A Recombinant Tail-Less Integrin β₄ Subunit Disrupts Hemidesmosomes, but Does Not Suppress α₆β₄-mediated Cell Adhesion to Laminins

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Abstract. To examine the function of the α₁β₄ integrin we have determined its ligand-binding ability and overexpressed two potentially dominant negative mutant β₄ subunits, lacking either the cytoplasmic or extracellular domain, in bladder epithelial 804G cells. The results of cell adhesion and radioligand-binding assays showed that α₁β₄ is a receptor for several laminin isoforms, including laminin 1, 2, 4, and 5. Overexpression of the tail-less or head-less mutant β₄ subunit did not suppress α₁β₄-mediated adhesion to laminins, as both types of transfectants adhered to these ligands in the presence of blocking anti-β₁ antibodies as well as the controls. However, immunofluorescence experiments indicated that the endogenous α₁β₄ integrin and other hemidesmosomal markers were not concentrated in hemidesmosomes in cells overexpressing tail-less β₄, while the distribution of these molecules was not altered in cells overexpressing the head-less subunit. Electron microscopic studies confirmed that cells overexpressing tail-less β₄ had a drastically reduced number of hemidesmosomes, while cells expressing the head-less subunit had a normal number of these structures. Thus, expression of a tail-less, but not a head-less mutant β₄ subunit leads to a dominant negative effect on hemidesmosome assembly without suppressing initial adhesion to laminins. We conclude that the α₁β₄ integrin binds to several laminins and plays an essential role in the assembly and/or stability of hemidesmosomes, that α₁β₄-mediated adhesion and hemidesmosome assembly have distinct requirements, and that it is possible to use a dominant negative approach to selectively interfere with a specific function of an integrin.

There are several reasons for believing that the α₁β₄ integrin, which has been implicated in binding to laminin 1 (subunit composition: α₁β₁γ₁) and laminin 5 (kalinin, epiligrin, nicein; α₁β₂γ₁) (Lee et al., 1992; Niessen et al., 1994), is characterized by unique intracellular interactions. The cytoplasmic domain of the β₄ subunit measures over 100 kD in molecular mass and bears no homology with the short cytoplasmic domains of the other known integrins (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). This portion of the molecule contains two pairs of type III fibronectin-like modules connected by a sequence (Connecting Segment) which appears to be the target of multiple potential regulatory mechanisms, including alternative splicing (Tamura et al., 1990) and proteolytic processing (Giancotti et al., 1992). Furthermore, in contrast to β₁ and β₃ integrins which localize to focal adhesions or otherwise interact with the actin filament system (Chen et al., 1985; Damsky et al., 1985; Giancotti et al., 1986a; Dejana et al., 1988), the α₁β₄ integrin is found concentrated in hemidesmosomes and, thus, may interact with the keratin filament system (Carter et al., 1990; Stepp et al., 1990; Sonnenberg et al., 1991).

By expressing various deletion mutant forms of the β₄...
that a critical function of the $\beta_4$ cytoplasmic domain is to mediate the association of the integrin with the hemidesmosomal cytoskeleton (Spinardi et al., 1993). As the extracellular domain of $\alpha_6\beta_4$ binds to basement membrane components, and the specialized cytoplasmic tail of $\beta_4$ subunit interacts with the hemidesmosomal cytoskeleton, this integrin may play a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system.

Despite their functional importance, relatively little is known about the molecular composition and mechanism of assembly of hemidesmosomes. In addition to $\alpha_6\beta_4$, hemidesmosomes contain another transmembrane protein, the Bullous Pemphigoid Antigen 2 (BPAG2) (Giudice et al., 1992), which may be involved in cell adhesion and hemidesmosome assembly. In fact, BPAG2 is a target of the pathogenic autoantibodies present in patients affected by Bullous Pemphigoid (Labib et al., 1986; Liu et al., 1993), a blistering skin disease characterized by fragmentation and disappearance of both hemidesmosomes and basement membrane (Schaumburg-Lever et al., 1972). The relative roles of the $\alpha_6\beta_4$ integrin and BPAG2 in the establishment of stable epidermal cell adhesion to the basement membrane and in the assembly of hemidesmosomes are presently unclear.

