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Purification and Characterization of Human Laminin-8

LAMININ-8 STIMULATES CELL ADHESION AND MIGRATION THROUGH $\alpha_3\beta_1$ AND $\alpha_6\beta_1$ INTEGRINS*

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Recently identified laminin isoforms containing the α4 chain have been shown to be expressed in the basement membrane of restricted organs such as heart, skeletal muscle, and blood vessels, especially those in embryos. We screened 38 human cell lines for the expression of the laminin α4 chain by reverse transcriptase-polymerase chain reaction and found that T98G glioblastoma cells express only α4, but not other α chains. Laminin-8, an isoform containing the α4 and β1 chains, was purified from conditioned medium of T98G cells by gel filtration and immunoaffinity chromatography using a monoclonal antibody against laminin β1 chain. The purified laminin isoform was composed of disulfide-linked 230-, 220-, and 200-kDa subunits, which immunoblot analysis identified as the β1, γ1, and α4 chains. Purified laminin-8 had cell adhesive activity comparable to laminin-1 but significantly weaker than laminin-5 and laminin-10/11. T98G cells adhering to laminin-8 became more elongated than those adhering to other laminin isoforms and extended multiple pseudopods. Cell adhesion to laminin-8 was abolished by an antibody against the integrin β_1 subunit or a combination of antibodies against the integrin α_3 and α_6 subunits, but not by either anti-α_3 or anti-α_6 antibody alone, suggesting that both $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins serve as adhesion receptors for laminin-8. Consistent with these observations, K562 erythroleukemic cells transfected with either integrin α_3 or α_6 cDNA were capable of adhering to laminin-8 when β_1 integrins were stimulated by the β_1-activating antibody 8A2. Despite its moderate cell adhesive activity, laminin-8 was significantly potent in promoting cell migration when compared with other laminin isoforms and fibronectin. Cell migration on laminin-8 was completely inhibited by a combination of antibodies against α_3 and α_6 integrins, and substantially inhibited by anti-α_3 antibody alone, suggesting that laminin-8-mediated cell migration is predominantly mediated by $\alpha_3\beta_1$ integrin. Given its potency to stimulate cell migration and preferential localization to the basement membrane of capillaries and embryonic tissues, laminin-8 may play a role in processes requiring enhanced cell migration during development, wound healing, and angiogenesis.

Laminins are the major class of basement membrane proteins, composed of three disulfide-linked subunits, α, β, and γ. To date, five α, three β, and three γ chains have been identified, combinations of which have been shown to give rise to at least 12 different laminin isoforms (1–4). These laminin isoforms are expressed in a tissue-specific and developmentally regulated manner, suggesting that they are functionally distinct (5, 6). The differences in biological activity among these isoforms, however, have yet to be defined.

Laminins have many biological functions, including promotion of cell adhesion and migration, control of cell proliferation and gene expression, maintenance of differentiation phenotypes, and stimulation of neurite outgrowth. Laminins mediate these functions through binding to cell surface receptors, particularly the integrin family of cell adhesion molecules. So far, nine integrin types, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$, have been shown to bind to laminins with distinct specificities (6, 7). For example, cell adhesion to laminin-1, the "classical laminin" purified from mouse Engelbreth-Holm-Swarm tumor, is mainly mediated by $\alpha_6\beta_1$ integrin, while adhesion to laminin-5 occurs through $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins (8–12). Laminin-2/4 is recognized by $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_7\beta_1$ integrins, depending on the cell type (13, 14). We purified laminin-10/11 from conditioned medium of lung carcinoma cells and showed that it interacts with $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins (15, 16). Recently, the α4 chain-containing laminin isoform, laminin-8, was reported to bind to $\alpha_6\beta_1$ integrin (17, 18).

The α4 chain is a truncated version of the laminin α chains like α3A (19, 20). The α4 chain is predominantly expressed in capillaries in brain, muscle, and bone marrow (19, 21–24). In the kidney, the α4 chain is present in nascent epithelial basement membrane of the renal vesicle and immature glomerular basement membrane, but is absent in the adult kidney (23, 25). The α4 chain is also localized to muscle basement membrane during muscle formation, but is absent in adult muscles, except at neuromuscular junctions (26, 27). Similarly, laminin-8 is the major laminin isoform in developing bone marrow (24). Despite its restricted expression and localization *in vivo*, the biological activity of the α4 chain-containing laminins has not been explored thoroughly due to the unavailability of sufficient purified laminin-8 for biochemical and functional analysis. In this study, we purified laminin-8 from conditioned medium of human glioma cells and characterized its biological activities, including cell adhesive and cell migration promoting properties and integrin binding specificity in comparison with other laminin isoforms and fibronectin.

EXPERIMENTAL PROCEDURES

Cell Culture—Human glioblastoma cell line T98G, human lung adenocarcinoma cell line A549, human gastric cancer cell line AZ-521, human hepatoma cell line HLF, and human gastric carcinoma cell line MKN-45 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human lung squamous carcinoma cell line RERF-

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LC-AI was purchased from RIKEN Gene Bank (Tsukuba, Japan). These cells were maintained in DMEM[1] containing 10% FBS. K562 human erythroleukemic cells transfected with cDNAs encoding human integrin α_{3A} or α_{6A} subunits were kindly provided by Dr. Arnoud Sonnenberg (The Netherlands Cancer Institute) and maintained in RPMI 1640 containing 10% FBS and 1 mg/ml Geneticin (13).

Antibodies—The mAb 8B12 against the human laminin α4 chain was produced by fusion of Sp2/O mouse myeloma cells with spleen cells from mice immunized with a glutathione *S*-transferase fusion protein containing the G1 domain of the laminin α4 chain (Ser^{835}-Cys^{1028} (20)). The mAb 8B12 was capable of binding to denatured laminin α4 chain on immunoblots, but not to intact α4 chain-containing laminin assembled with β1 and γ1 chains in an enzyme-linked immunosorbent assay, and was therefore unsuitable for immunoaffinity purification of intact laminins. The mAb 4F5 specific for the human laminin β1 chain was produced similarly to 8B12, except that spleen cells were obtained from mice immunized with human placental laminin (Chemicon, Temecula, CA). The specificity of 4F5 was determined by isolation and protein sequencing of peptides from a thermolysin digest of human placenta that selectively bound to 4F5-conjugated Sepharose 4B. The N-terminal amino acid sequence of the 4F5-binding 60-kDa fragment was ARQ*X*-DR*X*LPGHWGFP, identical to the sequence of the human laminin β1 chain (Ala^{848}-Pro^{862} (28)). The mAb 5D6 against the human laminin α5 chain, was produced by immunizing mice with commercially available human laminin-10/11 (Chemicon) purified from a pepsin digest of human placenta by immunoaffinity chromatography using the anti-α5 chain mAb 4C7 (29, 30). The mAb 5D6 was selected by positive reactivity with purified human laminin-10/11 (15) and negative reactivity with commercially available human laminin-2/4 (Chemicon). The mAb 2B10 against the laminin α3 chain was produced by immunizing mice with human laminin-5 purified from conditioned medium of MKN-45 cells (31). 2B10 was selected by reactivity with purified laminin-5 in an enzyme-linked immunosorbent assay, followed by specific binding to the laminin α3 chain as shown on immunoblots. mAbs against the human laminin α1 (5A3) and α5 (15H5) chains were produced in our laboratory as described previously (15). A mAb against the laminin β1 chain (DG10) was kindly provided from Dr. Ismo Virtanen (University of Helsinki, Finland). A hybridoma secreting mAb against the laminin β2 chain (C4), developed by Dr. Joshua Sanes (Washington University School of Medicine), was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). MAbs against the human laminin α2 chain (5H2) and human integrin α2 (P1E6) were purchased from Chemicon. MAbs against the human laminin γ1 chain (2E8 and number 22) were obtained from Chemicon and Transduction Laboratories (Lexington, KY), respectively. A mAb against the human integrin α_6 (GoH3) was purchased from Immunotech (Westbrook, ME). MAbs against the human integrin α3 (3G8), α5 (8F1), and β1 (4G2) chains were produced in our laboratory as described previously (16, 32). A mAb against integrin β1 (8A2) which activates β1 chain-containing integrins, was a gift from Dr. Nicholas Kovach (University of Washington, Seattle, WA).

