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Laminin isoforms differentially regulate adhesion, spreading, proliferation, and ERK activation of β1 integrin-null cells

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Abstract

The presence of many laminin receptors of the β1 integrin family on most cells makes it difficult to define the biological functions of other major laminin receptors such as integrin α6β4 and dystroglycan. We therefore tested the binding of a β1 integrin-null cell line GD25 to four different laminin variants. The cells were shown to produce dystroglycan, which based on affinity chromatography bound to laminin-1, -2/4, and -10/11, but not to laminin-5. The cells also expressed the integrin α6Aβ4A variant. GD25 β1 integrin-null cells are known to bind poorly to laminin-1, but we demonstrate here that these cells bind avidly to laminin-2/4, -5, and -10/11. The initial binding at 20 min to each of these laminins could be inhibited by an integrin α6 antibody, but not by a dystroglycan antibody. Hence, integrin α6Aβ4A of GD25 cells was identified as a major receptor for initial GD25 cell adhesion to three out of four tested laminin isoforms. Remarkably, cell adhesion to laminin-5 failed to promote cell spreading, proliferation, and extracellular signal-regulated kinase (ERK) activation, whereas all these responses occurred in response to adhesion to laminin-2/4 or -10/11. The data establish GD25 cells as useful tools to define the role integrin α6Aβ4A and suggest that laminin isoforms have distinctly different capacities to promote cell adhesion and signaling via integrin α6Aβ4A.
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Introduction

The biological effects of basement membranes are to a large extent due to laminins, large heterotrimers composed of α, β, and γ chains [1]. Combinations of chains can generate at least 14 different laminin isoforms, which are expressed in a tissue-specific manner [2–4].

To date, at least nine integrins have been shown to recognize various laminin isoforms. Six of these (α1β1, α2β1, α3β1, α6β1, α7β1, α9β1) belong to the β1 integrin subfamily. The α6 integrin is unique among the α-chains in that it also associates with β4, and integrin α6β4 is a major receptor for several laminins. Finally, integrins αvβ3 and αvβ5 recognize the α5 chain of laminin-10/11 [5–8]. A major non-integrin laminin receptor with particularly high affinity for laminin-1 and -2/4 is dystroglycan [9]. Laminin–integrin interactions stimulate several signal transduction cascades [10], but due to the multitude of ligands, receptors, and even splice variants of the receptors that may signal differentially [11], there are still several open issues. Gene targeting approaches in mice provide compelling evidence that laminins and their receptors have discrete functions already during embryogenesis [1,9,12,13], but the interpre-

tation of the mechanistic details of the generated phenotypes is complicated.

Cells can be confronted with individual laminin isoforms in vitro, but a large amount of laminin-binding β1 integrins are usually produced by cultured cells, and therefore the role of individual receptors may be difficult to dissect. Genetic approaches can be used to generate cells expressing a more limited or defined set of receptors [14–17]. GD25 cells lacking all β1 integrins have been successfully used to define the role of integrin αvβ3 for assembly of fibronectin or vitronectin [16,17]. Similar approaches should be particularly useful to clarify the roles of major non-β1 integrin laminin receptors such as integrin α6β4 and dystroglycan. However, previous studies have shown that β1 integrin-deficient GD25 cells bind poorly to laminin-1 [16], even though they express some form of integrin α6β4 [18]. We reasoned that GD25 cells might be used to dissect the role of non-β1 laminin receptors in cellular interactions with other laminin isoforms.

We found that laminin-1 was the only isoform of the four tested laminins that bound poorly to GD25 cells. Thus, GD25 cells bound well to laminins-2/4, -5, and -10/11. The initial GD25 cell binding to each of these laminins could be blocked by antibodies against integrin α6. This subunit can associate either with the β1 or the β4 subunit. Several splice variants of these subunits are known [11]. Because GD25 cells lack β1 integrin and we here demonstrate expression of only the α6A and β4A variants in these cells, it follows that initial GD25 cell binding to laminins-2/4, -5, and -10/11 is mediated by the α6Aβ4A integrin variant. Remarkably, only two of the three adhesive laminin isoforms initiated cell spreading, proliferation, and activation of extracellular signal-regulated kinase (ERK). Our results suggest fundamental differences between laminin isoforms as ligands mediating integrin α6β4 functions. We also provide evidence that GD25 cells express a 120-kDa form of dystroglycan and that GD25-derived dystroglycan can recognize laminin-1, -2/4, and -10/11.

Materials and methods

Proteins and antibodies

Mouse laminin-1 (EHS laminin) was purchased from Sigma (St. Louis, MO), and the human laminin-2/4 (merosin), plasma fibronectin, and the partially degraded human laminin-10/11 mixture [19] from GIBCO Life Technologies (Gaithersburg, MD). Laminin-5 was purified from the conditioned medium of the human gastric carcinoma line MKN45 by immunoaffinity chromatography using affinity-purified antibody against human laminin γ2 chain [20]. Based on molecular mass estimations, the laminin-5 preparation contains the expected chains, a 160- to 165-kDa α3A variant lacking the two last laminin globular (LG) domains, a 140-kDa β3 chain, and a 105-

kDa γ2 chain proteolytically cleaved in its N-terminus (Fig. 1). Intact laminin-10/11 was purified to homogeneity from conditioned medium of human lung adenocarcinoma A549 cells by immunoaffinity chromatography using monoclonal antibody against human laminin α5 chain as described [21].

The anti-mouse integrin α6 monoclonal antibody (GoH3) was a gift from Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam). The anti-α-dystroglycan monoclonal antibody IIH6, anti-dystrophin (XIXC2), and anti-β-dystroglycan polyclonal antibody AP83 were those described [22]. The anti-αvβ3 antibody LM 609 was from Chemicon International Inc. (Temecula, CA). The antihuman laminin β1 chain monoclonal antibody (114DG10B11) was a gift from Dr. I. Virtanen (University of Helsinki). The anti-mouse integrin β4 (346-11A) and anti-laminin β2 chain (C4) monoclonal antibodies were purchased from Pharmingen (San Diego, CA) or Developmental Studies Hybridoma Bank (University of Iowa). The antihuman laminin α5 chain monoclonal antibody 4C7 [23] was purchased from GIBCO Life Technologies. Monoclonal antibody 15H5 against human laminin α5 was described previously [21]. The monoclonal antibody (2B10) recognizing human laminin α3 subunit was produced by fusion of SP2/0 mouse myeloma cells with splenocytes from mice immunized with human laminin-5 purified by immunoaffinity chromatography using affinity-purified rabbit polyclonal antibody against human laminin γ2 chain [20]. Hybridomas were screened for reactivity with purified human laminin-5. The specificity of the antibodies was verified by immunoblotting and immunoprecipitation. Because the clone 2B10 was highly reactive with purified human laminin-5 (α3β3γ2) but not with a heterodimer of β3γ2 secreted by HT1080 fibrosarcoma cells, it recognized human laminin α3 chain (Sanzen,
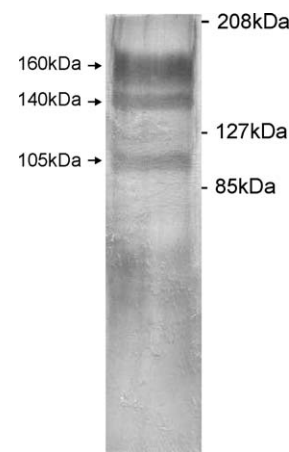

Fig. 1. Determination of the molecular masses of the three chains of laminin-5, purified from the medium of MKN45 human gastric carcinomas cells by immunoaffinity chromatography using rabbit polyclonal antibody against γ2 chain. The purified protein trimer was subjected to SDS-PAGE under reducing conditions and stained with silver. Molecular markers are denoted in kDa to the right, and the estimated molecular masses of the laminin-5 chains to the left.

N. and Sekiguchi, K., unpublished data). Anti phosphotyrosine antibody RC-20 was from BD Biosciences (Erembodegem, Belgium).

