Journal of Cell Science 113, 869-876 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1037

Integrin binding specificity of laminin-10/11: laminin-10/11 are recognized by α3β1, α6β1 and α6β4 integrins

Yamato Kikkawa[1], Noriko Sanzen[1,2], Hironobu Fujiwara[2], Arnoud Sonnenberg[3] and Kiyotoshi Sekiguchi[1,2,*]

[1]Research Institute, Osaka Medical Center for Maternal and Child Health, 840 Murodo, Izumi, Osaka 594-1101, Japan
[2]Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[3]Division of Cell Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
*Author for correspondence (e-mail: sekiguch@protein.osaka-u.ac.jp)

Accepted 14 December 1999; published on WWW 14 February 2000

SUMMARY

Laminin-10/11, the laminin isoforms containing the α5 chain, are major components of basement membranes of many fetal and adult tissues. Laminin-10/11 purified from the conditioned medium of human lung carcinoma cells were potent in mediating adhesion of the carcinoma cells in an integrin α3β1-dependent manner. To further define the type(s) of integrins involved in cell adhesion to laminin-10/11, we examined the effects of a panel of function-blocking anti-integrin antibodies on the adhesion of different cell types to laminin-10/11. Although anti-integrin β1 antibody inhibited the adhesion of all cell types tested, anti-α3 antibody inhibited the adhesion of carcinoma and glioma cells but not fibroblastic cells. Adhesion of fibroblastic cells was inhibited, however, by a combination of anti-α3 and anti-α6 antibodies, suggesting that both α3β1 and α6β1 integrins function as laminin-10/11 receptors in these cells. To explore this possibility, we examined the adhesion of K562 leukemic cells transfected with integrin α3 or α6 subunit to laminin-10/11 or other laminin isoforms. Laminin-10/11 were potent adhesive ligands for both the α3β1 and α6β1 transfectants, whereas laminin-5 was the preferred ligand for the α3β1 transfectants. Upon stimulation with the activating anti-integrin β1 antibody, both transfectants became more adherent to the substratum regardless of the type of laminins coated, although their preference for laminin isoforms remained unaltered. K562 cells transfected with α6 and β4 subunits were also capable of adhering to laminin-10/11, indicating that integrin α6β4 is another receptor for laminin-10/11. Even with lung carcinoma cells, the α6-containing integrins partly contributed to adhesion to laminin-10/11 at higher coating concentrations, although non-integrin receptor(s) might also be involved under such conditions. These results indicated that laminin-10/11 are potent and versatile adhesive ligands in basement membranes capable of binding to both α3β1 and α6β1 integrins with high avidity and also to α6β4 integrin.

Key words: Integrin, Laminin-10/11, Cell adhesion

INTRODUCTION

Interaction of epithelial and endothelial cells with basement membranes plays a critical role in induction and maintenance of their differentiation phenotypes. The biological activities of basement membranes can in large part be attributed to laminins, the major glycoproteins of basement membranes throughout the vertebrate body. Laminins are disulfide-linked heterotrimers of three distinct but distantly related subunit chains, α, β and γ. Eleven genetically distinct laminin chains, i.e. α1-5, β1-3 and γ1-3, have been identified in human and mouse (Engvall and Wewer, 1996; Koch et al., 1999). Combination of these chains generates at least 12 different laminin variants, expression profiles of which differ significantly in different tissues and at different developmental stages (Engvall, 1993; Miner et al., 1997; Koch et al., 1999). Differences in biological functions among these laminin variants, however, are poorly understood.

By binding to cell-surface integrins, laminins promote cell adhesion and exert profound influences on proliferation, differentiation and survival of cells (Mercurio, 1995; Delwel and Sonnenberg, 1996). It is likely that the effects of laminins on cellular behavior depend on the ability of integrins to participate in intracellular signaling (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). To date, at least nine integrins have been implicated in binding to various laminin isoforms (Mercurio, 1995; Delwel and Sonnenberg, 1996), although their binding specificities to laminin isoforms appear to overlap.

Recently, we purified and characterized laminin-10/11 from the conditioned medium of human lung carcinoma cells (Kikkawa et al., 1998). Laminin-10/11 is composed of α5, β1/β2 and γ1 chains (Miner et al., 1997). The laminin α5 chain was initially cloned in mouse and found to be more related to the *Drosophila* laminin α chain than to other laminin α chains (Miner et al., 1995). The α5 chain is widely expressed in adult tissues including the kidney, lung, placenta, heart, skeletal muscle and pancreas (Sorokin et al., 1997; Durkin et al., 1997;

Miner et al., 1997), and mice lacking the α5 chain were found to be embryonically lethal (Miner et al., 1998).

Laminin-10/11 were highly active in mediating cell adhesion to the substratum with potency comparable to that of laminin-5 (Kikkawa et al., 1998). Adhesion of human lung carcinoma cells onto laminin-10/11 was mediated by integrin α3β1 (Kikkawa et al., 1998), although integrin α6β1 was reported to be the major receptor for laminin-10/11 in human pancreatic carcinoma cells (Tani et al., 1999) and mouse hematopoietic cells (Gu et al., 1999). The reason for this apparent discrepancy may be that adhesion assays are dependent on cell types and it is difficult to find adherent cells that do not express integrin α3β1, making it necessary to study the contribution of other integrins, e.g. α6β1 and α6β4, by blocking α3β1 with antibodies or by transfecting those cells that express neither α3β1 nor α6β1 (e.g. the K562 erythroleukemic cells) with the integrin α subunit(s) to be examined. In the present study, we examined the integrin binding specificity of laminin-10/11 using fibroblastic cells and K562 cells transfected with integrin α3 or α6 subunits in combination with β4 subunit. Our results showed that the adhesion of fibroblastic cells to laminin-10/11 was mediated by both α3β1 and α6β1 integrins. Binding of α3β1 and α6β1 integrins to laminin-10/11 was also demonstrated with K562 transfectants and shown to be dependent on the activation status of these integrins. Integrin α6β4, when expressed on K562 cells, was also capable of mediating cell adhesion to laminin-10/11.

