Normal Human Keratinocytes Bind to the $\alpha3\beta4/5$ Domain of Unprocessed Laminin-5 through the Receptor Syndecan-1*

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Basal keratinocytes of the epidermis adhere to their underlying basement membrane through a specific interaction with laminin-5, which is composed by the association of $\alpha3$, $\beta3$, and $\gamma2$ chains. Laminin-5 has the ability to induce either stable cell adhesion or migration depending on specific processing of different parts of the molecule. One event results in the cleavage of the carboxyl-terminal globular domains 4 and 5 ($LG4/5$) of the $\alpha3$ chain. In this study, we recombinaently expressed the human $\alpha3LG4/5$ fragment in mammalian cells, and we show that this fragment induces adhesion of normal human keratinocytes and fibroascoma-derived HT1080 cells in a heparan- and chondroitin sulfate-dependent manner. Immunoprecipitation experiments with Na$_2$SO$_4$-labeled keratinocyte and HT1080 cell lysates as well as immunoblotting experiments revealed that the major proteoglycan receptor for the $\alpha3LG4/5$ fragment is syndecan-1. Syndecan-1 and syndecan-4 from keratinocytes also bound to $\alpha3LG4/5$. Furthermore we could show for the first time that unprocessed laminin-5 specifically binds syndecan-1, while processed laminin-5 does not. These results demonstrate that the $LG4/5$ modules within unprocessed laminin-5 permit its cell binding activity through heparan and chondroitin sulfate chains of syndecan-1 and reinforce previous data suggesting specific properties for the precursor molecule.

Laminins (LN)$_3$ are extracellular matrix glycoproteins composed of $\alpha$, $\beta$, and $\gamma$ chains assembled into a cross-shaped hetero- 

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Syndecan-1 Binding to Laminin α3LG4/5

Cells—To produce the recombinant protein, 293-EBNA cells were cultured in serum-free Dulbecco's modified Eagle's medium, and the conditioned medium was collected every 48 h. The medium was kept at −80 °C and proteolytic degradation was inhibited by the addition of 50 μg N-ethylmaleimide and 50 μg phenylmethylsulfonyl fluoride until use. For purification of rLG4/5, conditioned medium was applied to a 1-ml HiTrap heparin column (Amersham Biosciences), and elution was achieved using a linear NaCl gradient from 0.1 to 1.2 M. Protein purity was checked by SDS-PAGE where it was estimated to be greater than 90%. Protein concentration was determined by microprotein assay using bichinonic acid (BCA) (Pierce).

Purification of the Native α3LG4/5 Fragment As Well as the Processed and Unprocessed Native LN-5—The native LG4/5 was purified from conditioned NIH media as described previously (15). Processed LN-5 was purified from the culture medium of human SCC25 cells as described previously (15), and unprocessed LN-5 was purified according to the protocol described by Amano et al. (9). Briefly, 1 liter of conditioned medium from NIHs cultured in KEM-2 medium with 0.015 mM CaCl2 (Clonetics) was precleared with gelatin-Sepharose and applied to a 1-ml HiTrap heparin column. The bound materials were eluted with a linear NaCl gradient from 0.15 to 0.75 M. Fractions eluting around 0.5 M were precipitated using trichloroacetic acid and further analyzed for their unprocessed LN-5 by transfer to nitrocellulose followed by immunodetection with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) or Coomassie staining of the gel (Bio-Rad).

Flow Cytometry—Monolayer cells were harvested following exposure to PBS containing 5 mM EDTA, resuspended in PBS containing 2% BSA, and centrifuged. Cells (3 × 10^6/sample) were suspended in staining solution containing fluorescein isothiocyanate–goat anti-mouse IgG (Silenus, Eurobio, Les Ulis, France) or fluorescein isothiocyanate–goat anti-rabbit IgG (Jackson ImmunoResearch, Beckman Coulter, Roissy, France) depending on the primary antibody. After further incubation at 4 °C for 30 min, cells were washed, fixed, and analyzed by flow cytometry (FACScan, BD Biosciences). A 2 μg/ml propidium iodide solution was used as a dye for dead cells. Controls were carried out using isotype-matched antibodies or by omission of primary antibody.