Cell line and culture conditions

Mouse GD25 β1-null cells or GD25 cells transfected to express β1A-integrin [16,24] were grown in DMEM supplemented with 25 mM HEPES (pH 7.2), penicillin, streptomycin sulfate, fungizone, and 10% FBS (GIBCO Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell adhesion assays

Cell adhesion assays were performed as described previously [15]. To exclude effects of differential stickiness of proteins to the surface, 96-well immunoplates with high capacity to absorb proteins were used (Nunc, Roskilde, Denmark). Plates were incubated with laminin-1, -2/4, -5, -10/11, or fibronectin at 37°C for 1 h, and then blocked with PBS containing 1% bovine serum albumin for another hour at the same temperature. The cells were suspended in serum-free DMEM at a density of 4×10^5 cells/ml, and 50-μl aliquots of cell suspension were added to wells coated with varying concentrations of laminin isoforms or fibronectin, followed by incubation at 37°C for 1 h. Adhered cells and spread cells were stained with Giemsa and counted under a microscope. One square millimeter was counted in each well, three wells per assay, and at least three assays per experiment. Cells were considered to have adapted a spread morphology when they had become flattened with the long axis more than twice the diameter of the nucleus. Photographs of adhering cells were taken by a 1.2 million pixel digital camera system (Pixera corporation, Los Gatos, CA). For other cell adhesion assays, monoclonal antibodies against integrin α6 (GoH3), β4 (346-11A), α-dystroglycan (IIH6), and dystrophin (XIXC2) were individually preincubated with cells in serum-free DMEM at a density of 4×10^5 cells/ml at room temperature for 15 min. The preincubated cells were transferred onto plates precoated with laminin isoforms or fibronectin and further incubated at 37°C for 20 or 60 min. Attached cells were counted as described above.

Cell lysates, immunoprecipitation, and immunoblotting

For immunoprecipitation, GD25 cells were removed from culture flasks with PBS containing 10 mM EDTA and labeled by ECL protein biotinylation module (Amersham Biosciences, Buckinghamshire, UK). Labeled cells were washed twice with PBS and lysed with lysis buffer (20 mM Tris–HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1% NP-40) containing a protease inhibitor cocktail (Sigma). Lysates were clarified at 13,000 rpm and precleaned with Protein G-Sepharose (Amersham Bioscien-

ces). Protein G Sepharose and anti-mouse integrin α6 (GoH3) or β4 (346-11A) monoclonal antibodies were then added to the lysates. After incubation for 2 h at 4°C, the immune complexes were washed three times with lysis buffer and dissolved in SDS sample buffer. Precipitated proteins were separated on SDS-PAGE and transferred to PVDF membrane. Separated proteins were visualized with streptavidin horseradish peroxidase and ECL Western blotting detection reagents (Amersham Biosciences). For immunoblotting of integrin β4, cell lysates and precipitated proteins were separated on SDS-PAGE under reducing condition and transferred to PVDF membrane. Proteins on the membrane were reacted with monoclonal antibody against β4 followed by incubation with rabbit anti-rat IgG antibody conjugated with horseradish peroxidase (Sigma). Bound antibodies were visualized as described above.

For immunoblotting of phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2), GD25 cells grown to 80–90% confluency were washed twice with PBS and then incubated in medium without serum for 24 h. Cells were dissociated with trypsin/EDTA. Trypsin treatment was stopped by adding soybean trypsin inhibitor (1 mg/ml). Cells were washed with serum-free DMEM, adjusted to 1×10^6 cells/ml, and seeded on substratum coated with laminin isoforms or fibronectin. Following incubation for 1 h, cells were directly lysed with $2\times$ SDS sample buffer containing 2-mercaptoethanol. Whole lysates were subjected to SDS-PAGE and transferred to PVDF membranes. Proteins on the membrane were reacted with a phosphospecific ERK1/2 antibody (New England BioLab, Inc.) followed by incubation with secondary IgG antibody conjugated with horseradish peroxidase. Bound antibodies were visualized as described above. PVDF membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 0.1 M 2-mercaptoethanol) for 1 h at 60°C and reprobed with anti-ERK1/2 polyclonal antibody (New England BioLabs Inc.). To examine the effects of Wortmannin (Sigma) or PD98059 (New England BioLabs Inc.) on ERK activation, cells were treated with the pharmacological reagents for 10 min in suspension before plating on substrates. Signal intensities were quantified with the Fujifilm LAS 1000 image analyzer system and the Science Lab 2001 Image Gauge software version 4.0 (Fuji Photo Film Co, Japan).

To detect phosphorylated integrins, GD25 cells grown to 80–90% confluency were treated as described above and seeded on substratum coated with laminin-5 or laminin-10/11. Following incubation for 1 h, cells were washed twice with PBS and lysed with lysis buffer containing a protease inhibitor cocktail. Immunoprecipitation using anti-integrin β4 antibody (346-11A) followed by immunoblotting with the anti-phospho-tyrosine antibody RC-20, stripping, and reprobing with the anti-integrin β4 antibody was performed according to the above-described procedure.

RT-PCR

Total RNA was extracted by RNeasy Mini Kit (QIA-GEN, Hilden, Germany) and cDNA was synthesized using a First Strand Synthesis Kit (Amersham). The integrin α6 subunit was amplified using a previously described set of primers [25]. For integrin α6A and B subunits primer pair 1156/1157: 1156 (5′-ATCTCTCGCTCTTCTTTCCG-3′) and 1157 (5′-GACTCTTAACTGTAGCGTGA-3′). The integrin β4 subunit was amplified using three sets of primers of which two sets of primers were previously described [26]. Primer pair 679/680: 679 (5′-CGAGAGAGAGGCTATCA-TCAA-3′) and 680 (5′-GGACGTGCTCAGCACTCGGT-3′) for integrin β4C and primer pair 681/682: 681 (5′-CCACTCTCTGACACGCACAG-3′) and 682 (5′-TAGG-GATGTTGAGCCGATGCA-3′) for insert of integrin β4B. A set of previously described primers for integrin β4D was used [27]. MITGB409/MITGB412, nucleotides 5070–5158 of mouse integrin β4A: MITGB409 (5′-GGAGA-TATCCTTGGCTACCTG-3′) and MITGB412 (5′-TCAGGGTTGTCTCCGTCCAC-3′). PCR products were amplified in DNA engine RTC-200 (MJ Research, Incline Village, NV) in the presence of AmpliTaq Gold (Perkin-Elmer Life Sciences, Boston, MA). The conditions were: 95°C for 9 min, 40 cycles at 95°C for 1 min, 53°C (1156/1157), 55°C (679/680), or 57°C (681/682 and MITGB409/MITGB412) for 1 min, 72°C for 1 min, 72°C for 10 min. PCR products were analyzed by electrophoresis using 1.5% or 2.0% agarose gels.

Cell proliferation assay

A colorimetric Bromodeoxyuridine (BrdU) ELISA assay (Boehringer Mannheim, Mannheim, Germany) was used to measure cell proliferation. Cells were cultured in serum-free medium for 24 h. After cell dissociation, cells were adjusted to 1×10^6 cells/ml in serum-free DMEM containing BrdU. Cell suspension (100 μl) was seeded on substratum coated with Poly-L-lysine, laminin isoforms, or fibronectin. Following incubation for 3 h, incorporation of BrdU was detected according to the manufacturer's protocol.

Affinity chromatography on laminin columns and overlay assay

Laminin-2/4 or anti-mouse integrin β4 monoclonal antibody (346-11A) was coupled to CNBr-Sepharose 4B (Amersham). Columns were equilibrated with running buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM octyl-β-D-glucopyranoside (Sigma)). Biotin-labeled GD25 cells were lysed in running buffer containing 200 mM octyl-β-D-glucopyranoside and protein inhibitor cocktail and centrifuged at 13,000 rpm for 30 min. Cell lysates were passed over the anti-mouse integrin β4-Sepharose column to remove integrin α6β4 and then applied to the laminin-2/4-Sepharose column. The lami-nin-2/4-Sepharose column was washed with running buffer and eluted with running buffer containing 10 mM EDTA. For detection of biotin-labeled proteins and immunoblot-ting, fractions were separated on SDS-PAGE and transferred to PVDF membranes. Proteins on the membrane were reacted with streptavidin horseradish peroxidase or antibody against dystroglycan followed by incubation with secondary antibody conjugated with horseradish peroxidase. For overlay assays, biotin-labeled laminin-1 was incubated with membranes at 1 μg/ml overnight at 4°C.