To analyze the function of the $\alpha_6\beta_4$ integrin and its role in cell adhesion and assembly of hemidesmosomes, we have examined the spectrum of ligands recognized by $\alpha_6\beta_4$ and tested two distinct strategies for creating a dominant negative mutant $\beta_4$ subunit. We report that $\alpha_6\beta_4$ binds to several laminin isoforms. Introduction of a truncated tail-less $\beta_4$ subunit in cells possessing endogenous $\alpha_6\beta_4$ integrins and hemidesmosomes led to a dominant negative effect, while comparable expression of a $\beta_4$ molecule lacking almost the entire extracellular portion did not. Cells expressing the dominant negative tail-less form of $\beta_4$ were found to have a drastically reduced number of hemidesmosomes, but did not show defective adhesion to laminins.

Materials and Methods

Cell Lines, Antibodies, and Extracellular Matrix Molecules

Rat bladder carcinoma 804G cells (Izumi et al., 1981) were cultured in DMEM with 10% bovine calf serum (BCS). RAC-11P/SD cells (Sommenberg et al., 1993) were cultured in DMEM with 10% FBS.

The monoclonal antibody 3E1 recognizes the extracellular portion of human, but not rodent integrin $\beta_4$ subunit (Giancotti et al., 1992). The other subunit-specific antibodies used in this study were all raised by immunizing rabbits with synthetic peptides reproducing COOH-terminal sequences and were shown to cross-react with rodent integrins. The antibodies to $\alpha_2\beta_1$ and $\alpha_6\beta_4$ (Giancotti et al., 1992), $\alpha_1$ (Throne et al., 1993), $\alpha_1$ (Deffipili et al., 1992), $\alpha_3$ (Klein et al., 1993), $\alpha_5$ and $\beta_1$ (Giancotti and Ruoslahti, 1990), and $\alpha_5$ (Vogel et al., 1993) were previously described. The antibody to $\alpha_5\beta_1$ was generated and characterized in the laboratory of Erikki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). The adhesion-blocking rabbit antisera to affinity isolated rodent $\alpha_5\beta_1$ was previously described (Bernardi et al., 1987). The monoclonal antibody Ab-1, which binds to an epitope of $c-myc$ comprising the amino acid sequence EQK-LISEEDL (residues 410-419), was purchased from Oncogene Science, Inc. (Uniondale, NY). Human autoantibodies directed to the Bullous Pemphigoid Antigen 230 kD were provided by Jo-David Fine (University of North Carolina, Chapel Hill, NC). The BPAG2-specific rabbit polyclonal antiserum was raised by immunization with a GST-fusion protein comprising the major antigenic determinant of the mouse protein in the laboratory of Jouni Uitto (Thomas Jefferson University, Philadelphia, PA). The monoclonal antibodies 4C7 and 4E10 bind to the human $\alpha_5$ and $\beta_1$ laminin subunits, respectively (Eagavall et al., 1986). The monoclonal antibody C4 is directed to the $\beta_1$ laminin chain (Hunter et al., 1989). The rabbit polyclonal antibody R-1301 was raised by immunization with a synthetic peptide from the $\alpha_5$ laminin subunit (Paulson et al., 1991). The monoclonal antibody 5122 reacts with the 80-kD fragment of the same subunit (Leivo and Engvall, 1988). The monoclonal antibodies BM-165 (Rousselle et al., 1991) and BM-140 (Marinkovich et al., 1992a) recognize the human $\alpha_5$ and $\beta_1$ subunits, respectively.

