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Isolation and Characterization of Laminin-10/11 Secreted by Human Lung Carcinoma Cells

LAMININ-10/11 MEDIATES CELL ADHESION THROUGH INTEGRIN $\alpha3\beta1$*

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A panel of human tumor cell lines was screened for selective expression of laminin $\alpha5$ chain, a newly identified laminin subunit comprising laminin-10 ($\alpha5\beta1\gamma1$) and -11 ($\alpha5\beta2\gamma1$). The lung adenocarcinoma cell line A549 was found to express the $\alpha5$ chain at relatively high levels but no detectable amounts of other α chains. The laminin variants containing $\alpha5$ chain were purified from the conditioned medium of A549 cells by immunoaffinity chromatography using the anti-laminin monoclonal antibody 4C7 which was shown recently to recognize the laminin $\alpha5$ chain (Tiger, C.-F., Champliaud, M.-F., Pedrosa-Domellof, F., Thornell, L.-E., Ekblom, P., and Gullberg, D. (1997) *J. Biol. Chem.* 272, 28590–28595). The purified laminin variants consisted of three chains with molecular masses of 350, 220, and 210 kDa. The 350-kDa chain was specifically recognized by another anti-$\alpha5$ chain monoclonal antibody capable of recognizing denatured $\alpha5$ chain on immunoblots, whereas the 210-kDa chain was recognized by an anti-$\gamma1$ chain antibody. The purified $\alpha5$ chain-containing laminin variants (hereafter referred to as laminin-10/11) were highly active in mediating adhesion of A549 cells to the substratum with potency as high as that of laminin-5 and significantly higher than those of laminin-1, laminin-2/4, or fibronectin. Adhesion to substrata coated with laminin-10/11 was specifically inhibited by anti-integrin antibodies directed against the integrin $\alpha3$ or $\beta1$ subunit but not by those against $\alpha2$ or $\alpha6$ subunit, indicating that laminin-10/11 is specifically recognized by integrin $\alpha3\beta1$. Given the wide distribution of laminin-10/11 in the basement membrane of various tissue types and dominant expression of integrin $\alpha3\beta1$ in most epithelial cells, specific interaction of laminin-10/11 with integrin $\alpha3\beta1$ may play an important role in *in vivo* regulation of proliferation and differentiation of epithelial cells through the basement membrane.

Laminins are a family of basement membrane proteins implicated in diverse functions of epithelial and neuronal cells including adhesion, migration, proliferation, differentiation, and programmed cell death. Laminins are disulfide-linked heterotrimers of three distinct but distantly related subunit chains termed α, β, and γ. Nine genetically distinct laminin chains, *i.e.* $\alpha1$–5, $\beta1$–3, and $\gamma1$–2, have been identified in man and mouse (1). Combinations of these chains generate at least 11 different laminin variants, although the differences in biological functions among these variants are understood only poorly.

Interaction of cells with laminins is mediated by a variety of cell surface receptors including integrins, membrane-bound proteoglycans (*e.g.* dystroglycan), and other membrane glycoproteins, of which integrins are of crucial importance with respect to the control of growth and differentiation of cells by the basement membrane (2). To date, nine different integrins ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, $\alpha7\beta1$, $\alpha9\beta1$, $\alpha v\beta3$, and $\alpha?\beta8$) have been suggested to be receptors for laminins (3). Specificities of interactions of various laminin variants with integrins have been investigated extensively with laminin-1 (a prototype laminin purified from the EHS[1] tumor), laminin-2/4 (merosin), and laminin-5 (also referred to as kalinin, epiligrin, nicein, or ladsin), but those of other newly identified laminins, particularly those containing $\alpha4$ and $\alpha5$ chains, remain to be defined.

Laminin-10/11 is composed of $\alpha5$, $\beta1/2$, and $\gamma1$ chains (4). The laminin $\alpha5$ chain was cloned initially in mouse and found to be more related to a *Drosophila* laminin α chain than to other laminin α chains (5). cDNA clones encoding the G-domain of the human $\alpha5$ chain were isolated, and the gene encoding it has been mapped to chromosome 20q13.2-13.3 (6). In contrast to other laminin α chains, $\alpha5$ is expressed widely in adult tissues including placenta, heart, lung, skeletal muscle, kidney, and pancreas (4–10), suggesting that laminin-10/11 may be the major laminin isoforms in the adult basal laminae. Despite its wide distribution in the body, however, the biological functions and integrin binding specificity of laminin-10/11 are yet to be defined with purified proteins.

In the present study we screened a panel of human tumor cell lines for those selectively expressing laminin-10/11. One of the human lung adenocarcinoma cell lines, A549, was found to express the $\alpha5$ chain at high levels but no detectable amounts of other α chains. Purification of laminin-10/11 from the conditioned medium of A549 cells allowed us to characterize the cell adhesive activity and integrin binding specificity of these widely expressed laminin variants.

EXPERIMENTAL PROCEDURES

Materials—Laminin-1 was purified from mouse EHS tumor tissues by the method of Paulsson *et al.* as described previously (11). 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 $\gamma2$ chain (12). Human laminin-2/4 (merosin) was purchased from Chemicon

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[1] The abbreviations used are: EHS, Engelbreth-Holm-Swarm; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

(Temecula, CA). Plasma fibronectin was purified from outdated human plasma by gelatin-affinity chromatography (13).