Laminin-5 (100 μg) was bound to Sepharose-coupled anti-laminin γ2 polyclonal antibody. Laminin-10/11 (100 μg) was bound to Sepharose-coupled monoclonal antibody 114DG10B11 against laminin β1 chain and C4 monoclonal antibody against laminin γ2 chain. One milligrams of the antibodies and 1 ml of CNBr-Sepharose 4B were used for the coupling reactions (Amersham). Affinity chromatography was performed as described above. Protein concentrations were determined by the dye method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Results

Adhesion and spreading of β1 integrin-null GD25 cells on laminin isoforms

As reported previously [16], the GD25 β1 integrin-deficient cells attached and spread well on surfaces coated with 10 μg/ml of fibronectin, but not on surfaces coated with 10 μg/ml of laminin-1 (Fig. 2A). However, they readily attached on surfaces coated with 10 μg/ml of laminin-2/4, -5, or -10/11 (Fig. 2). The cells also spread on laminin-2/4 or -10/11 with no obvious differences in morphology (Fig. 2A). In sharp contrast, GD25 cells bound to laminin-5 acquired a rounded morphology and did not spread at all (Fig. 2A). GD25 cells transfected with β1 integrin attached and spread on both laminin-1 and laminin-5 (Fig. 2B), showing that the used laminin preparations were fully biologically active reagents for a control cell line.

In the initial morphological assays (Fig. 2), we used intact laminin-10/11 derived from culture supernatant [21]. Another laminin preparation, derived from human placenta by mild pepsin digestion and purification using antibody 4C7 [28] detecting laminin α5 [23], is a mixture of intact and partially degraded laminin-10 and -11 [19]. Because this preparation is widely used, we compared its adhesive activity with that of intact laminin-10/11 [21]. Immunoblotting of laminin α5 chain under nonreducing conditions showed that the molecular mass of intact laminin-10/11 was approximately 800 kDa, while the molecular mass of placenta-derived, pepsin-digested lam-inin-10/11 was approximately 650–750 kDa (Fig. 3A). Despite this difference, GD25 cells attached equally well to both preparations (Fig. 3B). The commercial pepsin-digested laminin-10/11 was slightly more active in
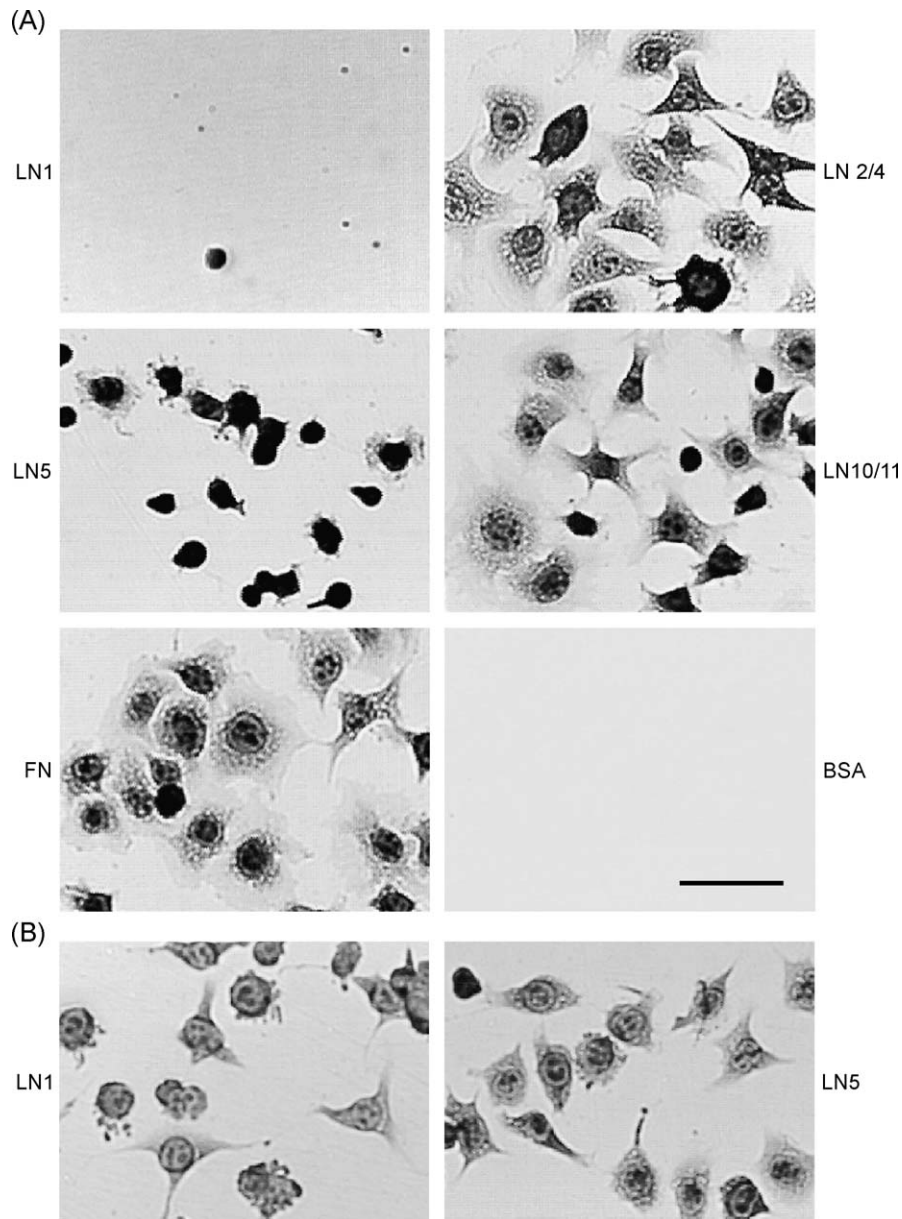
Fig. 2. Morphology of GD25 cells lacking the integrin β1 subunit on substrates coated with laminin isoforms or fibronectin. (A) Microtiter plates were precoated with 10 μg/ml of laminin-1 (LN1), laminin-2/4 (LN2/4), laminin-5 (LN5), laminin-10/11 purified from conditioned medium of human lung carcinoma (LN10/11), or 5 μg/ml of fibronectin (FN) and blocked with PBS containing 1%BSA. GD25 cells were incubated for 60 min at 37°C. Cells were rinsed with serum-free DMEM, fixed in methanol, stained with Giemsa, and photographed. (B) Microtiter plates were precoated with 10 μg/ml of laminin-1 or -5, and GD25 cells transfected with β1A integrin were incubated and analyzed as in A. Scale bar: 50 μm.

Fig. 3. Quantification of attachment and spreading of GD25 cells on laminin isoforms or fibronectin. (A) Intact laminin-10/11 purified from conditioned medium of human lung carcinoma or pepsin-digested laminin-10/11 purchased from GIBCO was separated on a 7.5% SDS polyacrylamide gel under nonreducing condition and immunoblotted with monoclonal antibody 15H5 against antihuman laminin α5 chain. The molecular mass was estimated by using the migration of EHS laminin-1 under nonreducing conditions (800 kDa) and reducing conditions (400 and 200 kDa) as markers. (B) Attachment (empty column) and spreading (full column) of GD25 cells to intact laminin-10/11 and pepsin-digested laminin-10/11. GD25 cells were incubated in 96-well microtiter plates precoated with 10 μg/ml of intact laminin-10/11 and pepsin-digested laminin-10/11 for 60 min at 37°C. Cells adhering to the plates were stained and counted under microscope. The spreading of GD25 cells was quantified as described in Materials and methods. Each column represents the mean of triplicate assays. Cell-attachment (C) and spreading (D) of GD25 cells to laminin isoforms and fibronectin. GD25 cells were incubated in 96-well microtiter plates precoated with increasing concentrations of laminin-1 (open circle), laminin-2/4 (open square), laminin-5 (closed circle), laminin-10/11 (closed square), or fibronectin (open triangle) for 60 min at 37°C. (E). Influence of the β1 integrin subunit on adhesion and spreading of GD25 cells on laminin-5. Attachment (open symbols) and spreading (closed symbols) of integrin β1-deficient GD25 cells (circle symbols) and GD25 cells expressing integrin β1A (square symbols). Each point represents the mean of triplicate assays. Bars indicate standard deviation.
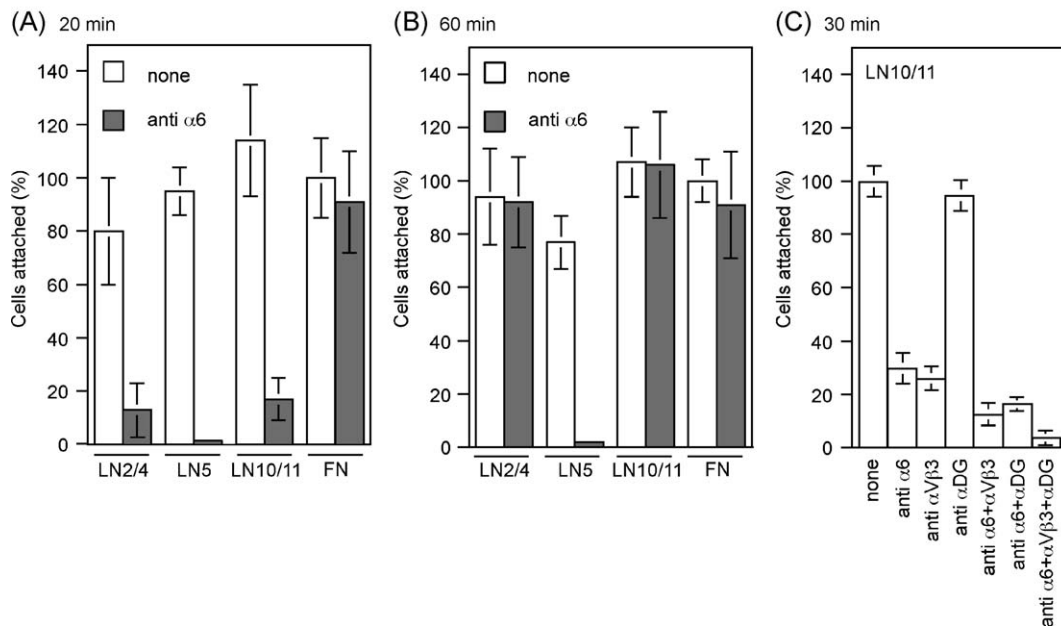
(A)