Cell Adhesion and Inhibition Assays—Multwell tissue culture plates (Costar, Dutscher, Brumath, France) were coated with the indicated concentrations of processed LN-5 or LG4/5 substrates by overnight adsorption at 4 °C. Wells were coated in indicator number of cells per well) in serum-free medium as detailed previously (15). The extent of adhesion was determined after fixation of adherent cells with 1% glutaraldehyde in PBS containing 0.1% crystal violet and absorbance measurements at 570 nm using an MR5000 enzyme-linked immunosorbent assay reader. A blank value corresponding to BSA-coated wells (>5% of maximal cell adhesion) was subtracted. Adherent cells were photographed using a phase-contrast microscope equipped with a camera (Olympus Corp., Lake Success, NY). For cell adhesion inhibition experiments with glycosaminoglycans (GAGs), the coated wells were preincubated with heparin, heparan sulfate (HS) from bovine intestinal mucosa, chondroitin 4-sulfate (C4S) from bovine trachea, or chondroitin 6-sulfate (C6S) from shark cartilage (Sigma) for 1 h at room temperature, and adhesion was measured in the presence of the inhibitors. In all experiments, each assay point was done in triplicate for each conditions.

Treatment of Cells with Enzymes Prior to Cell Adhesion and Interaction Studies—HT1080 cells and NIHs were treated with 25 μg/ml cycloheximide for 2 and 1 h, respectively. All the following experiments contained the same concentrations of cycloheximide. After detachment with PBS containing 5 mM EDTA, the cells were washed four times in PBS and suspended in the same medium. The resulting calcium concentrations were adjusted to 2 mM by treatment with 8 millimolars/ml heparinase I and/or 50 millimolars/ml chondroitinase ABC (Seikagaku America, Goger, Paris, France) for 1 h at 25 °C. For control experiments, cells were incubated in parallel with the corresponding buffers only. In cell adhesion experiments, the cells then were detached into the 96-well plates in the same medium (with 0.5 μg/ml trypsin (as below) in ice. Cell lysates were cleaved by centrifuga-

Experimental Procedures

Cells and Antibodies—Cells of NIHs were established from foreskin as described previously (12) and subcultured in KEM-2 medium (Clonetics, BioWhittaker, Villenave de Réon, France). Cells were harvested for subculturing or for subsequent experiments using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS). NIHs were used between passages 1 and 3. Fibrosarcoma cells (HT1080), skin epithelial cells (A343), mammary epithelial cells (HBL100), and melanoma cells (A375) were provided by Prof. Aumailley (Center for Biochemistry, University of Cologne, Cologne, Germany) and cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal calf serum. The HaCat human keratinocyte cell line, provided by Dr. Damour (Institut, Lyon, France), was grown in 50% Ham's F-12 and 50% Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal calf serum. Parental Chinese hamster ovary (CHO) cells, called CHO-K1, and mutant CHO-677, CHO-745, and CHO-618 cell lines, derived from Esko et al. (31), were obtained from the European Collection of Cultured Cells. The cells were maintained in Ham's F-12 medium (Invitrogen) supplemented with 2 mM glutamine, 1.5 mg/ml dextrose, and 10% fetal calf serum. The preparation and characterization of LN-5 antibodies, the monoclonal antibody (mAb) EM165 (anti-α3 chain), polyclonal antibodies (pAbs) 4101 (anti-α3, β3, and γ2 chains), mAb 6F12 (anti-β3 chain), and pAb against the native LG4/5 fragment (nLG4/5) have been described elsewhere (12, 27). Rabbit pAb H-174 against syndecan-1, pAb M-140 against syndecan-2, pAb M-300 against syndecan-3, pAb H-140 against syndecan-4, and mAb 6G9 against syndecan-4 were purchased from NeoMarkers (Burlington, MA). mAb MI15 against human syndecan-1 was from Dako (Cytomation, Trappes, France).

