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Changes in expression of monoclonal antibody epitopes on laminin-5r induced by cell contact

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SUMMARY

Laminin-5r is a basement membrane component that promotes rapid adhesion and hemidesmosome formation in epithelial cells. We raised monoclonal antibodies and identified their corresponding epitopes on the constituent chains of laminin-5r by western blotting. Using a combination of immunoprecipitation and ELISA assays, we determined that these epitopes are differentially exposed on two forms of the laminin-5r heterotrimer: soluble (passively adsorbed onto plastic) and cell-associated. Antibody 5C5 epitope is exposed on the cell-associated form, but not the soluble/passively adsorbed form of laminin-5r. Epitopes reactive with antibodies CM6, FM3, and TR1 are also preferentially exposed on cell-associated laminin-5r, such that reactivity of these antibodies with the cell-associated form is fourfold higher than with the soluble/passively adsorbed form in ELISA assays. Incubation of passively adsorbed laminin-5r with the human epithelial cell line SCC12 induced exposure of 5C5 and CM6, FM3, or TR1 epitopes. These data suggest that cells actively modify laminin-5r, perhaps during matrix assembly, and that the 5C5 epitope may serve as a marker for assembled laminin-5r matrix.

Key words: Extracellular matrix, Laminin, Monoclonal antibody

INTRODUCTION

Laminins are a family of extracellular matrix molecules found in the basement membrane of epithelial sheets. Laminins promote adhesion and migration of epithelial cells and may play a role in tumor progression and metastasis (Tryggvason, 1993; Kibbey et al., 1994; Sweeney et al., 1991). Laminin molecules form characteristic cross-shaped heterotrimers consisting of one α, one β, and one γ chain arranged as an α-helical, coiled coil 'core' with three short arms (Tryggvason, 1993; Yurchenco and Cheng, 1994); at present, at least 3α, 3β and 2γ subunits have been identified, which organize into at least 7 different laminin isoforms.

We have previously described a laminin isoform, laminin-5r, which is secreted by the rat carcinoma cell line 804G (Langhofer et al., 1993; Hormia et al., 1995) and is comprised of the α3, β3, and γ2 laminin subunits. Laminin-5r exists in two physical forms in vitro: a freely soluble form in aqueous solution (e.g. cell culture media) that readily coats surfaces (e.g. passively adsorbed onto cell culture plastic), and a cell-associated form (e.g. beneath epithelial cell monolayers). Both soluble/passively adsorbed and cell-associated laminin-5r induce rapid attachment and spreading of epithelial cells and induce formation of mature hemidesmosomes in vitro (Langhofer et al., 1993; Hormia et al., 1995).

How laminin-5r is assembled into basement membranes is unknown. Current models suggest that laminin 1 (also known as EHS laminin; Burgeson et al., 1994) heterotrimers self assemble in a Ca^{2+}-dependent manner to form an insoluble matrix within the basement membrane of epithelial cells. The amino-terminal globular domains of the short arms are thought to play critical roles in the polymerization of laminin 1 heterotrimers (Yurchenco and Cheng, 1993; Schittny and Schittny, 1993). Laminin-5r is closely related to human laminin 5 (J. Falk-Marzillier et al., unpublished; J. C. R. Jones et al., unpublished) and thus is distinguished from laminin 1 in that it lacks these amino-terminal globular 'assembly' domains in the α and γ chains (Ryan et al., 1994; Kallunki et al., 1992). It is therefore likely that the mechanism of laminin-5r assembly differs from that for laminin 1.

Like laminin-5r, the extracellular matrix molecule fibronectin exists in both a soluble and cell-associated form. Current models suggest that assembly of soluble fibronectin into an insoluble matrix requires interchain rearrangement of disulfide bonds in fibronectin dimers via a receptor-mediated interaction with the cell surface (reviewed by Mosher, 1993). Conformational changes detectable by monoclonal antibodies accompany fibronectin matrix assembly (Ugarova et al., 1995). We have recently observed that epithelial cells redistribute soluble laminin-5r during matrix assembly (J. C. R. Jones et

al., unpublished). It is therefore possible that assembly of laminin-5r matrix may involve cell-mediated modification and/or rearrangement of soluble laminin-5r, which may be reflected by changes in monoclonal antibody epitope exposure.

We examined this possibility by probing soluble and cell-associated laminin-5r with monoclonal antibodies, and found distinct patterns of reactivity that distinguished between these two forms. Incubation with the human epithelial cell line SCC12 caused the passively adsorbed form of laminin-5r to expose antibody epitopes characteristic of the cell-associated laminin-5r. These data suggest that cells actively modify laminin-5r in order to assemble it into a matrix.

MATERIALS AND METHODS

Cells

Rat 804G and human FGmet2 carcinoma cells were routinely passaged in DMEM or RPMI medium (Gibco, Grand Island NY), respectively, supplemented with 10% fetal calf serum (Gemini, Irvine CA) and 2 mM glutamine (BioWhittaker, Walkersville MD) at 37°C in a humidified incubator containing 5% CO_2. Human SCC12 carcinoma cells were routinely passaged in keratinocyte growth medium (Clonetics, San Diego, CA) lacking gentamycin. Human SCC25 carcinoma cells were grown in medium containing 3:1 DMEM:Ham's F12 medium (ICN, Costa Mesa CA) supplemented with 0.4 μg/ml hydrocortisone (Sigma, St Louis MO), 2 mM glutamine, and 10% fetal calf serum. 804G and FGmet2 cells were harvested for experiments by washing confluent plates with phosphate buffered saline (PBS) and incubating them in trypsin/versene (BioWhittaker) for 10 minutes at 37°C. Cells were suspended in culture medium containing 10% fetal calf serum to neutralize trypsin, centrifuged, and suspended in fresh culture medium. SCC12 cells were collected using the Trypsin System (Clonetics) as indicated by the manufacturer.