1. Purified LN10/11
2. Pepsin digested LN10/11

(B)

(C)

(D)

(E)
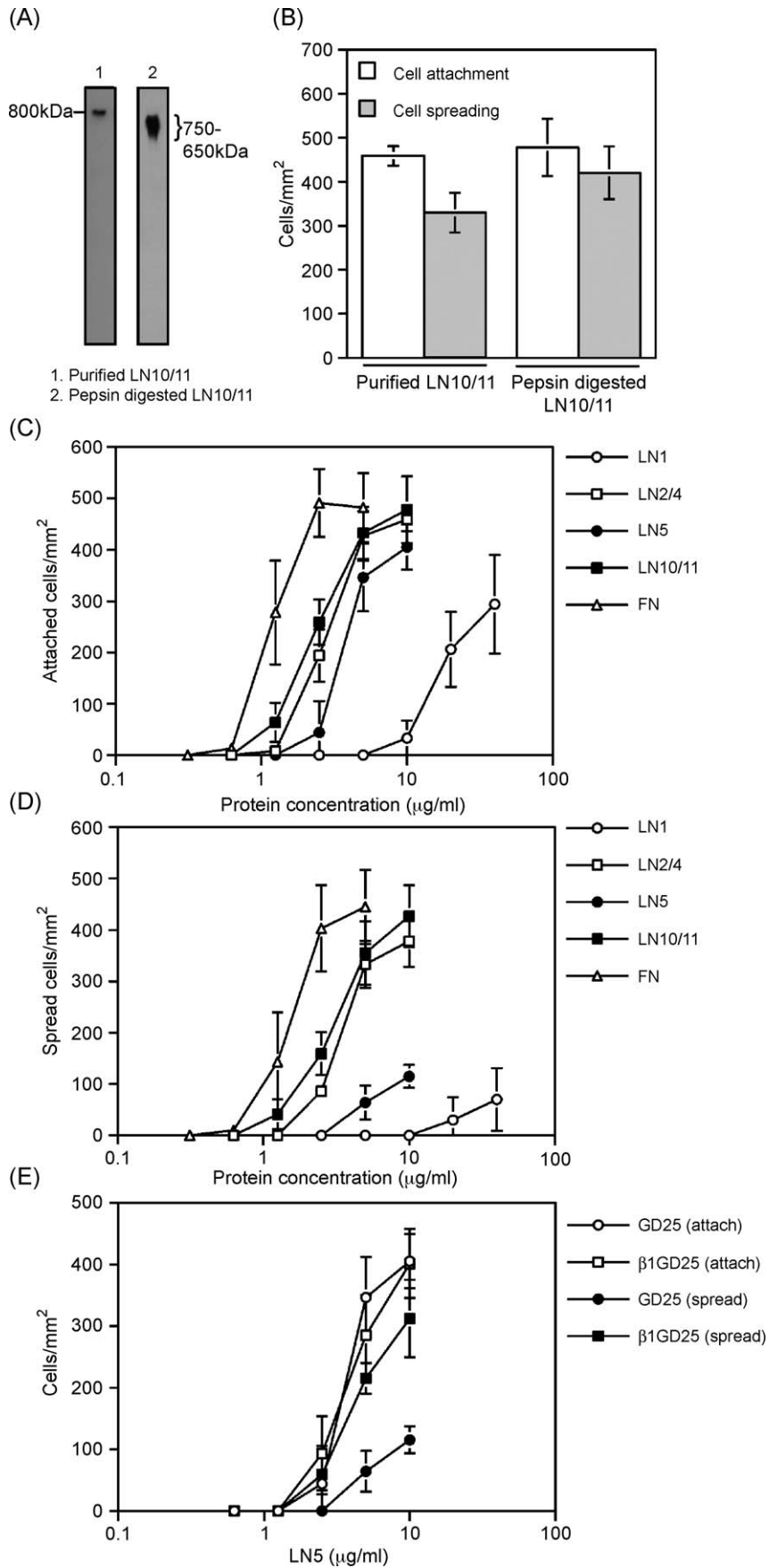
Fig. 4. Role of the integrin α6 subunit for adhesion of GD25 cells to laminin isoforms. Cells were preincubated with anti-α6 antibody (GoH3) and then incubated in 96-well microtiter plates coated with 10 μg/ml of laminin-2/4 (LN2/4), laminin-5 (LN5), laminin-10/11 (LN10/11), or 5 μg/ml of fibronectin for 20 min (A) or 60 min (B) in the absence or presence of GoH3 antibody at 10 μg/ml. The number of cells adhering to fibronectin in the absence of GoH3 was taken as 100%. Each column represents the mean of triplicate assays. In (C), cells were incubated on laminin-10/11 for 30 min in the presence of 10 μg/ml of antibody GoH3, LM 609 against αvβ3, IIH6 against dystroglycan, or in the presence of combinations of GoH3-LM 609 or GoH3-IIH6. Bars indicate standard deviation.

stimulating cell spreading (Fig. 3B). Both preparations were thus used in further studies here, both called laminin-10/11.

Quantitative analysis of cells adhering to surfaces coated with increasing concentrations of proteins showed that fibronectin was the most active in mediating adhesion of GD25 cells, followed by laminin-2/4 and -10/11. Laminin-5 was almost as adhesive as laminin-2/4 or -10/11, but laminin-1 was still a poor adhesive substrate at 20 μg/ml and some measurable binding could only be noted at significantly higher coating concentrations (Fig. 3C). Quantification of cell spreading revealed a distinct difference between the properties of laminin-5 and the two other adhesive laminins. Thus, most of the bound GD25 cells spread on fibronectin and on laminin-10/11 or laminin-2/4, but very few cells spread on laminin-5 (Fig. 3D) despite the distinct binding of these cells to laminin-5 (Fig. 3C). GD25 cells transfected with β1 integrin did not adhere more efficiently on laminin-5 than β1 integrin-deficient GD25 cells, whereas GD25 cell spreading on laminin-5 was significantly enhanced by transfection of the β1 integrin subunit (Fig. 3E).