Cell Lines and Culture Conditions—The human lung adenocarcinoma cell line A549 and other human tumor cell lines used in this study were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan), except for the human lung squamous carcinoma cell RERF-LC-AI and the cervix epidermoid carcinoma cell CaSki, which were obtained from RIKEN Gene Bank (Tsukuba, Japan) and American Type Culture Collection (Rockville, MD), respectively. These cells were grown in DMEM supplemented with 15 mM HEPES (pH 7.2), 100 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and 10% fetal bovine serum (JRH Bioscience, Lenexa, KS) unless otherwise indicated, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

RT-PCR and Isolation of cDNAs Encoding Human Laminin α Chains—Total RNA was extracted from cultured cells by the acid guanidinium isothiocyanate method (14). cDNA was synthesized using a First Strand Synthesis Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. A cDNA fragment encoding domain IIIb of the human laminin α5 chain was amplified by RT-PCR from CaSki cells using the primers 5′-TGTATCTGTCCACCACGCACTG-3′ (sense strand) and 5′-ACATCTTGAGCCCTGCACGTTC-3′ (antisense strand), which were modeled after the cDNA sequence 4258–4587 of mouse laminin α5 chain (5). The resulting 330-base PCR product was isolated on a low melting point agarose gel and ligated into *Eco*RV-cleaved pBluescript II KS(+). The nucleotide sequence of the amplified cDNA, deposited into GenBank under accession number AB010099, was 86% homologous to the corresponding sequence of mouse α5 chain cDNA. A pair of nested primers, 5′-GACTGCCTGCTGTGCCAGC-3′ (sense strand) and 5′-GGGGTAGCCATGAAAGCCCG-3′ (antisense strand), was used for routine amplification of the laminin α5 chain transcript by RT-PCR. The RNA transcripts encoding the domain IIIb of human laminin α1 chain were also amplified by RT-PCR using primers 5′-AAGTGTGAAGAATGTGAGGATGGG-3′ (sense strand; nucleotides 3020–3043 (7)) and 5′-CACTGAGGACCAAAGACATTTTCCT-3′ (antisense strand; nucleotides 3312–3336). Similarly, the transcripts encoding human laminin β1, β2, and γ1 chains were amplified using the PCR primers 5′-AACTGTGAGCAGTGCAAGCCGTTT-3′ (sense strand for β1 chain; nucleotides 1054–1077 (15)), 5′-CAACCAAATGGATCTTC-ACTGCTT-3′ (antisense strand for β1 chain; nucleotides 1278–1301), 5′-CACTGTGAGCTCTGTCGGCCCTTC-3′ (sense strand for β2 chain; nucleotides 1153–1176 (16)), 5′-CAAGGAGTGCTCCCAGGCACTG-TG-3′ (antisense strand for β2 chain; nucleotides 1427–1451), 5′-CACT-GTGAGAGGTGCCGAGAGAAC-3′ (sense strand for γ1 chain; nucleotides 1033–1056 (17)), and 5′-CATCCTGCTTCAGTGAGAGAATGG-3′ (antisense strand for γ1 chain; nucleotides 1203–1226). PCR products were amplified under the following conditions: 30 cycles at 94 °C for 1 min, 61 °C (α5 and β2 chains), 57 °C (γ1 chain), 55 °C (α1 chain) or 53 °C (β1 chain) for 1 min, and 72 °C for 1 min. PCR products were analyzed by electrophoresis using 2% agarose gels.

Monoclonal Antibodies—Monoclonal antibodies against human laminin α5 and α1 chains were produced by fusion of SP2/0 mouse myeloma cells with splenocytes from mice immunized with GST fusion proteins containing the IIIb domain of each laminin α chain. GST fusion proteins were expressed in *Escherichia coli* using pGEX4T-1 (Amersham Pharmacia Biotech) and purified on glutathione-Sepharose. Hybridomas were first screened for reactivity with GST fusion proteins used as immunogens and then selected for reactivity on immunoblots with intact human laminin α chains secreted by human lung carcinoma cells. Monoclonal antibodies against human laminin β1 chain (4E10) and γ1 chain (2E8) were purchased from Chemicon. Monoclonal antibodies against integrin α5 and β1 subunits, 8F1 and 4G2, were produced and characterized in our laboratory and were described previously (18). Monoclonal antibodies against laminin-2/4 were also produced by immunizing mice with human laminin-2/4 and screened for positive reactivity with reduced, denatured α2 chain on immunoblots. One of these antibodies, 10G1, specifically stained ~300-kDa α2 chain but not ~200-kDa β/γ chains. Monoclonal antibodies against human integrin α2 and α3 subunits, P1E6 and P1B5, respectively, were purchased from Life Technologies, Inc., and the monoclonal antibody against human integrin α6 subunit (GoH3) was from Cosmo Bio (Tokyo).