Production of the Recombinant α3LG4/5 Fragment in Mammalian Cells—A DNA fragment encoding the human laminin α3LG4/5 domain (nucleotides 4057–5124) was generated by PCR. cDNA was obtained by reverse transcription total RNA of SCC25 epithelial cells using the primer 5′-GCCATGGGTAATACTAGTGGTTTTGAAAGG. Two oligonucleotides flanking the desired sequence were designed. One, corresponding to the 5′-end of the domain, carried a SpeI site (underlined) (5′-CCCTAAGTGTGGTCACACTCCAAAGG), and the second, corresponding to the 3′-end of the domain, introduced a NotI site and a stop codon (3′-TCACACTCCAAACATGTCATCAGATGCTGT). The resulting PCR product of 1083 base pairs was restriction-digested with SpeI and NotI and was inserted in-frame with the BM-40 signal peptide in the mammalian expression vector pCPE-Pu (32). All inserts and borders were fully sequenced. Recombinant plasmid was introduced by electroporation into the human embryonic kidney cell line 293-EBNA (Invitrogen). The transfected cells were cultured in 5% of CO2 and grown to confluence. Secretion of the recombinant LG4/5 fragment (rLG4/5) into the medium was confirmed by SDS-PAGE of conditioned medium samples from transfected and wild type cells.

Purification of the Recombinant α3LG4/5 Fragment in Mammalian...
tion and immediately incubated with membranes containing LG4/5.

Immunoprecipitation—NHKs and HT1080 cells were metabolically labeled with 100 μCi/ml sodium [35S]sulfate (Na235SOSO4) (PerkinElmer Life Sciences) for 16 h. After labeling, the cell layers were washed with PBS and extracted with RIPA buffer on ice for 10 min. All the following procedures were performed at 4 °C. Extracts were incubated for 2 h with Gamma Bind-G Sepharose beads (Amersham Biosciences) previously incubated overnight with anti-syndecan-1 pAb (H-174), anti-syndecan-1 preincubated with GAGs or anti-nLG4/5 pAb-RL4/5 complex. The latter complex was obtained by incubating rLG4/5 with the beads carrying anti-nLG4/5 pAb for 2 h. In some experiments heparin was added during incubation. Beads carrying the immune complexes were washed three times with RIPA buffer, and finally the complexes were separated by heating at 95 °C in denaturing sample buffer followed by separation using a 3–10% SDS-polyacrylamide gradient gel under reducing conditions. The gel was subjected to autoradiography followed by image analysis with a Storm gel and blot imaging system (Amersham Biosciences). For reimmunoprecipitation experiments, immune complexes formed on the beads carrying the anti-nLG4/5 pAb–RL4/5 complex were eluted by heating at 95 °C in 50 mM Tris/HCl containing 1 mM NaCl, pH 7.4. The salt concentration of the eluate was brought to 0.25 M by dilution followed by reimmunoprecipitation overnight at 4 °C with beads carrying pAb H-174 and mAb 5G9 or beads carrying IgG from a preimmune rabbit. After incubation, the beads were washed with PBS-Tween (0.05%) and processed as above. In the experiments conducted with processed and unprocessed LN-5, the protocol was identical except that the mAb 6F12 against the laminin β3 chain was used to bind both forms of LN-5 to the Gamma Bind-G Sepharose beads.

For detection of syndecans by immunoblot, rLG4/5 and processed or unprocessed LN-5 were covalently bound to beads to avoid constraints produced by immunoglobulin detection. The ligands were conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences) at 1 mg of protein/ml of resin as described by the manufacturer. Beads were incubated with cell extracts and washed as described above. Beads were finally transferred in digestion buffer (20 mM sodium acetate containing 5 mM CaCl2, pH 7.0) and incubated with 8 milliunits/ml heparitinase I and 50 milliunits/ml chondroitinase ABC for 2 h at 25 °C. The samples were then prepared and subjected to electrophoretic migration in a 10 or 15% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose filter followed by immunodetection with Western Lightning Chemiluminescence Reagent Plus.