MATERIALS AND METHODS

Cell lines and culture conditions

The following human tumor cell lines were provided by the Japanese Cancer Research Resources Bank: A172 (glioma), A431 (vulva epidermoid carcinoma), A549 (lung adenocarcinoma), EJ-1 (bladder carcinoma), Detroit 529 (skin fibroblast), WI-38 (fetal lung fibroblast), and HT1080 (fibrosarcoma). These cells were grown in DMEM supplemented with 15 mM Hepes, pH 7.2, 100 i.u./ml penicillin, 0.1 mg/ml streptomycin sulfate and 10% FBS (JRH Bioscience, Lenexa, KS, USA) unless otherwise indicated, at 37°C in a humidified atmosphere containing 5% CO_2. K562 cells transfected with cDNAs encoding integrin α3 or α6 subunits and those cotransfected with cDNAs encoding integrin α6 and β4 subunits were prepared as described previously (Delwel et al., 1993, 1994; Niessen et al., 1994). Transfectants were grown in RPMI1640 supplemented with 100 i.u./ml penicillin, 0.1 mg/ml streptomycin sulfate and 10% FBS. Integrin expression profiles of the transfectants were verified by flow cytometry prior to cell adhesion assays.

Preparation of laminin 10/11 and other laminin isoforms

Laminin-10/11 were purified to homogeneity from conditioned medium of A549 cells by immunoaffinity chromatography (Kikkawa et al., 1998). Briefly, the conditioned medium of A549 cells was subjected to immunoaffinity chromatography with the monoclonal anti-human laminin antibody 4C7, which was recently shown to recognize the laminin α5 chain (Tiger et al., 1997). The proteins bound to the immunoaffinity column were eluted with 0.1 M triethylamine, pH 11.5, neutralized and dialyzed against PBS. The purified laminin-10/11 was verified not to contain any other laminin α chains by immunoblotting with monoclonal antibodies specific to α1 (Kikkawa et al., 1998), α2, α3 or α4 chains (N. Sanzen and H. Fujiwara, unpublished observations). Laminin-1 was purified from mouse EHS tumor by the method of Paulsson et al. (1987). Laminin-5 was purified from the conditioned medium of the human gastric carcinoma line MKN45 by immunoaffinity chromatography using affinity-purified rabbit polyclonal antibody against human laminin γ2 chain (Fukushima et al., 1998).

Monoclonal antibodies

The monoclonal antibody 3G8 recognizing human integrin α3 subunit was produced by fusion of SP2/0 mouse myeloma cells with splenocytes from mice immunized with the U251 human glioma cells, which have been shown to express a high level of integrin α3β1 (Fukushima et al., 1998). Hybridomas were screened for reactivity with integrin α3β1 purified from human placenta by immunoaffinity chromatography using antibodies against the cytoplasmic domain of the α3 subunit (N. Sanzen, manuscript in preparation). Specificity of this antibody was verified by the following criteria: (1) 3G8 was highly reactive with K562 cells transfected with the α3 subunit, but not with untransfected K562 cells; (2) 3G8 immunoprecipitated integrin α3β1 heterodimer from various cell lines including the HT1080 fibrosarcoma cells, the U251 glioma cells and K562 cells transfected with the α3 subunit, but not from untransfected K562 cells; (3) 3G8 strongly inhibited adhesion of A549 cells and K562 cells transfected with the α3 subunit to laminin-5 as was the case with P1B5, a well-characterized monoclonal antibody against integrin α3β1. The monoclonal antibody against integrin β1 subunits, 4G2, was produced and characterized in our laboratory (Manabe et al., 1997). The monoclonal antibody 8A2 against the integrin β1 subunit, which activates β1-containing integrins, was a gift from Dr Nicholas Kovach (University of Washington, WA, USA). Monoclonal antibodies against human integrin α2 and α3 subunits, P1E6 and P1B5, respectively, were purchased from Life Technologies, Inc. (Grand Island, NY, USA), and the monoclonal antibody against human integrin α6 subunit (GoH3) was from Cosmo Bio (Tokyo, Japan).

Cell adhesion assay

Cell adhesion assays were performed as described previously (Kikkawa et al., 1998). Briefly, 96-well microtiter plates (Nunc; Wiesbaden, Germany) were incubated with laminin-10/11, laminin-1 or laminin-5 at 37°C for 1 hour, and then blocked with PBS containing 1% bovine serum albumin for another 1 hour at the same temperature. For cell adhesion inhibition assays, monoclonal antibodies against different types of integrin were individually preincubated with cells in serum-free medium at a density of 4×10^5 cells/ml at room temperature for 15 minutes. The preincubated cells were transferred onto plates precoated with laminin-10/11, laminin-1 or laminin-5 and further incubated at 37°C for 20 minutes. After staining with Diff-Quik (International Reagents Corp., Kobe, Japan) or Crystal Violet, the attached cells were counted under a microscope or quantified as described previously (Kikkawa et al., 1998). In cell adhesion assays using K562 transfectants, cells were suspended in serum-free RPMI1640 at a density of 4×10^5 cells/ml, and preincubated with or without the stimulatory anti-integrin β1 monoclonal antibody 8A2 at 37°C for 10 minutes. 50-μl samples of the cell suspension were added to wells coated with varying concentrations of laminin-10/11, laminin-1 or laminin-5, followed by incubation at 37°C for 1 hour. The attached cells were stained with Diff-Quik and counted under a microscope. Photomicrographs of adhering cells were taken on Minicopy films (Fuji Photo Film Co., Ltd., Tokyo, Japan) with an Olympus IMT-2 microscope.