Adhesive Proteins—Mouse laminin-1 was purified from mouse Engelbreth-Holm-Swarm tumor tissues by the method of Paulsson *et al.* (33). Human laminin-5 was purified from conditioned medium of MKN45 cells by immunoaffinity chromatography using polyclonal antibodies against the human laminin γ2 chain (31). Human laminin-10/11 was purified from conditioned medium of A549 cells according to Kikkawa *et al.* (15), except that mAb 5D6, instead of 4C7, was used to conjugate Sepharose 4B in the immunoaffinity matrix. Human laminin-1 was isolated from conditioned medium of RERF-LC-AI cells on a 4F5 immunoaffinity column and used only for immunoblot analysis. Human laminin-2/4 (also referred to as merosin) was purchased from Chemicon. Human plasma fibronectin was purified from outdated plasma by gelatin affinity chromatography (34).

Screening of Cultured Cells for Expression of Laminin α Chains by RT-PCR—Total RNA was extracted from 38 human cell lines (13 lung carcinomas, 5 gastric carcinomas, 3 gliomas, 2 kidney carcinomas, 2 cervix carcinomas, 2 tropoblastomas, 2 endothelial cell lines, and one each of oral carcinoma, salivary grand carcinoma, rhabdomyosarcoma, leukemia, hepatoma, pancreatic carcinoma, epidermoid carcinoma, fibrosarcoma, and lung fibroblast) by the acid guanidinium isothiocyanate method (35) and used as templates for cDNA synthesis. cDNAs encoding each of five distinct laminin α chains were amplified by PCR using the following pairs of primers; 5′-AAGTGTGAAGAATGTGAGGATGGG-3′ (forward primer for α1; nucleotides 3020–3043 (36)) and 5′-CACTGAGGACCAAAGACATTTTCCT-3′ (reverse primer for α1; nucleotides 3312–3336); 5′-AAATGTACAGAGTGCAGTCGAGGTCA-3′ (forward primer for α2; nucleotides 3314–3339 (37)) and 5′-CAGTGGATGCCTTCCACATTCACCTT-3′ (reverse primer for α2; nucleotides 3458–3483); 5′-CACTGTGAACGCTGCCAGGAGGGCTA-3′ (forward primer for α3; nucleotides 280–305 (38)) and 5′-CAGCTACCTCCGAATTTCTGGGGATT-3′ (reverse primer for α3; nucleotides 466–491); 5′-CACTGTGAAAAGTGTCTGGATGGT-3′ (forward primer for α4; nucleotides 608–631 (20)) and 5′-CAGGTGCTTCCAATGAGGAAGGGG-3′ (reverse primer for α4; nucleotides 811–834); 5′-GACTGCCTGCTGTGCCAGC-3′ (forward primer for α5 (15)) and 5′-GGGGTAGCCATGAAAGCCCG-3′ (reverse primer for α5); 5′-AACTGTGAGCAGTGCAAGCCGTTT-3′ (forward primer for β1; nucleotides 1054–1077 (39)) and 5′-CAACCAAATGGATCTTCACTGCTT-3′ (reverse primer for β1; nucleotides 1278–1301); 5′-CACTGTGAGCTCTGTCGGCCCTTC-3′ (forward primer for β2; nucleotides 1153–1176 (40)) and 5′-CAAGGAGTGCTCCCAGGCACTGTG-3′ (reverse primer for β2; nucleotides 1427–1451); and 5′-CACTGTGAGAGGTGCCGAGAGAAC-3′ (forward primer for γ1; nucleotides 1033–1056 (41)) and 5′-CATCCTGCTTCAGTGAGAGAATGG-3′ (reverse primer for γ1; nucleotides 1203–1226). PCR products were analyzed by electrophoresis using 2% agarose gels.

Purification of Laminin-8—T98G cells were grown in 1,700-cm^2 roller bottles with DMEM containing 10% FBS. After the cells reached confluence, the medium was replaced with DMEM containing 5% FBS and harvested every 6 days. The conditioned medium (about 4 liters) was clarified by centrifugation and then precipitated with 45% saturated ammonium sulfate. The precipitates were dissolved in 10 ml of 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, clarified by centrifugation, and subjected to gel filtration on a Sepharose CL-4B column (2.5 × 120 cm) equilibrated in PBS containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM *N*-ethylmaleimide, and 1 mM EDTA. The Sepharose CL-4B column was calibrated prior to use with mouse laminin-1 (~800 kDa), human plasma fibronectin (~450 kDa), and mouse IgG (150 kDa). Fractions containing laminin-8 were detected by immunoblotting with anti-α4 mAb 8B12. These fractions were pooled and then applied to a 4F5-conjugated Sepharose 4B column equilibrated in PBS containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM *N*-ethylmaleimide, 5 mM EDTA, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The bound proteins were eluted with 0.1 M glycine-HCl (pH 2.4), immediately neutralized with 1.5 M Tris-HCl (pH 9.0), and dialyzed against PBS containing 1 mM EDTA.

Cell Adhesion Assay—Cell adhesion assays were performed as described previously (15) with minor modifications. Ninety-six-well microtiter plates (Nunc, Wiesbaden, Germany) were coated with 50 μl of increasing concentrations of cell adhesive proteins overnight at 4 °C, and then blocked with PBS containing 2% BSA at 37 °C for 1 h. 100-μl aliquots of cell suspension (3×10^5 cells/ml in serum-free DMEM containing 10 mM HEPES (pH 7.4) and 0.5% BSA) were added to each well of the coated plates and incubated at 37 °C for 30 min in a CO_2 incubator. After the plates were washed to remove unattached cells, attached cells were fixed with 3.7% formaldehyde, stained with Diff-Quik (International Reagents Corp., Kobe, Japan), and counted in three independent fields/well. For cell adhesion assays using K562 cells, cell suspensions (4×10^5 cells/ml in serum-free RPMI 1640 containing 10 mM HEPES (pH 7.4) and 0.5% BSA) were preincubated with or without 8A2, an integrin β1-activating mAb, for 10 min at room temperature. 50-μl aliquots of preincubated cell suspension were added to 96-well microtiter plates coated with increasing concentrations of various laminin isoforms and incubated at 37 °C for 30 min in a CO_2 incubator. Attached cells were counted as described above.

Cell adhesion inhibition assays were performed based on the cell adhesion assays. Cell suspensions (4×10^5 cells/ml in serum-free DMEM containing 10 mM HEPES (pH 7.4) and 0.5% BSA) were incubated with 20 μg/ml mAbs against different integrin isoforms for 20 min at room temperature. 50-μl aliquots of preincubated cells were added to wells that had been coated with different cell adhesive proteins and incubated for 30 min at 37 °C in a CO_2 incubator. Attached cells were then counted as described above.