Integrin α6β4 as a major GD25 cell adhesion receptor for three laminin isoforms

Integrin α6β4 has been reported to be a receptor for laminin-1, -2/4, and -5 [5,29,30]. K562 cells transfected

with α6 and β4 integrin-expressing constructs bind laminin-10/11, but the presence of β1 integrins on K562 cells made definite conclusions difficult [15]. To firmly establish that integrin α6β4 can attach cells to laminin isoforms in the absence of β1 integrins, we examined whether the anti-integrin α6 monoclonal antibody (GoH3) can block GD25 cell adhesion to laminin 2/4, -5, or -10/11. Adhesion onto surfaces coated with laminin-2/4, -5, or -10/11 was inhibited by the integrin α6 antibody in assays carried out for 20 min (Fig. 4A), but not with the dystroglycan IIH6 antibody (data not shown). In assays carried out for 60 min, the integrin α6 antibody still blocked cell adhesion to laminin-5. In contrast, the cells could overcome the inhibitory affect of the integrin α6 antibody when kept on laminin-2/4 or -10/11 for 60 min (Fig. 4B). Two possible explanations for this are receptor recycling [31] leading to reappearance of integrin α6β4 not bound to the antibody, or presence of additional receptors. To study whether additional receptors contributed to GD25 cell binding to laminin-10/11, we tested combinations of antibodies. In 60-min assays, the combination of antibodies against α6 integrin, dystroglycan, or integrin αvβ3 did not block GD25 cell adhesion to laminin-10/11 (data not shown). However, in adhesion assays carried out for 30 min, anti-αvβ3 decreased adhesion to laminin-10/11 by about 75%. The combination of anti-α6/αvβ3, anti-α6/dystroglycan, and the triple combination anti-α6/αvβ3/dystroglycan reduced cell adhesion to laminin-10/11 slightly more efficiently than the antibody against integrin

α6 (Fig. 4C). This is in line with recent findings that integrin αvβ3 and dystroglycan can recognize laminin-10/11 [7,8,32,33].

No inhibitory effect on cell adhesion to laminin isoforms -2/4, -5, or -10/11 was observed by the anti-integrin β4 monoclonal antibody 346-11A (data not shown). There are no well-characterized blocking antibodies against mouse integrin β4. To confirm that the partner of integrin α6 subunit in GD25 cells is the integrin β4 subunit, immunoprecipitation and immunoblotting were performed. GD25 cells were cell surface biotinylated and subjected to immunoprecipitation with anti-integrin α6 monoclonal antibody. As shown previously for GD25 cells [18], the antibody precipitated a protein complex, which by SDS-PAGE was resolved into two bands of 140 and 200 kDa, typical for the α6 and β4 integrin subunits (Fig. 5A). Immunoblotting of integrin β4 confirmed that the immune complex precipitated by anti-integrin α6 antibody contained a 200-kDa integrin β4 subunit (Fig. 5B).

Alternative splicing and post-translational modifications can produce several forms of the α6β4 integrin with different biological properties. To date, two cytoplasmic variants of integrin α6 subunit, α6A and α6B, have been identified [11,25]. RT-PCR using the primer set 1156/1157 revealed a band of 556 bp that corresponds to α6A (Fig. 5C). Five distinct mRNA variants of integrin β4 subunit are known, each altering the cytoplasmic domain, designated β4A [34], β4B [35], β4C [36], β4D [27], and β4E [37]. The 679/680 and 681/682 primer sets span the region, which is alternatively spliced to produce variants β4A, β4B, or β4C [26]. RT-PCR using these two primer sets revealed bands of

319 and 259 bp that correspond to the β4A variant. The primer set MITGB409/MITGB412 was used to distinguish between the β4A and β4D variant that contains a 21-bp deletion [27]. RT-PCR using this primer set revealed a band of 89 bp, which corresponds to β4A (Fig. 5D). Sequencing of the PCR product identified the sequence as β4A (not shown). Because the predicted molecular mass of integrin β4E subunit is 107 kDa [38] and we only detected a single 200-kDa β4 band by immunoprecipitation (Fig. 5A), the possibility of integrin β4E subunit was excluded. Hence, of the many possible integrin α6β4 variants, GD25 cells express only integrin α6Aβ4A.

Dystroglycan as a non-integrin laminin receptor of GD25 cells

Adhesion assays suggested some involvement of dystroglycan and integrin αvβ3 in GD25 cell binding to laminin-10/11. GD25 cell integrin αvβ3 has been described [16], but dystroglycan has not been demonstrated from GD25 cells. Here we characterize GD25 cell dystroglycan and its laminin-binding properties. After biotinylation of the cell-surface, a cell lysate was passed over a β4 integrin antibody-Sepharose column to remove integrin α6β4 and then applied to a laminin-2/4 Sepharose column. Two biotinylated proteins of molecular masses 120 and 43 kDa were eluted by a buffer containing 10 mM EDTA (Fig. 6A), suggesting that a small 120-kDa form of α-dystroglycan and the 43-kDa β-dystroglycan had been eluted. Immunoblotting with IIH6 antibody against α-dystroglycan and AP83 antibodies against β-dystroglycan
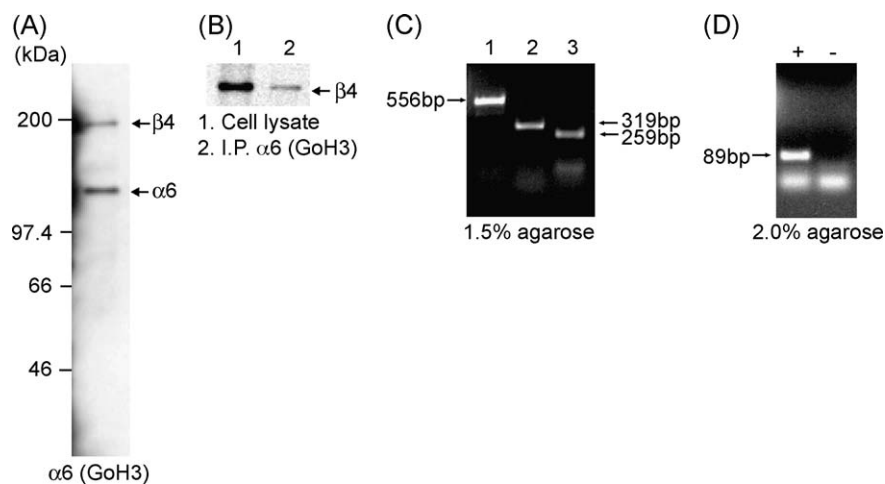

Fig. 5. Identification of the subunit associated with integrin α6 in GD25 cells lacking the integrin β1 subunit and splice variants of α6 and β4. (A) Cells were labeled with biotin and cell lysates were immunoprecipitated with monoclonal antibody against integrin α6 (GoH3). Immunoprecipitated proteins were separated on 7.5% gel under reducing conditions and transferred to PVDF membrane. Biotinylated proteins were detected as described in Materials and methods. (B) Cell lysates and immunoprecipitated proteins were subjected to SDS-PAGE under reducing condition and immunoblotted with an anti-integrin β4 antibody. (C) Analysis of alternative splice variants of integrin α6β4 expressed in GD25 cells. RT-PCR analysis using four different primer sets described in Materials and methods. The 1156/1157 (lane1) primer set spans the region that is alternatively spliced to produce integrin α6A and α6B subunits. The 556-bp band corresponds to integrin α6A subunit [25]. (Lane 2) The 679/680 (lane2) and 681/682 (lane3) primer sets span the region, which is alternatively spliced to produce variants β4A, β4B, or β4C [26]. The amplified 319- and 259-bp bands correspond to β4A, respectively. (D) The MITGB409/MITGB412 primer sets span the region that is alternatively spliced to produce the β4D variant that contains a 21-bp deletion [27]. The amplified 89-bp band correspond to β4A. –, no template cDNA.
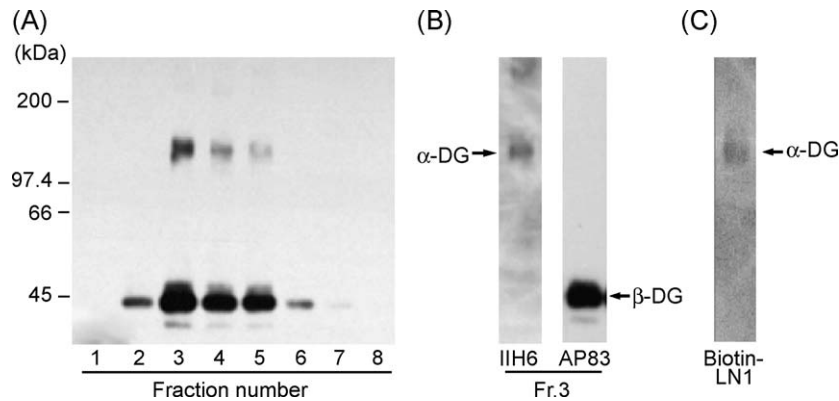
Fig. 6. Cell-surface proteins eluted from a laminin-2/4-Sepharose column. (A) Biotin-labeled GD25 cells were solubilized in lysis buffer containing 200 mM octyl-β-D-glucopyranoside and extract was passed through anti-integrin β4 antibody-Sepharose column. Flow through was applied to a laminin-2/4-Sepharose column. After washing, the column was eluted with buffer containing 10 mM EDTA. Fractions were separated on a 7.5% gel under nonreducing conditions and transferred to PVDF membrane. Biotinylated proteins were detected as described in Materials and methods. (B) Eluted proteins were also subjected to SDS-PAGE under nonreducing condition and immunoblotted with IIH6 antibody against α-dystroglycan or AP83 antibody against β-dystroglycan. (C) Detection of α-dystroglycan by a biotin–laminin-1 overlay assay. Biotin-labeled laminin-1 at 1 μg/ml in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$ was applied onto the same membrane as in B.