Interaction of Syndecan-1 with Immobilized Proteins—BSA or rLG4/5 (1 μg) were blotted on a nitrocellulose filter previously soaked in distilled water and, after blotting, saturated overnight with BSA (5 mg/ml). Membranes were rinsed in PBS and then incubated for 2 h at +4 °C in the various cell lysates. After repeated washes of the membranes with the RIPA buffer and one wash with PBS, syndecan-1 was immunodetected with pAb H-174 as described below. Biotinylation Procedure and Heparan Sulfate Immobilization—HS (from intestinal mucosa, Celsus) was biotinylated at the reducing end as described previously (33). Briefly HS resuspended in PBS at 1 mM was incubated for 24 h at room temperature with 10 mM biotin-long chain-hydrazine (Pierce). The mixture was then extensively dialyzed against water to remove unreacted biotin and freeze-dried. Two flow cells of an F1 sensor chip (Biacore) were activated with 50 μl of a mixture of 0.2 μl 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 0.05 μM N-hydroxysuccinimide before injection of 50 μl of streptavidin (0.2 mg/ml in 10 mM acetate buffer, pH 4). Remaining activated groups were blocked with 50 μl of 1 M ethanolamine, pH 8.5. Typically this procedure allowed the coupling of ~2500–3000 resonance units (RU) of streptavidin. Biotinylated HS, 50 μg/ml in HBS-P buffer (10 mM Hepes, 0.15 mM NaCl, 0.005% P20, pH 7.4), was then injected on one of the two streptavidin-activated surfaces (the other one being a negative control) to yield an immobilization level of 30–40 RU. Both flow cells were then conditioned with several injections of 2 mM NaCl. For binding and competitive analyses, rLG4/5, preincubated or not with GAGs, was injected over the HS surface at a flow rate of 80 μl/min after which the complexes formed were washed with HBS-P buffer. The sensor chip surface was regenerated twice with a 1-min pulse of 0.05% SDS followed by a 2-min pulse of 2 mM NaCl in HBS-P buffer.

Analytical Methods—The following procedures were performed as previously described: SDS-PAGE followed by the electrophoretic transfer of proteins to nitrocellulose with immunoblot analysis (15). Silver staining was carried out with the Silver Stain Plus kit according to the manufacturer’s instructions (Bio-Rad).

RESULTS

Production and Purification of Recombinant o3LG4/5 Fragment—rLG4/5 was purified from the culture medium of 293EBNA cells by affinity chromatography using a 1-ml HiTrap heparin column. Coomassie Blue staining of the fractions eluting at around 0.8 mM NaCl revealed a band of about 40 kDa (Fig. 1A, and B, which corresponds to the predicted size of rLG4/5. Immunoblot analysis of these fractions, using pAb against nLG4/5 (27), revealed the peptide as the rLG4/5 domain (Fig. 1C). As previously described for the native fragment, rLG4/5 bound to the heparin affinity column required a NaCl concentration of 0.8 mM to be eluted, demonstrating that a high affinity for binding to heparin is characteristic of this fragment. Comparison of native and recombinant LG4/5 by silver staining and immunoblot analysis revealed an identical electrophoretic pattern for both fragments (Fig. 1D).

Cell Binding Activity of the Recombinant o3LG4/5 Fragment—We tested the ability of rLG4/5 to induce adhesion of normal cells and different cell lines. As shown in Fig. 2A, rLG4/5 promoted adhesion of several cell lines including HT1080, HBL100, and A431 cells in a dose-dependent manner, while it induced only weak adhesion of A375 cells. Maximum
adhesion was measured as described under “Experimental Procedures.” Each point represents the average of triplicate determinations. A, dose-dependent cell adhesion to rLG4/5. Multiwell plates were coated with different concentrations of rLG4/5, and cell adhesion was measured as described under “Experimental Procedures.” Each point represents the average of triplicate determinations.

Multiwell plates were coated with rLG4/5 at 10 μg/ml. After saturation with 1% BSA, the wells were incubated with the indicated concentration of heparin for 1 h at room temperature, and the cells were seeded in the presence of the same concentration of heparin. The cells were incubated as in A, and the extent of adhesion was measured as above and expressed as percentage of adhesion in the absence of heparin. C, dose-response curves of different CHO cell lines adhered to rLG4/5. CHO-K1 is the control cell line, CHO-677 is deficient in HS and expresses more CS, CHO-745 lacks both HS and CS, and CHO-618 lacks all the GAGs. Wells were coated with rLG4/5 at the indicated concentrations, and after saturation with 1% BSA, the CHO cells were seeded at a density of 10^5 cells/well. Cells were incubated for 30 min at 37 °C, and their adhesion was measured as described under “Experimental Procedures.” Each point represents the average of triplicate determinations. Abs., absorbance.