Collection of cell-associated laminin-5r

804G cells were grown to confluency on 96-well plates or 150 mm plastic Petri dishes. The culture medium was removed and the cells were washed in sterile PBS. The cells were removed according to the method of Gospodarowicz (1984) by incubating them 2× 5 minutes in 20 mM sterile NH_4OH. The plates were extensively washed with PBS and distilled water and allowed to air dry. For recovering 804G matrix, each 150 mm Petri dish was incubated with 2 ml of collection buffer (10 mM Tris-HCl, pH 7.0, 0.1% SDS, 100 mM β-mercaptoethanol). After 15 minutes at 37°C the fluid was collected with a cell scraper and lyophilized. The matrix pellets were solubilized either directly into Laemmli sample buffer or collection buffer. Matrix was dialyzed against RIPA buffer prior to immunoprecipitation (see below).

Western blotting

Protein samples diluted in 2× sample buffer containing 50 mM dithiothreitol and 1.5 M β-mercaptoethanol were heated for 5 minutes at 95°C then separated by SDS-PAGE using 6% polyacrylamide gels (Novex, San Diego CA). Separated proteins were transferred to PVDF membranes (Bio-Rad, Hercules CA) using a Millipore Transblot system. Protein bands were visualized using Ponceau S stain (Sigma), then membranes were cut into strips and blocked 1 hour with blotto (5% nonfat dried milk in PBS, 0.2% Tween-20, 0.01% antifoam A (Sigma), 0.01% thimerosal). Primary antibodies diluted 1:1,000 in blotto were added for 1 hour at room temperature, then the strips were washed 2× with PBS, 0.2% Tween-20, 0.01% thimerosal (PBST). Secondary antibodies (goat anti-mouse IgG or anti-rabbit IgG, conjugated to horseradish peroxidase, Amersham) were diluted 1:10,000 in blotto and added for 1 hour at RT. Strips were washed 5× in PBS, 0.2% Tween-20, then developed using chemiluminescence (Amersham). Strips containing molecular mass standards (Mark 12, Novex) were stained 1 minute with Coomassie blue then destained in distilled water containing 45% methanol and 4.5% acetic acid.

Immunoprecipitation

804G or SCC25 cells between passages p20-p30 were split into 150 mm dishes in a medium containing 10% DMEM/90% Eagle's mimimal essential medium lacking methionine, cysteine, and glutamine (Sigma), 10% dialyzed FCS (Biowhittaker), 1% glutamine/penicillin/streptomycin (Irvine Scientific, Santa Ana CA) and labeled with 50 μCi/ml of tran^{35}S-label (ICN). Conditioned medium from SCC25 cells was used as a source of human laminin 5 because, unlike SCC12 cells, SCC25 cells have been shown previously to secrete laminin 5 in high amounts (Rousselle and Aumailley, 1994). After 48 hours the 804G conditioned medium was centrifuged 10 minutes at 3,000 ***g***. Immunoprecipitations were carried out with 500 μl dialysed conditioned medium diluted 1:1 with 2× RIPA buffer (2% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS, 300 mM NaCl, 100 mM Tris-HCl, pH 7.5) and 2 μl of polyclonal serum plus 5 μl anti-rabbit-IgG-agarose beads (Sigma), or 2 μl of purified monoclonal antibody plus 5 μl anti-mouse-IgG agarose beads (Sigma), respectively, per reaction. Reactions were incubated for 2 hours at 4°C, extensively washed in 1× RIPA buffer and eluted by boiling 5 minutes in Laemmli sample buffer containing β-mercaptoethanol. For chemical denaturation assays, radiolabelled conditioned medium was denatured by adding β-mercaptoethanol and SDS (100 mM and 0.1% final concentrations, respectively) for 20 minutes. Cell associated matrix was collected as described above. SDS and β-mercaptoethanol were removed from both conditoned medium and cell-associated matrix by dialysis against RIPA buffer. Samples were then analyzed by SDS-PAGE on 6% polyacrylamide gels, which were subsequently soaked 15 minutes in ENHANCE (DuPont), dried and exposed to X-ray film (Dupont/NEN, Boston MA). Labeled proteins bands were visualized on developed X-ray film.

ELISA assays

For passively adsorbed laminin-5r plates, 804G cell conditioned medium was diluted 1:4 in DMEM, 100 mM Hepes, pH 7.4, and plated (100 μl/well) in 96-well ELISA plates (Sarstedt, Newton NC). As a control, plates were also coated with unconditioned 804G medium at the same dilution. Plates were stored overnight at 4°C, then washed 2× with PBS (200 μl/well). For chemical denaturation assays, wells were treated for 5 minutes with 1 M acetic acid, 1 M KOH or 8 M urea, followed by two washes with PBS.

For cell-associated laminin-5r plates, 804G cells (25,000/well) were cultured in 96-well tissue culture plates (Corning, Cambridge MA). FGmet2 cells were grown in control plates. After 2 days, plates were washed 2× with PBS and cells removed by adding 20 mM NH_4OH for 5 minutes, followed by 2 washes with PBS. One set of 8 wells was treated for 5 minutes with 1 M acetic acid, followed by two washes with PBS.

Passively adsorbed plates were blocked for at least 2 hours with blotto. Cell associated plates were blocked with 3% bovine serum albumin (BSA)/PBST. Primary antibodies were diluted 1:2,000 in appropriate blocking buffer and 50 μl added to each well. After 1 hour, plates were washed 2× with PBST for passively adsorbed and 1%BSA/PBST for cell-associated plates. Goat (anti-mouse or anti-rabbit) secondary antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:2,000 in appropriate blocking buffer were added to each well. After 1 hour, wells were washed 4× with PBST then 2× with PBS. Wells were developed with 100 μl/well *o*-phenylenediamine (3 mg/ml; Sigma) and H_2O_2 (0.1%; Sigma) in 50 mM citrate, 50 mM Na_2HPO_4, pH 5.0. Development reaction was stopped with 4 N H_2SO_4. Plates were read at 490 nm using a Vmax plate reader (Molecular Devices,

Menlo Park CA). Values on control plates (averaged from 8 wells/antibody) were subtracted from those on experimental plates.