confirmed that the bands of 120 and 43 kDa contain α- and β-dystroglycan, respectively (Fig. 6B). The ability of GD25 cell-derived α-dystroglycan to bind other laminins was demonstrated by biotin–laminin-1 overlay of the same membrane. Biotin-labeled laminin-1 detected a band of 120 kDa (Fig. 6C).

It has been reported that dystroglycan recognizes laminin-10/11 but not laminin-5 [32,33], although it was predicted based on structural analyses that neither of these two variants should bind dystroglycan [39]. We therefore compared binding of GD25 cell dystroglycan to laminin-5 and -10/11 using affinity chromatography. Extracts of GD25 cells were applied either to a laminin-5- or -10/11 column, eluted by EDTA, run on a gel, and immunoblotted with AP83 β-dystroglycan antibody. This revealed that most of the dystroglycan in the cell extract bound to the laminin-10/11 column and was eluted with buffer containing EDTA. In contrast, only minimal binding of dystroglycan to the laminin-5-column was observed (Fig. 7). The laminin-5 preparation used in this study was composed of 160- to 165-, 140-, and 105-kDa subunits (Fig. 1). The 160- to 165-kDa band corresponds to the laminin α3A variant lacking the LG4-5 domains, the 140-kDa band to laminin β3 chain, and the 105-kDa band to the proteolytically cleaved γ2 chain [40,41]. Based on the molecular mass, we conclude that the laminin α3A chain lacks the LG4-5 modules. The corresponding LG modules are responsible for dystroglycan binding in some other laminins [33,39]. We can therefore not exclude the possibility that nonprocessed forms of laminin-5 bind dystroglycan. However, structural studies have predicted that none of the LG domains of laminin α3, the α-chain of laminin-5, should show high-affinity binding to dystroglycan [39]. Finally, it is important to note that proteolytic processing of laminin-5 is considered to be a physiological in vivo process.
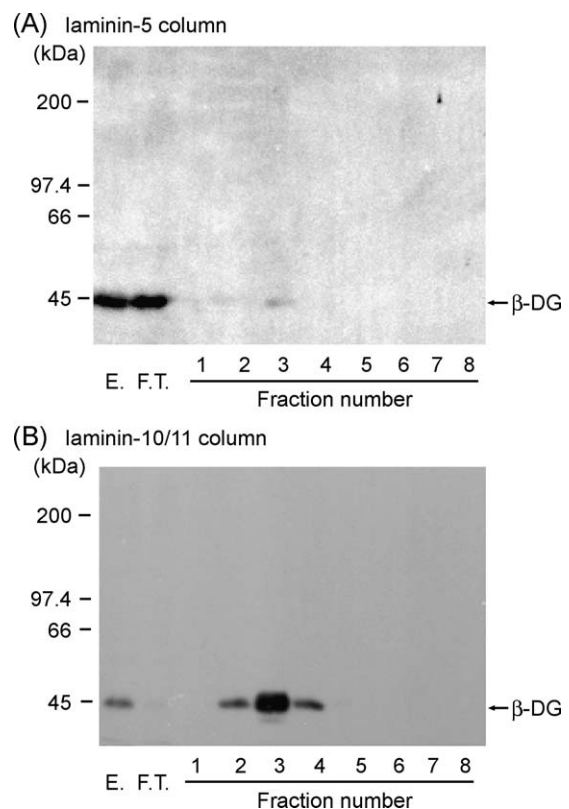

Fig. 7. Binding of dystroglycan to laminin-5 and laminin-10/11. One hundred micrograms of purified laminin-5 or laminin-10/11 was bound to 0.8 ml of Sepharose-coupled anti-laminin γ2 polyclonal antibody or to Sepharose-coupled anti-laminin β1/2 monoclonal antibodies. GD25 cells were solubilized in lysis buffer containing 200 mM octyl-β-D-glucopyranoside and extracts were passed over a laminin-5- column (A) or laminin-10/11-column (B). After washing, columns were eluted with buffer containing 10 mM EDTA. Fractions were separated on a 7.5% gel under nonreducing conditions and transferred to a PVDF membrane. Eluted proteins were subjected to SDS-PAGE under nonreducing conditions and immunoblotted with anti-β-dystroglycan antibody AP83.

Cell proliferation and MAP-kinase activation mediated by adhesion of GD25 cells to laminin isoforms

It is well known that extracellular matrix components can control cell growth and survival. To investigate whether growth can be stimulated by laminins in the absence of β1 integrins, incorporation of bromodeoxyuridine (BrdU) into DNA was measured. Quiescent cells were detached and held in suspension or replated on wells coated with laminin isoforms, fibronectin, or poly-L-lysine for 3 h. In cells plated on laminin-2/4, laminin-10/11, and fibronectin, incorporation of BrdU was enhanced, while a similar enhancement was not seen in cells plated on poly-L-lysine or laminin-5 (Fig. 8).

Cell proliferation induced by attachment to matrix components in vitro correlates both with cell spreading and activation of the mitogen-activated protein (MAP) kinase family [42,43]. We therefore investigated whether two members of the MAP-kinase family extracellular signal-regulated kinases 1 and 2 (ERK1/2) are stimulated by laminin isoforms in GD25 cells. Quiescent cells were detached and held in suspension, or replated on wells coated with laminin isoforms or fibronectin for 60 min. In cells plated on laminin-2/4 and on either one of the two laminin-10/11 preparations, phosphorylated forms of ERK1/2 increased markedly (2.5–3 times) in comparison to cells maintained in suspension. Only slight activation (1.8 times increase) was observed in cells replated on fibronectin, while no significant activation was observed in cells replated on laminin-1 or laminin-5 (Fig. 9). The kinetics of ERK1/2 activation was examined at different times after plating the cells on laminin-2/4 or laminin-10/11. Significant activation of ERK1 was detected at 30 min and sustained for over 120 min (data not shown).

GD25 cells used the same receptor, integrin α6β4, for initial adhesion to laminin-2/4, -5, and -10/11. Never-

Fig. 9. ERK1/2 activation in cells attached to laminin isoforms or fibronectin. Quiescent GD25 cells were either maintained in suspension or plated on wells coated with 40 μg/ml of laminin-1, 10 μg/ml of laminin-2/4, laminin-5, laminin-10/11 (1; intact laminin-10/11, 2; pepsin-digested laminin-10/11), or 5 μg/ml of fibronectin in serum-free medium for 60 min. SDS-PAGE sample buffer containing 2-mercaptoethanol was added to each well and cells lysed. Equal amounts of whole cell lysates were subjected to SDS-PAGE, activated ERK1 and ERK2 were detected by immunoblotting with phospho-ERK1/2-specific polyclonal antibody (upper panel). The same membrane was stripped, and total ERK1/2 was detected by using ERK1/2 polyclonal antibody (middle panel). Quantitative measurements of the phosphorylation status of ERK1/2. Phosphorylation of ERK1/2 in cells kept in suspension was set as 1.0 (lower panel).

theless, GD25 cell binding to laminin-5 failed to induce spreading of or ERK activation in GD25 cells, whereas cell binding to laminin -2/4 or -10/11 induced these events. Integrin α6β4 signaling probably requires both localization of the receptor to focal contacts and tyrosine phosphorylation of the integrin subunits. We therefore examined whether integrin β4 was present in adhesion complexes in cells on the different substrates and whether it was phosphorylated. In cells plated on laminin-10/11, integrin β4 was present together with filamentous actin in focal adhesion structures, whereas in cells plated on laminin-5, no such structures could be detected (Fig. 10). Cells plated on laminin-10/11 also displayed tyrosine phosphorylation of integrin β4 as well as tyrosine phosphorylation of several proteins brought down by immunoprecipitation with anti-integrin β4 antibodies. One band corresponds in size to integrin α6, indicating phosphorylation also of this subunit. In sharp contrast, no tyrosine phosphorylation of integrin β4 was evident in cells plated on laminin-5 (Fig. 10).

