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Characterization of a Tight Molecular Complex between Integrin $\alpha 6\beta 4$ and Laminin-5 Extracellular Matrix

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In many adult epithelia, e.g., epidermis or intestine, adhesion of epithelial cells to basement membrane requires the integrin $\alpha 6\beta 4$ and laminin-5 (Ln-5). In the absence of one or the other, extensive blistering and exfoliation occur. While $\alpha 6\beta 4$ was reported to be a receptor for Ln-5, this interaction is poorly understood. We characterize complexes between $\alpha 6\beta 4$ and Ln-5 in cell-free preparations of extracellular matrix (ECM) from the epithelial cell line, 804G. By microsequencing, Ln-5 and $\alpha 6\beta 4$ were the major proteins in this ECM and were likely engaged in receptor/ligand complexes because, by immunofluorescence, $\alpha 6\beta 4$ was colocalized with Ln-5 both in cell monolayers and in cell-free ECM preparations, but they disappeared after preincubation of the monolayers with $\alpha 6\beta 4$ or Ln-5 function-blocking antibodies. The $\alpha 6\beta 4$/Ln-5 complexes were resistant to dissociation by extreme pH, urea, chaotropes, eDTA, non-ionic detergents, and b-mercaptoethanol. They were only dissociated by strong anionic detergents, e.g., 1% SDS, suggesting receptor/ligand interactions based on high affinity or avidity. We propose that these $\alpha 6\beta 4$/Ln-5 complexes may provide links between plasma membrane and basement membrane that resist mechanical stress and support epithelial integrity.

The basement membrane (BM) underlying epithelial tissues plays an important role in the organization and maintenance of tissues and organs as well as in development, tissue morphogenesis and wound healing. Laminin-5 (Ln-5) is an essential component of several epithelial basement membranes (Burgeson et al., 1994, Timpl, 1996). Genetic evidence indicates that Ln-5 mutations may be incompatible with survival, by causing extensive blistering and exfoliation of skin and gut epithelium (Uitto and Pulkkinen, 1996). Ln-5 is a member of the growing family of laminins, which form cross- or T-shaped heterotrimeric protein complexes through the combination of an α, β, and γ chain. Their expression is regulated tightly during development, morphogenesis and growth. At present, the cloning of five α-, three β-, and two γ-chains has been reported, which combine into at least 11 different disulfide-bonded heterotrimers (Timpl, 1996). Ln-5 was described independently as either epiligrin, kalinin, nicein, or ladsin (Carter et al., 1991, Rousselle et al., 1991, Verrando et al., 1987, Mizushima et al., 1996). Based on DNA sequence information and antigenic comparisons, these molecules are likely to be very similar or identical (Marinkovich et al., 1993). Compared to laminin-1 (Ln-1), the best characterized member of the family, all three chains of Ln-5 lack the N-terminal domains thought to be involved in self-polymerization and binding to nidogen, another component of the basement membrane (Timpl, 1996).

Ln-5 appears to mediate diverse cellular behavior. Defects in genes encoding Ln-5 subunits (lama3, lamb3, and lamc2) (Aberdam et al., 1994, Pulkkinen et al., 1994, Kivirikko et al., 1995) and/or its receptor integrins (Vidal et al., 1995, Brown et al., 1996) as well as autoantibodies directed against Ln-5 (Kirtschig et al., 1995, Yancey et al., 1995) have been reported to be associated with blistering skin diseases, such as junctional epidermolysis bullosa or cicatricial pemphigoid, pointing to a critical role of these molecules in maintaining epithelial integrity. Consistent with this, Ln-5 has been reported to promote rapid adhesion and spreading of various cell lines (Rousselle and Aumailley, 1994, Hormia et al., 1995, Xia et al., 1996) and to induce the formation of mature hemidesmosomes (Langhofer et al., 1993, Hormia et al., 1995), an important adhesive feature of epithelia. In apparent contrast to this static adhesion promoting activity, Ln-5 was reported to serve as a cell scattering factor for carcinoma cells (Kikkawa et al., 1994). To extend these observations we have shown that MMP2 protease can convert Ln-5 from a static to a migratory substrate

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Abbreviations: BM, basement membrane; BP, bullous pemphigoid; ECM, extracellular matrix; Fn, fibronectin; IIF, indirect immunofluorescence; Ln, laminin.