Screening of Human Tumor Cells for Expression of Laminin Variants—31 human tumor-derived cell lines (13 lung carcinomas, 4 gastric carcinomas, 3 cervix carcinomas, 3 gliomas, 2 kidney carcinomas, 2 choriocarcinomas, 1 fibrosarcoma, 1 hepatoma, 1 oral carcinoma, and 1 pancreatic carcinoma) were grown to confluence in 15-cm culture dishes with DMEM containing 10% fetal bovine serum. The conditioned media were harvested and clarified by sequential centrifugation at 1,500 × *g* for 10 min and 15,000 × *g* for 30 min followed by precipitation with ammonium sulfate at 40% saturation. The resulting precipitates were collected by centrifugation at 15,000 × *g* for 40 min and then dissolved in and dialyzed against 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. The precipitates were screened for the expression of laminin α chains by immunoblotting with antibodies specific to each α chain.

Purification of Laminin 10/11—The human lung adenocarcinoma cell line A549 was grown to confluence in 1,700-cm^2 roller bottles with DMEM containing 10% fetal bovine serum (400 ml/bottle). After the cells reached confluence, the conditioned medium was harvested once every 6 days and clarified by centrifugation. Pooled conditioned medium (3–5 liters) was first precipitated with 40% ammonium sulfate and then dissolved in and dialyzed against phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). The precipitated proteins were subjected to immunoaffinity chromatography with the monoclonal anti-human laminin antibody 4C7 which was shown recently to recognize the laminin α5 chain (19). The affinity column was prepared by coupling 1 mg of 4C7 IgG purified from ascites (Life Technologies, Inc.) using protein G-Sepharose 4B (Amersham Pharmacia Biotech) to CNBr-Sepharose 4B (Pharmacia). The bound proteins were eluted from the 4C7 column with 0.1 M triethylamine (pH 11.5), neutralized, and dialyzed against phosphate-buffered saline.

Electrophoretic Analysis and Immunoblotting—SDS-PAGE was carried out on 4% gels under nonreducing or reducing conditions (20). For immunoblotting, proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Proteins on the membrane were reacted with chain-specific monoclonal antibodies followed by incubation with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (EY Laboratories, San Mateo, CA). Bound antibodies were visualized with ECL Western blotting detection regents (Amersham Pharmacia Biotech).

Cell Adhesion Assay—Cell adhesion assay was performed as described previously (21). Briefly, 96-well microtiter plates (Nunc, Wiesbaden, Germany) were incubated with different types of laminins or fibronectin at 37 °C for 1 h and then blocked with phosphate-buffered saline containing 1% bovine serum albumin for another h at the same temperature. A549 cells were trypsin treated and suspended in serum-free DMEM at a density of 3 × 10^5 cells/ml; then 0.1 ml of the cell suspension was added to each well of the plates followed by incubation at 37 °C for 1 h. The attached cells were fixed and stained with a 0.4% crystal violet in methanol (w/v) for 30 min. After washing with distilled water, the stained cells were extracted with 0.1 M citrate in 50% ethanol. The absorbance of each well of the plates was measured at 590 nm with a model 3550 microplate reader (Bio-Rad). Photomicrographs of cells stained with Diff-Quik (International Reagents Corp., Kobe, Japan) were taken on Minicopy films (Fuji Photo Film Co., Ltd., Tokyo) with an Olympus IMT-2 microscope (Olympus Optical Co., Ltd., Tokyo).

To identify the receptor for laminin-10/11, monoclonal antibodies against different types of integrins were preincubated individually with A549 cells in a volume of 0.05 ml of incubation solution (4 × 10^5 cells/ml) at room temperature for 15 min. The preincubated cells were transferred onto plates precoated with different proteins and then incubated further at 37 °C for 30 min. After staining with crystal violet, the attached cells were quantified as described above.

Determination of Protein Concentration—Protein concentration was determined by the dye method using a Bio-Rad protein assay kit.

RESULTS

Screening of Human Tumor Cell Lines for Expression of Laminin α5 Chain—To purify and characterize human laminin-10/11, we screened by RT-PCR a panel of 31 human tumor cell lines for expression of the laminin α5 chain. The PCR primers were designed according to the nucleotide sequence of human α5 chain cDNA encoding the IIIb domain, which had been cloned by RT-PCR from total RNA extracted from human cervix epidermoid carcinoma cells using primers modeled after the mouse α5 cDNA sequence (5). Expression of the laminin α1 chain, the α chain of the classical laminin-1, was also screened by RT-PCR in parallel to select cells expressing α5 but not α1. One of the human lung adenocarcinoma cell lines, A549, was found to express the α5 chain mRNA at relatively high levels but not that of α1 chain, although another lung carcinoma cell line, RERF-LC-AI, expressed α1 but not α5 (Fig. 1*A*).

