiquitous of basement membranes (Timpl and Brown, 1994; Aumailley and Krieg, 1996). They are formed by the association of three different gene products, the α, β, and γ chains (for nomenclature see Burgeson et al., 1994). Up to now 11 laminin isoforms (laminin 1 to 11) resulting from the heterotrimeric combinations of five α, three β, and two γ chains have been identified (for references see Miner et al., 1997) and additional chains may exist. The expression of laminin chains is spatio-temporally regulated (Ekblom et al., 1990; Lentz et al., 1997; Miner et al., 1997; Tiger et al., 1997) suggesting that different isoforms might have distinct roles to fulfill. One major function of laminin is to control cell behavior by interacting with integrins (Aumailley et al., 1996), transmembrane receptors involved in the bidirectional transfer of signals between the extracellular matrix and the cell interior (Hynes, 1992; Clark and Brugge, 1995). Ligation of integrins to extracellular targets leads to a series of events including clustering of the receptors, recruitment of focal-adhesion linker proteins, actin polymerization, and a phosphorylation cascade (Burridge et al., 1988; Luna and Hitt, 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993; Clark and Brugge, 1995).

Detailed studies of laminin 1 (α1β1γ1) isolated from the Engelbreth-Holm-Swarm tumor (EHS) have shown that the native molecule contains two major integrin binding domains (Aumailley et al., 1996). One is located on the short arms of the native molecule and is the target for α1β1 or α2β1 integrins (Hall et al., 1990; Goodman et al., 1991; Pfaff et al., 1994; Colognato-Pyke et al., 1995). The other, which is more strongly conformation dependent, has been mapped to a proteolytic fragment of laminin 1, fragment E8, encompassing the end of the long arm and the three first carboxy-terminal G domains (Aumailley et al., 1996; Goodman et al., 1987; Deutzmann et al., 1990). Fragment 8 as well as native laminin 1 are ligands for cells expressing α6β1 or for a restricted number of cell types expressing α7β1 integrins (Aumailley et al., 1996; Sonnenberg et al., 1990a; Hall et al., 1990; Kramer et al., 1991; von der Mark et al., 1991). In addition, the most carboxy-terminal region of laminin 1 has affinity for α-

dystroglycan, a component of the dystrophin-glycoprotein complex (Henry and Campbell, 1996).

Investigations of laminin 5 (α3β3γ2) have indicated that all the cell binding sites are conformation-dependent (Rousselle et al., 1995) and that two integrins only, α3β1 or α6β1, mediate initial cell adhesion to laminin 5 (Delwel et al., 1993, 1994; Rousselle and Aumailley, 1994). Although it might not be involved in the initial step of cell adhesion to laminins (Carter et al., 1990), another integrin, α6β4, is particularly important for stable, in vivo anchorage of basal keratinocytes which bind only to basement membranes containing epithelial laminins (Vidal et al., 1995; van der Neut et al., 1996; Niessen et al., 1996; Dowling et al., 1996). Mainly due to difficulties in the isolation of the proteins, information on other laminin isoforms is more limited. Laminin 2 (α2β1γ1), 4 (α2β2γ1), and partially characterized isoforms isolated from bovine kidney also induce cell adhesion (Brown et al., 1994; Forsberg et al., 1994), and interact with cells specifically transfected to express α3β1 or α6β1 integrins (Delwel et al., 1993, 1994). The contribution of these various integrins in mediating cell adhesion to the different laminin isoforms and in transducing laminin signals might not be identical.

Integrins are thought to transduce signals from the extracellular matrix to the cell interior at focal adhesion sites (Burridge et al., 1988; Juliano and Haskill, 1993; Clark and Brugge, 1995), but all integrins are not necessarily present in focal contacts (Hynes, 1992). These stuctures are multimolecular complexes formed by the association of a given extracellular matrix ligand, its receptors, and intracellular cytoskeletal linker proteins. They therefore provide a physical link between the exterior and the inside of the cell suitable for the transmission of signals elicited by the extracellular matrix. However, in spite of the different extracellular matrix ligands and the many integrins playing a role in cellular interaction and signal transduction, no ligand- or integrin-specific focal adhesion complexes have been described. Since laminins 1 and 5 are ligands for different sets of integrins, they provide a model to address this question. In particular, α3β1 integrins interact with laminin 5 only and direct binding to laminin 1 could not be demonstrated (Sonnenberg et al., 1991; Delwel et al., 1993, 1994; Rousselle and Aumailley, 1994). We have now used laminin 1 or 5 substrates to study the role of α3β1 and α6β1 integrins in the formation of focal adhesion complexes. Our choice of primary human skin fibroblasts was based on the fact that: (1) they are normal cells, (2) they express, among others, the α3, α6, and β1 integrin subunits, and (3) they do not express the β4 integrin subunit, so that α6β4 dimers are absent (Sonnenberg et al., 1990b; P. Rousselle, unpublished results). So, even not necessarily physiological in the context of adhesion to laminins, normal human skin fibroblasts represent a simplified and well-suitable model to study focal adhesion formation under the control of α3β1 and α6β1 integrins. Immunofluorescence analysis of several focal adhesion-associated elements during cell attachment to laminin 1 or 5 showed the formation of substrate-specific and morphologically different focal adhesions. In particular, laminin 1 induced clustering of integrins and cytoskeletal-associated molecules into thick, short aggregates, while on laminin 5 these elements were clustered into thin, discontinuous-line structures. Furthermore we could demonstrate that the pattern of focal adhesions is dependent on the occupancy of α3β1 integrins by either its extracellular matrix ligand (laminin 5) or antibodies against the integrin α3 subunit. Our results indicate that the supramolecular assembly of focal adhesion structures is laminin isoform-specific and dictated by the nature of the integrins present in the complexes.

MATERIALS AND METHODS

Cell cultures

Human skin fibroblast cultures were initiated by explant outgrowth from the dermis of neonatal foreskin as previously described (Aumailley et al., 1982). Established lines of human ovarian carcinoma (OVCAR-4), or mammary epithelial (HBL100) cells have been previously described (Aumailley et al., 1989; Rousselle and Aumailley, 1994). The cells were subcultured and maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum, 200 mM glutamine and a cocktail of antibiotics. Cells were harvested for subculturing or for experiments using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS). Normal human skin fibroblasts were used between passages 3 and 10. Cell culture reagents were from Seromed-Biochrom (Polylabo, Strasbourg, France) and plastic ware from Falcon (Dutscher, Brumath, France).