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(Giannelli et al., 1997). Additionally, others have reported that processing by proteases may affect Ln-5 functions (Goldfinger, 1998).

We and others reported previously that the epithelial rat bladder carcinoma cell line 804G deposits an ECM rich in Ln-5 (Langhofer et al., 1993, Hormia et al., 1995, Baker et al., 1996) and forms hemidesmosomes *in vitro* (Riddelle et al., 1991). Hemidesmosomes provide tight contact of epithelial cells to the underlying basement membrane (Schwarz et al., 1990, Quaranta and Jones, 1991). They appear by transmission electron microscopy as electron-dense structures at the plasma membrane, which constitute sites of attachment for intermediate filaments of the keratin types 5 and 14 (Borradori and Sonnenberg, 1996, Fuchs, 1997). Hemidesmosomes are multimolecular complexes; several of their components have been identified including two transmembrane proteins, bullous pemphigoid antigen (BP) 180 and the integrin $\alpha6\beta4$ (Giudice et al., 1991, Kajiji et al., 1989, Tamura et al., 1990). Cytoplasmic components include the BP230, intermediate filament-associated protein 300, plectin or hemidesmosomal protein1, the 6A5 antigen and keratins (Borradori and Sonnenberg, 1996). The integrin $\alpha6\beta4$ is therefore in a position to form transmembrane linkages between the intermediate filament cytoskeleton and the ECM of the basement membrane (Hynes, 1992).

Integrin $\alpha6\beta4$ belongs to a family of transmembrane receptor proteins that mediate cell-cell and cell-ECM interactions (Engel, 1992). They are heterodimers comprising an α and β subunit, which can be found in different pairwise combinations. For example, the integrin $\alpha6$ can combine with either the integrin $\beta1$ or $\beta4$ subunit forming functionally distinct heterodimers that act as receptors recognizing various laminins including Ln-1 and Ln-5 (Lee et al., 1992, Niessen et al., 1994, Spinardi et al., 1995). Beside integrin $\alpha6\beta4$, 804G cells express integrins $\alpha2\beta1$, $\alpha3\beta1$, and to a minor extent $\alpha_v\beta1$, but no $\alpha6\beta1$ (Spinardi et al., 1995).

It had been observed previously that integrin $\alpha6\beta4$ and Ln-5 co-localize (Langhofer et al., 1993) and are involved in the nucleation of hemidesmosomes (Jones and Green, 1991, Baker et al., 1996). Transfection experiments confirmed that $\alpha6\beta4$ is a receptor for Ln-5 (Niessen et al., 1994).

Here, we describe a complex between integrin $\alpha6\beta4$ and Ln-5, which is detected in the cell-free 804G cell ECM, apparently reflects a receptor/ligand interaction, and is resistant to strong ionic and hydrophobic treatments. These observations may aid our biochemical understanding of epithelial interactions with the basement membrane.

MATERIALS AND METHODS

Cell culture. The rat bladder carcinoma 804G cell line was maintained in DMEM (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA.), 2 mM glutamine (BioWhittaker, Walkersville MD) and penicillin (20U/ml)/ streptomycin (20 mcg/ml; Irvine Scientific, CA).

804G matrix preparation. Confluent 804G cells were removed by incubating them 2 × 5 min in 20 mM sterile NH_4OH at room temperature (RT) followed by gently scraping and extensive washing according to Gospodarowicz (Gospodarowicz, 1984). The remaining 804G matrix preparation was directly scraped into reducing Laemmli sample buffer, boiled, and applied to SDS-PAGE. In some cases, prior to this final scraping in Laemmli sample buffer, cell-free matrix was incubated for 1 h at RT with indicated buffers (Fig. 4).

Antibodies. The polyclonal antisera J18 and 0668B to denatured or non-denatured 804G cell-deposited matrix, respectively, were prepared as previously described (Langhofer et al., 1993, Plopper et al., 1996). Integrin $\alpha6$ antibodies 6845 and 1407 against the cytoplasmic tail recognize the 30 kd light chain of $\alpha6$ under reducing conditions and integrin $\beta4$ antibodies 5710 and 6945 were generated as previously described (Kajiji et al., 1989, Tamura et al., 1990). Horseradish peroxidase conjugated antibodies were purchased from Amersham (Rockford, IL). Goat-anti-mouse FITC antibody was obtained from Cappel (West Chester, PA), anti-rabbit biotin antibody and Texas Red Streptavidin were purchased from Zymed, San Francisco, CA.