To confirm the selective expression of the α5 chain by A549 cells, the conditioned medium of A549 cells was analyzed for

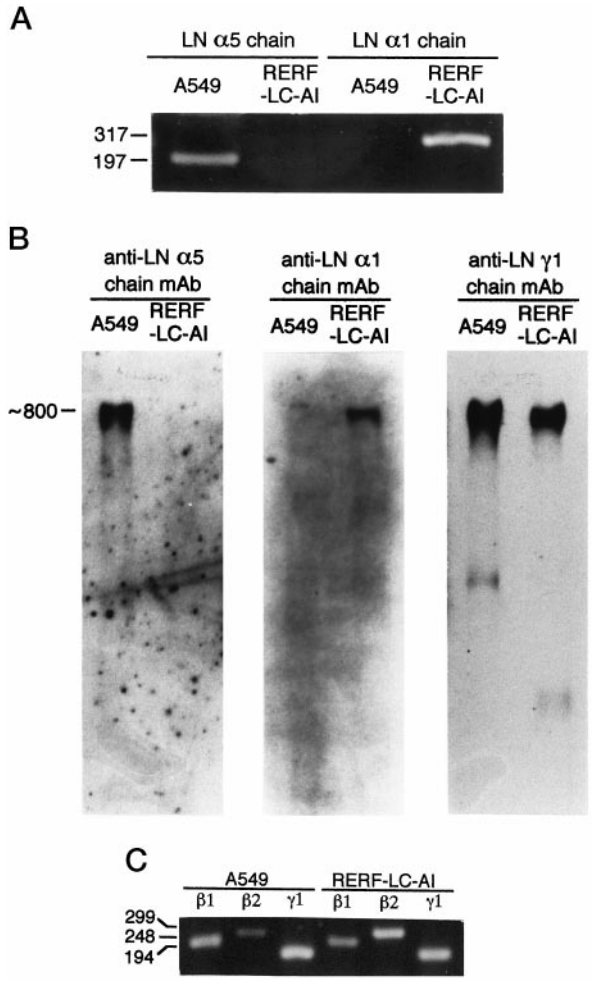

FIG. 1. **Expression of laminin-10/11 in human lung carcinoma cells.** *Panel A*, detection of transcripts encoding laminin α5 and α1 chains by RT-PCR. Transcripts encoding the IIIb domain of the α5 and α1 chains were amplified by RT-PCR from two human lung carcinoma cell lines, A549 and RERF-LC-AI, using the primer sets described under "Experimental Procedures." The expected sizes of the amplified cDNA fragments corresponding to the α5 and α1 chains were 197 and 317 base pairs, respectively. *Panel B*, detection of laminin (*LN*) α5, α1, and γ1 chains by immunoblotting. The conditioned media of A549 and RERF-LC-AI cell lines were subjected to SDS-PAGE on 4% polyacrylamide gels under nonreducing conditions and transferred onto polyvinylidene difluoride membranes followed by immunostaining with monoclonal antibodies against human laminin α5 chain (15H5), α1 chain (5A3), or γ1 chain (2E8). The anti-γ1 chain antibody was used as a panspecific antibody detecting all of the known laminin variants except laminin-5 (α3β3γ2). The position of the nonreduced EHS laminin-1 (~800 kDa) is indicated in the *left margin*. *Panel C*, expression of laminin β1, β2, and γ1 chains in A549 and RERF-LC-AI cells. Transcripts encoding β1, β2, and γ1 chains were amplified by RT-PCR by using the primer sets described under "Experimental Procedures." The expected sizes of the amplified cDNA fragments corresponding to the β1, β2, and γ1 chains were 248, 299, and 194 base pairs, respectively.

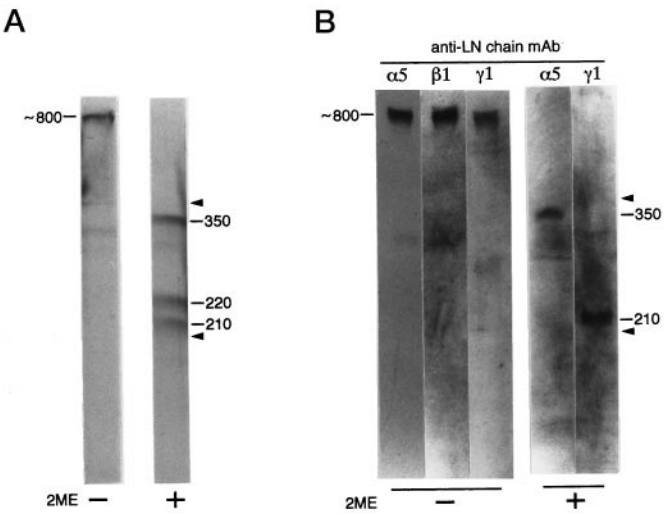

FIG. 2. **SDS-PAGE and immunoblotting analyses of purified laminin-10/11.** *Panel A*, laminin-10/11 purified from conditioned medium of A549 cells was subjected to SDS-PAGE using 4% polyacrylamide gels under nonreducing and reducing conditions. Proteins were visualized by silver staining. *Panel B*, proteins separated by SDS-PAGE under nonreducing (*2ME*−) and reducing (*2ME*+) conditions were transferred onto polyvinylidene difluoride membranes followed by immunostaining with monoclonal antibodies specific for α5, β1, and γ1 chains. Positions of EHS laminin-1 under reducing conditions (400 and 200 kDa) are indicated in the *right margin*.