Sandwich capture ELISA

Primary monoclonal antibodies were diluted to 2 μg/ml in 100 mM carbonate buffer (pH 8.8), then plated overnight at 4°C in 96-well ELISA plates. Wells were emptied by shaking, then blocked for 2 hours at room temperature with blotto. Wells were emptied by shaking, then were incubated for 2 hours at room temperature with 804G cell conditioned medium (diluted 1:50 in blotto) to capture laminin-5r. Following three washes with PBST, captured laminin-5r was detected by probing with biotinylated monolonal antibodies (2.5 μg/ml in blotto) for 1 hour at room temperature, followed by 1 μg/ml streptavidin conjugated to horseradish peroxidase (Pierce) for 30 minutes. Finally, wells were washed four times with PBST and twice with PBS, and developed as described above.

Cell contact ELISA

Tissue culture plates (96-well) coated with passively adsorbed 804G conditioned medium as described above were washed 2× with PBS. SCC12 cells (25,000/well) were added to these plates as well as to control, uncoated plates. At indicated times, plates were processed as for cell-associated ELISA assays. One set of 8 wells on each plate was treated for 5 minutes with 1 M acetic acid to denature laminin-5r; these wells were incubated with 0668B polyclonal antibody. Background values averaged from 8 wells/antibody on control plates were subtracted. For each time point, monoclonal values were expressed as a percentage of the corresponding polyclonal average for acid-treated wells.

Proteolysis ELISA

ELISA plates were coated for 2 hours at room temperature with purified, soluble laminin-5r (generously provided by Desmos, Inc., La Jolla, CA) (100 ng/ml in carbonate buffer). Wells were washed twice with PBS, then incubated for 30 minutes at 37°C with 3-fold serial dilutions of purified trypsin (100 ng/ml, diluted in 50 mM acetic acid; Sigma) or purified chymotrypsin (100 ng/ml, diluted in 20 mM phosphate buffer, pH 7.5; Sigma). Wells were washed twice with PBS then incubated 30 minutes with 1 μg/ml aprotinin in PBS to inactivate residual protease activity. Following two washes with PBS, wells were blocked and incubated with antibodies as described for the passively adsorbed laminin-5r ELISA assay described above.

Ammonium sulfate precipitation of soluble laminin-5r

Soluble laminin-5r was collected by ammonium sulfate precipitation of serum-free 804G conditioned medium. Solid ammonium sulfate was added to conditioned medium to a final concentration of 30% saturation and stirred overnight at 4°C. Precipitated material was removed from the supernatant by centrifugation at 10,000 ***g*** for 30 minutes. Solid ammonium sulfate was added to 40% saturation and the solution was stirred overnight at 4°C. Precipitated laminin 5r was collected by centrifugation at 10,000 ***g*** for 30 minutes, dissolved and dialyzed in PBS. Fibronectin was removed by mixing with gelatin Sepharose (Pharmacia, Alameda CA) for 30 minutes at room temperature. The supernatant was then concentrated using an Amicon stirred cell (Amicon, Beverly MA) and a 100,000 molecular mass filter. Protein concentration was determined using the microBCA assay (Pierce, Rockford IL).

Monoclonal antibody production

Female Balb/cByJ (6-8 weeks old) were given intraperitoneal injections of 100 μg of ammonium sulfate-precipitated laminin-5r dissolved in 0.5 ml RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton MT). This injection was repeated two and four weeks later. Mouse sera were then collected from tail veins and screened by cell-associated ELISA assay. Positive mice were boosted for three consecutive days with intraperitoneal injections of 100 μg of ammonium sulfate-precipitated laminin-5r dissolved in PBS. The next day, mice were sacrificed by CO_2 asphyxiation and the splenocytes were fused to SP2/0 mouse myeloma cells using established methods. Hybridomas were selected using HAT medium (Sigma) and wells were screened by cell-associated ELISA assay. Positive wells were expanded and cloned twice in succession by sorting single cells into 96-well plates using a Becton-Dickinson FACStar fluorescence activated cell sorter (Becton-Dickinson, Bedford MA) equipped with an Automated Cell Deposition Unit. Single cells were sorted on the basis of side and forward light scatter. Clones were screened by cell-associated ELISA assay; all secondary clones screened were positive. The DF2 and TR1 lines were cloned an additional two times. Specificity of monoclonal antibodies for laminin-5r was determined by ELISA assay of immunodepleted 804G conditioned medium (not shown). Isotypes of antibodies were determined using isotype-specific secondary antibodies (Southern Biotechnology Associates, Inc.) in a cell-associated ELISA assay; 5C5, BH5, CM6, DF2, FM3, and TR1. CM6, FM3 and TR1 were determined to be mouse isotype IgG_1, DF2 is mouse isotype IgG_{2a}, and BH5 is mouse isotype IgG_{2b}. Ascites were induced in Balb/cByJ mice and ascites fluid collected as previously described (Harlow and Lane, 1988).

Characterization of the 5C5 epitope

5C5 recognizes a 150 kDa protein band in 804G cell matrix (Langhofer et al., 1993; Hormia et al., 1995). This band was identified as the α3 chain of laminin-5r by screening an 804G cell expression library with 5C5, plus direct protein sequencing of the 150 kDa band from both soluble and cell-deposited laminin-5r (J. C. R. Jones et al., unpublished; J. Falk-Marzillier et al., unpublished). 5C5 also recognizes a 135 kDa band that has been identified by direct amino acid sequencing as a truncated form of the α3 chain (J. Falk-Marzillier et al., unpublished).

Polyclonal antibody production

The polyclonal antibody J18 has been described previously (Langhofer et al., 1993). For production of the 0668B polyclonal antibody, 804G cells were directly grown on nitrocellulose (Bio-Rad) until confluent. Cells were removed according to the method of Gospodarowicz (1984). For the first immunization a 2 cm × 2 cm piece of nitrocellulose was subcutaneously implanted into male New Zealand white rabbits (6-8 weeks). Booster injections with cell-associated 804G matrix (20 μg per immunization) were subsequently given four and eight weeks later. Blood was collected from ear veins, incubated for 1 hour at 37°C, then stored overnight at 4°C. Following centrifugation for 30 minutes at 3,000 ***g*** to remove cells, the serum was divided into aliquots and stored at −70°C.