Cell Migration Assay—Cell migration on substrates coated with laminins or fibronectin was examined by time lapse video microscopy using the image processing software Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Glass-bottom culture dishes fitted with ϕ 8-mm coverslips were coated with 250 μl of cell adhesive proteins overnight at 4 °C, and blocked with PBS containing 2% BSA at 37 °C for 1 h. 4-ml of cell suspension (1×10^4 cells/ml in DMEM containing 1% FBS) was

[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; mAb, monoclonal antibody; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis.

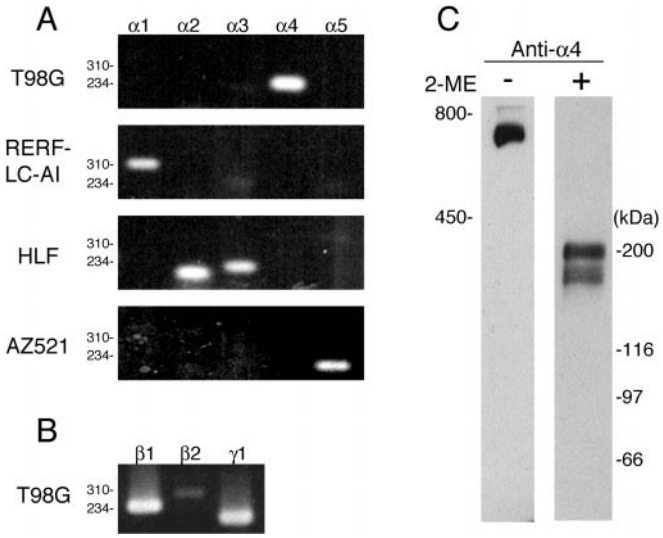

FIG. 1. **Expression of laminin-8 in human tumor cell lines.** *A,* transcripts for α1, α2, α3, α4, and α5 chains of human laminin were amplified by RT-PCR from T98G human glioblastoma cells, RERF-LC-AI human lung carcinoma cells, HLF human hepatoma cells, and AZ521 human gastric carcinoma cells, using the primers described under "Experimental Procedures." The predicted size of the amplified cDNAs were 317 (α1), 168 (α2), 212 (α3), 227 (α4), and 197 (α5) base pairs. *B,* transcripts for β1, β2, and γ1 chains of human laminin were amplified by RT-PCR from T98G cells, using the primers described under "Experimental Procedures." The predicted size of the amplified cDNAs were 248 (β1), 299 (β2), and 194 (γ1) base pairs. *C,* the α4 chain-containing laminins in conditioned medium of T98G cells were detected by immunoblot analysis. Conditioned medium of T98G cells was separated by SDS-PAGE on 4% gels under nonreducing conditions or on 5.5% gels under reducing conditions. The separated proteins were transferred onto PVDF membranes followed by immunostaining with 8B12, a mAb against the laminin α4 chain. Shown in the margin are the positions of molecular weight markers.

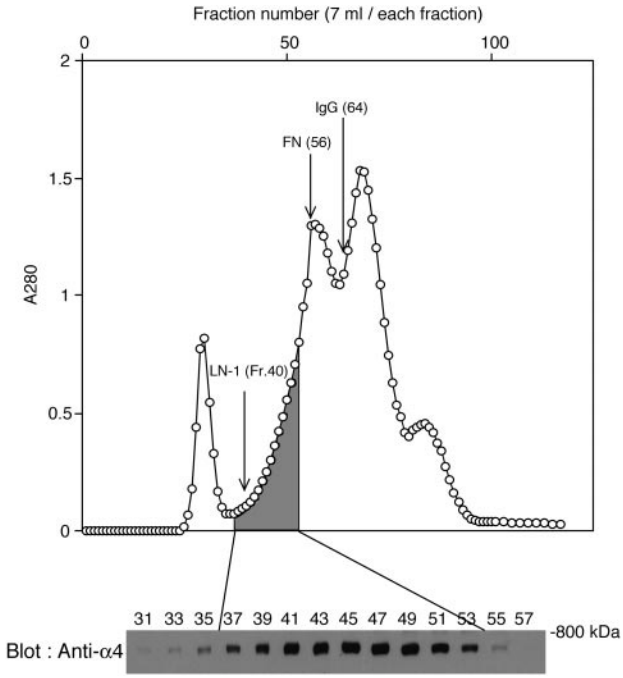

FIG. 2. **Size fractionation of conditioned medium of T98G cells by gel filtration.** Conditioned medium of T98G cells (about 4 liters) was precipitated with 45% saturated ammonium sulfate, and the precipitates were subjected to gel filtration on a Sepharose CL-4B column (2.5 × 120 cm). The column was calibrated with mouse laminin-1 (~800 kDa), human plasma fibronectin (450 kDa), and mouse IgG (150 kDa) prior to use. *Arrows* indicate the positions where laminin-1, fibronectin, and IgG eluted. Fractions containing laminin-8 were detected by immunoblotting with anti-α4 chain mAb 8B12 under nonreducing conditions. Fractions 37–53 were pooled and subsequently subjected to immunoaffinity chromatography.

added to each coated dish and incubated at 37 °C for 30 min to allow the cells to attach. The dishes were then placed in a built-in CO_2 incubator on the stage of the Zeiss Axiovert 25 microscope, and subjected to time lapse video microscopy at 10-min intervals for 8 h. Cell migration was quantified by tracing the position of the nucleus of migration cells using Image-Pro Plus.

For cell migration inhibition assays, cell suspensions (2×10^4 cells/ml in DMEM containing 1% FBS) were incubated with 20 μg/ml mAbs against different integrin isoforms for 20 min at room temperature. 2-ml of preincubated cell suspension was added to the precoated glass-bottom culture dishes and incubated at 37 °C for 30 min to allow cells to attach. Cell migration images were taken as described for cell migration assays for 8 h.

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out according to Laemmli (42) using 4 or 5.5% acrylamide gels. Separated proteins were visualized by silver staining or transferred onto PVDF membranes. The membranes were probed with mAbs against individual laminin chains, followed by visualization using the ECL detection kit (Amersham Pharmacia Biotech).

RESULTS

Screening of Human Cell Lines for Selective Expression of the Laminin α4 Chain—To purify laminin isoforms containing the α4 chain from conditioned medium of cultured cells, we screened 38 human cell lines for selective expression of the laminin α4 chain by RT-PCR. We found that one of the glioma cell lines, T98G, expresses only one of the five known laminin α chains, the α4 chain (Fig. 1*A*). Failure to detect RNA transcripts for other α chains was not due to inappropriate PCR conditions, since these transcripts were clearly detected in other cell lines, including the α1 chain in RERF-LC-AI, α2 and α3 chains in HLF, and α5 chain in AZ521. T98G cells also expressed β1 and γ1 chains, but only a low level of the β2 chain (Fig. 1*B*), indicating that laminin-8 (α4β1γ1) is the major laminin isoform expressed in T98G cells.

The expression of laminin-8 in T98G cells was confirmed at the protein level by immunoblot analysis of conditioned medium of T98G cells using mAb 8B12 produced by immunizing mice with a recombinant G1 domain of the human α4 chain. The mAb 8B12 specifically detected a ~650-kDa band under nonreducing conditions, while reducing conditions resulted in a major 200-kDa band and a faint 180-kDa band (Fig. 1*C*). The 200-kDa band is consistent with the molecular mass of the laminin α4 chain as predicted by its amino acid sequence (19, 20), confirming that T98G cells synthesize and secrete the α4 chain as a disulfide-linked heterotrimer with β1 and γ1 chains.