Cell adhesion substrates and anti-integrin antibodies

Laminin-nidogen complex (the laminin 1 substrate) purified from the EHS tumor of the mouse and the elastase digestion-derived fragment 8 (Paulsson et al., 1987) were kindly provided by Dr R. Timpl (Max-Planck Institute, Martinsried, Germany). Laminin 5 was purified from the culture medium of human SCC25 cells by affinity chromatography on laminin α3 chain-specific mAb BM165 as previously described (Rousselle et al., 1991; Rousselle and Aumailley, 1994). Human collagen IV from placenta was a gift from Dr K. Kühn (Max-Planck Institute, Martinsried, Germany). Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). All substrates were kept frozen at –20°C.

The following mAbs against integrin subunits were used: P4C10 against β1, P1E6 against α2, and P1B5 against α3, all from Telios (distributed through Gibco BRL, Cergy Pontoise, France), K20 against β1 (Immunotech, Marseille, France) and GoH3 against α6 (kindly given by Dr A. Sonnenberg, The National Cancer Institute, Amsterdam, The Netherlands, or from Cappel, distributed by Speci, Sainte Foy Lès Lyon, France). Other mAbs included F-VII against human vinculin (a gift from Dr M. Glukhova, CNRS UMR144, Paris, France), 8d4 against talin (Sigma Immunochemicals, St Quentin-Fallavier, France), and mAβ165 against paxillin (a generous gift from Dr C. Turner, State University of New York, Syracuse, NY, USA).

Cell adhesion and inhibition assays

Multiwell tissue culture plates (96-well, Costar, Dutscher, France) were coated with serial dilutions of laminin substrates (0-20 μg/ml, 100 μl/well) by overnight adsorption at 4°C. After saturation of the wells with 1% bovine serum albumin (BSA, fraction V, Sigma Chimie) the plates were immediately used for cell adhesion assays in serum-free medium as detailed previously (Aumailley et al., 1989). The extent of adhesion was determined after fixation of the adherent cells with 1% glutaraldehyde in PBS, by staining with 0.1% Crystal Violet, and colour reading at 570 nm with an ELISA reader (MR5000 Dynatech, Guernsey, Channel Islands). A blank value corresponding to BSA coated wells (≤5% of maximal cell adhesion) was automatically subtracted. Adherent cells were photographed using a phase contrast microscope equipped with camera (Olympus, OSI, Paris, France). For adhesion inhibition experiments with anti-integrin antibodies, cells in suspension were mixed with dilutions of antibody

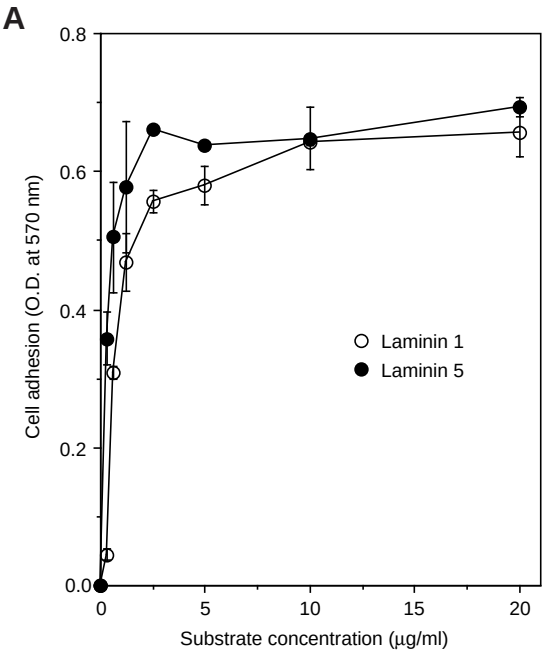
prior to plating onto the coated wells. For all experiments, each assay point was derived in triplicate.

Immunofluorescence staining of focal adhesions

Round glass coverslips deposited on the bottom of 24-well plates (Costar, Dutscher) were coated with laminin 1, fragment E8, laminin 5, collagen IV, or fibronectin (2-10 μg/ml), at 4°C overnight. Cell adhesion to the coats was carried out as described above for 60 minutes. In some experiments cell adhesion to the coated coverslips was performed in the presence of anti-integrin antibodies used at function-blocking concentrations (P1B5, 1:400; P1E6, 1:2,000). Adherent cells were fixed with 2% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.2% Triton X-100 for 1 minute, rinsed several times with PBS, and incubated for 45 minutes with the first antibody. Cy3-conjugated second antibodies against mouse or rat immunoglobulins (Jackson, distributed through Immunotech) were applied together with fluorescein isothiocyanate-phalloidin (Sigma Immunochemicals) for another 45 minutes. The glass coverslips were mounted onto slides in 9:1 (v/v) glycerol/PBS and the cells were observed by epifluorescence with an Universal or an Axiophot Zeiss microscope. Photomicrographs were taken on Kodak T-Max 400 film.