expression of α5 and α1 chains by immunoblotting with monoclonal antibodies specific to each laminin α chain. The monoclonal antibodies were produced by immunizing mice with recombinant GST fusion proteins containing the IIIb domain of either the α5 or α1 chain. Immunoblotting with the monoclonal antibody against the α5 chain (clone 15H5) specifically detected a protein band migrating at the ~800-kDa region in the conditioned medium of A549 cells but not that of RERF-LC-AI cells (Fig. 1*B*). In contrast, the monoclonal antibody against the α1 chain (clone 5A3) specifically stained a protein band migrating at the same region in the conditioned medium of RERF-LC-AI cells, but not that of A549 cells. The ~800-kDa protein secreted by A549 cells was strongly reactive with a monoclonal antibody specific to the laminin γ1 chain and migrated only slightly above the α1-containing laminin variant(s) secreted by RERF-LC-AI cells. Immunoblotting with antibodies specific for the α2 or α3 showed that no detectable amounts of these laminin α chains were expressed by A549 cells (data not shown). Because the anti-γ1 chain antibody did not detect any bands corresponding to the molecular masses of the α4 chain-containing laminin-8 or laminin-9 (*i.e.* 500–600 kDa) in the conditioned medium of A549 cells, it is likely that A549 cells express only laminin-10 (α5β1γ1) or laminin-11 (α5β2γ1) among the 11 laminin variants identified to date. Because laminin-10 and laminin-11 differ in their β chain types, we examined the expression of laminin β1 and β2 chains in A549 and RERF-LC-AI cells by RT-PCR (Fig. 1*C*). The results showed that both β1 and β2 chains were expressed in both cell types, indicating that A549 cells expressed both laminin-10 and laminin-11. The relative amounts of the PCR products for β1 and β2 chains also indicated that A549 cells expressed more β1 chain than β2 chain, whereas RERF-LC-AI cells expressed more β2 than β1. These results suggest that laminin-10 is the major laminin variant expressed in A549 cells.

Purification of Laminin-10/11—Laminin-10/11 in the conditioned medium of A549 cells was purified by fractionation with 40% ammonium sulfate followed by immunoaffinity chromatography using the monoclonal antibody 4C7, which was previously considered to recognize the α1 chain but has recently been shown to recognize the α5 chain (19). The protein eluted from a 4C7-Sepharose column gave a single band with molecular mass of ~800 kDa on SDS-PAGE under nonreducing conditions and three bands with molecular masses of 350, 220, and 210 kDa under reducing conditions (Fig. 2*A*). The nonreduced ~800-kDa band was recognized by the anti-α5 monoclonal antibody 15H5 and also by monoclonal antibodies specific for the β1 or γ1 (Fig. 2*B*), confirming that the purified ~800-kDa protein was either laminin-10 or a mixture of laminin-10 and laminin-11. In support of this conclusion, the 350-kDa and 210-kDa bands on the reducing gel were specifically stained by monoclonal antibodies specific for the α5 chain (350-

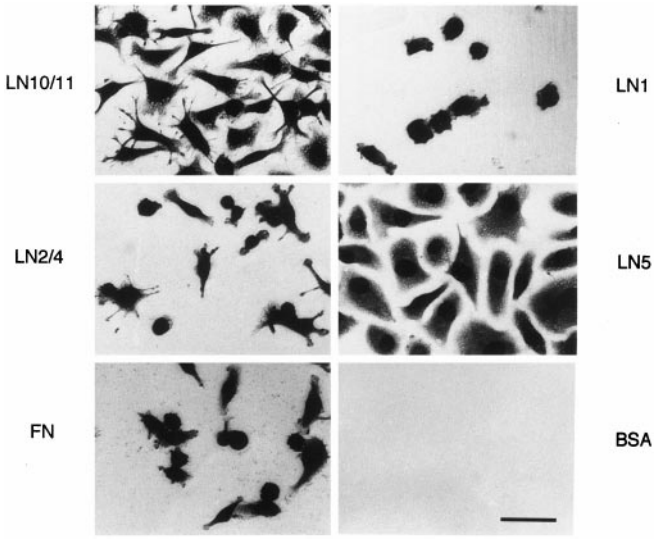

FIG. 3. **Attachment and spreading of A549 cells on surfaces coated with laminin-10/11 and other adhesive proteins.** A549 cells (2×10^4) were seeded onto 96-well microtiter plates coated with laminin-10/11 (*LN10/11*), mouse laminin-1 (*LN1*), laminin-2/4 from human placenta (*LN2/4*), laminin-5 (*LN5*), or fibronectin (*FN*) and incubated for 60 min at 37 °C. The protein concentration used for coating was 5 nM. The cells were rinsed with DMEM, fixed in methanol, and stained with Diff-Quik. *Bar*, 50 μm.