Purification of Laminin-8—Laminin-8 secreted by T98G cells was purified from conditioned medium by ammonium sulfate precipitation, gel filtration, and immunoaffinity chromatography using mAb 4F5 specific for the laminin β1 chain. Conditioned medium of T98G cells was precipitated with 45% saturated ammonium sulfate, and the precipitates were subjected to gel filtration on a Sepharose CL-4B column (Fig. 2). Fractions containing trimeric laminin-8 were detected by immunoblotting with 8B12, an anti-α4 chain mAb, pooled, and subjected to immunoaffinity chromatography using 4F5. The eluate from the 4F5-Sepharose column was found by silver staining to contain a single 650-kDa species under nonreducing conditions (Fig. 3*A*). The ~650-kDa band was stained with mAbs specific for the α4, β1, and γ1 chains, confirming that the purified protein was laminin-8. Under reducing conditions, three bands of 230, 220, and 200 kDa were resolved, each identified as the β1, γ1, and α4 chains, respectively, by immunoblot analysis (Fig. 3*B*). A faint 180-kDa band was also detectable by immunoblotting with anti-α4 mAb. Since the 180-kDa band was also detectable in fresh conditioned medium (Fig. 1*C*), it could be a proteolytically processed α4 chain or an

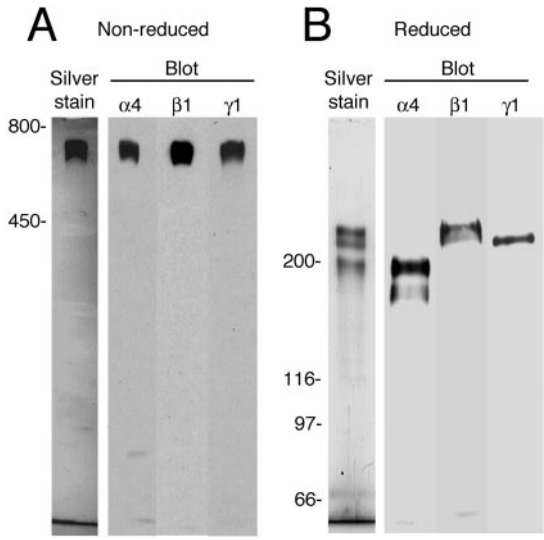

FIG. 3. **SDS-PAGE and immunoblot analysis of purified laminin-8.** Laminin-8 purified from conditioned medium of T98G cells was analyzed by SDS-PAGE on 4% gels under nonreducing conditions (*A*) or on 5.5% gels under reducing conditions (*B*). Separated proteins were visualized by silver staining or transferred onto PVDF membranes followed by staining with mAbs against laminin α4 (8B12), β1 (DG10), or γ1 (2E8 under nonreducing conditions and mAb number 22 under reducing conditions) chains. The positions of molecular size markers are shown in the *left margin*.

alternatively spliced variant. Since no bands near 150 kDa were detected by silver staining under reducing or nonreducing conditions, the purified laminin-8 seemed to be devoid of nidogen-1.

To confirm the absence of other laminin isoforms in the purified laminin-8, we examined the reactivity of the purified laminin-8 with mAbs specific for each laminin chain by immunoblotting. Purified laminin-8 was strongly stained by a mAb against the α4 chain, but not by mAbs against the α1, α2, α3, α5, or β2 chains (Fig. 4*A*), demonstrating the absence of other α chain-containing laminin isoforms and the β2 chain-containing laminin-9 (α4β2γ1). When a mAb against the γ1 chain was used as a probe against purified laminin-8, only the 650-kDa band was labeled, while 700–800-kDa bands were detected in purified laminin-1, laminin-2/4, and laminin-10/11 samples (Fig. 4*B*). The absence of a 700–800-kDa band in laminin-8 purified from conditioned medium of T98G cells further confirmed the absence of any contaminating laminin isoforms.

Cell Adhesive Activity of Laminin-8—Cell adhesive activity of laminin-8 was compared with that of other laminin isoforms (*i.e.* laminin-1, laminin-2/4, laminin-5, and laminin-10/11) and fibronectin using T98G cells as substrate. Laminin-8 was a less potent mediator of cell adhesion than laminin-5 and laminin-10/11, and was comparable to laminin-1 (Fig. 5). Half-maximal levels of T98G cell adhesion were achieved at a substrate coating concentration of 2.5 nM for laminin-5 and laminin-10/11, and 5 nM for laminin-8 and laminin-1.

Cell adhesion to laminins is mainly mediated by integrins. We examined the effects of function blocking mAbs against various integrin subunits on adhesion of T98G cells to laminin-8 to determine the major integrin type(s) that serves as its adhesion receptor(s) (Fig. 6). As previously demonstrated (9, 10, 15, 31), cell adhesion to laminin-1, laminin-5, and fibronectin is specifically inhibited by mAbs against integrins α_6, α3, and α5, respectively. The mAb against integrin β1 also inhibit cell adhesion to these proteins. In contrast, the adhesion of T98G cells to laminin-8 is not inhibited by any single mAb, except for anti-β1 mAb. When combined, however, mAbs against α3 and α_6 were found to strongly inhibit the adhesion of T98G cells to laminin-8, indicating that T98G cells adhere to laminin-8 through both α3β1 and α_6β1 integrins.

To confirm the dual integrin specificity of laminin-8, we examined the laminin-8 adhesion of K562 human erythroleukemic cells transfected with integrin α3 or α_6 in the absence or presence of 8A2, an anti-β1 activating mAb (Fig. 7). Untransfected K562 cells, shown to express only the α5 subtype of β1 integrin (13, 43), could not adhere to all laminin isoforms examined, regardless of the absence or presence of the β1-activating mAb. K562 cells expressing the integrin α_6 subunit together with endogenous β1 were competent in adhering to laminin-8 and, to a lesser extent, to laminin-5, but not to laminin-1, in the absence of mAb 8A2. Upon β1 integrin stimulation by 8A2, α_6-transfected cells became highly adherent to all these laminin isoforms with similar potencies. In contrast, α3-transfected cells expressing α3β1 integrin were only capable of adhering to laminin-5, and did not respond to laminin-1 or laminin-8 in the absence of mAb 8A2 stimulation. Upon 8A2 stimulation, however, α3-transfected cells became adherent to laminin-8. Adhesion of the stimulated α3-transfected cells to laminin-8 was not due to increased nonspecific adhesiveness of these cells, as they remained nonadherent to laminin-1. These results confirmed that both α3β1 and α_6β1 integrins could serve as the adhesion receptors for laminin-8, but that the latter may act as the preferred receptor for laminin-8, since α_6-transfected, but not α3-transfected K562 cells adhere to laminin-8 without stimulation by 8A2.

Cell Migration Promoting Activity of Laminin-8—Laminins have been shown to stimulate cell migration during development and in many pathological processes (44–46). We examined the ability of laminin-8 to promote cell migration by using time lapse video microscopy to track T98G cells migrating on dishes coated with different concentrations of laminin-8 or other adhesive proteins. Among the six different adhesive ligands examined, laminin-8 was most potent in promoting cell migration, attaining maximal activity at coating concentrations of >10 nM without a significant decline up to 80 nM (Fig. 8). Laminin-10/11 was also very potent in promoting cell migration, attaining maximal activity at coating concentrations of 5–10 nM. Laminin-2/4 and laminin-5 were of roughly equal potency, lower than laminin-8 and laminin-10/11, while laminin-1 was weaker still. Fibronectin was barely active, if at all, in promoting migration of T98G cells.