Determination of protein concentration

Protein concentration was determined by the dye method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

Adhesion of fibroblastic cells to laminin-10/11 is mediated by both α3β1 and α6β1 integrins

We previously found that adhesion of the A549 human lung carcinoma cells to laminin-10/11 was mediated by integrin

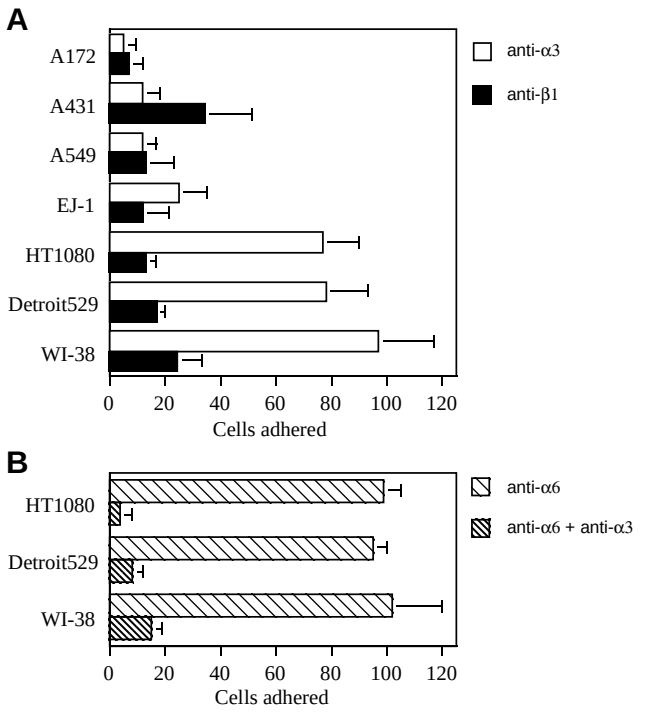

Fig. 1. Effects of anti-integrin antibodies on adhesion of different cell types to laminin-10/11. (A) The following cells were preincubated with either anti-α3 antibody (3G8; white columns) or anti-β1 antibody (4G2; black columns) and then incubated in 96-well microtiter plates coated with 5 nM laminin-10/11 for 20 minutes in the presence of the antibodies: A172, glioma; A431, vulva epidermoid carcinoma; A549, lung adenocarcinoma; EJ-1, bladder carcinoma; Detroit 529, skin fibroblast; WI-38, fetal lung fibroblast; and HT1080, fibrosarcoma. (B) Three fibroblastic cells were preincubated with anti-α6 antibody (GoH3; white hatched columns) or with a mixture of anti-α3 and anti-α6 antibodies (black hatched columns) and incubated in 96-well plates for 20 minutes as described above. The concentration of the antibodies added was 10 μg/ml. Cells did not attach to wells that were uncoated but blocked with 1% bovine serum albumin under the assay conditions. The numbers of adhering cells are expressed as percentages of the number of cells adhering in the absence of monoclonal antibodies, which accounts for 70-75% of input cells. Each column represents the mean ± s.d. of triplicate assays.

α3β1 (Kikkawa et al., 1998). To extend this observation to other cell types, we examined whether function-blocking antibodies against integrin α3 and β1 subunits could inhibit the adhesion to laminin-10/11 of seven distinct cell lines, i.e. three carcinoma cell lines of different origins, one glioma cell line, one fibrosarcoma cell line and two normal fibroblast strains. The adhesion of glioma cells and three distinct carcinoma cell types was strongly inhibited by both antibodies, whereas the adhesion of fibrosarcoma cells and normal fibroblasts was inhibited by anti-β1 antibody but not by anti-α3 antibody (Fig. 1A). Since these fibroblastic cells express functionally active integrin α3β1 (i.e. adhesion of these cells to laminin-5 was inhibited by anti-a3 antibody; data not shown), it seemed likely that other β1-containing integrin(s) were involved in the adhesion of fibroblastic cells to laminin-10/11. When added individually, none of the antibodies against integrin α2, α4 or α5 subunits inhibited the adhesion of fibroblastic cells to laminin-10/11 (data not shown), as was the case with anti-α6 antibody (Fig. 1B). However, when combined with anti-α3 antibody, the anti-α6 antibody, but not anti-α2 or anti-α5 antibodies, strongly inhibited the adhesion of fibroblastic cells to laminin-10/11 (Fig. 1B). These results indicated that not only integrin α3β1 but also α6β1 serves as a receptor for laminin-10/11 in fibroblastic cells.

Adhesion of K562 transfectants expressing integrin α3β1 or α6β1 to laminin-10/11

To confirm that both α3β1 and α6β1 integrins were capable of binding to laminin-10/11, we examined the adhesion of K562 erythroleukemic cells transfected with integrin α3 or α6 cDNAs (Delwel et al., 1994) to laminin-10/11 as compared with adhesion to laminin-1 and laminin-5 (Fig. 2). Untransfected K562 cells were shown to express α5β1 and minute amounts of αvβ1, but no significant amounts of any laminin-binding integrins such as α3β1, α6β1, α6β4 and α7β1 (Delwel et al., 1993, 1994; A. Sonnenberg, data not shown). Absence of functional laminin-binding integrins on K562 cells was further confirmed by their inability to adhere to laminin-1, laminin-2/4 or laminin-5 (Delwel et al., 1994). Since adhesion of the K562 transfectants to laminins was dependent on the activation status of β1-containing integrins (Delwel et al., 1994), cell adhesion assays were performed in the absence or presence of the activating anti-β1 monoclonal antibody 8A2 (Kovach et al., 1992). Transfectants expressing integrin α3β1 adhered moderately to laminin-10/11 and laminin-5 at relatively high coating concentrations (e.g. 10-20 nM) without 8A2 stimulation, but they became more adherent to both substrates after stimulation, reaching the maximum levels at 10 nM. Laminin-5 was a slightly better substrate than laminin-10/11 for unstimulated α3 transfectants, but no significant difference was observed between laminin-10/11 and laminin-5 after integrin stimulation. Adhesion of α3 transfectants to laminin-10/11 and laminin-5 was inhibited by the antibody against integrin α3 subunit (e.g. 3G8), but not by the antibodies against other integrin α subunits, confirming the specificity of the adhesion of α3 transfectants (data not shown). The α3 transfectants did not adhere to laminin-1 even after stimulation.

In contrast, the K562 transfectants expressing integrin α6β1 adhered to laminin-10/11, but not to laminin-5 or laminin-1, without integrin stimulation. They became more adherent to all three laminin isoforms after stimulation, with laminin-10/11 being most potent among the three isoforms and attaining the maximum level of adhesion at 10 nM. Specificity of the adhesion of α6 transfectants was confirmed by inhibition assays using the antibody against integrin α6 subunit (data not shown). These results clearly showed that laminin-10/11 was a potent ligand for integrin α6β1, confirming that laminin-10/11 was capable of mediating cell adhesion through both α3β1 and α6β1 integrins.