Gel electrophoresis and immunoblotting. 804G ECM preparations were separated on 6% or 12. 5% SDS-PAGE according to Laemmli (Laemmli, 1970). To ensure reducing conditions during electrophoresis 750 mM β-mercaptoethanol (Sigma, San Louis, MO) was added to the upper chamber. The separated polypeptides were subsequently transferred (semi-dry) to polyvinylidene fluoride (PVDF; Bio-Rad, Hercules, CA) membrane and processed after Ponceau S staining (Sigma) either for Western blotting or direct amino acid sequencing. Blocking and antibody dilutions were done in blotto (5% nonfat dry milk in PBS, 0.2% Tween-20; PBST). Primary rabbit antisera diluted 1:200 (6845, 1407 and 6945) or 1:5000 (0668B) were incubated for 1 h at RT followed by washing in PBST. Secondary horseradish peroxidase coupled antibodies were incubated for another hour at RT in a 1:10.000 dilution followed by washing. Detection of specifically stained polypeptides was performed using enhanced chemiluminescence ECL (Amersham) reagent and exposing to autoradiography film (DuPont, NEN, Boston, MA).

Automated gas phase amino acid analysis. N-terminal protein sequencing was achieved directly from the PVDF membrane using an Applied Biosystem Sequencer Model 494 at the Scripps Core Protein Facility. Internal protein sequences were obtained by a tryptic in-gel digest of a preparative Coomassie Brilliant Blue stained gel followed by an HPLC separation of the resulting fragments.

Indirect immunofluorescence. Confluent 804G cell monolayers grown on glass coverslips were either fixed in ice-cold acetone for 10 min at RT or removed with 20 mM NH_4OH as described above and blocked in 0.1% BSA/ 0.3% Triton-X-100/PBS. Primary antibodies were incubated in a 1:1000 dilution (CM6) or 1:20 (6845) in 50 mM Tris Saline/2% BSA at 37°C in a wet chamber. Controls included the omission of primary antibodies or use of irrelevant IgG to determine non-specific binding of secondary antibodies. After washing with PBS, the coverslips were incubated with secondary antibody (goat-anti-mouse FITC, 1:125) or (anti-rabbit biotin, 1:150) for 1 h at 37 °C followed by incubation with Texas Red Streptavidin (1:150) for 1 h at RT. Coverslips were mounted in SlowFade antifade buffer (Molecular Probes, Eugene, OR) and viewed on a Zeiss Axiovert microscope.

Cell-antibody incubation studies. 804G cells were grown to confluency, washed and incubated with 804G conditioned medium supplemented with the mAbs CM6 or TR1 (100 μg/ml), or the antisera 5710, 0668B, or normal rabbit serum (NRS) (1:25). After 2 h cells were washed, removed with 20 mM NH_4OH and the remaining matrix components were subsequently processed for Western blotting.