We also examined the role of phosphoinositide 3-OH (PI-3) kinase and MEK for ERK activation by laminin-2/4 and -10/11. MEK is the only known immediate upstream regulator of ERK activation. As expected, the MEK
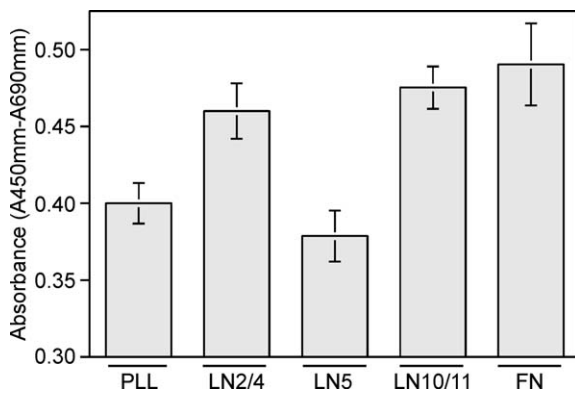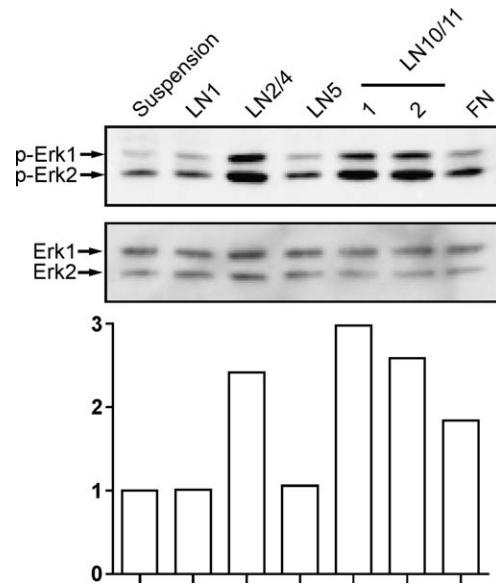

Fig. 8. Induction of DNA synthesis upon adhesion of GD25 cells on laminin substrata. Quiescent GD25 cells were replated onto wells coated 10 μg/ml of poly-L-lysine (PLL), laminin-2/4, laminin-5, laminin-10/11, or 5 μg/ml of fibronectin in serum-free DMEM containing 10 μM of BrdU. Cells were incubated for 3 h. BrdU incorporation into DNA was measured colorimetrically. Each column represents the mean of triplicate assays. Bars indicate standard deviations.
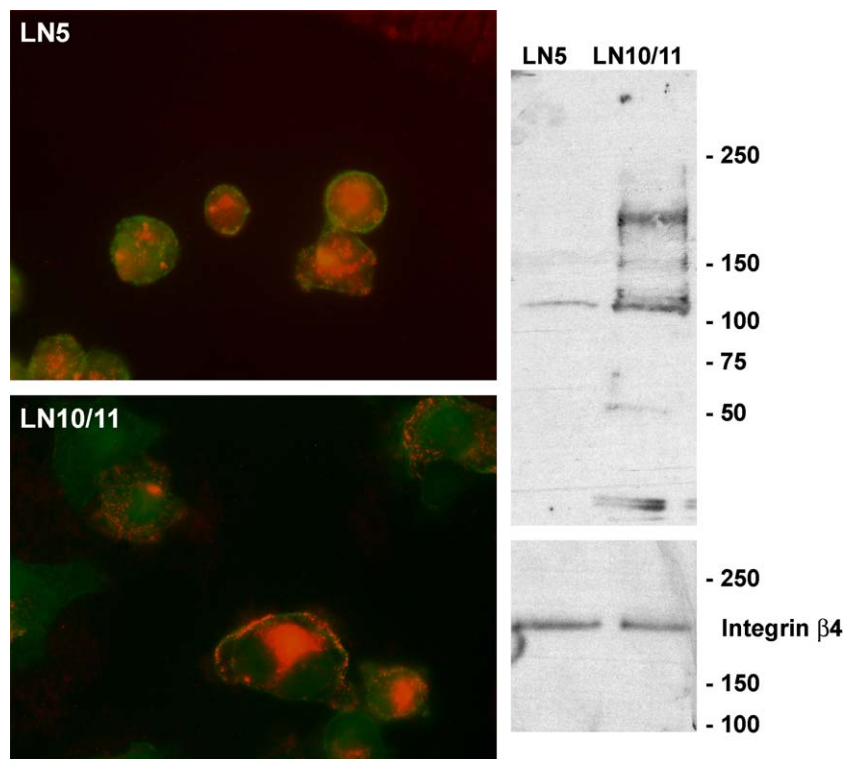
Fig. 10. Integrin β4 in GD25 cells plated on different laminin substrates. Localization of integrin β4 (red) and actin (green) as detected by mAb 346-11A and FITC-labeled phalloidin staining of cells plated on laminin-5 or laminin-10/11 (left panel). Whereas very little integrin β4 (red) can be seen on the surface of cells plated on laminin-5 (left top panel), brilliant staining for integrin β4 (red) can be seen on the surface of many cells plated on laminin-10/11 (left bottom panel). Immunoprecipitation of integrin β4 from cells plated on laminin-5 or laminin-10/11 followed by SDS-PAGE and immunodetection with antibody RC-20 reacting with phosphotyrosine residues (upper right panel) and mAb 346-11A detecting integrin β4 (lower right panel).

inhibitor PD98059 inhibited ERK1/2 activation. The level of phosphorylated ERK1/2 induced by laminins significantly declined also by the PI-3 kinase inhibitor Wortmannin, suggesting activation of ERK1/2 through PI-3 kinase (Fig. 11).

Discussion

The α6β4 integrin is an essential component of hemidesmosomes [30], and its interactions with laminins may enhance cancer cell survival [44,45]. Altered expression of
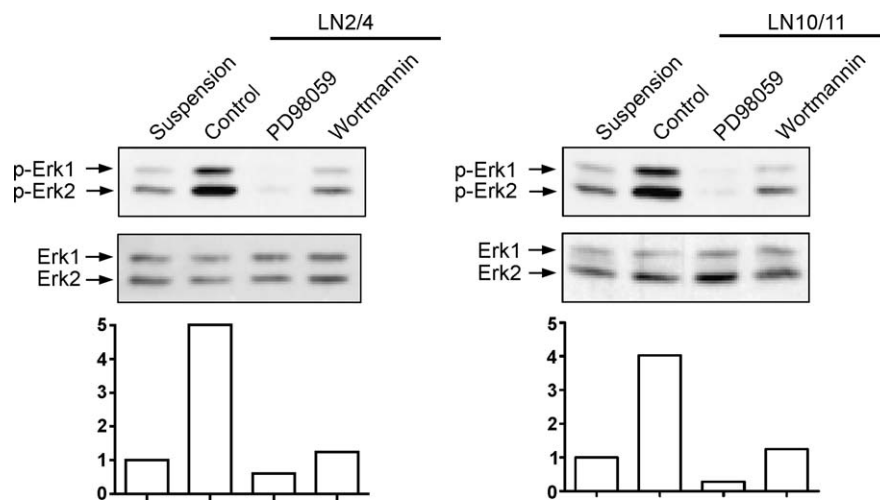

Fig. 11. Involvement of MEK and phosphatidynol 3-OH kinase in activation of ERK. Effects of MEK inhibitor PD980595 and PI3 kinase inhibitor Wortmannin on ERK1/2 activation induced by laminin-2/4 or laminin-10/11. A suspension of quiescent GD25 cells was incubated with 100 μM of PD98059 or 100 nM of Wortmannin for 10 min. The cells were maintained either in suspension or plated on wells coated with 10 μg/ml laminin-2/4 and laminin-10/11 in serum-free medium for 60 min. Activation of ERK1/2 was analyzed as described in Fig. 9.

several laminin isoforms is frequently seen in cancer and could significantly influence tumor invasion, angiogenesis, and metastasis [46]. It is therefore important to understand how laminin isoforms influence cells through this receptor. As shown here, β1 integrin-null GD25 cells can be used to dissect the role of different laminin isoforms as integrin α6β4 ligands. These cells bind poorly to laminin-1 [16], but in the present study we demonstrate distinct, integrin α6β4-dependent binding of GD25 cells to laminin-2/4, -5, and -10/11. Although the cells attached equally well to these three laminin isoforms using the same receptor, the cellular response to the binding varied significantly. Thus, the GD25 interaction with laminin-2/4 and -10/11 activated ERK and induced cell spreading and proliferation, whereas adhesion to laminin-5 did not induce any of these cellular responses.