It was noted that the α6 transfectants not only attached to but spread on laminin-10/11 without integrin stimulation (Fig. 3). Upon stimulation, they spread more extensively with pseudopod-like extension on laminin-10/11. Similar extended morphology was observed when the stimulated α3 transfectants adhered to laminin-10/11. In contrast, both α3 and α6 transfectants assumed cobblestone-like morphology with little pseudopod-like extension on laminin-5 when stimulated by 8A2, consistent with the previous observation

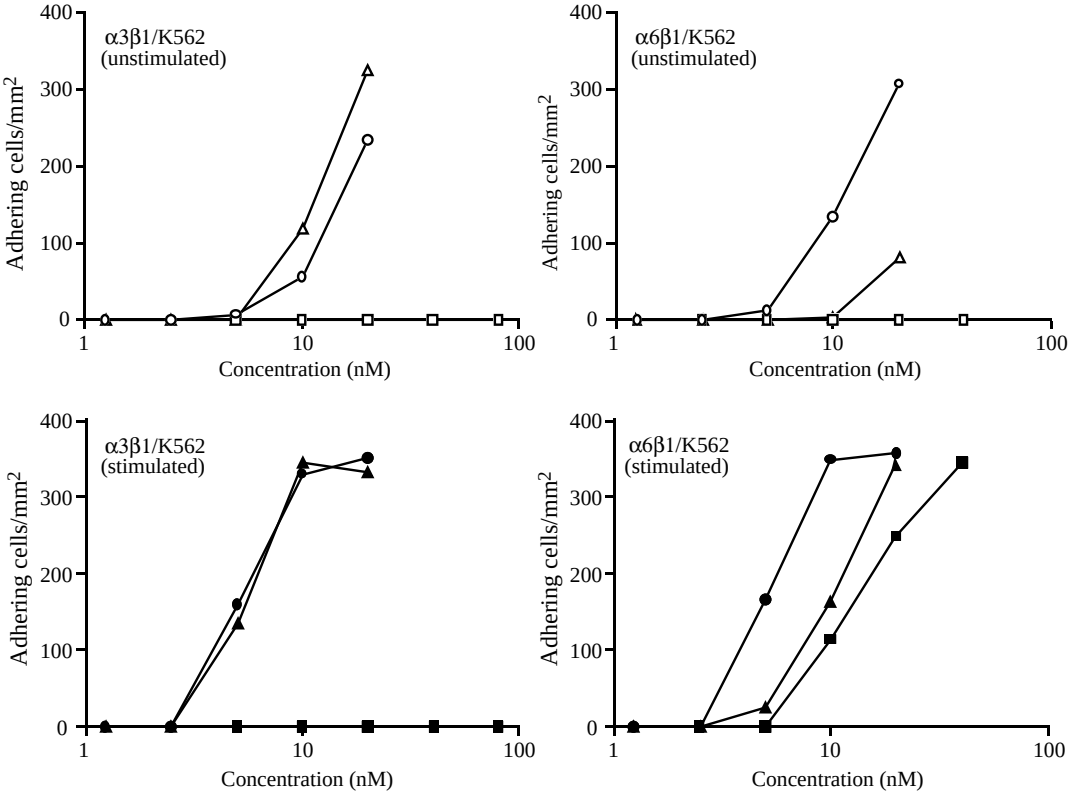

Fig. 2. Adhesion of K562 transfectants expressing integrin α3β1 or α6β1 to different laminin isoforms. K562 transfectants expressing α3β1 (α3β1/K562) or α6β1 (α6β1/K562) were incubated in the 96-well microtiter plates precoated with increasing concentrations of laminin-1 (squares), laminin-5 (triangles) or laminin-10/11 (circles) for 60 minutes at 37°C in the absence (open symbols) or presence (closed symbols) of the activating anti-β1 antibody 8A2. The concentration of 8A2 added was 1:5000 dilution of ascites. The cells adhering to the plates were stained and counted as described in Materials and Methods. Each point represents the mean of triplicate assays.

that lung carcinoma cells adhered equally well to laminin-10/11 and laminin-5 but differed significantly in their morphology when spread (Kikkawa et al., 1998).

Integrin α6β4 also mediates cell adhesion to laminin-10/11

In epithelial cells where both integrin β1 and β4 subunits are expressed, the integrin α6 subunit preferentially dimerizes with the β4 subunit on the cell surface (Giancotti et al., 1992). Since integrin α6β4 serves as a receptor for laminin-5 and is involved in hemidesmosome formation in keratinocytes (Borradori and Sonnenberg, 1996), we examined whether integrin α6β4 could also mediate cell adhesion onto laminin-10/11 using K562 cells cotransfected with cDNAs encoding integrin α6 and β4 subunits (Niessen et al., 1994). As shown in Fig. 4, the α6β4 transfectants adhered to not only laminin-5 but also laminin-10/11. The adhesion of the α6β4 transfectants to laminin-10/11 was inhibited by the antibody against integrin α6 subunit, as was the case with adhesion to laminin-5. The antibody against integrin β4 subunit that was capable of inhibiting the adhesion to laminin-5 failed to inhibit the adhesion to laminin-10/11. However, the anti-β4 antibody inhibited the adhesion to laminin-10/11 when combined with the antibody against the β1 subunit, suggesting that the α6β4 transfectants expressed not only integrin α6β4 but also α6β1 due to dimerization of the α6 subunit with the endogenously expressed β1 subunit (Niessen et al., 1994). These results indicated that the integrin α6β4 also serves as an adhesion receptor for laminin-10/11.