Human plasma fibronectin, mouse laminin 1 (purified from Engelbreth-Holm-Swarm tumor), and human placental laminin were purchased from GIBCO BRL (Gaithersburg, MD). The human placental laminin was purified according to Elirg et al. (1990). Electrophoretic and immunoblotting analyses conducted in our laboratory have shown that it contains the $\alpha_2$, $\beta_1$, and $\gamma_1$ chains, corresponding to laminin 4. The same conclusion was reached in a recently published study (Sommenberg et al., 1993). Immunoblotting with monoclonal antibodies to the human $\alpha_1$, $\beta_1$, $\alpha_3$, and $\beta_1$ chains have indicated that this preparation was not contaminated by laminin 1, 5, 7, or 8. Laminin 2 was purified to homogeneity from mouse heart in the laboratory of Mats Paulsson (University of Bern, Switzerland) by using previously published procedures (Paulson et al., 1991). Human laminin 5 was purified to homogeneity from keratinocyte cell culture medium in the laboratory of Robert Burgeson (Harvard Medical School, Boston, MA). Laminin 5 matrices were prepared as described previously (Sommenberg et al., 1993). Electrophoretic analysis and immunoblotting with antibodies to laminin isoforms and other adhesive ligands have shown that laminin 5 is by far the predominant protein in matrices of RAC-11P/SD cells (Sommenberg et al., 1993). Our own electrophoretic analysis indicated that the 165-kD $\alpha_5$ chain, the 155-kD $\gamma_1$ chain, the 140-kD $\beta_1$ chain, and the 105-kD $\gamma_1$ chain degradation product represent more than 95% of the Coomassie blue stainable proteins present in such matrices. Most of the residual material migrates as a band of $\sim$190 kD and possibly represents the precursor of the $\alpha_5$ chain.

Constructs and Transfections

Expression constructs encoding wild-type and mutant truncated human $\beta_4$ subunits were assembled in the eukaryotic expression vector pRC-CMV (Invitrogen Corp., San Diego CA) and were previously described (Spinardi et al., 1993). The plasmid pcMV-$\alpha_4$, $\alpha_4$ 854-1752 directs the expression of a $\beta_4$ molecule lacking almost the entire cytoplasmic domain. The plasmid pcMV-$\beta_4$, $\beta_4$ 470-660 encodes a $\beta_4$ subunit in which most of the extracellular sequences were replaced by a $c-myc$ epitope tag.

Rat bladder carcinoma 804G cells stably expressing a tail-less (clones B13, B23, and B29) or a head-less (clone F28) human $\beta_4$ subunit were obtained by transfection with pcMV-$\beta_4$, $\beta_4$ 854-1752 and pcMV-$\alpha_5$, $\alpha_5$ 70-660, respectively (Spinardi et al., 1993). The clone expressing a full-length human $\beta_4$ subunit (clone A2) was previously described. The control cell lines (clones Z10 and Z32) were generated by transfection with pRC-CMV alone. The transfected cell lines were maintained in DMEM-10% BCS supplemented with 400 ng/ml of G418 and cultured for at least 48 h in the absence of the selective agent before all experiments.

Immunoprecipitation and Western Blotting Analysis

For immunoprecipitation of cell surface molecules, intact cells were labeled in suspension with $^{125}$I by the lactoperoxidase- $\text{H}_2\text{O}_2$ method, as previously described (Giancotti and Ruoslahti, 1990). After washing, the cells were extracted for 45 min on ice with lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.01% Aprotinin (Sigma Chem., Co., St. Louis, MO), 4 $\mu$g/ml Peptatin A (Sigma), 10 $\mu$g/ml Leupeptin (Sigma), 1 mM PMSF and 10 mM EDTA. For immunoprecipitation of integrins other than $\alpha_6\beta_4$, lysis buffer contained 1 mM CaCl$_2$, 1 mM MgCl$_2$, and no EDTA. The immunoprecipitations were performed as previously described (Giancotti and Ruoslahti, 1990) and analyzed by SDS-PAGE.
separating gels contained 6% acrylamide-bisacrylamide. Autoradiography was performed with X-Omat AR films (Eastman Kodak Co., Rochester, NY). For Western blotting analysis, confluent monolayers were extracted on ice with lysis buffer. The extracts were clarified and their protein content was determined by the BCA assay (Pierce, Rockford, IL). Samples containing 6 mg of total proteins were immunoprecipitated with an excess of the β4 cytoplasmic peptide antibody. Samples containing 100 μg total proteins were precipitated with 6 μl of acetic acid. Both types of samples were separated by SDS-PAGE and subjected to immunoblotting according to previously published procedures (Giancotti et al., 1992). Bound antibodies were detected by incubation with [125I]-Protein A (ICN Biomedicals, Irvine, CA) and autoradiography or chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