RESULTS

Substrate concentration-dependent curves showed that within 30 minutes laminin 1 or 5 induce adhesion of normal human skin fibroblasts to the same extent (Fig. 1A) followed by distinct spreading of the cells. To better visualize cell morphology, fibroblasts adhering to laminin coated glass coverslips were stained with fluorescein isothiocyanate-phalloidin at different time points (15, 30, 60 and 120 minutes) after seeding. On both substrates the cell morphology progressively changed from round, at 15 minutes, to fully spread, at 60 minutes. At this time point, staining of fibrillar actin revealed a well developed network, with many actin cables running longitudinally across the cell body (Fig. 1B). However, the overall cell morphology was different, with irregular cell margins, numerous spikes, and filopodia-like elongated processes on laminin 1, while on laminin 5 large lamellae-like areas developed giving the cell margins a regular and smooth appearence (Fig. 1B). This difference was observed after shorter (30 minutes) or longer (120 minutes)



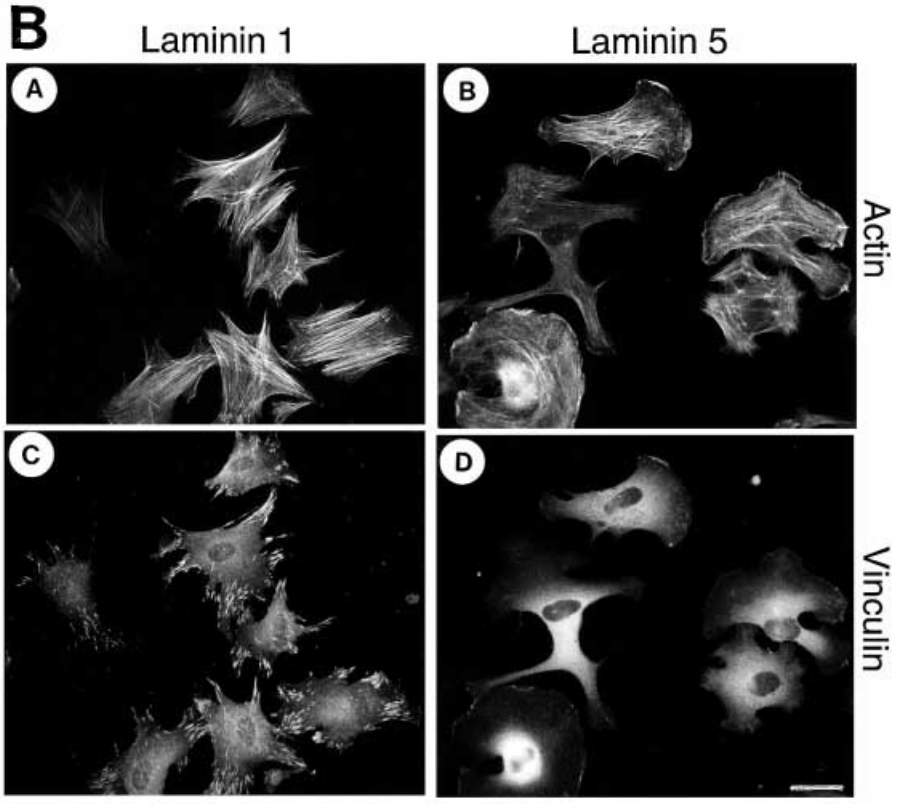


Fig. 1. Dose-dependent adhesion and spreading of normal human skin fibroblasts on laminin 1 or 5. (A) Multiwell plates were coated with different concentrations of laminin 1 (○) or 5 (●) as indicated on the figure. Cell adhesion was measured after 30 minutes using a colorimetric reaction as described in Materials and Methods. Each point represents the average of triplicate wells. (B) Glass coverslips were coated with laminin 1 or 5. After 60 minutes of adhesion adherent fibroblasts were double-stained with fluorescein-phalloidin (A,B) and with mAb against vinculin followed by Cy3-conjugated second antibody (C,D). The specimen were observed with a ×40 objective. Bar, 15 μm.

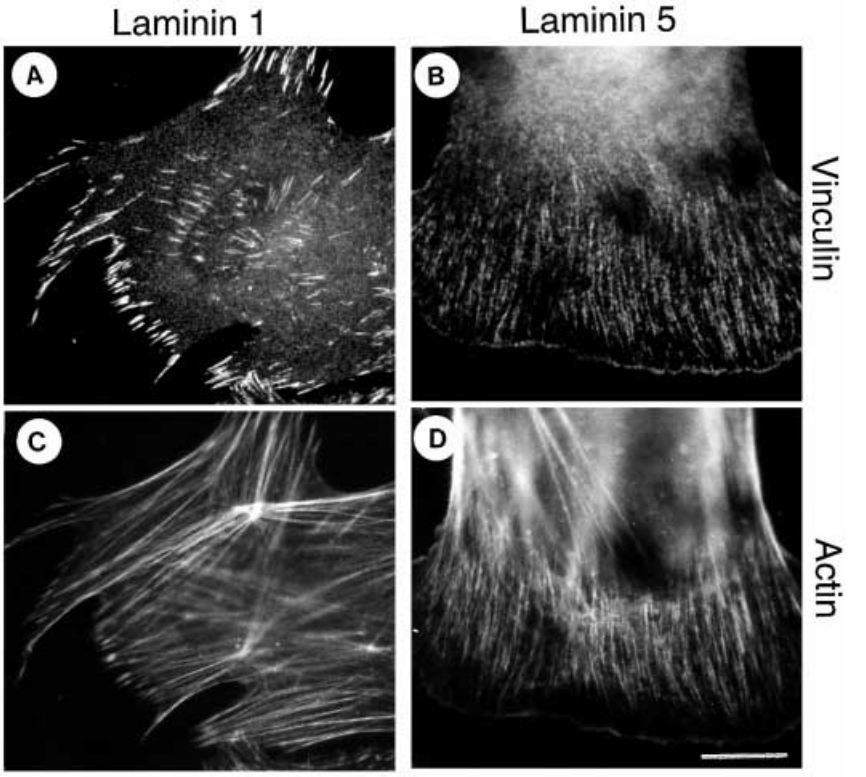


Fig. 2. Detailed morphology of focal adhesions formed by normal human skin fibroblasts on laminin 1 or laminin 5 substrates. Adherent fibroblasts were processed for visualization of vinculin (A,B) or fibrillar actin (C,D) as described in Fig. 1B. The specimens were observed under oil immersion with a ×100 objective. Bar, 5 μm.