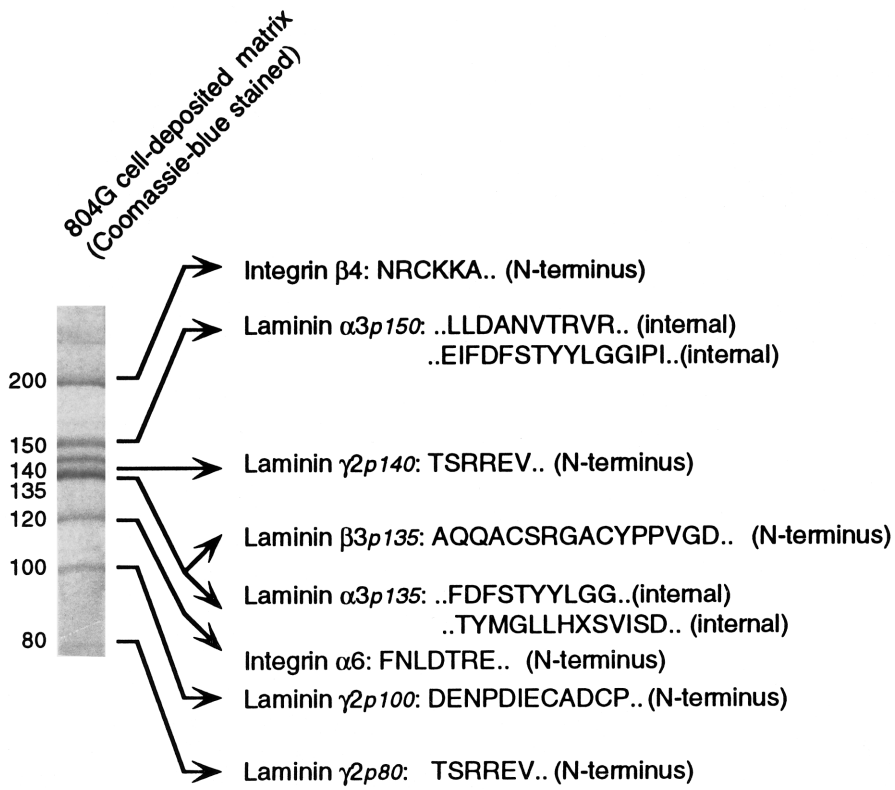

FIG. 1. Laminin-5 and integrin $\alpha 6\beta 4$ are the two major components of 804G matrix preparations. Amino acid sequence analysis of 804G ECM components. 804G matrix was prepared by NH_4OH extraction, separated on 6% SDS-PAGE, and transferred to PVDF membrane. All visible protein bands after staining with Ponceau Red were subjected to aminoterminal sequencing. N-terminally blocked polypeptides underwent a limited in-gel digest and resulting peptides were subjected to sequencing. Cysteines not identified in standard Edman degradation were derived from respective cDNA sequence data.

RESULTS

Integrin $\alpha 6\beta 4$ and Ln-5 are the major components of cell-free 804G ECM preparations. To analyze the protein composition of 804G ECM, cell-free 804G ECM preparations obtained by NH_4OH extraction as described in Materials and Methods, were solubilized in reducing Laemmli sample buffer and resolved by SDS-PAGE. All visible bands were analyzed by protein microsequencing (Fig. 1). The M*r* 150- and the M*r* 135 bands yielded internal tryptic peptides sequences identical to rat Ln-5 $\alpha 3$ chain (Baker et al., 1996) and homologous to human Ln-5 $\alpha 3$ chain (Ryan et al., 1994), indicating that they represent M*r* variants of the rat Ln-5 $\alpha 3$ subunit, possibly related to each other by posttranslational processing.

The M*r* 140 and M*r* 80 bands yielded the same N-terminal sequence, identical to the human $\gamma 2$ Ln-5 subunit (Fig. 1) (Kallunki et al., 1992). In addition, the M*r* 100 band amino-terminus, corresponded to amino acid 415-426 of the human $\gamma 2$ subunit (Vailly et al., 1994). Therefore, at least three forms of the $\gamma 2$ subunit exist in Ln-5 deposited by 804G cells.

The N-terminal sequence of the M*r* 135 polypeptide was homologous to the amino-terminus of human Ln-5 $\beta 3$-chain (Gerecke et al., 1994).

Thus, by microsequencing, all 6 bands detected in 804G ECM by Ln-5 polyclonal antisera (Fig. 4A) correspond to Ln-5 subunits.

The N-terminus of the M*r* 200 and 120 polypeptides (Fig. 1), which by Western blotting were not related to Ln-5 (not shown), were identical to the $\beta 4$ and $\alpha 6$ integrin subunits, respectively (Fig. 1) (Tamura et al., 1990). The ratio of $\alpha 6\beta 4$ subunits to Ln-5 subunits, estimated by their relative staining intensity, varied somewhat in several preparations, but consistently approached 1:1.

In conclusion, Ln-5 and $\alpha 6\beta 4$ were the only proteins detectable by microsequencing in our cell-free 804G ECM preparation, supporting the possibility of a specific association.

Integrin $\alpha 6\beta 4$ association with Ln-5 results from receptor–ligand interactions. Confluent 804G monolayers were acetone-fixed and double-stained with antibodies to $\alpha 6\beta 4$ and Ln-5, respectively. Alternatively, cell monolayers were removed by lysis with 20 mM NH_4OH, and the remaining cell-free ECM was fixed and immunostained.