Monoclonal antibody purification

Ascites fluid was delipidated by mixing with dextran sulfate (average molecular mass 8,000; Sigma) and precipitation with 0.5 M $CaCl_2$. Delipidated ascites fluid was passed over immobilized Protein A (Macroprep; Bio-Rad) and antibodies were eluted with 50 mM sodium citrate, 100 mM NaCl, pH 6.0. Eluted fractions were immediately exchanged into PBS using a G25 Sephadex column (Pharmacia).

Antibody biotinylation

Purified monoclonal antibodies were incubated with NHS-LC-biotin (Pierce) in 100 mM sodium borate buffer (pH 8.8) for 3.5 hours at room temperature, then exchanged into PBS using a Bio-Rad 10DG disposable desalting column. Biotinylated antibodies were sterile filtered and stored at −80°C.

Immunofluorescence microscopy

Fresh tongue tissue from male Sprague-Dawley rats was snap-frozen in liquid nitrogen and 20 μm sections were cut using a microtome. Frozen sections were processed for immunofluorescence microscopy

as described previously (Klatte et al., 1989). Briefly, sections were stained with monoclonal antibodies followed by goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (Southern Biotechnology Associates, Inc.). Fluorescence images were visualized and photographed using a Zeiss Photomicroscope III (Zeiss, Thornwood NY).

RESULTS

The 5C5 epitope is only exposed in cell-associated laminin-5r

We tested the monoclonal antibody 5C5 with soluble and cell-associated laminin-5r in ELISA, western blot and immunoprecipitation assays. In ELISA assays, 5C5 reacted strongly with the cell-associated form of laminin-5r, but did not react with the soluble form (passively adsorbed onto plastic) (Fig. 1). Treatment of passively adsorbed laminin-5r with acid, base, or urea failed to induce 5C5 reactivity in the ELISA (Fig. 1). These results suggested that the 5C5 epitope may be expressed exclusively on cell-associated laminin-5r. However, by western blot 5C5 reacted with the α3 subunit (150 and 135 kDa bands) of both soluble and cell-associated laminin-5r (Fig. 2), indicating that the 5C5 epitope is in fact expressed on both soluble and cell-associated laminin-5r, but may be differentially exposed. In agreement, 5C5 immunoprecipitated cell-associated, but not soluble laminin-5r (Fig. 3).

An explanation for these results is that the portion of the α3 chain containing the 5C5 epitope (amino acids 291-1061; J. C. R. Jones et al., unpublished) assumes such an orientation in native soluble laminin-5r, that it is not accessible to antibody. Neither denaturation, nor passive adsorption, by themselves or in combination, are capable of exposing the epitope. However, the 5C5 epitope is readily accessible in cell-associated laminin-5r. This raises the possibility that cells modify laminin-5r so as to expose this epitope.

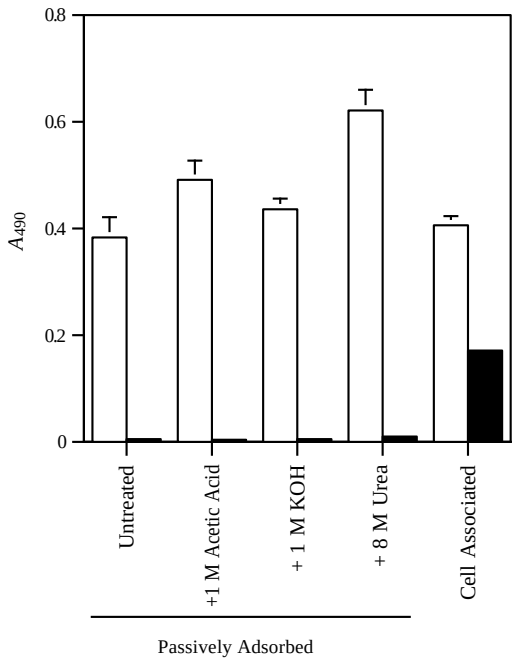

Fig. 1. The 5C5 epitope is expressed only on cell-associated laminin-5r. ELISA assays were performed on passively adsorbed and cell-associated laminin-5r using 5C5 (■) and 0668B (□) antibodies. Note that denaturation of passively adsorbed laminin-5r with 1 M acetic acid, 1 M KOH, or 8 M urea increased polyclonal antibody reactivity but failed to induce 5C5 reactivity. Values shown represent statistical mean ± s.e.m. (*n*=8).

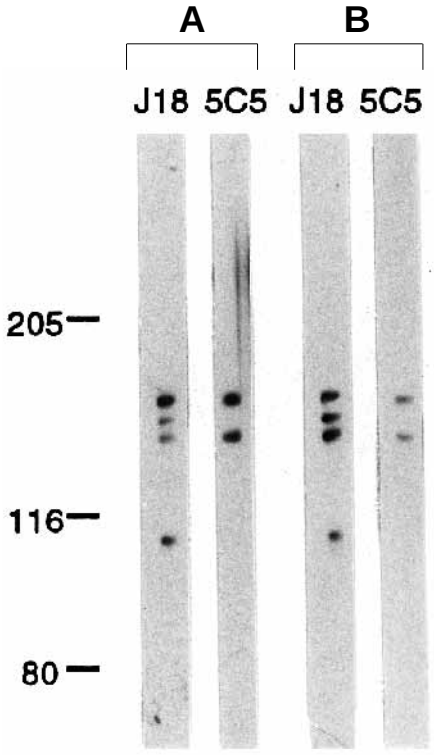

Fig. 2. The 5C5 epitope is present in both soluble and cell-associated laminin-5r. Western blot of soluble (A) and cell-associated (B) laminin-5r with the monoclonal antibody 5C5 and the polyclonal anti-laminin-5r antibody J18. Migration of molecular mass standards is indicated at left (in kDa).