adhesion for all three cell types was obtained at concentrations of 10 μg/ml. The strong binding capacity of rLG4/5 to heparin suggested that adhesion might be mediated by cell surface HSPGs. This was verified by adding soluble heparin, which strongly inhibited cell adhesion to rLG4/5 (Fig. 2B). Inhibition was dose-dependent with half-maximal inhibition achieved in the range of 0.05–0.1 μg/ml. The involvement of cell surface HSPGs in cell adhesion to rLG4/5 was verified by the use of CHO cells known to express HSPG receptors as well as mutants deficient in GAG synthesis. Wild type CHO-K1 cells were able to adhere to rLG4/5 in a dose-dependent manner (Fig. 2C). Three mutant cell lines derived from CHO-K1 were tested in adhesion experiments: CHO-677, deficient in HS but synthesizing more CS, CHO-745, which lacks both HS and CS, and CHO-618, deficient in all the GAGs (31). While mutant CHO-618 cells did not adhere at all to rLG4/5, the two mutant cells CHO-677 and CHO-745 exhibited a moderate to very low interaction with rLG4/5, respectively (Fig. 2C). Because NHKs are in direct contact with LN-5 in vivo and because they are normal cells, their adhesion capacity to rLG4/5 was analyzed and compared with that of HT1080 cells (Fig. 3). Both these cell types were chosen for further characterization of the receptor involved in the interaction. Integrin-mediated adhesion to processed LN-5 lacking the LG4/5 domain was also performed. NHKs attached to rLG4/5 in a dose-dependent manner (Fig. 3A), but the adhesion was lower compared with that obtained with processed LN-5. HT1080 cells revealed a similar profile (Fig. 3B). Microscopic analysis of the cells after staining with crystal violet revealed that cell morphology was markedly different (Fig. 3C). Cells plated on wells coated with processed LN-5 exhibited a typical spread phenotype with numerous cell-cell contacts. In contrast, NHKs and HT1080 cells attached but failed to spread on rLG4/5 resulting in few intercellular contacts. Occasionally both NHKs and HT1080 cells produced numerous cytoplasmic processes resembling filopodia as shown by arrows in Fig. 3C. Detachment of HT1080 cells and NHKs by trypsin treatment (Fig. 4A) or detachment with 5 mM EDTA followed by heparitinase I and chondroitinase ABC treatment prior to the cell adhesion experiment largely reduced their adhesion to rLG4/5 (Fig. 4B), suggesting a non-integrin-mediated interaction. The use of 5 mM EDTA for cell detachment preserved their adhesion capacity and was used as the control. As expected, the integrin-mediated NHK and HT1080 cell adhesion to LN-5 was totally inhibited by incubation of the cells with 10 mM EDTA (Fig. 4C). Adhesion was partially affected when the cells were subjected to adhesion to rLG4/5 in the same conditions. To the contrary, heparin totally inhibited adhesion of NHKs and HT1080 cells to rLG4/5, but it had no effect when cells were plated on purified LN-5, confirming involvement of different types of cellular interaction on these two related substrates.

For further characterization of the GAGs involved in the
interaction, rLG4/5-coated wells were preincubated with free GAG chains prior to NHK and HT1080 cell adhesion. As shown in Fig. 5, A and B, the HS greatly inhibited adhesion of both cell types to rLG4/5 at the concentration of 100 μg/ml. While C4S produced partial but significant inhibition of cell adhesion at the same concentration (42% for HT1080 cells and 58% for NHKs), C6S had no effect. To further characterize the roles of HS and CS in cell binding, rLG4/5-coated wells were preincubated with different amounts of free HS and CS alone or in combination prior to HT1080 cell adhesion (Fig. 5C). The concentration of HS (10 μg/ml) used in this experiment produced 43% inhibition. Similar to results shown in Fig. 5A, about 20% of the binding was inhibited if 50 μg/ml C4S alone was used, and combining HS and C4S completely abrogated adhesion suggesting an additive effect of the two tested GAGs. As expected, combining HS and C6S did not result in a stronger inhibiting effect than HS used alone. These results suggest that both HS and C4S could be involved in NHK and HT1080 cell adhesion to rLG4/5 and that each of them alone is sufficient for the interaction.