To measure the expression of recombinant and endogenous integrins in the various clones, the radioactivity of immunoreactive bands in polyacrylamide gels and nitrocellulose filters was quantified by a Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Adhesion Assay

Adhesion assays were performed essentially as previously described (Giancotti et al., 1985). After coating, all plates were blocked with PBS-0.1% BSA (Sigma). To avoid synthesis and secretion of adhesion proteins during the assay, the cells were treated with 20 μM cycloheximide (Sigma) for 1 h and with 1 μM monensin (Sigma) for 5 min before the assay. Cells were detached by incubation in 0.25% trypsin (GIBCO BRL) for 5 min. After washing and with 1 μM monensin (Sigma) for 5 min before the assay. Cells were washed, and solubilized with 10 ml of lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 200 mM n-Octyl-β-D-Glucopyranoside (Calbiochem, San Diego, CA), 5 mM EDTA, 0.01% Aprotinin, 4 μg/ml Pepstatin A, 10 μg/ml Leupeptin, 1 mM PMSF, and 1 mM MnCl2 for 45 min at 0°C. Extracts were clarified by centrifugation at 15,000 rpm and incubated with 1 ml of Sepharose-normal IgG to remove proteins which bind nonspecifically. The extracts were then applied to an affinity matrix prepared by cross-linking 5 μg of purified 3E1 monoclonal antibody to 1 ml of Sepharose-Protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). After 1 h of incubation at 4°C, the unbound material was removed and the column was washed with 25 bed volumes of 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM n-Octyl-β-D-Glucopyranoside. The integrins were eluted with 5 bed volumes of 50 mM Triethylamine, pH 10, 150 mM NaCl, 50 mM n-Octyl-β-D-Glucopyranoside into tubes containing neutralizing buffer (10% by volume 1 ml Tris-HCl, pH 7.4). Peak fractions were analyzed by SDS-PAGE and Coomassie blue staining, and pooled. Protein concentration was estimated by comparison with known amounts of BSA. The purity of the results was as previously described (Giancotti et al., 1986d).

Radioligand-binding Assay

The partially recombinant wild-type and tail-less α6β4 integrins used in radioligand-binding experiments were purified from the transfected 804G clones A12 and B13, respectively. Approximately 1.5 × 108 cells were used for each purification. Cells were harvested by using 5 mM EDTA, washed, and solubilized with 10 ml of lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 200 mM n-Octyl-β-D-Glucopyranoside (Calbiochem, San Diego, CA), 5 mM EDTA, 0.01% Aprotinin, 4 μg/ml Pepstatin A, 10 μg/ml Leupeptin, 1 mM PMSF, and 1 mM MnCl2 for 45 min at 0°C. Extracts were clarified by centrifugation at 15,000 rpm and incubated with 1 ml of Sepharose-normal IgG to remove proteins which bind nonspecifically. The extracts were then applied to an affinity matrix prepared by cross-linking 5 μg of purified 3E1 monoclonal antibody to 1 ml of Sepharose-Protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). After 1 h of incubation at 4°C, the unbound material was removed and the column was washed with 25 bed volumes of 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM n-Octyl-β-D-Glucopyranoside. The integrins were eluted with 5 bed volumes of 50 mM Triethylamine, pH 10, 150 mM NaCl, 50 mM n-Octyl-β-D-Glucopyranoside into tubes containing neutralizing buffer (10% by volume 1 ml Tris-HCl, pH 7.4). Peak fractions were analyzed by SDS-PAGE and Coomassie blue staining, and pooled. Protein concentration was estimated by comparison with known amounts of BSA. The purity of the results was consistently higher than 95%.