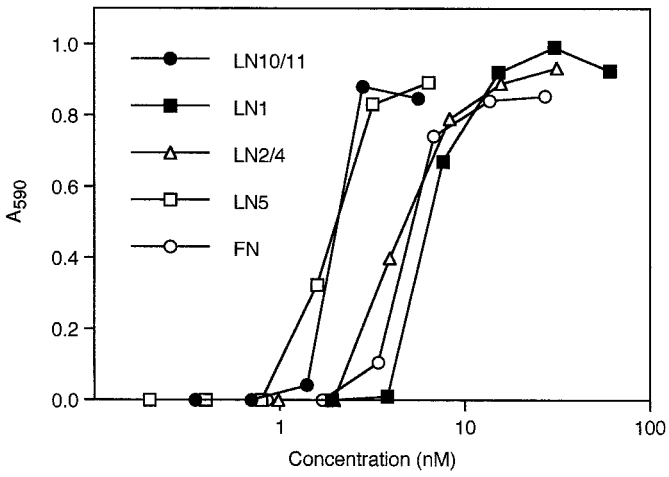

FIG. 4. **Adhesion of A549 cells onto surfaces coated with laminin-10/11 and other adhesive proteins.** 96-well microtiter plates were coated with increasing concentrations of laminin-10/11 (*LN10/11*, ●), mouse laminin-1 (*LN1*, ■), laminin-2/4 from human placenta (*LN2/4*, △), laminin-5 (*LN5*, □), or fibronectin (*FN*, ○) and incubated with A549 cells at 37 °C for 1 h. After incubation, cells attached to the surfaces were quantified by crystal violet staining as described under "Experimental Procedures." Each point represents the mean of triplicate assays.

kDa band) and for the γ1 chain (210-kDa band), respectively (Fig. 2*B*). Weak reactivity of the anti-β1 monoclonal antibody with reduced protein prevented identification of the subunit type(s) of the 220-kDa chain (data not shown). However, the apparent size of the chain was consistent with that of the β1 or β2 chain, supporting the conclusion that the laminin variants purified from the conditioned medium of A549 cells were α5 chain-containing laminin-10/11. The purified laminin-10/11 did not show any detectable bands at ~150 kDa, indicating that nidogen/entactin was not associated with the purified protein.

Cell Adhesion Activity and Receptor Binding Specificity of Laminin-10/11—The cell adhesive activity of the purified laminin-10/11 was compared with those of other laminin variants (*i.e.* laminin-1, laminin-2/4, and laminin-5) and fibronectin using A549 cells. A549 cells readily attached and spread onto surfaces coated with laminin-10/11, as was the case with surfaces coated with laminin-5 (Fig. 3). Cells spread on the laminin-10/11-coated surface assumed an elongated, spindle-shape morphology with thin processes, as opposed to the cells on the laminin-5-coated surface which displayed a well spread cobblestone-like morphology with greater cell-substratum contact area. Cells were less adherent to the surfaces coated with laminin-1, laminin-2/4, or fibronectin with limited cell spreading at the same coating concentration. In support of this conclusion, quantitative analysis of cells adhering to surfaces coated with increasing concentrations of different adhesion proteins showed that laminin-10/11 and laminin-5 were almost equally active in mediating adhesion of A549 cells, exhibiting maximal activity at concentrations as low as 3 nM. At this concentration, other laminin variants as well as fibronectin were barely capable of supporting cell adhesion (Fig. 4). The coating concentrations for half-maximal adhesion were ~2 nM for laminin-10/11 and laminin-5, 4 nM for laminin-2/4, 5 nM for fibronectin, and 6 nM for laminin-1. Similar results were also obtained with other cell types including A172 human glioma cells and A431 human epidermoid carcinoma cells (data not shown).

Because cell adhesion onto the laminin-coated substratum is mediated predominantly by the integrin family of adhesion receptors, we examined the effects of function-blocking monoclonal antibodies against various integrin subunits on adhesion of A549 cells onto the surfaces coated with laminin-10/11 or other adhesive proteins (Fig. 5). Adhesion onto surfaces coated with laminin-1, laminin-5, and fibronectin was specifically inhibited by antibodies against integrin α6, α3, and α5 subunit, respectively, and also by the antibody against integrin β1 subunit, consistent with previous reports (12, 21, 22). Interestingly, adhesion of A549 cells onto laminin-10/11-coated surfaces was inhibited completely by anti-integrin α3 antibody and by anti-integrin β1 antibody, but not by antibodies against other α subunits including anti-α6 antibody. These results indicated that cell adhesion onto laminin-10/11 is mediated by integrin α3β1, as is the case with laminin-5. Specific inhibition of the laminin-10/11-mediated cell adhesion by anti-integrin α3 subunit antibody was also observed with A172 glioma cells (data not shown).

DISCUSSION

The newest laminin α chain identified to date, α5, has been established as the most widely expressed α chain in mammalian tissues. The anti-human laminin monoclonal antibody 4C7, which was reported initially to be directed against the α1 chain, has been shown to recognize the α5 chain (19), resolving the previous discrepancy in histological distribution between mouse laminin-1 and its human counterpart defined by 4C7 (2). Immunohistochemical studies using 4C7 and other antibodies specific for mouse α5 chain showed that the α5 chain is localized in basement membranes of a wide variety of epithelial tissues and of blood vessels (4, 23, 24). Despite the ubiquitous distribution of the α5 chain, however, the biological functions of the laminin variants containing the α5 chain remain to be determined, mainly because these laminin variants have not been purified in intact form. This study was performed to characterize the biological activities of laminin-10/11 using purified, intact proteins.