To further test this hypothesis, we examined HT1080 and NHK adhesion to rLG4/5 after digestion of HS by heparitinase I and C4S by chondroitinase ABC (Fig. 5, D and E). For both cell types, heparitinase I treatment as well as chondroitinase treatment of the cells partially abrogated cell adhesion to rLG4/5; however, heparitinase I was more efficient. Incubation of rLG4/5 with C4S prior to cell adhesion totally abrogated adhesion of the heparitinase I-treated cells, while it did not modify that of chondroitinase ABC-treated cells. Treatment of the substrate with C6S had no effect in any case. Inversely incubation of rLG4/5 with HS totally inhibited cell adhesion of chondroitinase ABC-treated cells as well as that of heparitinase I-treated cells. These results suggest that HS and C4S may bind the same site with different affinities.

The Biacore technology was used to confirm and to extend the analysis of the rLG4/5 interaction with HS in vitro. Surface plasmon resonance was used to measure changes in refractive index caused by the binding of rLG4/5 to immobilized biotylated HS. Injection of rLG4/5 (1 μg/ml, 27 nM) over an activated sensor chip containing 30–40 RU of HS gave a signal of 350 RU, whereas injection of the protein over a control surface (containing streptavidin only) led to a signal of no more than 40 RU (not shown). Using this binding assay, inhibition analysis
Identification of Syndecan-1 as the Major Receptor for α3LG4/5 Fragment—As cell adhesion to rLG4/5 was totally inhibited by soluble heparin or prevented by trypsinization or treatment of cells with heparitinase I and chondroitinase ABC, we looked for heparan sulfate-type cell surface receptors. We set up a cell lysis system in which receptors were solubilized while retaining the ability to interact with rLG4/5. Among the several lysis buffer systems tested, the RIPA buffer provided the best compromise between solubilization of the receptor of interest and the absence of precipitation. These lysates were therefore used in solid phase assays. Biotinylated rLG4/5 bound to coated HT1080 and NHK cell lysates in a dose-dependent fashion (not shown). Immunoprecipitation experiments were therefore performed to identify the receptor. Capture of the proteoglycan-type receptor was carried out by incubating Na$_3$SO$_4$-labeled NHK or HT1080 cell lysates with beads that were immunologically covered with rLG4/5. Autoimmunoprecipitation of the bound material revealed a unique diffuse band of $\approx 250$ kDa for both cell types (Fig. 7, A and B, lane 1). To identify the bound receptor, immunoprecipitations were performed concomitantly with anti-syndecan antibodies, and an analysis of the electrophoretic pattern revealed syndecan-1 and possibly syndecan-4 as potential candidates (Fig. 7, A and B, lanes 2 and 3). The identity of the rLG4/5-bound proteoglycan-type receptor as syndecan-1 and/or syndecan-4 was verified by dissociation of the complex followed by reimmunoprecipitation of the eluate with the corresponding antibodies (Fig. 7, A and B, lanes 5 and 6). Depletion of syndecan-1 from the HT1080 cell lysate did not improve the detection of the syndecan-4 binding to LG4/5 (not shown). As expected, binding of syndecan to rLG4/5 was prevented by heparin for both HT1080 and NHK cell lysates (Fig. 7C).

As syndecan-2 and -4 were shown to interact with recombinantly expressed α3LG4 (28), we analyzed by flow cytometry the level of expression of the four different syndecans at the cell surface of the NHKs and HT1080 cells used in this study (Fig. 8). As a control, we included the keratinocyte cell line HaCat. The expression pattern was analyzed and revealed that for the