Role of the α6-containing integrins in adhesion of A549 cells to laminin-10/11

Flow-cytometric analysis using antibodies against integrin α6 and β4 subunits showed that the A549 lung adenocarcinoma cells express α6β4 on the cell surface (data not shown). A549 cells are also likely to express functionally active integrin α6β1, since the adhesion of A549 cells to laminin-1 was inhibited by the antibodies against integrin α6 or β1subunits (Kikkawa et al., 1998). The presence of α6β1 and α6β4 integrins on A549 cells suggested that these α6-containing integrins could be involved in the adhesion of A549 cells to laminin-10/11, although the anti-α3 antibody alone inhibited the A549 adhesion to laminin-10/11 (Fig. 1; also Kikkawa et al., 1998). To explore this possibility, we examined the inhibitory effects of the anti-α3 and -α6 antibodies on the adhesion of A549 cells to laminin-10/11 in more detail by increasing the coating concentration of laminin-10/11 (Fig. 5). Inhibition of A549 adhesion by the anti-α3 antibody became less pronounced when substrates were coated with >10 nM laminin-10/11. The inhibition by the anti-α3 antibody was, however, potentiated when the anti-α3 antibody was combined with the anti-α6 antibody, which was not inhibitory when added alone. These results indicated that the α6-containing integrins could play an auxiliary role in A549 adhesion to laminin-10/11, which became detectable when the substrates were coated with higher concentrations of laminin-10/11. It was also noted that the adhesion of A549 cells to laminin-10/11 was only partially inhibited by a mixture of anti-α3 and anti-α6 antibodies when substrates were coated with >10 nM laminin-10/11, suggesting that the non-integrin-type receptor(s) such as α-dystroglycan were also involved in the adhesion of A549 cells to laminin-10/11 under these conditions.

DISCUSSION

Defining the integrin binding specificities of extracellular

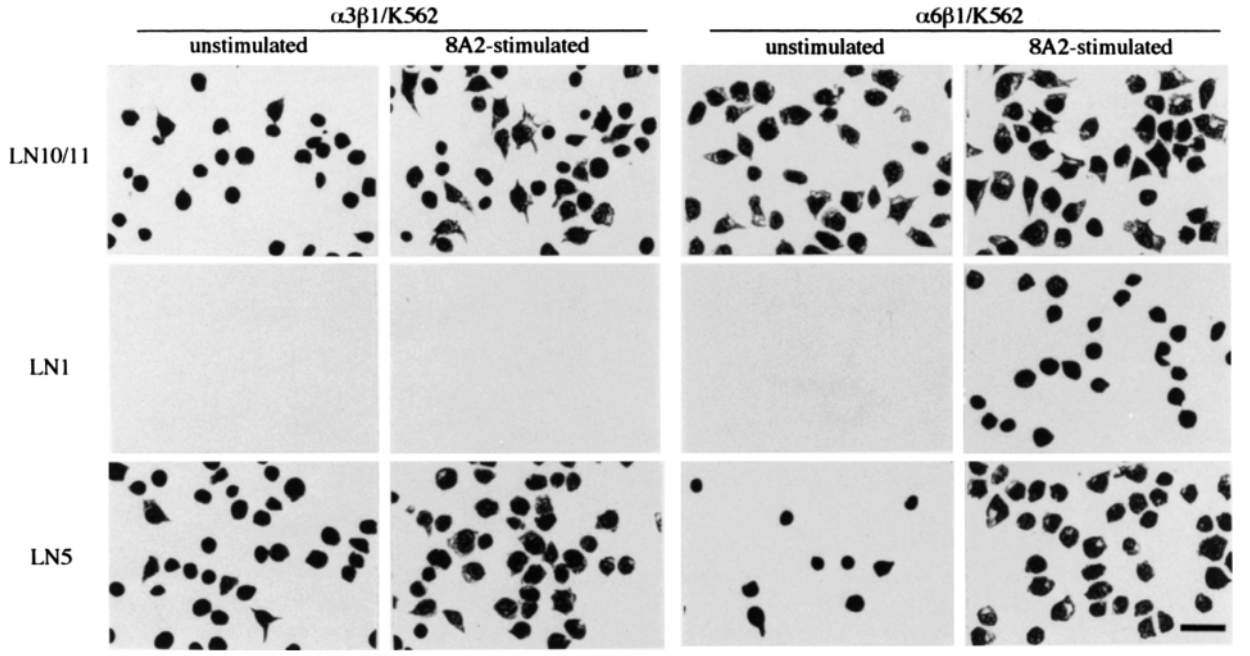

Fig. 3. Attachment and spreading of K562 transfectants on substrata coated with different laminin isoforms. K562 transfectants expressing α3β1 (α3β1/K562) or α6β1 (α6β1/K562) were preincubated with or without the activating anti-β1 antibody 8A2 for 10 minutes, and then incubated for 60 minutes at 37°C in the 96-well microtiter plates precoated with 20 nM laminin-1 (LN1), laminin-5 (LN5) or laminin-10/11 (LN10/11) in the absence or presence of 8A2 at 1:5000 dilution of ascites. Cells were rinsed with serum-free DMEM, fixed in methanol and stained with Diff-Quik. Bar, 50 μm.

adhesive proteins, e.g. laminins, by cell adhesion assays has often been obscured by the presence of multiple receptors on the cell surface and the distinct repertoires of integrins on different cell types. We previously reported that integrin α3β1 was the primary receptor for laminin-10/11 on the basis of adhesion assays with A549 lung adenocarcinoma cells (Kikkawa et al., 1998). Here, we provide evidence that integrin α6β1 could also serve as a potent receptor for laminin-10/11 when assayed with fibroblastic cells and K562 cells transfected with the integrin α6 subunit. First, adhesion of fibroblastic cells to laminin-10/11 was not inhibited by either the anti-α3 or anti-α6 antibody alone, but was strongly inhibited when these antibodies were combined, indicating that both α3β1 and α6β1 are operating as functional receptors for laminin-10/11 on fibroblastic cells. Even with A549 cells, integrin α6β1 could serve as an auxiliary receptor when the substratum was coated with >10 nM laminin-10/11, although non-integrin-type receptor(s) were also operating under these conditions. Interestingly, adhesion through non-integrin-type receptor(s) also became evident with fibroblastic cells (e.g. HT1080 cells) when the substratum was coated with laminin-10/11 at higher (>10 nM) concentrations, while neither anti-α3 nor anti-α6 antibody alone, but only the combination of these, could inhibit the adhesion of these cells at lower (1-5 nM) concentrations (H. Fujiwara, unpublished observation). Second, K562 cells expressing α6β1 were capable of adhering to laminin-10/11, as was the case with those expressing α3β1. Potency of laminin-10/11 as an adhesive ligand for integrin α6β1 was also indicated by the observation that K562 cells expressing α6β1 not only attached to but spread on laminin-10/11 without stimulation by the activating anti-β1 antibody 8A2. We also showed that integrin α6β4 was capable of mediating adhesion of K562 cells when the cells were transfected with both cDNAs