incubation time on the substrates (not shown). Double-labeling with antibodies against vinculin indicated that for cells on laminin 1 vinculin aggregates were segregated into thick patches, mostly at the ends of the actin microfibrils (Fig. 1B and Fig. 2). In contrast, on laminin 5 vinculin clustering was barely seen at low magnification (Fig. 1B). At higher magnification, vinculin aggregates appeared as tiny streaks aligned with a long portion of actin microfibrils, much different to those in cells adhering to laminin 1 (Fig. 2). Immunofluorescence labeling of two other focal adhesion-associated components, talin or paxillin, showed that they were organized similarly to vinculin according to a substrate-specific pattern (Fig. 3). In order to assess whether the substrate-characteristic pattern of staining was specific for normal human skin fibroblasts, two human cell lines, ovarian carcinoma (OVCAR-4) and mammary epithelial (HBL100) cells, were processed under the same conditions. Observation revealed the same difference in the stainings of cells adhering to laminin 1 or 5 (Fig. 4). Moreover, clustering of the β1 and α6 integrin subunits (Fig. 5) was similar to that observed for cytoskeletal linker proteins. On laminin 1, indirect immunofluorescence labeling of integrin subunits decorated thick and well individualized short patches (Fig. 5) which colocalized with the termini of actin stress fibers (not shown). In contrast, on laminin 5 the two integrin subunits were organized into tiny aligned dots (Fig. 5) associated with a long portion of actin microfilaments (not shown). A similar discontinuous line-pattern was seen when cells plated on laminin 5 were stained with antibodies against the α3 integrin subunits, while on laminin 1 such staining resulted in a more diffuse labeling and distinct decoration of focal adhesions was infrequently observed (Fig. 5).

For a wide range of normal cells or established cell lines it has been shown that the α6β1 integrin is the major receptor for laminin 1 (Aumailley et al., 1996; Sonnenberg et al., 1990a; Deutzmann et al., 1990) and that α3β1 integrin does not bind to laminin 1 (Sonnenberg et al., 1991; Delwel et al., 1994). In contrast, the α3β1 integrin is the major receptor for laminin 5 (Carter et al., 1991; Rousselle and Aumailley, 1994; Delwel et al., 1994). By cell adhesion-inhibition studies with function blocking antibodies, we therefore assessed if that was also true for normal human skin fibroblasts. Incubating the cells with antibodies directed against the β1 integrin subunit resulted in a complete inhibition of cell adhesion to laminin 1 or 5 (Fig. 6) indicating that, in the model used here, fibroblast adhesive interactions with both substrates are entirely mediated by integrins of the β1 family. Antibodies directed against the α6 integrin subunit decreased fibroblast adhesion to laminin 1 but not to laminin 5, while antibodies against the α3 integrin subunit had no effect on cell adhesion to laminin 1 but decreased cell adhesion to laminin 5. When the cells were incubated with a combination of antibodies against the α3 and the α6 integrin subunits, adhesion to laminin 5 was totally prevented (Fig. 6) as we have previously reported for other cells, including the OVCAR-4 or HBL100 lines (Rousselle and Aumailley, 1994). In the case of laminin 1, combining the antibodies did not result in a more pronounced inhibition of cell adhesion than that observed with antibodies against the α6 integrin subunit alone (Fig. 6) and the residual adhesion was attributed to α1β1 and α2β1 integrin binding to the short arms of laminin 1 (not shown) in agreement with previous results (Languino et al., 1989; Goodman et al., 1991; Pfaff et al., 1994; Colognato-Pyke et al., 1995).

The fact that α3β1 integrin contributes to cell adhesion to laminin 5 but not to laminin 1 suggested either that occupancy of this integrin or the presence of additional integrin binding

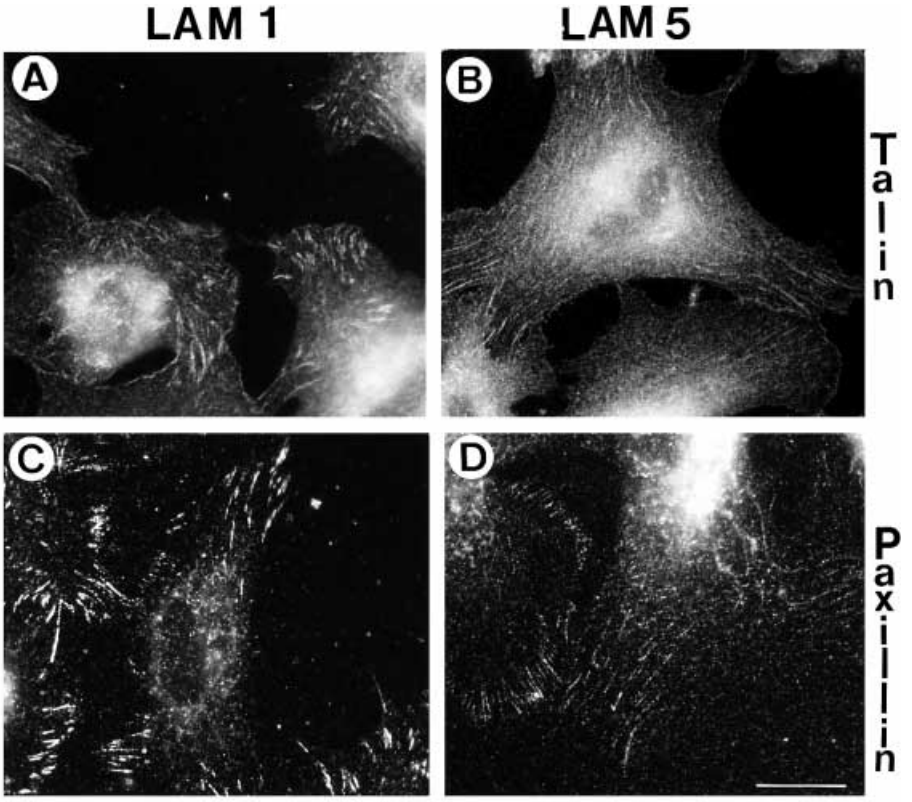


Fig. 3. Indirect immunofluorescence staining of talin or paxillin in normal human skin fibroblasts on laminin 1 or 5. Normal human skin fibroblasts were seeded on laminin 1 (A,C) or 5 (B,D) coats. After 60 minutes of adhesion the cells were processed for immunofluorescence staining of talin (A,B) or paxillin (C,D). The specimen were observed with a ×100 objective under oil immersion. Bar, 5 μm.