Cell migration promoting activity of all six adhesive ligands except laminin-8 declined at higher coating concentrations, consistent with previous observations (47–49) that cell migration speed was dependent on the adhesive strength of cells to substratum, giving a biphasic dose-response curve when substrates are coated with increasing concentrations of adhesive ligands. Laminin-8 also strongly promoted migration of other cell types, including A549 human lung adenocarcinoma cells, to a similar degree as was demonstrated with T98G cells (data not shown).

T98G cells migrating on laminin-8 assumed an elongated morphology with frequent pseudopod extension in the direction of cell migration (Fig. 9). Small lamellipodia-like structures were observed at the tip of the pseudopods. The cells plated on laminin-8-coated substrates were capable of easily detaching from the substratum at the rear, allowing them to crawl smoothly. A similar elongated morphology with pseudopods extended in the direction of cell migration was observed with cells plated on substrates coated with laminin-2/4 and laminin-10/11, but not with laminin-1, laminin-5, or fibronectin. Cells on laminin-1, laminin-5, or fibronectin-coated substrates assumed rather round morphologies with wide lamellipodia extending in multiple directions from time to time, thereby pre-

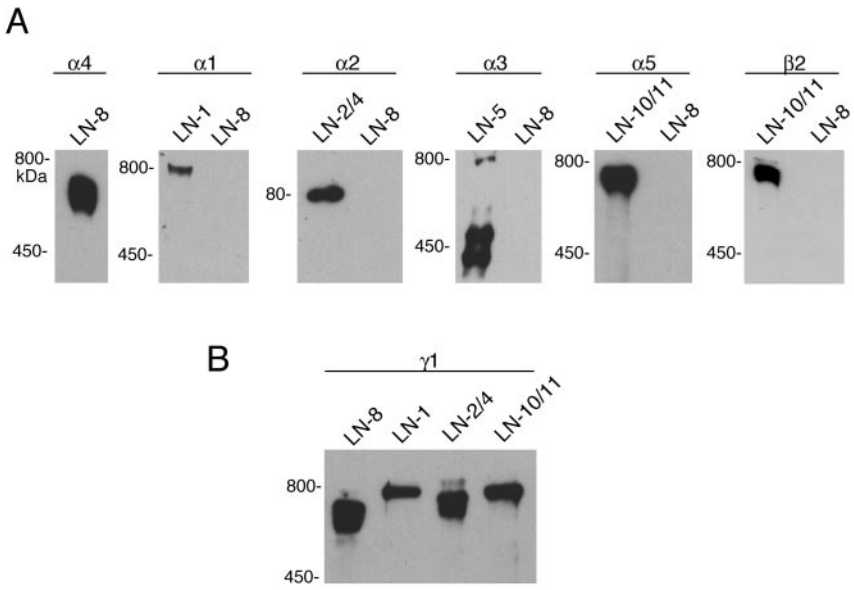

FIG. 4. **Immunoblot analysis of purified laminin-8 with mAbs against distinct laminin chains.** *A,* purified laminin-8 was subjected to immunoblotting with mAbs against distinct laminin α chains or with a mAb against the β2 chain to confirm the absence of other laminin isoforms. Equal amounts (0.4 μg/lane) of purified human laminin-1 (*LN-1*), laminin-2/4 (*LN-2/4*), laminin-5 (*LN-5*), laminin-8 (*LN-8*), and laminin-10/11 (*LN-10/11*) were separated by SDS-PAGE on 4% gels under nonreducing conditions and transferred onto PVDF membranes followed by staining with mAbs against the laminin α1 (5A3), α2 (5H2), α3 (2B10), α4 (8B12), α5 (15H5), or β2 (C4) chains. The mAb against the laminin α2 chain reacts with the 80-kDa fragment derived from the C-terminal region of the α2 chain. *B,* equal amounts (0.4 μg/lane) of purified human laminin-1 (*LN-1*), laminin-2/4 (*LN-2/4*), laminin-8 (*LN-8*), and laminin-10/11 (*LN-10/11*) were similarly subjected to immunoblotting with anti-γ1 mAb 2E8 as described above. The positions of molecular size markers are shown in the *left margin*.

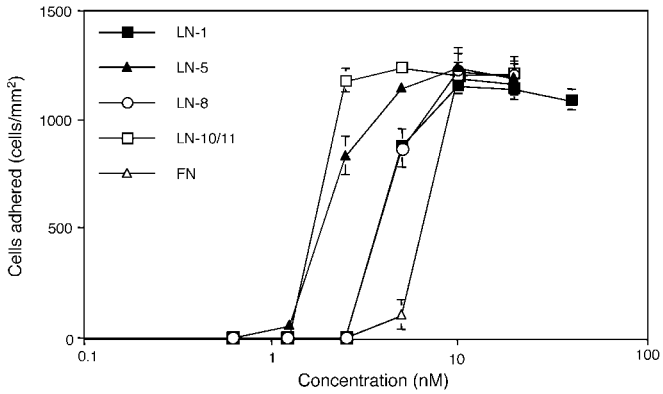

FIG. 5. **Cell adhesive activity of laminin-8.** T98G cells were incubated at 37 °C for 30 min on 96-well microtiter plates coated with increasing concentrations of laminin-1 (*LN-1*), laminin-5 (*LN-5*), laminin-8 (*LN-8*), laminin-10/11 (*LN-10/11*), or fibronectin (*FN*). The plates were washed with serum-free DMEM three times to remove unattached cells, after which attached cells were fixed and counted under a microscope. *Bars* represent the standard deviation of triplicate assays.

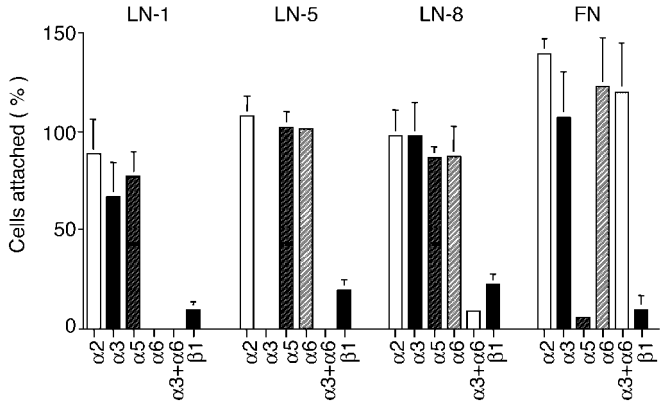

FIG. 6. **Effects of anti-integrin mAbs on adhesion of T98G cells to laminin-8 and other adhesive proteins.** Ninety-six-well microtiter plates were coated with 8 nM laminin-1 (*LN-1*), 2.5 nM laminin-5 (*LN-5*), 8 nM laminin-8 (*LN-8*), or 8 nM fibronectin (*FN*). The coating concentrations of adhesive ligands were chosen to obtain near-maximal levels of cell adhesion in the absence of blocking mAbs. T98G cells were preincubated with the following function-blocking mAbs against integrin subunits at a concentration of 20 μg/ml IgG for 20 min at room temperature and then added to the precoated wells: α_2, anti-integrin α_2 subunit mAb (P1E6); α3, anti-integrin α3 subunit mAb (3G8); α5, anti-integrin α5 subunit mAb (8F1); α_6, anti-integrin α_6 subunit mAb (GoH3); and β1, anti-integrin β1 subunit mAb (4G2). After 30 min incubation at 37 °C, cells attached to the substrates were counted under a microscope. The number of adhering cells is expressed as a percentage of the number of cells adhering in the presence of control mouse IgG. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

venting them from becoming polarized. There was a close correlation between the ability of the substrates to polarize cells and their potency in promoting cell migration.