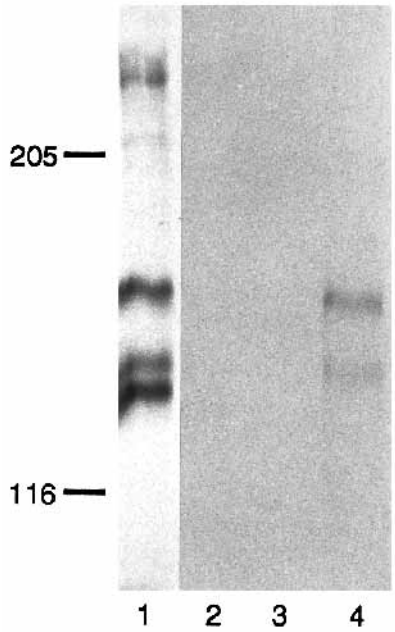

Fig. 3. 5C5 does not immunoprecipitate soluble laminin-5r. Immunoprecipitation of laminin-5r from 804G conditioned medium with the polyclonal antibody 0668B (lane 1) and the monoclonal antibody 5C5 (lane 2). As controls, 804G conditioned medium (lane 3) and cell-associated laminin-5r (lane 4) were solubilized in 0.1% SDS/100 mM β-mercaptoethanol, dialysed in PBS, and laminin-5r was precipitated with the 5C5 antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by fluorography. Migration of molecular mass standards is indicated at left (in kDa). Note that 5C5 precipitated only cell-associated laminin-5r.

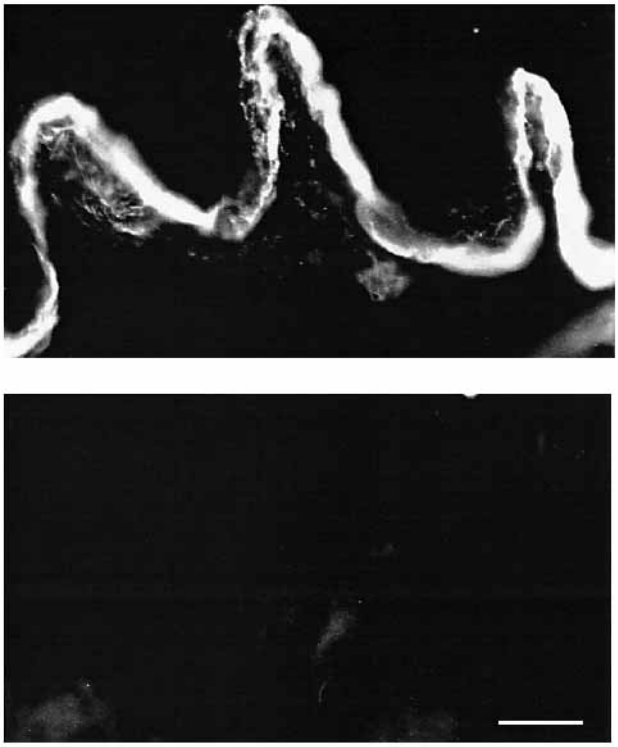

Fig. 4. The 5C5 epitope is expressed in vivo. Rat tongue frozen sections were stained with the 5C5 antibody followed by fluorescein-conjugated secondary antibodies (top). Staining with secondary antibody alone was used as a control (bottom). The photos are aligned so that the epidermis is located at the top. Note prominent staining of the basement membrane by 5C5. Bar, 10 μm.

In support of this possibility, we stained unfixed, non-denatured frozen rat tongue tissue sections with antibody 5C5, and found strong reactivity with basement membranes in contact with the germinal cell layer of the oral mucosa (Fig. 4), indicating that in vivo cell-associated laminin-5r is also 5C5 reactive.

Cells induce exposure of the 5C5 epitope in soluble laminin-5r

To test directly whether cells induced 5C5 epitope exposure, we plated SCC12 human epidermal carcinoma cells on passively adsorbed laminin-5r. We then removed the cells with 20 mM NH_4OH (Gospodarowicz, 1984), at one day intervals for up to three days, and monitored reactivity of 5C5 by ELISA assay. After 48 and 72 hours, 5C5 was strongly positive in laminin-5r coated wells that had been incubated with SCC12 cells. In contrast, 5C5 did not react in control uncoated wells incubated with SCC12 cells. Note that SCC12 cells secrete human laminin-5, which is unreactive with 5C5 but can be detected with a polyclonal serum, 0668B (Fig. 5).

From these results we conclude that cells modified passively adsorbed laminin-5r to induce exposure of the 5C5 epitope, perhaps as a result of matrix assembly.

New monoclonal antibodies distinguish between two forms of laminin-5r

To further examine the differences between soluble and cell-associated laminin-5r, we developed five new monoclonal antibodies against cell-associated laminin-5r. We found that these antibodies reacted with the following bands in western blots: CM6 recognized bands of 200, 150 and 135 kDa, DF2 recognized bands of 140, 135, and (weakly) 100 kDa, FM3 recognized a single band of 135 kDa, and TR1 recognized bands of 140, 135, and 100 kDa; BH5 reacted only weakly with the 135 kDa band (Fig. 6). This pattern was identical in blots of both soluble (not shown) and cell-associated laminin-5r (Fig. 6). Using direct amino acid sequencing, we have identified these

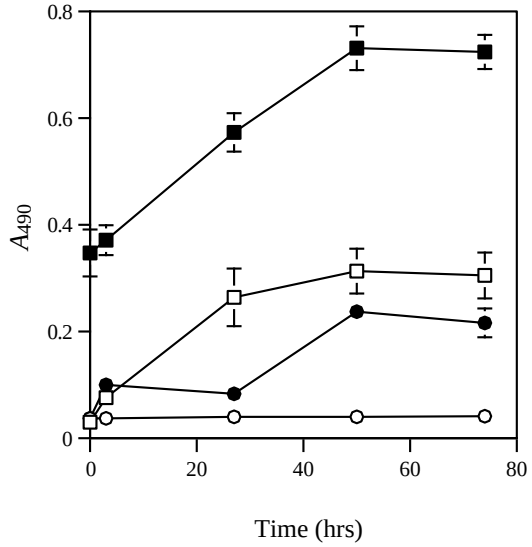

Fig. 5. SCC12 cells induce expression of the 5C5 epitope on passively adsorbed laminin-5r. SCC12 cells were cultured in uncoated 96-well plates (open symbols) or on plates coated with passively adsorbed laminin-5r (filled symbols) for the indicated times and then prepared for cell-associated ELISA assays, using 5C5 (○,●) or the polyclonal antibody 0668B (□,■) as probes. Values represent statistical mean ± s.e.m. for each time point (n=8).