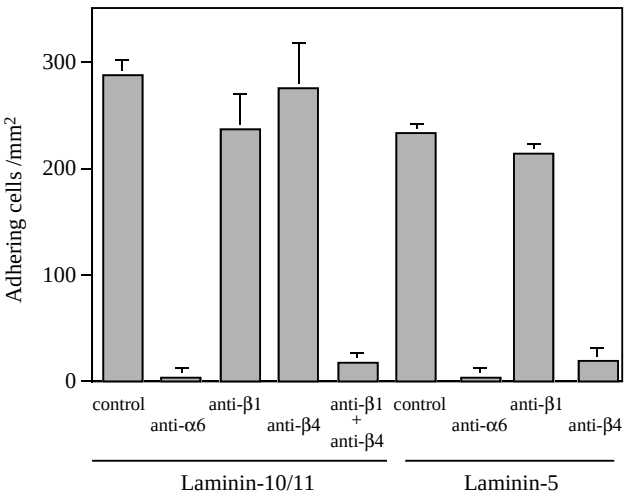

Fig. 4. Adhesion of K562 transfectants expressing integrin α6β4 to laminin-10/11 and laminin-5. K562 transfectants expressing integrin α6β4 were preincubated with or without the following function-blocking anti-integrin antibodies at a concentration of 10 μg/ml for 10 minutes at room temperature, and then incubated in the wells of microtiter plates precoated with 20 nM laminin-10/11 or laminin-5 for 20 minutes at 37°C in the presence or absence of the antibodies: α6, anti-α6 subunit antibody (GoH3); β1, anti-β1 subunit antibody (4G2); β4, anti-β4 subunit antibody (ASC-9). Cells attached to the substrates were stained and counted as described in Materials and Methods. Each column represents the mean ± s.d. of triplicate assays.

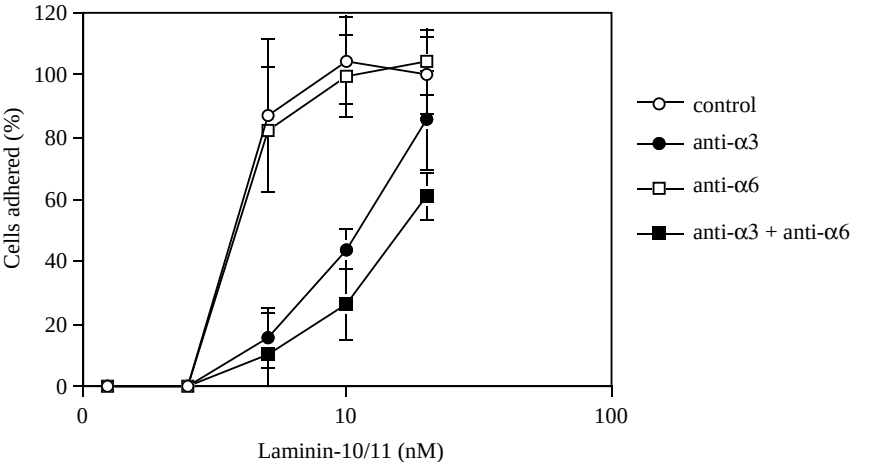

Fig. 5. Effects of anti-integrin antibodies on the adhesion of A549 lung carcinoma cells to substrata coated with increasing concentrations of laminin-10/11. A549 cells were incubated on the wells of microtiter plates precoated with increasing concentrations of laminin-10/11 for 20 minutes at 37°C in the absence or presence of anti-α3 (3G8) and anti-α6 (GoH3) antibody alone or in combination. The concentration of the antibodies added was 10 μg/ml. Cells attached to the substrates were stained and counted as described in Materials and Methods. The number of cells adhered to substrates in the absence of antibody was taken as 100%. Each point represents the mean of triplicate assays.

encoding α6 and β4 subunits. The role of integrin α6β4 as a functional receptor for laminin-10/11, however, remains to be defined, since adhesion of A549 cells was strongly inhibited by the antibody against the integrin β1 subunit when the substratum was coated with lower concentrations of laminin-10/11. These results, taken together with those of our previous study (Kikkawa et al., 1998), indicate that laminin-10/11 is a versatile adhesive ligand in basement membranes capable of binding to both α3β1 and α6β1, the two major laminin-binding integrins expressed on a variety of epithelial and mesenchymal cells.

The laminin α5 chain, the α chain of laminin-10/11, is considered to be evolutionarily most related to the α3 chain among the five laminin α chains (Doliana et al., 1997). Thus, a full-sized α3 chain, α3B, was recently identified in human and mouse (Miner et al., 1997; Doliana et al., 1997), showing the highest degree of homology to the α5 chain at the amino acid level. In support of the close relationship between the laminin α3 and α5 chains, our observations indicated that the laminin isoforms containing the α3 and α5 chains, i.e. laminin-5 and laminin-10/11, have similar specificities toward integrins, since laminin-5 has been shown to be recognized by integrins α3β1 (Carter et al., 1991; Weitzman et al., 1993; Delwel et al., 1994; Rouselle and Aumailley, 1994), α6β1 (Delwel et al., 1993, 1994; Rouselle and Aumailley, 1994) and α6β4 (Niessen et al., 1994), as was the case with laminin-10/11. The binding affinities to α3β1 and α6β1 integrins, as well as the dependence on their activation status were, however, distinctly different for laminin-5 and 1aminin-10/11. Laminin-10/11, but not laminin-5, was capable of mediating significant adhesion of K562 cells expressing α6β1 without stimulation by the anti-integrin β1 antibody 8A2. The α6 transfectants became adherent to laminin-5 upon stimulation by 8A2, but the adhesive affinity was still lower than that for laminin-10/11. In contrast, laminin-5 was more potent than laminin-10/11 in mediating adhesion of the unstimulated α3 transfectants, while both laminin-5 and laminin-10/11 became equally potent after stimulation by 8A2. Consistent with our results, Delwel et al. (1994) reported that adhesion of the α6 transfectants to laminin-5 was strongly dependent upon stimulation of the β1 integrins, whereas the α3 transfectants were highly adherent to laminin-5 without stimulation.