To explore whether both $\alpha 3\beta 1$ and $\alpha_6\beta 1$ integrins are involved in cell migration on laminin-8, we examined the effects of function-blocking mAbs against different integrin subunits on T98G cell migration on laminin-8-coated substrates. The mAb against α3 integrin strongly inhibited laminin-8-mediated cell migration, but an anti-α_6 integrin mAb was only marginally inhibitory (Fig. 10). The combination of α3 and α_6 integrin mAbs completely inhibited cell migration. These results indicate that cell migration on laminin-8 is predominantly driven by the interaction of $\alpha 3\beta 1$ integrin with laminin-8.

DISCUSSION

In this study, we purified laminin-8 from conditioned medium of T98G cells by affinity chromatography using a mAb specific for the laminin β1 chain. T98G cells were used as a source of laminin-8 because these cells express only the α4 subtype of laminin α chain. Selective expression of the α4 chain was confirmed at both the RNA and protein level by RT-PCR and immunoblot analysis. Although mAb 8B12 recognizes only denatured, and not intact, α4 chains, and cannot be used as an affinity ligand for purification, selective expression of the α4 chain in T98G cells allowed us to purify laminin-8 by affinity chromatography using mAb against the laminin β1 chain. The identity of the purified laminin-8 was confirmed by its positive reactivity with mAbs against the α4, β1, and γ1 chains. The absence of other contaminating laminin isoforms was established by negative reactivity to a panel of mAbs recognizing each one of the other four known α chains. Furthermore, laminin-9 ($\alpha 4\beta 2\gamma 1$) was shown to be absent from the purified laminin-8, as evidenced by a lack of reactivity with a mAb against the β2 chain. Immunoblotting of purified laminin-8 with an anti-α4 chain mAb detected a major 200-kDa band, together with a minor 180-kDa band. The 180-kDa band was also detectable in conditioned medium of T98G cells, suggesting that the 180-kDa α4 chain could be a proteolytically processed form or an alternatively spliced form. A similar 180-kDa α4 chain

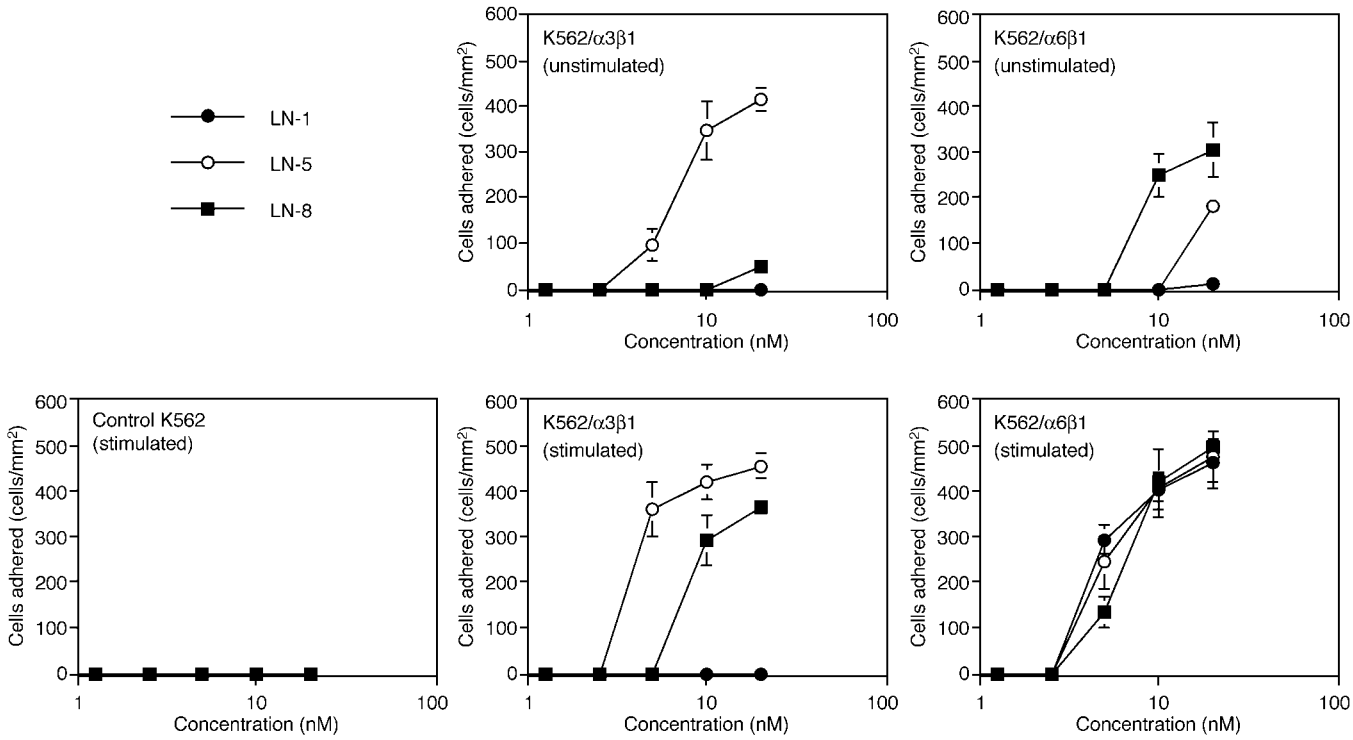

FIG. 7. **Adhesion of K562 transfectants expressing $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins to different laminin isoforms.** Control K562 cells and those expressing integrin $\alpha3\beta1$ (K562/$\alpha3\beta1$) or $\alpha_6\beta1$ (K562/$\alpha_6\beta1$) were incubated for 30 min at 37 °C with or without the stimulatory mAb 8A2 (1:10,000 dilution of ascites) in 96-well microtiter plates precoated with increasing concentrations of laminin-1 (*LN-1*), laminin-5 (*LN-5*), and laminin-8 (*LN-8*). Cells adhering to the substrates were fixed, stained, and counted as described under "Experimental Procedures." Each *point* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

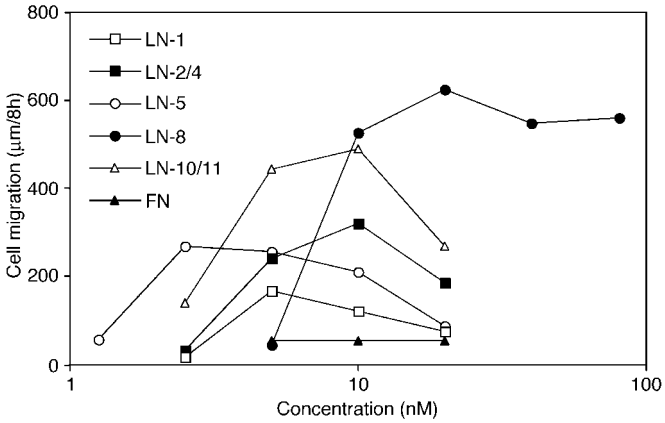

FIG. 8. **Cell migration promoting activity of laminin-8.** Glass-bottom culture dishes were precoated with increasing concentrations of laminin-1 (*LN-1*), laminin-2/4 (*LN-2/4*), laminin-5 (*LN-5*), laminin-8 (*LN-8*), laminin-10/11 (*LN-10/11*), or fibronectin (*FN*). T98G cells were added to the precoated dishes and the migrating cells were tracked by time lapse video microscopy at 37 °C for 8 h. Cell migration paths were traced and quantified using the Image-Pro Plus image-processing software. Each *point* represents the mean of migration distances of 10 different migrating cells.

was also detected in laminin-8 purified from platelets (17).