The strategies employed to purify laminin-10/11 were as follows. First, we selected a human cell line that expresses only α5 chain by screening more than 30 different human tumor cell lines by RT-PCR and immunoblotting. Conditioned medium of cultured cells is superior to tissue extracts as a source of intact

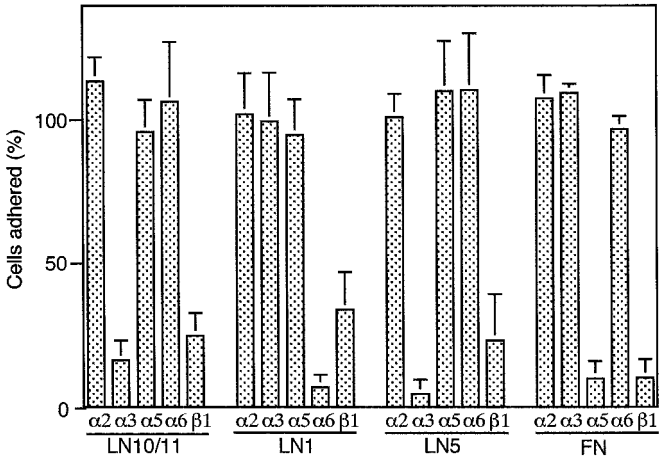

FIG. 5. **Effects of anti-integrin monoclonal antibodies on adhesion of A549 cells to plastic substrates coated with laminin-10/11 and other adhesive proteins.** Wells of microtiter plates were coated with 4 nM laminin-10/11 (*LN10/11*), 30 nM laminin-1 (*LN1*), 4 nM laminin-5 (*LN5*), or 12 nM fibronectin (*FN*). A549 cells were preincubated with the following function-blocking monoclonal antibodies against integrin subunits at a 100 × dilution for ascites or at a concentration of 10 μg/ml IgG for 15 min at room temperature and were then added to the precoated wells: α2, anti-integrin α2 subunit antibody (P1E6); α3, anti-integrin α3 subunit antibody (P1B5); α5, anti-integrin α5 subunit antibody (8F1); α6, anti-integrin α6 subunit antibody (GoH3); β1, anti-integrin β1 subunit antibody (4G2). After a 30-min incubation, cells attached to the substrates were quantified by crystal violet staining as described under "Experimental Procedures." The numbers of adhering cells are expressed as percentages of the number of cells adhering in the absence of monoclonal antibodies. Each *column* represents the mean of triplicate assays. *Bars*, standard deviation.

laminins because the laminin variants reactive with the 4C7 antibody can be solubilized only after proteolytic digestion (*e.g.* pepsin digestion) but not by neutral salt or EDTA extraction, which instead solubilizes laminin-2/4 (25, 26). Second, we produced a monoclonal antibody that specifically recognizes human α5 chain on immunoblots. The availability of such a monoclonal antibody is crucial to identify the α5 chain because final verification of purified laminin-10/11 requires immunoblotting under reducing conditions. No such monoclonal antibodies recognizing reduced, denatured human α5 chain have been reported to date. We also produced a monoclonal antibody that specifically recognizes α1 chain on immunoblots. The α1 chain has a molecular mass similar to that of α5 chain, and therefore it is important to distinguish these two α chains with specific antibodies. Previous confusion regarding the specificity of 4C7 antibody also made it crucial to distinguish α1 and α5 chains on immunoblots. Third, we employed affinity chromatography with 4C7-Sepharose to ensure the authenticity of the purified protein. Based on these strategies, we selected A549 cells as a source for human laminin-10/11 and purified them on a 4C7-Sepharose column. In a separate experiment, we also purified laminin-10/11 by conventional procedures for purification of laminins from tissues, *i.e.* size fractionation on Sepharose 4B-CL, heparin-Sepharose chromatography, and ion exchange chromatography with HiTrap Q-Sepharose. The resulting laminin-10/11 preparation contained some contaminant proteins but exhibited essentially identical cell adhesive and integrin binding activities as observed with those purified by 4C7 immunoaffinity chromatography.[2]

The laminin-10/11 thus purified gave a single band migrating at ~800 kDa under nonreducing conditions and consisted of three chains of 350, 220, and 210 kDa. Based on the reactivity with monoclonal antibodies specific for α5, β1, or γ1 chain on immunoblots, we concluded that the 350-kDa chain was α5 and the 210-kDa chain was γ1. Other α chains including α1, α2, and α3 were not detectable in the purified laminin-10/11. The absence of a 500–600-kDa protein in the purified laminin-10/11 also made it unlikely that the α4 chain-containing laminin variants copurified with laminin-10/11. Weak reactivity of the anti-β1 monoclonal antibody with reduced, denatured protein failed to identify the 220-kDa chain, but the 220-kDa chain is considered to be a mixture of β1 and β2 chains because both transcripts encoding β1 and β2 chains were detectable in A549 cells by RT-PCR. The presence of the β1 chain was verified by positive staining of the unreduced ~800-kDa band with the anti-β1 antibody.

The relative molecular mass of the α5 chain estimated from SDS-PAGE (350 kDa) was significantly smaller than the mass of mouse α5 chain (450 kDa) calculated from the amino acid sequence predicted from the cDNA (5), raising the possibility that the α5 chain expressed in A549 cells is processed post-translationally proteolysis as observed with the α2 and α3 chains (27, 28). Consistent with our observation, the α5 chains expressed in human choriocarcinoma cells (19) and in mouse endothelial cells (24) were also found to be significantly smaller than the predicted mass. It is also possible that the 350-kDa form of α5 chain was generated by alternative RNA splicing, as has been reported for the α3 chain (29).