In addition to the differences in adhesive affinities toward α3β1 and α6β1 integrins, the morphologies of attached cells were significantly different between laminin-5 and laminin-10/11. We previously showed that A549 cells adhered to the substratum coated with laminin-10/11 assumed an elongated, spindle-shaped morphology with thin projections, whereas the cells on laminin-5 assumed a well-spread, cobblestone-like morphology with no projections (Kikkawa et al., 1998). Similarly, the K562 transfectants differed in their morphology when spread on substrata coated with either laminin-5 or laminin-10/11, displaying more elongated morphology with occasional projections on laminin-10/11 but not on laminin-5. The differences in the morphology of attached cells could be due to either the differences in the types of integrins involved (e.g. engagement of integrin α6β1 on laminin-10/11) or possible involvement of other non-integrin-type laminin receptors such as α- dystroglycan, although binding of laminin-10/11 or laminin-5 to α-dystroglycan remains to be defined.

Although intact laminin-10/11 has been purified to homogeneity only from the conditioned medium of human lung carcinoma cells (Kikkawa et al., 1998), a commercially available ‘human laminin’ prepared from the pepsin digest of placenta was shown to be a mixture of partially degraded laminin-10 and laminin-11 (Ferletta and Ekblom, 1999). We also found that the commercial human laminin (Chemicon, Temecula, CA, USA) contained the α5 chain, but not α1, α2, α3 or α4 chains, based on the immunoreactivity with monoclonal antibodies specific to each α chain (our unpublished observations). Ferletta and Ekblom (1999) showed that the commercial human laminin was a potent adhesive ligand for two epithelial cell lines, adhesion of which was not inhibited by antibodies against integrin α3, α6 or β1 chain nor the antibody against α-dystroglycan. Although they did not examine the inhibitory effects of these antibodies in combination, they suggested that the cell lines used in their study might use as yet unidentified high-affinity receptor(s) for laminin-10/11 or multiple integrin isoforms in combination with α-dystroglycan for strong adhesion onto the substratum coated with the commercial laminin-10/11. It should also be noted that the laminin purified from bovine kidney (Lindblom et al., 1994) may contain laminin-10/11 as a major component. Bovine kidney laminin contained a novel 375 kDa α chain, which migrated slightly faster than the α1 chain upon SDS-

PAGE, consistent with the electrophoretic behavior of the α5 chain. The α5 chain-containing laminins were highly expressed in glomeruli and all tubules in the adult kidney (Miner et al., 1997; Sorokin et al., 1997). Bovine kidney laminin was shown to serve as an adhesive ligand for both α3β1 and α6β1 integrins (Delwel et al., 1994), again consistent with the adhesive properties of intact laminin-10/11 as demonstrated in the present study. The identity of bovine kidney laminin as laminin-10/11, however, remains to be confirmed.

Recently, mice lacking the laminin α5 chain were produced (Miner et al., 1998). These α5 chain-deficient mice exhibited multiple developmental defects including failure of anterior neural tube closure (exencephaly) failure of digit separation (syndactyly) and dysmorphogenesis of the placental labyrinth, and died late in embryogenesis. Histochemical and ultrastructural examination showed that these defects were closely associated with the defects in the basement membrane that normally contain laminin-10/11 (Miner et al., 1998). Interestingly, the mice lacking both integrin α3 and α6 subunits were found to exhibit developmental defects overlapping with those of mice lacking the laminin α5 chain, including exencephaly and syndactyly (Arcangelis et al., 1999). Mice deficient in either integrin α3 subunit or α6 subunit alone did not show such severe phenotypes and survived to birth (Kreidberg et al., 1996; Georges-Labouesse et al., 1996). The defects in the limb and neural tube of the α3 and α6 double mutant mice were closely associated with the defects in the basement membrane, particularly a marked reduction of the laminin α5 chain deposited in the basement membrane. These observations in mutant mice indicated that both α3β1 and α6-containing integrins are the functional receptors for laminin-10/11 in vivo, consistent with our results obtained with in vitro cell adhesion assays.

We thank Drs Nicholas Kovak, Toshinaga Maeda, Yuji Fukushima for providing valuable reagents used in this study. This work was supported by Coordination Funds from Science and Technology Agencies of Japan and the grants from Uehara Memorial Foundation and Novartis Foundation for the Promotion of Science.

REFERENCES

Arcangelis, A. D, Mark, M., Kreidberg, J., Sorokin, L. and Georges-Labouesse, E. (1999). Synergistic activities of α3 and α6 integrins are required during apical ectodermal ridge formation and organogenesis in the mouse. *Development* **126**, 3957-3968.

Borradori, L. and Sonnenberg, A. (1996). Hemidesmosomes: roles in adhesion, signaling and human disease. *Curr. Opin. Cell Biol.* **8**, 647-656.

Carter, W. G., Ryan, M. C. and Gahr, P. J. (1991). Epiligrin, a new cell adhesion ligand for integrin α3β1 in epithelial basement membranes. *Cell* **65**, 599-610.

Clark, E. A. and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-239.

Delwel, G. O., de Melker, A. A., Hogervorst, F., Jaspars, L. H., Fles, D. L. A., Kuikman, I., Lindblom, A., Paulsson, M., Timpl, R. and Sonnenberg, A. (1994). Distinct and overlapping ligand specificities of the α3Aβ1 and α6Aβ1 integrins: recognition of laminin isoforms. *Mol. Biol. Cell* **5**, 203-215.

Delwel, G. O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R. and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin α6 subunit in transfected K562 cells: activation-dependent adhesion and interaction with isoforms of laminin. *J. Biol. Chem.* **268**, 25865-25875.