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**Fig. 6.** Surface plasmon resonance analysis of the rLG4/5-HS interactions. A, LG4/5 (1 μg/ml) was preincubated with HS at (from top to bottom) 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 50, or 100 μg/ml and injected over a HS-activated surface at a flow rate of 80 μl/min. B, LG4/5 (1 μg/ml) was preincubated with C4S at (from top to bottom) 0, 10, 25, 50, or 100 μg/ml and injected over a HS-activated surface as in A. C, LG4/5 (1 μg/ml) was preincubated with C6S at (from top to bottom) 0, 10, 25, 50, or 100 μg/ml and injected over a HS-activated surface as in A. Binding response was recorded as a function of time and normalized. The response at the end of the injection phase (300 s) was 350 RU in the absence of competitor. Injection of LG4/5 in the same condition (1 μg/ml, no competitor) over the streptavidin surface gave a response of no more than 40 RU (not shown).
three cell types, syndecan-1 was the most intensively expressed syndecan. Syndecan-4 was also expressed in the three cell types although in a much lower amount. While syndecan-2 was expressed in a very low amount in HaCat cells only, syndecan-3 could not be detected in any of the three tested cell types.

To confirm the identity of the syndecans bound to rLG4/5, we performed detection by immunoblotting after treatment of the bound proteoglycan receptor with heparitinase I and chondroitinase ABC (Fig. 9). Immunoblotting with the pAb H-174 against syndecan-1 revealed an intense positive band migrating around 96 kDa, which corresponds to the molecular mass of the syndecan-1 core protein. Immunoblotting of the same material electrophoretically separated on a 15% gel with pAb H-140 against syndecan-4 and pAb M-140 against syndecan-2 did not reveal any positive band in the position of the core protein of either syndecan-4 or syndecan-2 (not shown), suggesting that these receptors were not present in sufficient amount to be detected. To study the mechanism of syndecan-1/LG4/5 binding, intact as well as HS-free, CS-free, and HS+CS-free GAGs from HT1080 and HaCat cells were compared to reveal the possible role of HS and CS in syndecan-1 binding (Fig. 9B). HT1080 and HaCat cells were either untreated or treated with heparitinase I, chondroitinase ABC, or both to degrade HS, CS, or both. BSA- or rLG4/5-containing membranes were incubated in intact or enzyme-treated cell lysates, and bound syndecan-1 was detected by immunoblotting. Treatment of the cells with either heparitinase I or chondroitinase ABC only partially prevented interaction of the syndecan-1 with LG4/5, while digestion of both HS and CS prevented the interaction. These results suggest that syndecan-1 expressed on these cells contains both HS and CS and confirm our cell adhesion experiment suggesting that both HS and CS bind LG4/5 independently.

Unprocessed LN-5 Is a Ligand for Syndecan-1—We then addressed the question of whether unprocessed LN-5, contain-
Fig. 10. Syndecan-1 from NHKs binds to unprocessed LN-5. A, immunoblot analysis of unprocessed LN-5 from NHK culture media. As described by Amano et al. (9), NHK culture medium was affinity-chromatographed on HiTrap heparin, and 100-μl aliquots from the fractions eluted with 0.45 M NaCl were trichloroacetic acid-precipitated and analyzed by 6% SDS-PAGE under reducing conditions (lanes 1, 3, and 5) and compared with processed LN-5 (lanes 2, 4, and 6). Immunoblot analysis was performed with mAb BM165 against an epitope within LG1–3 (lanes 1 and 2), pAb against nLG4/5 (lanes 3 and 4), and pAb 4101 against α3, β3, and γ2 chains of LN-5 (lanes 5 and 6). Reactions were visualized by chemiluminescence. After stripping, the same membrane was repetitively used for detection with the antibodies described above. Molecular mass markers are shown on both sides. Arrows on the right show molecular masses of unprocessed α3 chain (190 kDa), processed α3 chain (165 kDa), unprocessed γ2 chain (155 kDa), β3 chain (140 kDa), and processed γ2 chain (105 kDa). B, immunoprecipitation analysis of NHK cell lysate binding to unprocessed LN-5. A cell lysate of Na2SO4-labeled NHKs was immunoprecipitated with beads immunologically covered with unprocessed (lane 1) or processed LN-5 (lane 2). For molecular mass comparison, the lysate was also immunoprecipitated with beads carrying pAb H-174 against syndecan-1 (lane 3) or mAb 5G9 against syndecan-4 (lane 4). Precipitated samples were subjected to SDS-PAGE followed by autoradiography as described under "Experimental Procedures." C, NHK cell lysates were incubated with beads covered without protein (lane 1) or with rLG4/5 (lane 2), unprocessed LN-5 (lane 3), and processed LN-5 (lane 4). After washes, bound material was digested with heparitinase I and chondroitinase ABC. Electrophoretic analysis of the bound material was performed on an 8% SDS-polyacrylamide gel under reducing conditions followed by immunoblotting with the pAb H-174 against syndecan-1. The migration positions of molecular mass markers are shown on the right.