Both $\alpha3\beta1$ and $\alpha_6\beta1$ integrins function as the major cell surface receptors for laminin-8, based on the following observations: 1) adhesion of T98G cells to laminin-8 was not inhibited by any single mAb against the integrin α_2, $\alpha3$, $\alpha5$, or α_6 chain, but inhibited by an anti-$\beta1$ integrin mAb or a combination of anti-$\alpha3$ and anti-α_6 mAbs. 2) Wild-type K562 cells could not adhere to laminin-8 even after stimulation of $\beta1$ integrins by 8A2, whereas K562 cells transfected with $\alpha3$ or α_6 integrin subunits could adhere to laminin-8. α_6-Transfected cells adhered to laminin-8 without $\beta1$ integrin stimulation, but $\alpha3$-transfected cells needed $\beta1$ integrin activation to adhere to laminin-8, indicating that although both $\alpha3\beta1$ and $\alpha_6\beta1$ could serve as adhesion receptors for laminin-8, $\alpha_6\beta1$ might be the preferred laminin-8 receptor. The role of $\alpha_6\beta1$ integrin as the laminin-8 receptor was recently reported using platelet laminin-8 (17) and recombinant laminin-8 (18). Geberhiwot *et al.* (17) showed that platelet adhesion to laminin-8 is inhibited by anti-α_6 mAb alone, in contradiction to our present observations. This could be due to a very low level, if at all, of expression of $\alpha3\beta1$ integrin in platelets (50), while platelets have been shown to express $\alpha_6\beta1$ integrin that is functionally active in mediating platelet adhesion to laminin-1 (51).

Recently, Kortesmaa *et al.* (18) produced recombinant laminin-8 in a mammalian expression system by triple transfection of human laminin $\alpha4$ and $\gamma1$, and mouse $\beta1$ chains, with an acidic FLAG-tag attached to the C terminus of the $\alpha4$ chain. They showed that K562 cells expressing $\alpha_6\beta1$ integrin could adhere to recombinant laminin-8 without integrin stimulation. They also reported that K562 cells expressing $\alpha3\beta1$ integrin adhere poorly to recombinant laminin-8, although they did not show the data, nor did they indicate whether the assays were done with or without integrin stimulation. Their results are consistent with our observations, except that they did not indicate whether $\alpha3$-transfected cells could adhere to recombinant laminin-8 with integrin stimulation. They also reported, however, that adhesion of HT1080 cells to recombinant laminin-8 was strongly inhibited by the anti-integrin α_6 mAb alone, while our data show that both anti-$\alpha3$ and anti-α_6 mAbs are necessary to inhibit adhesion of T98G cells to laminin-8. This apparent discrepancy is not due to contamination of our laminin-8 preparation, which has been determined by immunoblot analysis to be free of laminin isoforms containing $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ chains, as well as the $\beta2$ chain-containing laminin-9. Laminins have been shown to undergo post-translational modifications such as proteolytic processing and *N*- and *O*-linked gly-

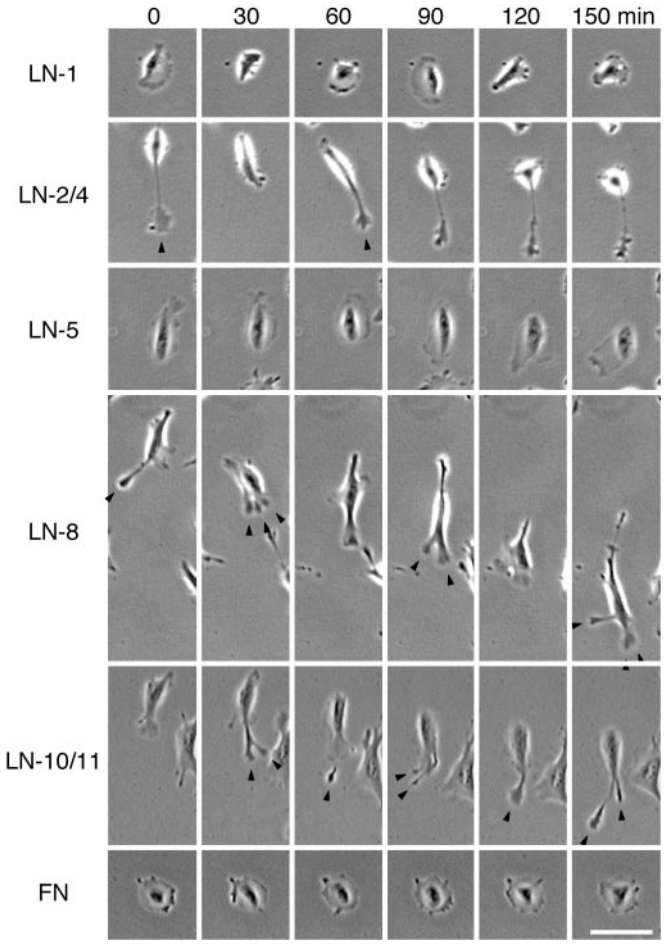

FIG. 9. **Morphology of T98G cells migrating on the substrates coated with laminin-8 and other adhesive proteins.** T98G cells were plated onto glass-bottom culture dishes precoated with 10 nM laminin-1 (*LN-1*), laminin-2/4 (*LN-2/4*), laminin-5 (*LN-5*), laminin-8 (*LN-8*), laminin-10/11 (*LN-10/11*), or fibronectin (*FN*). Cells were incubated on the substrates for 30 min to allow them to attach, after which migrating cells were recorded by time lapse video microscopy at 30-min intervals for 2.5 h. *Arrowheads* indicate pseudopods extended in the direction of cell migration. *Bar*, 100 μm.

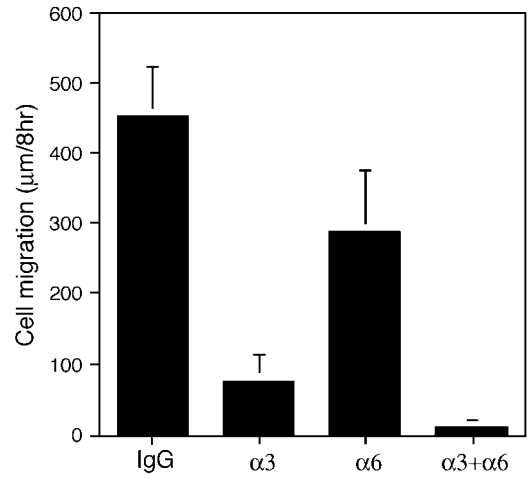

FIG. 10. **Effects of anti-integrin mAbs on migration of T98G cells adhering to laminin-8.** Glass-bottom culture dishes were precoated with 20 nM laminin-8. T98G cells were preincubated with the following function blocking mAbs against integrin subunits at a concentration of 20 μg/ml IgG for 20 min at room temperature and then added to the precoated dishes: IgG, control mouse IgG; α3, anti-integrin α3 subunit mAb (3G8); and α_6, anti-integrin α_6 subunit mAb (GoH3). Cell migration images were acquired by time lapse video microscopy at 10-min intervals for 8 h. Cell migration was quantified as described under "Experimental Procedures." Each column represents the mean of migration distances of 10 different migrating cells. *Bars* represent the standard deviation.

cosylation (52, 53), which may explain the discrepancy in the integrin-binding specificity between the recombinant laminin-8 and the laminin-8 that we purified. The recombinant laminin-8 was produced in human embryonic kidney cells, while our laminin-8 was produced in human glioma cells. Cell type-dependent glycosylation and/or proteolytic processing of subunit chains may therefore modify the integrin-binding specificity of laminin-8. Differences in elution protocols for immunoaffinity purification of laminin-8 may also explain the apparent discrepancy in integrin-binding specificity between the recombinant laminin-8 and our laminin-8 preparation. Although the recombinant laminin-8 was eluted from an anti-FLAG immunoaffinity column under nondenaturing conditions using the FLAG peptide, our laminin-8 was eluted from an anti-β1 mAb column under denaturing conditions using 0.1 M glycine-HCl (pH 2.4). It should be also noted that the recombinant laminin-8 is a hybrid protein consisting of human α4 and γ1 chains assembled with a mouse β1 chain, with an acidic FLAG-tag attached to the C terminus of the α4 chain. It is possible that such non-physiological features associated with the recombinant expression system also modify the integrin-binding specificity of laminin-8.