Laminins 1, 2, 4, and 5 and fibronectin were radiolabeled by the iodogen method and separated from free iodine by Sephadex G25 (Pharmacia) gel filtration. Protein peak fractions were analyzed by SDS-PAGE. The specific activity of each preparartion was determined by counting in a gamma counter a TCA-precipitated aliquot of the peak fractions. Specific activities were 1.3 × 106 CPMs/μg for laminin 1, 1.9 × 106 CPMs/μg for laminin 2, 3.3 × 106 CPMs/μg for laminin 4, 0.5 × 106 CPMs/μg for laminin 5, and 9.9 × 106 CPMs/μg for fibronectin.

Purified integrins were diluted to 0.25 μg/ml with PBS containing 1 mM MnCl2. Membrane microtiter wells (Microtest III, Falcon) were coated with 100 μl of receptor solution (25 ng) overnight at 4°C. After blocking with 2% BSA, the wells were incubated with the indicated amounts of radiolabeled matrix molecules diluted in PBS containing 1 mM MnCl2 for 4 h at room temperature. When indicated, synthetic peptides or EDTA were included. At the end of incubation, the wells were washed five times with PBS, 1 M Na2SCN and counted in a gamma counter. Non-specific binding was defined as the amount of radioligand which bound to wells coated with BSA only and was subtracted from each dose point.

To determine the dissociation constant between laminin 4 and the wild-type or truncated tail-less integrin, displacement experiments were carried out. A single concentration of radiolabeled laminin 4 (100 ng/ml) was added to each well in presence of increasing concentrations of cold ligand (0-2.5 μg/ml). The results were subjected to Scatchard analysis.

**Immunofluorescence**

The 804G transfecants were cultured for ~48 h on glass coverslips, and then either fixed directly with cold methanol for 2 min or treated with PBS containing 0.2% Triton X-100 for 5 min on ice before fixation with methanol. Cells were stained for 45 min with the various antibodies. The purified anti-human β4 3E1 and anti-c-myc Ab-1 monoclonal antibodies were used at 5 μg/ml. The anti-BPAG1 human serum and the α6 cytoplasmic domain rabbit serum were diluted 1:200. The anti-BPAG2 fusion protein IgGs were used at 25 μg/ml. The affinity-purified β4 cytoplasmic peptide antibodies were used at 5 μg/ml. After extensive washing, the cells were incubated for 45 min with 0.3-1 μg/ml of affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse, anti-rabbit, or anti-human IgGs (Molecular Probes Inc., Eugene, OR). The coverslips were mounted in Citifluor (Chemical Laboratory of the University of Kent, Canterbury, UK).

For double immunostaining with the 3E1 or Ab-1 monoclonal antibody and the anti-BPAG2 rabbit IgGs, the coverslips were incubated first with the monoclonal antibody followed by Texas red (TR)-conjugated goat anti-mouse IgGs, and then with the anti-BPAG2 IgGs followed by affinity-purified FITC-labeled goat anti-rabbit IgGs (Molecular Probes). For double immunostaining with the 3E1 or Ab-1 monoclonal antibody and the anti-BPAG1 human serum, the coverslips were incubated first with the monoclonal antibody followed by affinity-purified TR-conjugated goat anti-mouse IgGs (Molecular Probes), and then with the anti-BPAG1 serum followed by FITC-labeled goat anti-human IgGs. All secondary antibodies used were species-specific. Samples were examined with a Zeiss Axioskop Fluorescence Microscope.

**Electron Microscopy**

Cells grown on laminin 4-coated Aclar plastic coverslips were rinsed in PBS and fixed overnight at 4°C in 0.05 M Sodium Phosphate buffer, pH 7.0, containing 2% Glutaraldehyde and 0.1 M Sucrose. After washing in 0.1 M Phosphate buffer, the coverslips were incubated in 2% Osmium Tetroxide in 0.1 M Phosphate buffer for 1 h and incubated in Epon (Miller and Bacon, 1989). To obtain cross-sections of the cells, pieces of the embedded coverslips were reembedded in Epon in the appropriate orientation for sectioning. Ultrathin sections (50-65 nm) were collected on copper grids and counterstained with Uranyl Acetate and Reynold’s Lead Citrate. Sections were analyzed on a Philips 201 electron microscope.