Immunoprecipitation and blotting showed that GD25 cells produced the α6 subunit coupled to a 200-kDa β4 chain. Two splice variants of α6 and five β4 variants have been described. Because these may have different biological properties, we determined the nature of the splice variants expressed by GD25 cells. RT-PCR showed that GD25 cells produced only the α6A and β4A variant. These findings combined with antibody perturbation assays identified integrin α6Aβ4A as the main initial receptor for GD25 cell adhesion to all three laminins (laminin-2/4, -5, and -10/11). It is well established that integrin α6β4 is a receptor for laminin-1, -2/4, and -5 [5,6]. The current findings, taken together with previous studies with cells expressing both β4 and β1 integrins [15], clearly establish integrin α6β4 as one major receptor for cell binding to laminin-10/11.

Cell adhesion to extracellular matrix can in vitro activate numerous intracellular signaling cascades. It is well known that matrix-dependent ERK activation is frequently coupled to cell spreading [43] by not yet fully clarified mechanisms. Both laminin binding β1 integrins and integrin α6β4 have been linked to the ERK pathway [10,47], but it is not quite clear which receptors are used in laminin-mediated ERK activation and if all laminins can activate ERK. Different laminins may use different receptors for ERK activation. Our recent data [32] support the proposal of Wei et al. [48] that integrin α6Aβ1, but not α6Bβ1, is coupled to ERK activation. In both reports, the studied cells lacked the β4 subunit. In the present study, we instead used cells that lack β1 integrin and express the α6A subunit coupled to β4A. We found that integrin α6Aβ4A-dependent cell adhesion to laminin-2/4 and -10/11 correlated with cell spreading and ERK activation. A significant finding was that cell binding to laminin-10/11 stimulated integrin β4A phosphorylation. Nevertheless, one cannot conclude that laminin-2/4 and -10/11 mediated cell spreading and ERK activation was mediated by integrin α6Aβ4A. Co-receptors may be responsible for these events and could include integrin αvβ3, which was recently shown to interact with laminin-10/11 [7,8]. Another co-receptor is dystroglycan, but our recent data from other cell types suggest that laminin

binding to dystroglycan decreases integrin α6Aβ1-mediated ERK activation [32].

A most intriguing finding was that laminin-5 was completely inactive in stimulating cell spreading, integrin β4 phosphorylation, ERK activation, or cell proliferation, even though it was a cell-adhesive α6β4 ligand. This is in apparent conflict with some major proposals in the field. Based mainly on one system using keratinocytes [10], it has been proposed that ligation of integrin α6β4 to laminin-5 recruits Shc/Grb2-mSOS and subsequently activates the MEK-ERK kinase pathway through Ras [49–51]. Different laminins might use different α6β4 variants for ERK activation, and in future studies it will now be important to determine the α6β4 isoform expressed by the cells employed in various assays. Also, the type of laminin-5 used should be clearly indicated. An obvious possibility is that laminin-5 needs co-receptors to induce cell spreading and to activate the ERK pathway. Our findings that laminin-5 induced cell spreading of β1-integrin-expressing GD25 cells support this view. Moreover, it was recently shown that laminin-5 can activate ERK and cell spreading through integrin α3β1 [53]. GD25 cells or modified GD25 cells expressing various types of β1 or β4 integrins should be excellent tools to dissect these issues further.

To begin to understand some of the upstream pathways of laminin-induced ERK activation in GD25 cells, we tested some inhibitors of signaling molecules. In some cells, signaling along the Ras-ERK cascade is blocked at the level of the activation of either Raf or MEK in absence of attachment [54,55]. Integrins may remove this block, perhaps by activating Rac or PI-3 kinase [56]. In fact, laminin-10/11 has recently been shown to strongly activate Rac and Akt, downstream of phosphoinositide 3-OH (PI-3) kinase, in lung carcinoma cells expressing both β1 and β4 integrins [57], but the responsible receptors remain to be identified. In GD25 cells, the activation of ERK1/2 by laminin-2/4 and laminin-10/11 seems to be dependent on PI-3 kinase because the PI3 kinase inhibitor Wortmannin could block this activation. Our findings with GD25 cells indicate that both laminin-10/11 and -2/4 can activate ERK in the absence of β1 integrins, and suggest PI-3 kinase as one upstream regulator.

The exact role of dystroglycan for GD25 cell interactions with the laminins remains unclear, but several findings emphasize the importance to further study this laminin receptor in the GD25 model system. The dystroglycan antibody did not block the initial GD25 cell adhesion to laminins, and yet affinity chromatography revealed that both laminin 2/4 and -10/11 bound to a complex identified as dystroglycan. GD25 cells were shown to produce a 120-kDa α-dystroglycan subunit and a 43-kDa β-dystroglycan subunit. The GD25 cell-derived α-dystroglycan is smaller than those found in many adult tissues, but smaller isoforms with capacity to bind laminin-1 have been demonstrated, particularly in embryonic tissues [58]. The recognition of laminin-10/11 is notable because the laminin globular modules of the

α5 chain of laminin-10/11 lack the typical calcium-binding sites required for dystroglycan binding to α2 laminin [39]. However, the recognition of dystroglycan by laminin-10/11 is in agreement with recent findings using other approaches. Overlay assays have demonstrated that purified laminin-10/11 can bind muscle and kidney dystroglycan, and low affinity binding was also demonstrated in solid phase assays [32,33]. It is important to note that the function-blocking antibody IIH6 has been described to block interactions between laminin-1 and dystroglycan, and it does not necessarily act as an efficient blocking antibody of dystroglycan binding to other laminins. Therefore, it cannot be excluded that dystroglycan is involved in GD25 cell adhesion to laminins.

Considering the current results that GD25 cells express two major laminin-1 receptors—dystroglycan and integrin α6β4—it is surprising that GD25 cells bind so poorly to laminin-1. We could show that laminin-1 can bind to a 120-kDa form of dystroglycan isolated from GD25 cells in affinity chromatography assays, but neither this binding nor the presence of integrin α6β4 was sufficient to allow GD25 cells to adhere to laminin-1 in cell attachment assays. Transfection of β1 integrin to these cells leads to expression of several β1 integrins including α6β1 and confers cell adhesiveness to laminin-1 [16,59], confirming that integrin α6β1 is a major receptor for cell adhesion to laminin-1. However, several mouse and human hematopoietic cell types use α6β1 integrin to adhere to laminin-10/11, but nevertheless do not adhere to laminin-1 [60,61]. Binding of cells to laminin-10/11 is thus in several settings less dependent on co-receptors or some integrin activation processes than binding of cells to laminin-1.

In summary, laminin isoforms interact with integrin α6β4 in more distinct fashions than previously recognized. GD25 cells are thus powerful tools to dissect the role of laminin isoforms as ligands for integrin α6β4. Finally, we demonstrate that GD25 cells express dystroglycan, which can bind laminins. The lessons from the GD25 cell system could be relevant for other cell types. Integrin α6β4 is involved carcinoma progression [44,45]. In normal skin, laminin-5 and laminin-10/11 have essential but non-overlapping functions, with laminin-5 promoting epidermal adhesion and laminin-10 promoting epithelial development [30,62,63]. Our data raise the possibility that this in part is due to their differential ability to interact with integrin α6β4.

Acknowledgments

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