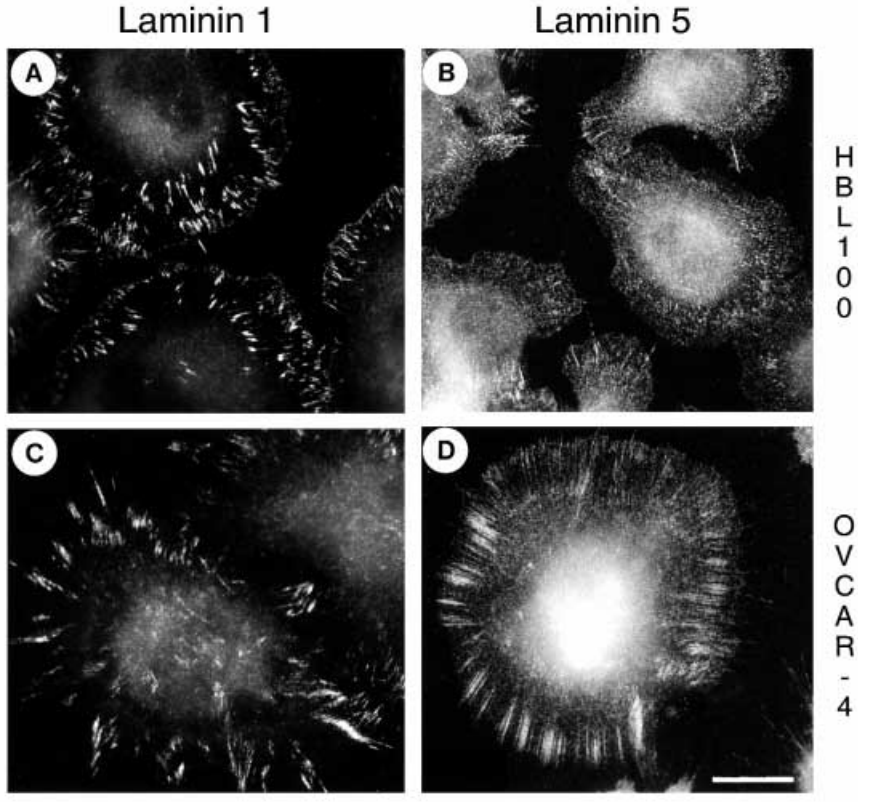


Fig. 4. Indirect immunofluorescence labeling of vinculin in OVCAR-4 and HBL100 cells adhering to laminin 1 or 5. Glass coverslips were coated with laminin 1 (A,C) or 5 (B,D). After 60 minutes of adhesion to laminin coats, adherent HBL100 (A,B) or OVCAR-4 (C,D) cells were incubated with mAb against vinculin followed by Cy3-conjugated second antibody (C,D). The specimens were observed with a ×100 objective under oil immersion. Bar, 5 μm.

sites on the short arms of laminin 1 could modulate the morphology of focal adhesion. To test these hypotheses normal human skin fibroblasts, OVCAR-4, or HBL100 cells were seeded on laminin 1 coats in the presence of mAb P1B5, a function-blocking antibody against the α3 integrin subunit. Under these conditions cell adhesion was not affected but the

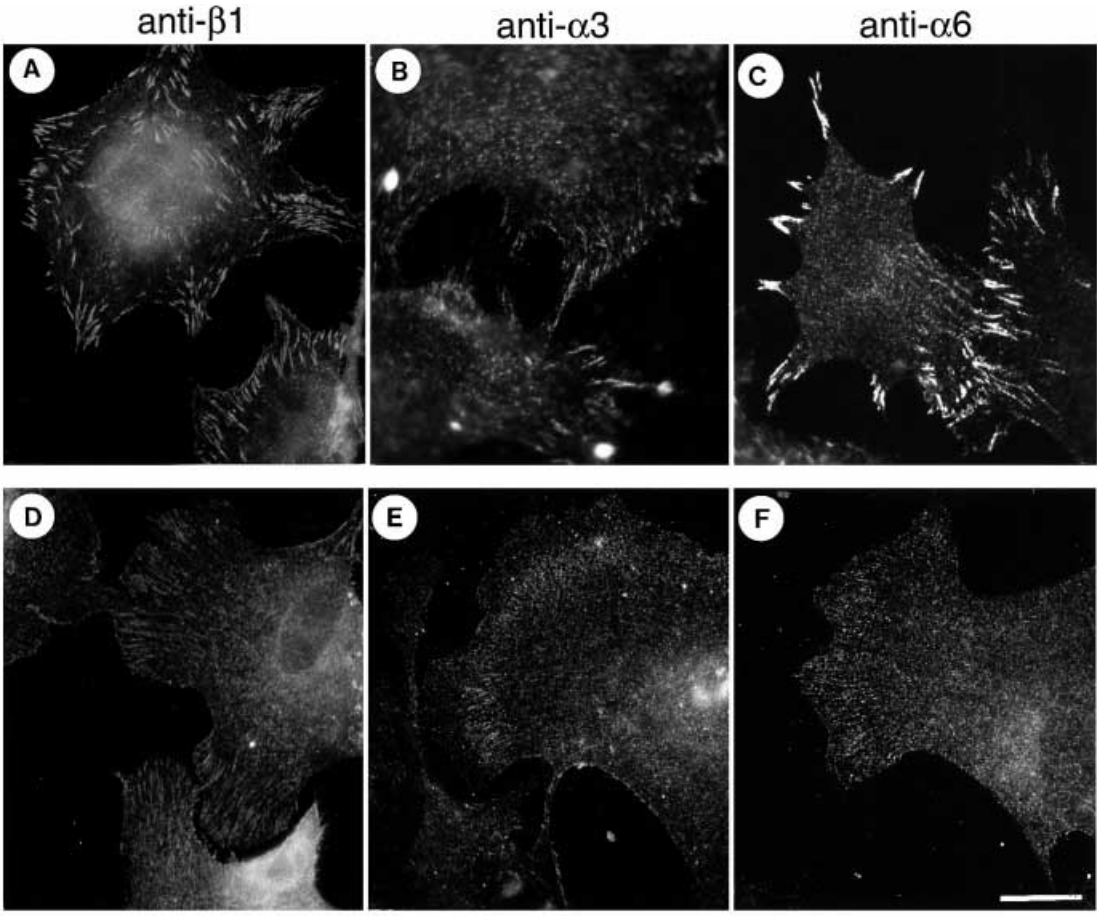


Fig. 5. Indirect immunofluorescence staining of integrin subunits in human skin fibroblasts after adhesion to laminin 1 or 5. After adhesion to glass coverslips coated with laminin 1 (A-C) or 5 (D-F), fibroblasts were incubated with mAbs against β1 (K20; A,D), α3 (P1B5; B,E), or α6 (GoH3; C,F) integrin subunit, followed by the appropriate Cy3-conjugated second antibody. In all cases fibrillar actin was visualized by fluorescein-phalloidin (not shown). Bar, 5 μm.