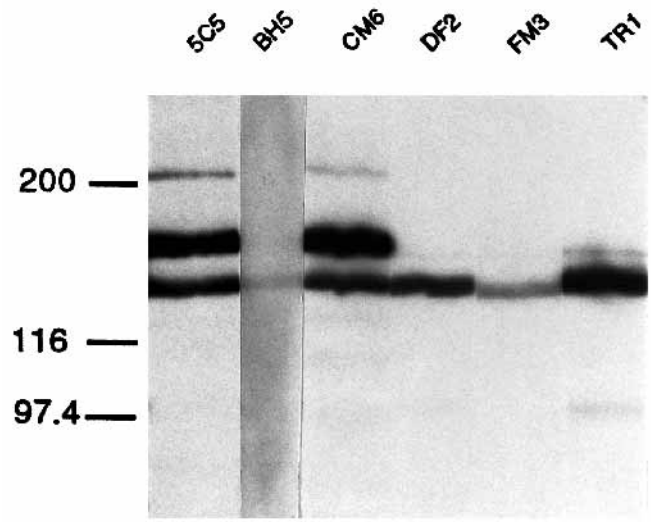

Fig. 6. New monoclonal antibodies recognize laminin-5r. Western blot of cell-associated laminin-5r with polyclonal antibodies (J18, 0668B) and the indicated monoclonal antibodies. Migration of molecular mass standards is indicated at left (in kDa).

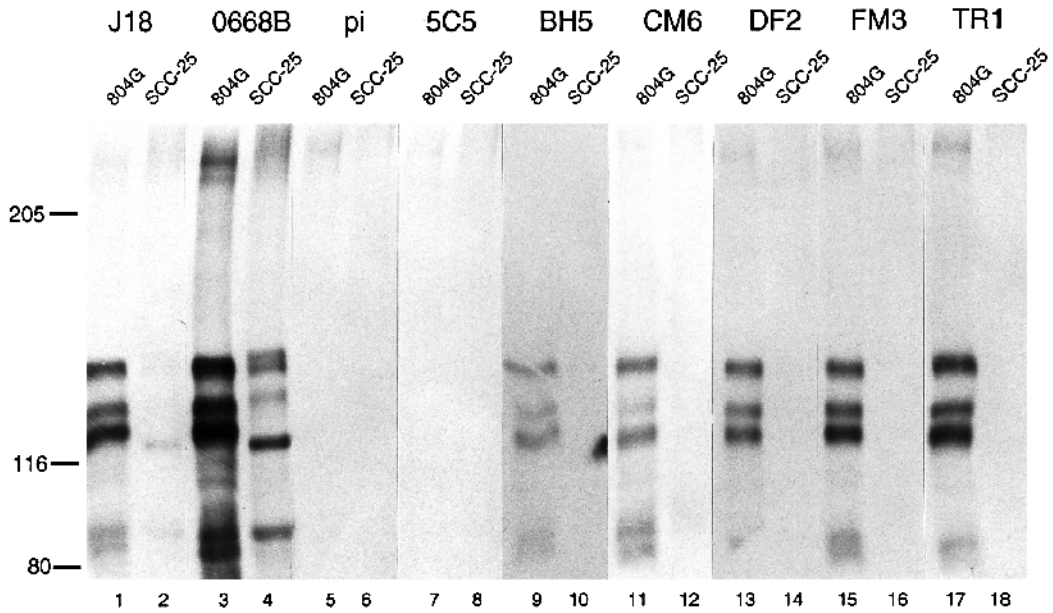

Fig. 7. New monoclonal antibodies immunoprecipitate soluble laminin-5r. Indicated antibodies were added to ^{35}S-labelled conditioned medium from rat 804G (odd numbered lanes) or human SCC25 cells (even numbered lanes) and resulting immunoprecipitations were separated by SDS-PAGE and analyzed by fluorography. Note that the polyclonal antibodies J18 and 0668B cross-react with human laminin 5 but that the monoclonal antibodies do not. Note also that preimmune serum from rabbit 0668 (pi) fails to precipitate laminin-5r or human laminin 5. Migration of molecular mass markers is indicated at left (in kDa).

bands as follows: two forms of α3 (150 and 135 kDa), one form of β3 (135 kDa) and three forms of γ2 (140, 100, and 80 kDa) (J. Falk-Marzillier et al., unpublished). The α3 chain exists as a 200 kDa precursor (Matsui et al., 1995; Marinkovich et al., 1992); both 5C5 and CM6 may recognize this 200 kDa band (Fig. 6). Based on these observations, we conclude that CM6 recognizes the α3 chain, BH5 and FM3 recognize the β3 chain, and DF2 and TR1 may recognize epitopes cross reactive with β3 and γ2.

Unlike 5C5, all of these antibodies immunoprecipitated soluble laminin-5r, indicating that each of these epitopes is exposed on soluble laminin-5r (Fig. 7). They did not cross react with human laminin 5 (Fig. 7).

To test whether these antibodies recognized distinct epitopes, we performed a sandwich capture ELISA assay wherein each antibody was coated in ELISA wells and used to capture soluble laminin-5r from 804G cell conditioned medium. Captured laminin-5r was then detected with biotinylated monoclonal antibodies and streptavidin/horseradish peroxidase. Capturing of soluble laminin-5r by one antibody did not interfere with detection by any of the others, with the exception of the DF2 and TR1 antibodies (Fig. 8). Capturing of laminin-5r with DF2 blocked detection by TR1 and vice versa, suggesting that these antibodies recognize a common epitope, consistent with our observation that they recognize common bands in western blots (Fig. 6). We conclude that the CM6, FM3 and DF2/TR1 epitopes are distinct from one another on laminin-5r.

We used the CM6, FM3 and TR1 antibodies in ELISA assays to directly compare the monoclonal antibody epitope expression in passively adsorbed and cell-associated forms of laminin-5r. We found that while all three antibodies reacted with both forms of laminin-5r, they were fourfold more reactive with the cell-associated form of laminin-5r (Fig. 9).