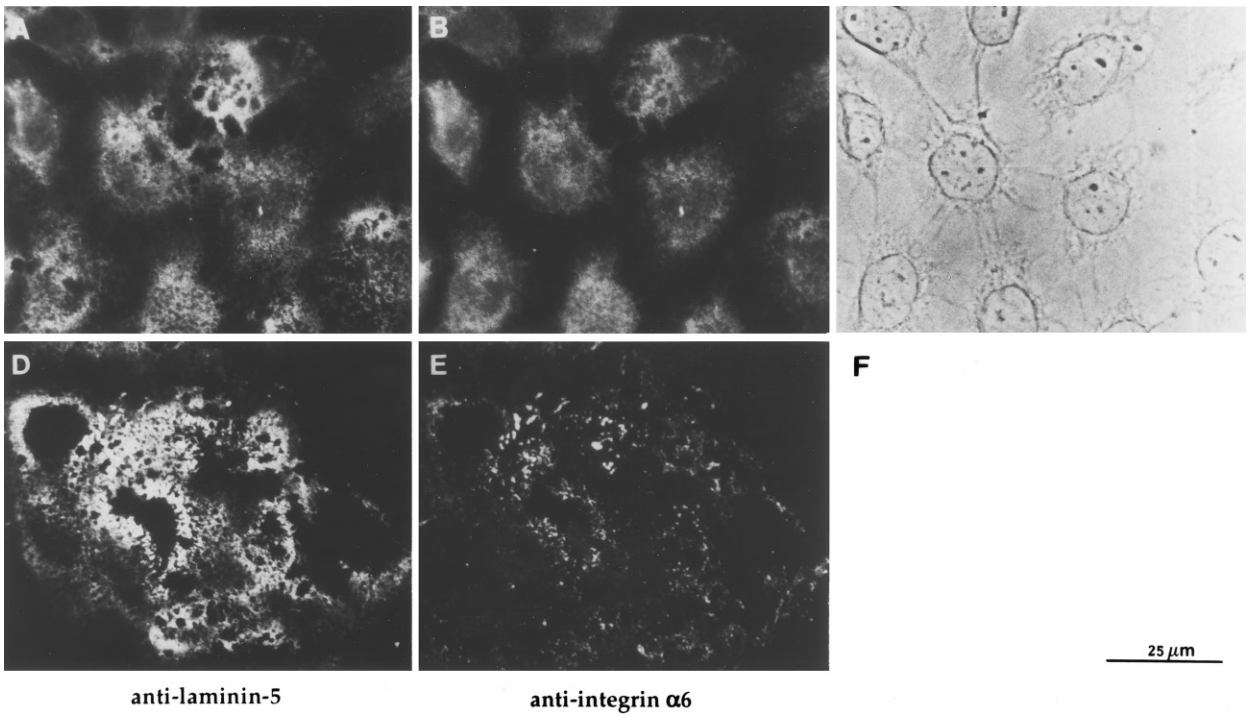

FIG. 2. Laminin-5 and its receptor integrin $\alpha 6\beta 4$ co-localize and remain associated in cell-free 804G matrix preparations. Double-label immunofluorescence of 804G cells (A–C) or 804G matrix preparations (D–F). 804G cells were grown to confluency and either fixed with acetone (A–C) or removed by detergent extraction. Coverslips were doubled-stained with mouse monoclonal antibodies to laminin-5 (CM6) and rabbit anti-integrin $\alpha 6$ antibodies (6845). Primary antibodies were visualized by the use of fluorescein-conjuated rabbit anti-mouse IgG (A, D) or biotinylated goat anti-rabbit IgG followed by staining with Texas Red (B, E). Panels C and F show the respective phase contrast images of 804G cells and 804G matrix preparations. Bar, 25 μm.

Anti-Ln-5 antibodies outlined a pattern of Ln-5 deposition (Fig. 2 A, D) consistent with the described "swiss-cheese" pattern (Langhofer et al., 1993), in both the acetone-fixed cells and the cell-free ECM preparations.

Anti-$\alpha 6$ (Fig. 2 B, E) or anti-$\beta 4$ (not shown) antibodies were also positive with both fixed cells and cell-free ECM, and in both cases the staining co-localized exactly with that of anti-Ln-5 antibodies, by double IIF.