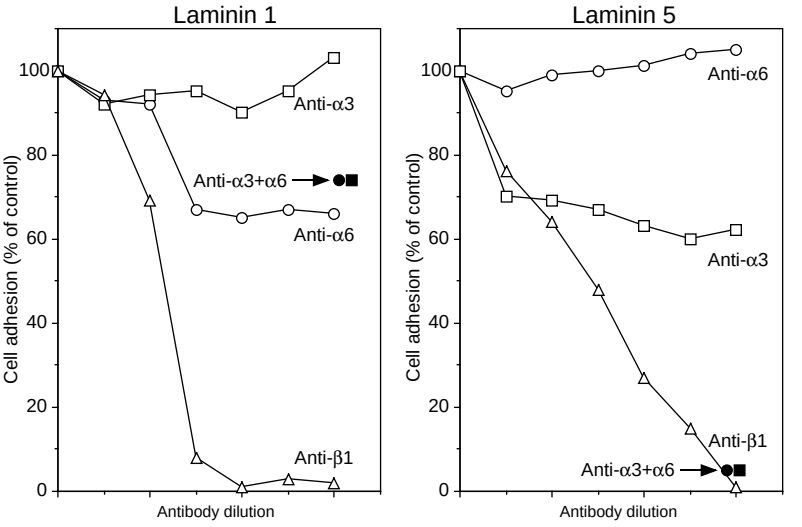


Fig. 6. Inhibition of fibroblast adhesion to laminin 1 or 5 by function-blocking anti-integrin antibodies. Multiwell plates were coated with an optimal concentration of laminin 1 or 5 and post-coated with 1% bovine serum albumin. Fibroblasts were seeded in the presence of serial 1:2 dilutions of mAbs directed against integrin subunits as indicated on the figure. The highest concentration used were as follows: P4C10 at 1:500 (anti-β1); P1B5 at 1:200 (anti-α3); GoH3 at 1:10 (anti-α6). Filled symbols indicate adhesion in the presence of the combination P1B5 at 1:200 and GoH3 at 1:10. Cell adhesion was measured after 30 minutes using a colorimetric reaction as described in Materials and Methods. Each point represents the average of triplicate wells. Adhesion in the absence of antibodies was set as 100% and values reported on the graph express the percentage of adherent cells in the presence of antibodies.

fibroblasts developed lamellipodia and immunofluorescence labelings of vinculin (shown for normal human skin fibroblasts, Fig. 7B) or α6 integrin subunit (shown for OVCAR-4 and HBL100 cells, Fig. 8) were distinctly altered and were similar to that observed for cells plated onto laminin 5 (compare Figs 2 and 7 for normal human skin fibroblasts and Figs 4 and 8 for OVCAR-4 and HBL100 cells). In control experiments replacement of mAb P1B5 by mAb P1E6, a function-blocking antibody against the α2 integrin subunit, did not alter the morphology of focal adhesion structures (Fig. 7F). Furthermore, when fibroblasts were seeded on fragment E8 (a proteolitically derived fragment of laminin 1), the major cell binding site and the ligand of the α6β1 integrin, in the absence or presence of mAb P1B5 (Fig. 7), vinculin labeling was identical to that observed on laminin 1 under the same conditions (Fig. 7; compare A to C and B to D). By contrast,