Cell contact increases exposure of monoclonal antibody epitopes on soluble laminin-5r

We also examined whether, like 5C5, exposure of these monoclonal antibody epitopes could be induced in passively adsorbed laminin-5r by cell contact. Within hours of SCC12 cell plating, reactivity of all three antibodies increased substantially (Fig 10). Like 5C5, these antibodies did not react with SCC12-derived matrix (Fig. 10), because they are rat-specific (Fig. 7). These data suggested that SCC12 cell contact induced exposure of these epitopes by modifying passively adsorbed laminin-5r to form the cell-associated conformation.

Numerous cell types secrete proteases that digest extracellular matrix components. To determine whether the cell-induced modification of laminin-5r was caused by proteolysis, we incubated soluble laminin-5r with serial dilutions of purified trypsin or chymotrypsin and probed for antibody epitope expression by ELISA assay. Low concentrations (3-30 ng/ml) of either protease increased polyclonal antibody reactivity but failed to increase reactivity with any of the monoclonal antibodies, including 5C5; the results from trypsin

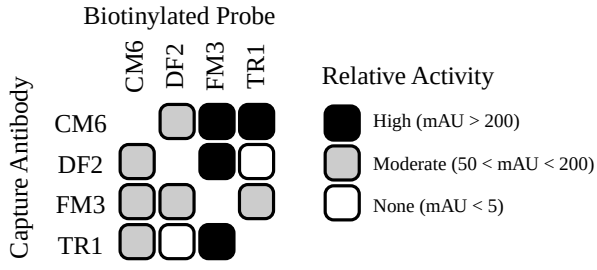

Fig. 8. New monoclonal antibodies recognize distinct epitopes. Sandwich ELISA assays were performed with unconjugated monoclonal antibodies used to capture soluble laminin-5r from 804G cell conditioned medium. Captured laminin-5r was probed with biotinylated monoclonal antibodies, followed by horseradish peroxidase conjugated to streptavidin. Reactions were repeated in quadruplicate on separate plates and the average levels were classified as high, moderate, or none based on absorption at 490 nm (mAU = milliabsorbance units at 490 nm). Note that capturing with DF2 interfered with detection by TR1 and vice-versa.

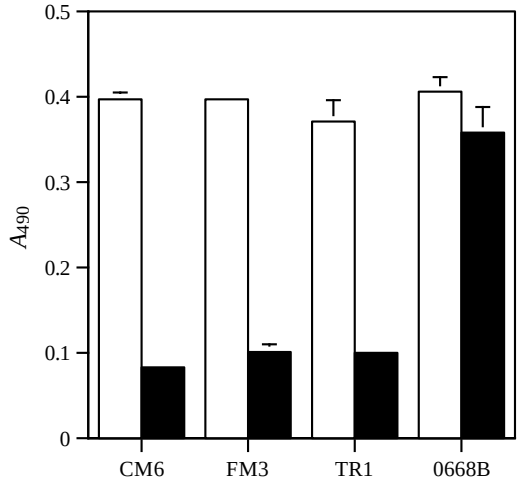

Fig. 9. New monoclonal antibodies distinguish between soluble/passively adsorbed (■) and cell-associated (□) laminin-5r. ELISA assays were performed with indicated antibodies on passively adsorbed and cell-associated laminin-5r. Values expressed represent statistical means ± s.e.m. (*n*=8).

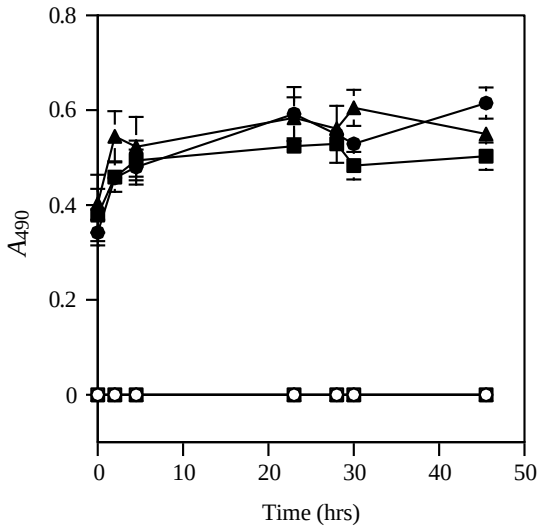

Fig. 10. SCC12 cells induce expression of monoclonal epitopes on passively adsorbed laminin-5r. SCC12 cells were cultured in uncoated (control) plates (open symbols) or on passively adsorbed laminin-5r (filled symbols) for the indicated times and then prepared for cell-associated ELISA assays. Values are expressed as statistical mean ± s.e.m. (*n*=8). (●,○) CM6; (▲,△) FM3; (■,□) TR1. △ obscured by ○,□ because △ values all equal zero.

treatment are shown in Fig. 11. In fact, increasing concentrations of protease generally decreased monoclonal antibody reactivites. We therefore conclude that digestion of laminin-5r by serine proteases does not induce appearance of any of the monoclonal antibody epitopes.

In summary, we have used monoclonal antibodies against laminin-5r to demonstrate that the soluble (passively adsorbed onto plastic) and cell-associated forms of laminin-5r express distinct surface epitopes: the 5C5 epitope is only expressed on the cell-associated form, while the CM6, FM3, and TR1 epitopes are exposed in both forms but predominate in the cell-