To investigate this point further, we utilized function-blocking antibodies; monoclonal CM6, which reacts with the Ln-5 $\alpha 3$ chain, and antiserum 5710 to $\alpha 6\beta 4$ (Jones et al., 1991). Incubation of confluent 804G monolayers for 2 hours with these antibodies caused no detectable decrease in deposited Ln-5, as determined by Western blotting (not shown). However, $\alpha 6\beta 4$ was not detectable in ECM preparations of cells treated with CM6 (Fig. 3, lane 1), and was significantly decreased in those treated with 5710 (Fig. 3, lane 2). Control antibodies, including a non-blocking anti-Ln-5 antibody (TR1), normal rabbit serum, or a non-blocking anti-Ln-5 antiserum (0668B), showed no effect (Fig. 3, lanes 3–6). Thus, co-localization by IIF (Fig. 2) and inhibition of co-localization by functional antibodies, indicate that $\alpha 6\beta 4$ remains associated with Ln-5 in the cell-free 804G ECM preparations because of a receptor-ligand interaction.

Molecular complexes between $\alpha 6\beta 4$ and Ln-5 are based on both hydrophobic and ionic protein interactions. To characterize some of the chemical properties of the association between integrin $\alpha 6\beta 4$ and Ln-5, cell-free 804G ECM preparations were exposed to treatments known to dissociate protein-protein interactions, solubilized and probed by immunoblotting (Fig. 4).

Anti-Ln-5 antibodies (Fig. 4A) reacted approximately the same, indicating that none of the dissociating conditions caused removal of deposited Ln-5 from the culture plates. That Ln-5 could be removed from the plates was shown by treatment with a proteolytic enzyme, trypsin (Fig. 4A, lane 2).

Integrin $\alpha 6$ was removed by strong anionic detergent (1% SDS) (Fig. 4B, lane 15) and, as expected, trypsin (Fig. 4B, lane 11). However, it remained in the ECM under all other dissociating conditions tested (Fig. 4B, lanes 10, 12–14, 16–18), which included extreme pH (0.2 M glycine, pH 2.5; 20 mM NH_4OH, pH 12), which disrupts protein electrostatic interactions, chaotropes (3M KSCN), urea (8M), and non-ionic detergents (1% Triton-X100), which disrupt hydrophobic interactions. The $\alpha 6\beta 4$/Ln-5 complexes were also resistant (Fig. 4B) to reducing agents (0.1 M β-mercaptoethanol) and EDTA (15mM). These results indicate that both hydro-

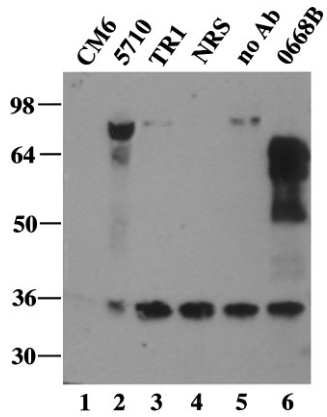

FIG. 3. The integrin $\alpha6\beta4$/laminin-5 complex can be disrupted by incubation of 804G monolayers with a blocking Ln-5 specific monoclonal antibody (CM6). 804G cell monolayers were incubated for 2 h with indicated antibodies and subsequently removed with NH_4OH. The remaining matrix preparation was analyzed by Western blotting for the presence of integrin $\alpha6\beta4$ (only $\alpha6$ shown). (CM6: blocking anti-Ln-5 mAb; 5710: anti-integrin-$\beta4$ specific rabbit serum; TR1: non-blocking anti-Ln-5 mAb; NRS: normal rabbit serum; noAb: no antibody; 0668B: non-blocking anti-Ln-5 specific rabbit serum).

phobic and ionic interactions contribute to forming the complex between $\alpha6\beta4$ and Ln-5 detected in the 804G ECM. Furthermore, because the dissociating conditions tested are harsh for protein-protein interactions, the $\alpha6\beta4$/Ln-5 complex is probably based on either high affinity or high avidity interactions.

To relate these tight complexes to hemidesmosome assembly, we looked for their presence in cells that assemble either complete hemidesmosomes (human squamous cell carcinoma, SCC25) or no hemidesmosomes (human pancreatic carcinoma FGmet-2) (Hopkinson, 1994). In both cases, $\alpha6\beta4$ was found associated with cell-free ECM, suggesting that formation of the tight complexes preceeds or is independent of hemidesmosomes.