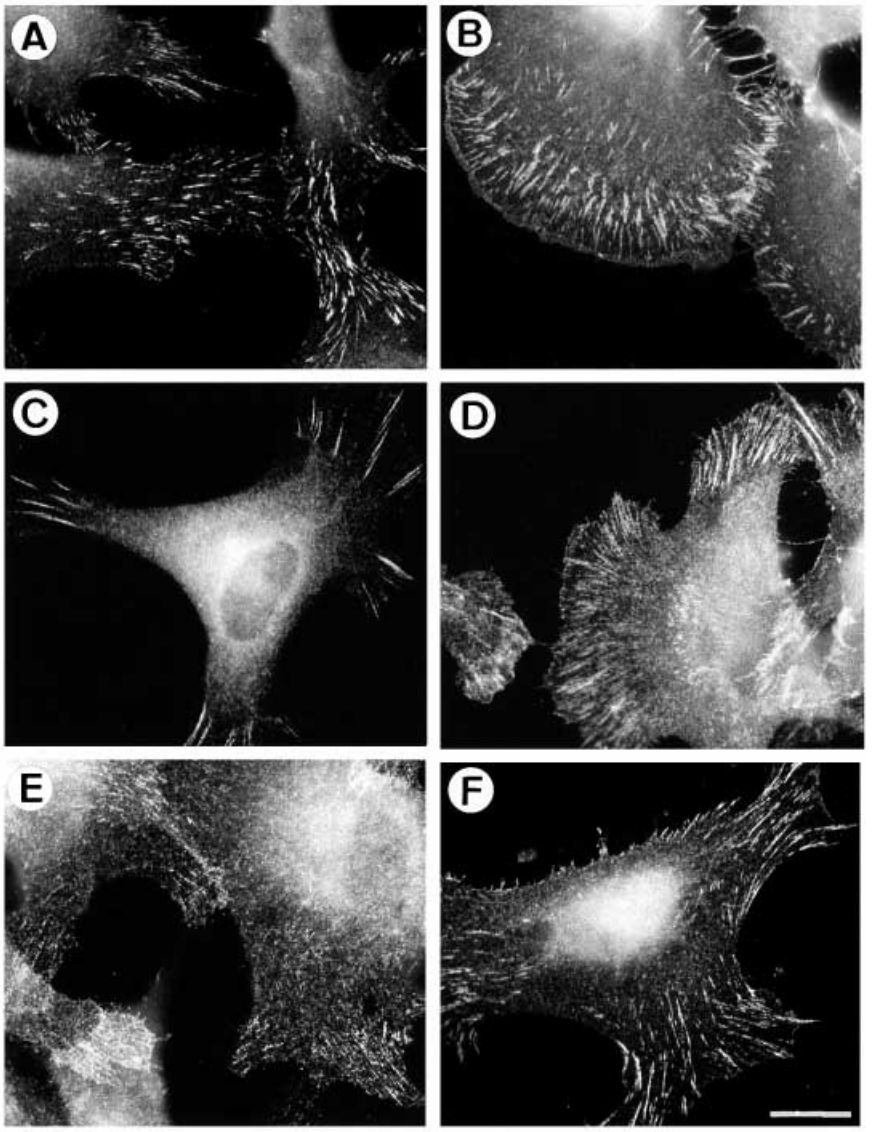


Fig. 7. Indirect immunofluorescence staining of focal adhesions formed by fibroblasts adherent to laminin 1or to fragment E8 in absence or in presence of antibodies against α2 or α3 integrin subunits. Fibroblasts were seeded on glass coverslips coated with laminin 1 (A,B,E,F) or fragment E8 (C,D), in the absence (A,C,E), or presence of antibodies against α3 (B,D) or α2 (F) integrin subunits. After adhesion the cells were processed for immunofluorescence visualization of vinculin (A-E) or α2 integrin subunit (F). Specimens were observed under oil immersion. Bar, 5 μm.

antibody occupancy of α3β1 integrins on cells seeded on collagen IV or fibronectin did not modify the focal adhesions formed on these substrates (not shown). Finally, labeling of the α2 integrin subunit in fibroblasts adherent to laminin 1 (Fig. 7) indicated a clustering different from that observed when the major laminin 1 receptor α6β1 integrin was involved. As a positive control, fibroblasts seeded on collagen IV coats were processed in parallel for indirect immunofluorescence staining of α2 integrin subunit (not shown). Observation of the specimen revealed that on collagen IV, in contrast to laminin 1, α2 integrins were clustered in large patchy focal adhesions as previously reported (Mercier et al., 1996).

DISCUSSION

We have investigated focal adhesions formed by cells on laminin 1 and laminin 5 isoforms. Both induced a comparable concentration-dependent adhesion of normal human skin fibroblasts. This confirms and extends previous work showing that laminin 5 is a very potent adhesion substrate for a wide range of normal, transfected, or neoplastic cells (Carter et al., 1991; Wayner et al., 1993; Rousselle and Aumailley, 1994; Delwel et al., 1994).

Using different types of cells, including lines transfected with specific integrin cDNAs, two integrins, α3β1 and α6β1, were found to participate in cell adhesion to laminin 5 (Carter et al., 1991; Rousselle and Aumailley, 1994; Delwel et al., 1994). Based on inhibition experiments with function-blocking anti-integrin antibodies, the contribution of α3β1 integrins to the cellular interactions with laminin 5 was found to be more significant than that of α6β1 integrins. Indeed, adhesion to laminin 5 was not affected by antibodies against α6 integrin subunit used alone, and was completely or partially inhibited by antibodies against α3 integrin subunit in, respectively, OVCAR-4 or HBL100 cells (Rousselle and Aumailley, 1994), which express both α3β1 and α6β1 integrins (Sonnenberg et al., 1990). In this report, similar cell adhesion inhibition assays were performed with normal human skin fibroblasts. The results showed that α6β1 integrins, but not α3β1, mediated

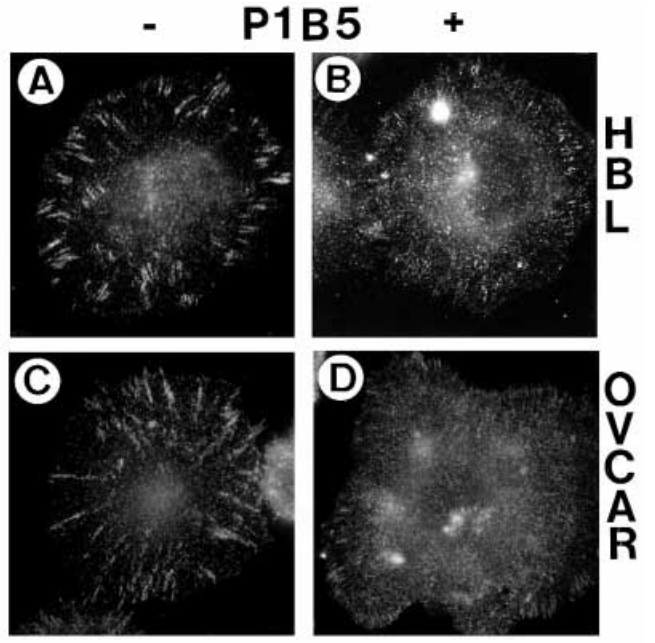


Fig. 8. Antibody occupancy of α3β1 integrins affect α6β1 integrin clustering on laminin 1. OVCAR-4 or HBL100 cells were plated on laminin 1 coats in the absence (A,C) or presence (B,D) of mAb P1B5 (1:400) against the α3 integrin subunit. After 60 minutes of incubation, adherent cells were fixed and processed for indirect immunofluorescence staining of α6 integrin subunit. Bar, 5 μm.

fibroblast adhesion to laminin 1, while both integrins were involved in the adhesion of fibroblasts to laminin 5 (Fig. 6). This agrees with previous results obtained by inhibition assays of other type of cells (Sonnenberg et al., 1990; Rousselle and Aumailley, 1994) and with affinity chromatography experiments showing binding of α6β1 integrins, but not α3β1, to laminin 1 (Sonnenberg et al., 1991). Together these results show that the α3β1 and α6β1 integrins have isoform-specific affinity.