Although both anti-α3 and anti-α_6 integrin mAbs were needed to inhibit adhesion of T98G cells to laminin-8, cell migration on laminin-8-coated substrate was strongly inhibited by anti-α3 mAb alone. It seems, therefore, that $\alpha3\beta1$, but not $\alpha_6\beta1$, integrin is the major adhesion receptor through which laminin-8 mediates cell migration. In support of this notion, $\alpha3\beta1$ integrin has been shown to serve as the principal adhesion receptor in migration of various cell types. For example, migration of neurons along radial glial fibers during development of the cerebral cortex is dependent on $\alpha3\beta1$ integrin (54). Migration of keratinocytes during wound healing is also mediated primarily by $\alpha3\beta1$ integrin with concomitant deposition of laminin-5 (46). Integrin $\alpha3\beta1$ has also been shown to serve as the major integrin receptor operating in the migration and invasion of various tumor cells (31, 55). The prevalence of $\alpha3\beta1$ integrin in cell migration processes may explain the varied responses to different laminin isoforms. The observation that laminin-1 is a much weaker promoter of cell migration than laminin-8, even though they have similar cell-adhesive activity, may be because laminin-1 does not act through $\alpha3\beta1$ integrin as an adhesion substrate. In contrast, laminin-2/4, laminin-5, and laminin-10/11, all of which are ligands for $\alpha3\beta1$ integrin (12, 15, 31), were more potent than laminin-1 in promoting cell migration, further supporting the role of $\alpha3\beta1$ integrin as a preferred receptor for this specific activity.

For a cell to migrate, the cell needs to extend membrane ruffles in the direction of cell migration and become polarized. T98G cells adhering to laminin-8 assumed a highly polarized morphology with extension of multiple pseudopods in the direction of cell migration. A similar polarized morphology was also observed in cells adhering to laminin-10/11, and, to lesser extent, in cells adhering to laminin-2/4. In contrast, laminin-1 and fibronectin, both of which were very weak promoters of cell migration, did not induce morphological polarization, consistent with the close correlation between the ability of cells to become polarized and to migrate (48). Although the mechanisms of cell polarization are not well understood, it seems likely that the Rho family of small GTPases, particularly Rac and Cdc42, play critical roles in this phenomenon through promoting lamellipodia and filopodia formation (56, 57). It has been established that activation of Rac and Cdc42 is involved in

extension of lamellipodia and filopodia, respectively. Thus, the prominent cell polarization with multiple pseudopod extension observed to be induced by laminin-8 may be associated with activation of Rac and/or Cdc42. Ligation of $\alpha3\beta1$ integrin by anti-$\alpha3$ mAbs has been shown to induce curtain-like lamellipodia in skin fibroblasts adhering to laminin-1, which usually is associated with the adoption of a typical fibroblastic morphology with dense focal contacts (58). This suggests that ligation of $\alpha3\beta1$ integrins transduces signals that activate Rac. Consistent with this view, $\alpha3\beta1$ integrin has been shown to negatively regulate actin cytoskeletal reorganization. For example, ablation of $\alpha3\beta1$ by gene targeting in keratinocytes and kidney epithelial cells resulted in enhanced stress fiber formation and denser focal contacts (59, 60). Since Rac has been shown to down-regulate Rho activity (61, 62), it seems likely that $\alpha3\beta1$ integrin transduces signals that activate Rac, which in turn down-regulates Rho and suppresses formation of stress fibers and focal contacts. For a cell to migrate, it needs to detach from the substratum at its rear edge. Formation of focal contacts stabilized by stress fibers is disadvantageous for this cell detachment. Enhanced cell migration on laminin-8 may be due to the suppression of this stabilization mechanism. Indeed, cells migrating on laminin-8-coated substrates were not only highly polarized, but also frequently became rounded during migration (data not shown), consistent with this proposed mechanism.

Cell migration speed has been shown to exhibit a biphasic dependence on adhesive ligand concentration, regardless of integrin expression level or integrin-ligand binding affinity (49). Our data also show that cell migration on various types of laminin isoforms, except laminin-8, follows a biphasic curve as the substratum was coated with increasing concentrations of the adhesive ligands. The reason why cell migration speed did not decline when laminin-8 was used at high coating concentrations (*i.e.* up to 80 nM) is not clear, but it is conceivable that formation of focal contacts and stress fibers, the major suppressor of cell detachment at the rear, was not induced under these conditions, possibly due to the signals transduced by the $\alpha3\beta1$ integrin. The relatively low binding affinity of laminin-8 toward $\alpha3\beta1/\alpha_6\beta1$ integrins, as revealed by the relatively weak cell adhesive activity of laminin-8, may also contribute to the facilitation of rear cell detachment, even at high coating concentrations. Further studies are needed, however, to better understand the molecular mechanisms of signaling pathways through $\alpha3\beta1$ and $\alpha_6\beta1$ integrins on laminin-8 and other laminin isoforms.

Laminin isoforms containing the $\alpha4$ chain have been shown to be expressed strongly in embryonic and regenerating tissues, where cells are actively migrating. For example, in developing kidney, invading vessels that are destined to generate the capillary loops of the glomeruli are surrounded by a basement membrane rich in $\alpha4$- but not $\alpha5$-containing laminin isoforms (23). During the late stages of kidney development, arteriolar basement membranes lose $\alpha4$ and acquire the $\alpha5$ chain (23). In the adult, expression of $\alpha4$ chain-containing laminins is restricted to the capillary basement membrane (22, 23, 26). Given the remarkable potency of laminin-8 to promote cell migration, selective expression of $\alpha4$ chain-containing laminins in invading blood vessels during organogenesis and in capillary basement membranes in the adult suggests that laminin-8 is involved in endothelial cell migration during these processes. The role of laminin-8 as a potent stimulator of cell migration is consistent with the expression pattern of the $\alpha4$ chain in muscle, where it is expressed exclusively during development and in the neuromuscular junctions of adults (26, 27). In addition, the expression of the $\alpha4$ chain is up-regulated in regenerating muscle following a traumatic crushing injury (63). Furthermore, laminin-8 is one of the major components of neural crest cell migration pathways. These pathways have been shown to be blocked by an antibody against a laminin-8-agrin complex (45, 64), indicating that laminin-8 is also involved in migration of neural crest cells. Since these cells do not express α_6 subunit-containing integrins, the primary candidate for an integrin operating during neural crest cell migration may be $\alpha3\beta1$ (50), the laminin-8 receptor most prominent in promoting cell migration. Further studies on the tissue distribution of laminin-8 under both normal and pathological conditions, as well as the effects of laminin-8 on many other biological processes involving vast cell migration, should shed light on the roles of laminin-8 in cell migration during development and tissue regeneration.

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Purification and Characterization of Human Laminin-8: LAMININ-8 STIMULATES CELL ADHESION AND MIGRATION THROUGH α3β1 AND α6 β1INTEGRINS

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