DISCUSSION

In the present study, we investigated biochemical properties of a complex between integrin $\alpha6\beta4$ and Ln-5, detected in the ECM deposited by the epithelial cell line, 804G. Ln-5 and $\alpha6\beta4$ were the major components in 804G ECM preparations. Several lines of evidence indicated that they were engaged in a complex, based on receptor-ligand interaction: i) their amounts were stoichiometrically comparable, by protein staining; ii) double immunostaining for $\alpha6\beta4$ and Ln-5 was entirely overlapping, both before and after removal of cells; iii) organization of the immunostaining was "swiss-cheese" patterned, characteristic of hemidesmosomes; iv) treatment of cells with anti-Ln-5 or -$\alpha6\beta4$ function-blocking antibodies disrupted $\alpha6\beta4$ complexing with Ln-5.

While $\alpha6\beta4$ has been already reported to be a receptor for Ln-5 (Niessen et al., 1994), there has been little characterization of their interaction. In our hands, the $\alpha6\beta4$/Ln-5 receptor-ligand complexes were resistant to extraction procedures with base or detergent (Fig. 4B), survived strong ionic and hydrophobic solvents (Fig. 4B), and could be dissociated only under conditions relatively harsh for protein-protein interactions, such as SDS (Fig. 4, lane 15) or combined high-pH/non-ionic detergent treatment (not shown). There are precedents

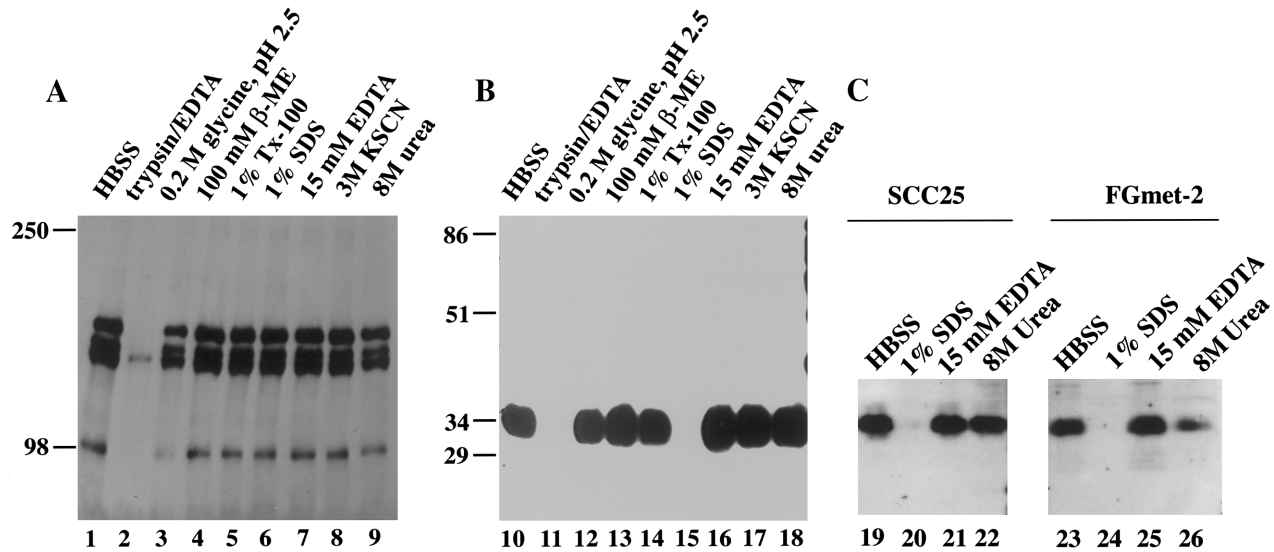

FIG. 4. Laminin-5 and its receptor integrin $\alpha6\beta4$ form complexes resistant to various ionic and hydrophobic solvents. Cell-free 804G (A and B), SSC25 and FGmet-2 (C) matrix preparations were further extracted with indicated protein–protein interaction dissociating buffers (see Materials and Methods, and Results, for further details) and analyzed upon solubilization by Western blotting using Ln-5 or integrin $\alpha6$ specific antibodies. Controls include treatment of 804G matrix with Hanks buffer alone or removal of 804G cells by trypsinization.