Both laminin substrates induced a distinct spreading of the cells and a similar organization of fibrillar actin as shown by staining with fluorescein-conjugated phalloidin. The overall cell shapes adopted on laminin 1 or 5 were, however, different. In particular, cell contours were smoother on laminin 5, with numerous lamellipodia, whereas filopodia-like structures developed on laminin 1. In addition, vinculin, a marker of mature focal contacts (Burridge et al., 1988), appeared differently organized. For cells adhering to laminin 1, or to its proteolytically derived fragment E8, indirect immunofluorescence labeling of vinculin showed that it was clustered into short and thick patches localized at the termini of actin stress fibers as described for cells on fibronectin or collagens (Fath et al., 1989; Woods and Couchman, 1992; Mercier et al., 1996). By contrast, in cells adhering to laminin 5, antibodies against cytoskeletal linker proteins decorated thin, punctuate structures extending long distances along the actin microfilaments running longitudinally across the cells. The β1 integrin subunits too were clustered into focal adhesions in similar patterns as for cytoskeletal linker molecules on the different laminin isoforms. This could reflect different organisation of the supramolecular complexes formed at the focal contacts by integrin cytoplasmic domains and cytoskeletal linker proteins. Similar observations were made for the α6 integrin subunit, indicating that, besides mediating the interactions with both ligands, it also participates in the formation of focal adhesions. Interestingly, indirect immunofluorescence staining of the α2 integrin subunit revealed that it segregated to adhesion structures differing from those containing the α6 integrin subunits. The α2β1 integrin is a classical collagen receptor which can also interact with the short arms of laminin 1, however, with low affinity (Languino et al., 1989; Pfaff et al., 1994). This may explain the difference seen between staining of α2 or α6 integrin subunits.

In contrast, although staining of the α3 integrin subunit revealed that it was recruited along the actin microfilaments in cells adhering to laminin 5, this integrin did not occur in the focal adhesions formed by cells adhering to laminin 1, in agreement with previous work showing no direct binding of α3β1 integrin to laminin 1 (Sonnenberg et al., 1991; Delwel et al., 1994; Rousselle and Aumailley, 1994). Moreover, occupancy of α3β1 integrins with an α3-directed function-blocking antibody, resulted in a major change in the clustering of vinculin or α6 integrin subunits in cells adhering to laminin 1 or fragment E8. Alteration of focal adhesion morphology by antibody occupancy of α3β1 integrins was specific for laminin 1 and not observed in cells adherent to collagen IV or fibronectin. These results indicate that the nature of the integrin α subunits clustered into the adhesion structures affects the supramolecular organization of integrin subunits and cytoskeletal linker proteins. In particular, clustering of α6β1 integrins, other focal adhesion-associated components, and α3β1 integrins itself are dependent on occupancy of the later by its natural ligand, laminin 5, or by a specific antibody. This observation may explain conflicting reports on the presence of α3 integrin subunits in focal adhesions. Indeed, the presence of α3 integrin subunits in adhesion structures has been observed in only some cell types such as keratinocytes (Carter et al., 1990) or kidney mesengial cells (Grenz et al., 1993), but not in many others, including fibroblasts (Carter et al., 1991; Elices et al., 1991), which may, respectively, have or not have α3β1 integrin ligands in their matrix.

Furthermore, occupancy of α3β1 integrins specifically affects α6β1 integrins and not the integrins involved in cell adhesion to collagen IV or fibronectin. Although the functional role of the intracellular domains of α integrin subunits is still elusive it has been suggested that they regulate integrin clustering by preventing indiscriminate recruitment of the subunits, presumably by hampering β tail interactions with cytoskeletal components (Briesewitz et al., 1993; Weitzman et al., 1997). Along the same lines, it has been shown that detection of α3 integrin subunit into focal contacts is favored by chemical cross-linking of the integrin to the extracellular matrix followed by extraction of cytoskeletal components (DiPersio et al., 1995) and the authors proposed the hypothesis that the α3 integrin subunit may be masked by interacting with focal adhesion components. Our data indicate that, when ligated to its natural ligand, α3β1 integrins are integrated into focal adhesions in such a state that not only renders these integrins available for immunological detection but also, and most importantly, allows for a direct or indirect regulation of the clustering of α6β1 integrins and of focal adhesion-associated components. It has been recently postulated that

αIIbβ3 integrins inhibit the function of both α2β1 and α5β1 integrins by a trans-dominant mechanism (Diaz-Gonzalez et al., 1996). Here we have provided the first evidence for a cross-talk between α3β1 and α6β1 integrins when both are ligated, a process which is likely due to a trans-dominant regulation of α6β1 by α3β1 integrins.

The formation of adhesion complexes is crucial for signal delivery from the extracellular matrix to the cell interior and for the inside-out transfer of information (Burridge et al., 1988; Juliano and Haskill, 1993; Sastry and Horwitz, 1993; Clark and Brugge, 1995). A different ultrastructural arrangement of the molecules within the adhesion complexes could be linked to the transmission of distinct signals. Cells can develop morphologically different cell adhesion structures, such as focal contacts (Burridge et al., 1988; Woods and Couchman, 1992), podosomes (Tarone et al., 1985), or point contacts (Tawil et al., 1993) which differ in molecular composition. Here we described two morphologically different types of focal adhesions, which are laminin isoform-specific. At a molecular level they both contain vinculin, talin, paxillin, α6 and β1 integrin subunits, but differ by the presence of α3 integrin subunit, and they are related to a different cell shape, i.e. the presence of filopodia or lamellipodia. The reorganization of the actin cytoskeleton is under the control of many factors, including small GTP-binding proteins of the Rho family (Hall, 1994). In particular, Rac or Cdc42 have been shown to regulate the formation of, respectively, lamellipodia or filopodia (Machesky and Hall, 1996). Occupancy of the integrin α3β1 by laminin 5 could possibly trigger one of the small GTP-binding proteins which would explain the difference in cell morphology that we have observed, but further studies are required to support this hypothesis.

In conclusion, by interacting with different integrins, laminin 1 and 5 have the property to induce isoform specific focal adhesions. Since expression of laminin chains varies with time and developmental stage, the presence of a given laminin isoform may provide tissue-specific signals to the resident cells.

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