Numerous recent reports indicate that distinct biological events can be assigned to LN-5 depending on the level of processing of its α3 and γ2 chains. Fully processed LN-5 induces stable adhesion of NHKs and hemidesmosome formation, while unprocessed LN-5 is related to migratory situations (34). To understand the physiological significance of the carboxyl-terminal processing of the LN-5 α3 chain, we have recombinantly expressed the entire LG4/5 domain, which, in normal keratinocyte cultures, is rapidly removed after secretion and deposition of LN-5 into the ECM. 293-EBNA-expressed rLG4/5 shares the high affinity to heparin with the native domain and induced adhesion of several cell types including NHKs (7, 27). Moreover, in a manner comparable to nLG4/5, cell adhesion to rLG4/5 was specifically inhibited by heparin, suggesting the involvement of cell surface HSPGs. In addition, the abolition of NHK and HT1080 cell adhesion to rLG4/5 by trypsinization supported the speculation that the cell surface receptors are HSPGs and not integrins, which are trypsin-resistant. Inhibition studies showed that HT1080 and NHK cell adhesion to rLG4/5 occurs in a HS- and CS-dependent manner and that C4S participates in the interaction. This was suggested by cell adhesion studies using CHO cell mutants lacking the ability to introduce HS and CS chains upon their cell surface proteoglycans as well as by inhibition of NHK and HT1080 cell adhesion when they were treated with heparitinase I and chondroitinase ABC. We show that HS and C4S interact with LG4/5. Heparin alone blocked cell binding entirely, which indicates that the HS and C4S chains both bind to the same site in rLG4/5 as heparin. Moreover, our interaction studies demonstrate that HS and C4S bind the same site in rLG4/5 with different affinities with the affinity of HS being higher than that of C4S. A low binding of C6S to rLG4/5 could also be detected with the Biacore apparatus but was not detected in cell adhesion studies suggesting a very low affinity of this interaction. We have subsequently identified syndecan-1 as a major receptor for rLG4/5 by immunoprecipitation of Na2SO4-labeled NHK and HT1080 cell lysates with beads carrying rLG4/5. We also detected syndecan-4 as a receptor for rLG4/5 in NHK cell lysates, a finding in accordance with a recent study suggesting that fibroblast-produced syndecan-4 binds to a recombinantly expressed α3LG4 fragment (28). Probably due to its insufficient amount, binding of syndecan-4 from HT1080 cells could not be detected in our experiments. Indeed
evaluation of the cell surface expression level of the four different syndecans in HT1080 cells and NHKs revealed syndecan-1 as the most abundant, while the expression of syndecan-4 was low. We also found a high level of syndecan-1 in HaCat cells, a result at variance with previous findings, which reported an absence of syndecan-1 mRNA in these cells (28). Our result was confirmed by immunoblot detection of syndecan-1 from HaCat cell lysate as a major receptor bound to rLG4/5 in a manner comparable to that of HT1080 and NHK lysates.

Our results provide the first evidence that primary keratinocytes use syndecan-1 for adhesion to α3LG4/5. As expected from the cell adhesion study, the binding of Na2SO4-labeled syndecans from NHKs to rLG4/5 was prevented by heparin. As syndecan-1 core protein has been shown to carry either both HS and CS (36, 37) or HS alone (38), our experiments show that these two GAG chains bind independently to rLG4/5 with different affinities. A recent report describes the characterization of these two GAG chains bind independently to rLG4/5 with different affinities. A recent report describes the characterization of these two GAG chains bind independently to rLG4/5 with different affinities. A recent report describes the characterization of these two GAG chains bind independently to rLG4/5 with different affinities. A recent report describes the characterization of these two GAG chains bind independently to rLG4/5 with different affinities.

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