for strong and specific protein-protein interactions that withstand similar denaturing conditions, including the assembly of gap junction proteins into pore-forming connexons (Stauffer et al., 1991). However, the interactions of integrins with their ligands have not been characterized extensively in this respect. Integrins left behind in the "footprints" of migrating cells (Palecek et al., 1997) have been taken as an indication of the "strength" of interaction between integrins and matrix Orlando and Cheresh (Orlando and Cheresh, 1991) found that purified radiolabeled $\alpha v\beta 3$ bound to its ligand, vitronectin, irreversibly over time. Carter and his colleagues described the "Stable Adhesion Contacts" in adherent keratinocytes after extraction with urea and high salt (Carter et al., 1990). These contacts contained the hemidesmosomal antigen, BP230, and were co-distributed with Ln-5 (epiligrin) in the ECM. While no $\alpha 6\beta 4$ was immunodetected in those contacts, perhaps because of epitope denaturation, it is likely that they are similar or identical in nature to the Ln-5/$\alpha 6\beta 4$ complexes we describe here. Side-by-side comparisons will be necessary to appreciate the extent of such similarity. Nonetheless, we have extended the observations by Carter, and have provided a model system in which the interaction between $\alpha 6\beta 4$ and Ln-5 can be characterized in some detail.

It will be interesting to determine whether these complexes can occur between purified $\alpha 6\beta 4$ and Ln-5, or require living cells. Our data indicated that incubation of living cells with anti-Ln-5 function-blocking monoclonal antibody, CM6 at 37°C readily disrupted the complex (Fig. 3), whereas in cell-free 804G ECM, the complex was not disrupted by CM6 (not shown). This result may be explained by rapid turnover in living cells, possibly related to hemidesmosome remodeling. Additional experiments are necessary to test whether establishment of the Ln-5/$\alpha 6\beta 4$ complex also requires living cells.

Three forms of $\beta 4$ are generally detectable in whole-cell lysates, with M*r* 200, 180 and 150, respectively, due to proteolysis of the intracellular domain (Kajiji et al., 1989, Giancotti et al., 1992). Of these, only the M*r* 200 was detectable in the Ln-5/$\alpha 6\beta 4$ complexes (not shown), suggesting that proteolysis of the $\beta 4$ cytoplasmic domain may be a way for preventing or disengaging $\alpha 6\beta 4$ complexing with Ln-5.

The 804G ECM has been involved in the formation of mature hemidesmosomes *in vitro* (Langhofer et al., 1993, Rousselle and Aumailley, 1994, Hormia et al., 1995, Baker et al., 1996). Our data indicate that Ln-5 is mostly, if not entirely, responsible for this activity. Coomassie staining indicated that 804G cells assemble an ECM consisting almost entirely of Ln-5 $\alpha 3\beta 3\gamma 2$ heterotrimers. Two $\alpha 3$ (M*r* 150 and 135) and three $\gamma 2$ (M*r* 140, 100 and 80 kD) forms, distinguished by apparent molecular weight, may occur because of post-translational processing, perhaps proteolysis (Vailly et al., 1994). Immunoprecipitations with anti-$\alpha 3$ antibody indicated that these alternative forms are all part of heterotrimers (not shown), as previously noted (Marinkovich et al., 1992, Champliaud et al., 1996). Their functional significance with respect to cell functions remains to be elucidated. We showed that the metalloprotease MMP2 can convert Ln-5 from a static adhesive to a migratory substrate, by clipping the small arm of the $\gamma 2$ chain (Giannelli et al., 1997). Thus, Ln-5 subunits of different size may confer distinctive properties to the heterotrimer.

We cannot rule out formally the presence of functionally important components in the 804G ECM, which were not detected by protein staining. Such putative components may or may not be associated physically to Ln-5. We did test for other ECM molecules, particularly ones found in basement membranes (not shown). The absence of the Ln-1 chains $\alpha 1$, $\beta 1$, $\gamma 1$ effectively excludes the presence of Ln-6 or Ln-7 isoforms, since they contain at least one of these chains, as described (Champliaud et al., 1996). Abundant fibronectin was detectable in the 804G conditioned medium, but not in the ECM, consistent with tumor cells secreting but not assembling fibronectin (Hynes, 1990).

The integrin $\alpha 6\beta 4$ uniquely interacts with intermediate filaments, forming a transmembrane link between the cytokeratin network and the ECM. The complex Ln-5/$\alpha 6\beta 4$ may extend this link to the basement membrane, such that its strength may influence the resistance of epithelia to detachment, eg, by mechanical stresses. Thus, evaluating the strength of the Ln-5/$\alpha 6\beta 4$ complex may be useful to better characterize the blistering occurring in genetic diseases such as junctional epidermolysis bullosa.

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