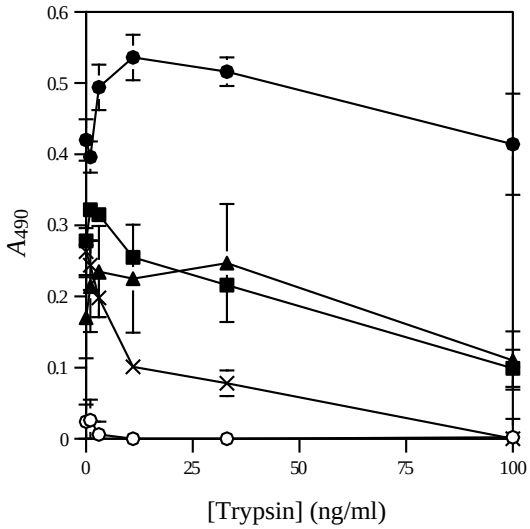

Fig. 11. Proteolysis does not increase exposure of the 5C5 (○), CM6 (×), FM3 (■), or TR1 (▲) epitopes. ELISA plates coated with soluble laminin-5r were treated with the indicated concentrations of purified trypsin, incubted with 1 μg/ml aprotinin to block protease activity, then prepared for ELISA assay. (●) 0668B. Values are expressed as statistical mean ± s.d. (*n*=3).

associated form. Importantly, cell contact with passively adsorbed laminin-5r induces unveiling of the 5C5 epitope and increases exposure of the CM6, FM3 and TR1 epitopes, suggesting that cells may modify soluble laminin-5r to produce the cell-associated form. Treatment of laminin-5r with serine proteases fails to induce unveiling of the 5C5 epitope and does not increase exposure of the CM6, FM3, or TR1 epitopes.

DISCUSSION

Laminin-5r exists in two different physical forms in vitro: as a soluble molecule, capable of passively adsorbing onto surfaces (e.g. tissue culture plastic), and as a cell-associated molecule found directly beneath cell culture monolayers. In this study, we examined the expression of monoclonal antibody epitopes on the molecular surface of both forms of laminin-5r. We found that these epitopes are differentially exposed on soluble vs cell-associated laminin-5r in ELISA and immunoprecipitation assays, and are induced by cell contact.

All monoclonal antibodies tested in this study preferentially bound the cell-associated form of laminin-5r in ELISA assays. This is to be expected, because these antibodies were selected with screening assays using cell-associated laminin-5r. However, since cell-associated laminin-5r is probably a part of an assembled extracellular matrix, it is also possible that these antibodies could be useful markers for matrix assembly (e.g. Ugarova et al., 1995). Consistent with this possibility, the monoclonal antibody 5C5 reacted with cell-associated, but not soluble, laminin-5r in either ELISA or immunoprecipitation.

The lack of 5C5 reactivity in these assays could be trivial if 5C5 were a low affinity antibody that fails to bind antigen under immunoprecipitation (or ELISA) assay conditions. This seems unlikely, though, since 5C5 does immunoprecipitate cell-associated laminin-5r. Furthermore, the 5C5 epitope is

easily detected on the α3 chain of the soluble form in western blots. Therefore, a more likely explanation is that the native conformation of soluble laminin-5r may be such that the 5C5 epitope is sterically hindered from interacting with antibody.

Exposure of the 5C5 epitope on soluble laminin-5r is not due simply to denaturation. Both by ELISA and immunoprecipitation, none of the denaturing conditions we tested induced 5C5 reactivity. A major difference between these assays is that in western blots the individual subunits are physically immobilized on PVDF membranes, which greatly hinders refolding of these subunits, prior to removal of denaturing conditions and testing with antibody. In contrast, these subunits are completely soluble during immunoprecipitation, which allows them to at least partially refold following dialysis of SDS and reducing agents. Therefore, it is likely that partial refolding of the α3 chain blocks immunoprecipitation of this chain from 804G conditioned medium.

Three new monoclonal antibodies reported in this study did not display the clear-cut selectivity of 5C5, yet preferentially bound the cell-associated over the soluble form of laminin-5r in ELISA assays. It is possible that the cell-associated form, being part of an assembled matrix, exists in a more fibrous conformation, which favors exposure of these antibody binding sites, whereas the soluble form may be more globular. It should be interesting to prepare antibodies that preferentially or selectively react with the soluble form, and then map a set of epitopes specific to this form; these efforts are currently under way in our laboratory.

Appearance of monoclonal antibody epitopes is a convenient marker for surface conformation of proteins. Reports of conformation specific changes in epitope expression are relatively common (e.g. Frelinger et al., 1991; VanCott et al., 1994; Takeda et al., 1994) but they have not been described for laminins. Some evidence suggests that laminin 1 epitopes are lost during formation of kidney basement membrane (Abrahamson and St John, 1992), although this may be due to changes in expression of laminin subunits rather than changes in conformation of these subunits (Miner and Sanes, 1994; Vanden Heuvel and Abrahamson, 1993). Modification of laminin conformation is therefore a relatively new observation which deserves to be explored in further detail.

Our data support a model wherein the transition from the soluble form to the cell-associated form does not occur spontaneously (e.g. by passive adsorption onto substrate). Rather, cells appear to actively modify the soluble/adsorbed form, perhaps during a process of matrix assembly. We observed that cell-mediated modification of laminin-5r is rapid and may involve several steps. We observed significant increases in four distinct monoclonal antibody epitopes (5C5, CM6, FM3, TR1) after SCC12 cells were plated on passively adsorbed laminin-5r. However, the exposure of these epitopes varied over the course of three days, suggesting that laminin-5r may assume multiple configurations during its modification.

The mechanism of this modification is unknown. At least two possibilities exist: proteolytic processing and rearrangement of disulfide bonds. Serine proteases do not appear to induce this modification (Fig. 11). However, there are over 100 cysteine residues in the three chains of human laminin 5 (Ryan et al., 1994; Kallunki et al., 1992; Gerecke et al., 1994), and disulfide bonds are rearranged during the receptor-mediated assembly of fibronectin matrices (Hynes, 1990; Mosher et al., 1992), making modification by disulfide rearrangment possible during the assembly of laminin-5r matrix.

An important point to be addressed is whether the antigenic distinction between soluble and cell-associated laminin-5r is mirrored in vivo, e.g. in tissues. Cell-associated laminin-5r is reactive with 5C5 antibody in tissue sections. A source of soluble laminin-5r from tissues is not obvious, however. While a soluble form of human laminin-5 was reported in amniotic fluid (Chan et al., 1995), we were not able to detect it in rat amniotic fluid. More studies are necessary to clarify these issues, e.g. by using monoclonal antibodies to identify the pathways and mechanisms by which cells secrete and assemble laminin-5 matrices.

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