Using purified laminin-10/11, we demonstrated that laminin-10/11 is a highly adhesive protein, as potent as laminin-5 in mediating cell attachment and spreading onto the substratum. Cell adhesion onto laminin-10/11-coated surfaces was mediated by integrin α3β1 but not by α6β1. Integrin α3β1, once thought to be a promiscuous receptor recognizing laminin-1, collagen, and fibronectin with low affinities, has been shown to recognize laminin-5 preferentially (21, 22, 30–32). Our results indicate that integrin α3β1 is a dominant surface receptor recognizing both laminin-5 and laminin-10/11, both of which are major constituents of basement membranes of a wide variety of epithelial tissues. Although our results provide the first clear evidence demonstrating the integrin binding specificity of laminin-10/11, there have been previous reports on identification of integrin types binding to human laminin. Gehlsen *et al.* (33) reported that integrin α3β1 was specifically bound by an affinity column of human laminin which was prepared from placenta after pepsin digestion followed by immunoaffinity chromatography with a B1 (β1) chain-specific monoclonal antibody (34). Although the laminin used in their study appeared to be a mixture of truncated forms of laminin variants containing the β1 chain, its strong reactivity with 4C7 (25) indicated that the human laminin from pepsinized placenta contained a significant amount of laminin-10/11 in truncated form. In support of this, we found that human placental laminins obtained from different commercial sources, either purified after pepsin digestion or EDTA extraction, were strongly reactive with our anti-α5 monoclonal antibody on immunoblots.[2] The specific binding of integrin α3β1 to an affinity column of human laminin from pepsinized placenta is therefore consistent with our conclusion that laminin-10/11 is specifically recognized by integrin α3β1. Essentially an identical approach was also taken by Sonnenberg *et al.* (35) to identify laminin-binding integrin types, resulting in a similar conclusion except that human laminin could also bind to integrin α6β1 with lower affinity. The apparent discrepancy between these two previous reports may have been caused by the differences in the proportion of laminin-10/11 relative to other contaminant laminin variants.

Among three other laminin variants examined in this study, laminin-10/11 seems to be more related to laminin-5 than to other laminin variants (*i.e.* laminin-1 and laminin-2/4) in ad-

[2] Y. Kikkawa, unpublished observation.

hesive properties. Laminin-5 has been reported to be most potent in mediating adhesion of keratinocytes (32), endothelial cells (36), and glioma cells (12) among various adhesive proteins including laminin-1, laminin-2/4, fibronectin, and vitronectin. Our results showed that laminin-10/11 has potency comparable to that of laminin-5 in mediating cell adhesion to the substratum. Furthermore, both laminin variants seem to be specifically recognized by integrin $\alpha3\beta1$, although laminin-5 can also be recognized by integrin $\alpha6\beta4$ which plays an important role in hemidesmosome assembly (37). Furthermore, the α chains of laminin-10/11 and laminin-5, *i.e.* $\alpha5$ and $\alpha3$, seem to be evolutionally the most related among five different laminin α chains (38). A full sized laminin $\alpha3$ chain, $\alpha3$B, was identified recently in mouse and human, showing the highest homology to the $\alpha5$ chain at the amino acid level (4, 38). Despite these similarities, however, it should be noted that morphologies of cells adhering onto surfaces coated with either laminin-10/11 or laminin-5 were significantly different. Cells on laminin-10/11-coated surfaces assumed an elongated morphology with multiple thin processes, and those adhering to laminin-5-coated surfaces assumed a cobblestone-like morphology. This clear distinction in adhering cell morphology suggests that the signals transduced from substrate-adsorbed laminin-10/11 and laminin-5 through integrin $\alpha3\beta1$ are functionally different. The differences in signaling events could be either quantitative, *i.e.* simply the result of differences in the binding affinity of these laminin variants with integrin $\alpha3\beta1$, which in turn determines the magnitude of cytoplasmic signals elicited by the ligand-ligated integrin, or qualitative, *i.e.* the result of differences in the involvement of coreceptors such as dystroglycan and integrin $\alpha6\beta4$, which also bind to substrate-bound laminin variants (39, 40). Furthermore, integrin $\alpha3\beta1$ has been shown to associate with transmembrane-4 superfamily proteins (41, 42) and EMMPRIN (43). These integrin-associated membrane proteins could be involved in the regulation of signaling events mediated by ligand-ligated integrin $\alpha3\beta1$, leading to different cell morphologies on substrata coated with different laminin variants.

In summary, we purified laminin-10/11 from the conditioned medium of A549 cells and demonstrated that it is highly competent in mediating cell adhesion to the substratum in an integrin $\alpha3\beta1$-dependent manner. Given that laminin-10/11 are the predominant laminin variants of most epithelial tissues and that integrin $\alpha3\beta1$ is the most abundant integrin receptor expressed in epithelial cells of different tissue types, specific interaction of integrin $\alpha3\beta1$ with laminin-10/11 may play a central role not only in the adhesion of epithelial cells to underlying basement membranes but also in the regulation and maintenance of the differentiated phenotypes of epithelial cells *in vivo*.

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Isolation and Characterization of Laminin-10/11 Secreted by Human Lung Carcinoma Cells: LAMININ-10/11 MEDIATES CELL ADHESION THROUGH INTEGRIN α3β1

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