Delwel, G. O. and Sonnenberg, A. (1996). Laminin isoforms and their integrin receptors. In *Adhesion Receptors as Therapeutic Targets* (ed. M. A. Horton), pp. 9-36. Boca Raton, New York: CRC Press.

Doliana, R., Bellina, I., Bucciotti, F., Mongiat, M., Perris, R. and Colombatti, A. (1997). The human α3β is 'full-sized' laminin chain variant with a more widespread tissue expression than the truncated α3a. *FEBS lett.* **417**, 65-70.

Durkin, M. E., Loechel, F., Mattei, M.-G., Gilpin, B. J., Albrechtsen, R. and Wewer, U. M. (1997). Tissue-specific expression of the human laminin α5-chain, and mapping of the gene to human chromosome 20q13.2-13.3 and to distal mouse chromosome 2 near the ragged (Ra) mutation. *FEBS lett.* **411**, 296-300.

Engvall, E. (1993). Laminin variants: why, where and when? *Kidney Int.* **43,** 2-6.

Engvall, E. and Wewer, U. M. (1996). Domains of laminin. *J. Cell. Biochem.* **61**, 493-501.

Ferletta, M. and Ekblom, P. (1999). Identification of laminin-10/11 as a strong cell adhesive complex for a normal and a malignant human epithelial cell line. *J. Cell. Sci.* **112**, 1-10.

Fukushima, Y., Ohnishi, T., Arita, N., Hayakawa, T., and Sekiguchi, K. (1998). Integrin α3β1-mediated interaction with laminin-5 stimulates adhesion, migration, and invasion of malignant glioma cells. *Int. J. Cancer* **76**, 63-72.

Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A. and Le Meur, M. (1996).Absence of integrin α6 leads to epidermolysis bullosa and neonatal death in mice. *Nat. Genet.* **13**, 370-373.

Giancotti, F. G. and Ruoslahti, E. (1999). Integrin signalling. *Science* **285**, 1028-1032.

Giancotti, F. G., Stepp, M. A., Suzuki, S., Engvall, E. and Ruoslahti, E. (1992). Proteolytic processing of endogenous and recombinant β4 integrin subunit. *J. Cell Biol.* **118**, 951-959.

Gu, Y., Sorokin, L., Durbeej, M., Hjalt, T., Jönson J.-I. and Ekblom, M. (1999). Characterization of bone marrow laminins and identification of α5-containing laminins as adhesive proteins for multipotent hematopoietic FDCP-mix cells. *Blood* **93**, 2533-2542.

Kikkawa, Y., Sanzen, N. and Sekiguchi, K. (1998). Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells: laminin-10/11 mediates cell adhesion through integrin α3β1. *J. Biol. Chem.* **273**, 15854-15859.

Koch, M., Olson, P. F., Albus, A., Jin, W., Hunter, D. D., Brunken, W. J., Burgeson, R. E. and Champliaud, M.-F. (1999). Characterization and expression of the laminin γ3 chain: a novel, non-basement membrane-associated, laminin chain. *J. Cell Biol.* **145**, 605-617.

Kovach, N. L., Carlos, T. M., Yee, E. and Harlan, J. M. (1992). A monoclonal antibody to β1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol.* **116**, 499-509.

Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C. And Jaenisc, R. (1996). α3β1 integrin has a crucial role in kidney and lung organogenesis. *Development* **122**, 3537-3547.

Lindblom, A., Marsh, T., Fauser, C., Engel, J. and Paulsson, M. (1994). Characterization of native laminin from bovine kidney and comparison with other laminin variants. *Eur. J. Biochem.* **219**, 383-392.

Manabe, R., Oh-e, N., Maeda, T., Fukuda, T. and Sekiguchi, K. (1997). Modulation of cell-adhesion activity of fibronectin by the alternatively spliced EDA segment. *J. Cell Biol.* **139**, 295-307.

Mercurio, A. M. (1995). Laminin receptors: achieving specificity though cooperation. *Trends Cell Biol.* **5**, 419-423.

Miner, J. H., Lewis, R. M. and Sanes, J. R. (1995). Molecular cloning of a novel laminin chain, α5, and widespread expression in adult mouse tissues. *J. Biol. Chem.* **270**, 28523-28526.

Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G. and Sanes, J. R. (1997). The laminin α chains: expression, developmental transitions, and chromosomal locations of α1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel α3 isoform. *J. Cell Biol.* **137**, 685-701.

Miner, J. H., Cunningham, J. and Sanes, J. R. (1998). Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin α5 chain. *J. Cell Biol.* **143**, 1713-1723.

Niessen, C. M., Hogervorst, F., Jaspars, L. H., de Melker, A. A., Delwel, G. O., Hulsman, E. H. M., Kuikman, I. and Sonnenberg, A. (1994). The α6β4 integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.* **211**, 360-367.

Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K. and

Engel. J. (1987). Laminin-nidogen complex. Extraction with chelating agents and structural characterization. *Eur. J. Biochem.* **166**, 11-19.

Rouselle, P. and Aumailley, M. (1994).Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors. *J. Cell Biol.* **125**, 205-214.

Sorokin, L. M., Pausch, F., Frieser, M., Kroger, S., Ohage, E. and Deutzmann, R. (1997). Developmental regulation of the laminin α5 chain suggests a role in epithelial and endothelial cell maturation. *Dev. Biol.* **189**, 285-300.

Tani, T., Lehto V.-P. and Virtanen, I. (1999). Expression of laminins 1 and 10 in carcinoma cells, and comparison of their role in cell adhesion. *Exp. Cell Res.* **248**, 115-121.

Tiger, C.-F., Champliaud, M.-F., Pedrosa-Domellof, F., Thornell, L.-E., Ekblom, P. and Gullberg, D. (1997). Presence of laminin α5 chain and lack of laminin α1 chain during human muscle development and in muscular dystrophies. *J. Biol. Chem.* **272**, 28590-28595.

Weitzman, J. B., Pasqualini, R., Takada, Y. and Hemler, M. E. (1993). The function and distinctive regulation of the integrin VLA-3 in cell adhesion, spreading, and homotypic cell aggregation. *J. Biol. Chem.* **268**, 8651-8657.