**Results**

Overexpression of Truncated β4 Integrin Subunits

Previous results indicated that a truncated tail-less human β4 subunit (Δ cyto 874-1752) combines with endogenous α6 and reaches the cell surface, but is not incorporated in hemidesmosomes. In contrast, a truncated head-less β4 subunit (Δ exo 70-660) does not associate with endogenous α6, but is transported to the cell surface and recruited in hemidesmosomes (Spinardi et al., 1993). We reasoned that the integrin containing the tail-less recombinant β4 subunit could exert a dominant negative effect by competing with endogenous wild-type α6β4 for adhesive ligands. Conversely, the head-less recombinant β4 subunit could compete with the endogenous α6β4 receptor for binding to cytoskeletal elements or regulatory factors (Fig. 1).

To test the potential dominant negative effect of truncated β4 subunits in hemidesmosome-forming cells, we selected 804G clones with potential for high level expression of either the tail-less (Δ cyto 874-1752) or the head-less (Δ exo 70-660) human β4 subunit, as described in Materials and
Methods. The transfectants selected for study included: clones B13, B23, and B29, expressing the tail-less $\beta_4$ subunit; clone F28, expressing the head-less $\beta_4$ molecule; and clones Z10 and Z32, transfected with the selection marker alone.

The level of expression of recombinant tail-less $\beta_4$ and endogenous wild-type $\beta_4$ in the various B clones was examined by immunoprecipitation (Fig. 2 A). Control clones Z10 and Z32, clone A12 which expresses a full-length human $\beta_4$ subunit and clones B13, B23, and B29 carrying the tail-less human $\beta_4$ subunit were labeled at the surface with $^{125}$I and extracted. Samples containing equal amounts of TCA-precipitable counts were immunoprecipitated with saturating amounts of the 3E1 monoclonal antibody, reacting selectively with the extracellular domain of human $\beta_4$ (Fig. 2 A, left). The 3E1 monoclonal antibody did not bind to any membrane protein in control cells, but immunoprecipitated high levels of recombinant full-length $\beta_4$ from clone A12, similarly high levels of tail-less $\beta_4$ from clone B13, and lower levels from clones B23 and B29. In accordance with previous results indicating that the $\alpha_6$ subunit is poorly labeled by cell surface iodination, the 110-kD $\alpha_6$ subunit associated with full-length and tail-less recombinant $\beta_4$ could be detected only upon prolonged exposure of the gel.

To compare the expression of recombinant tail-less $\beta_4$ with that of endogenous wild-type $\beta_4$, the various clones were also immunoprecipitated with excess amount of a polyclonal antiserum raised against a synthetic peptide reproduc-
ing the cytoplasmic domain of α6a. The anti-α6a antiserum was selected because α6a is the only α6 subunit isoform expressed in 804G cells (unpublished results). As shown in Fig. 2 A (right), the antiserum immunoprecipitated two types of α6 heterodimers from the B clones: those containing the 100-kD recombinant tail-less β6 molecule and those containing the 200-kD wild-type endogenous β6 subunit. Phosphorimager analysis indicated that the recombinant tail-less β6 subunit was 1.6 times more abundant than endogenous β6 at the surface of clone B13, but endogenous β6 was 3.2 and 3.4 times more abundant than the recombinant molecule in clones B23 and B29, respectively. It was concluded that tail-less β6 is overexpressed in clone B13, but not in clones B23 and B29.

The level of recombinant β6 lacking the extracellular domain expressed in clone F28 was examined by performing immunoblotting experiments with an antibody raised against a synthetic peptide designed after the COOH terminus of human β6. This β6 cytoplasmic domain antibody was expected to react well with both the human head-less β6 molecule and the endogenous rat wild-type β6 subunit, because its target sequence is conserved in rodents and humans (Kennel et al., 1993). To obtain two distinct measurements of different sensitivities, cells of the control clone Z10 and the head-less β6 expressing clone F28 were either directly extracted in sample buffer or immunoprecipitated with excess amount of the β6 cytoplasmic domain antibody before immunoblotting. The β6 cytoplasmic domain antibodies bound to the recombinant head-less β6 subunit in clone F28 and reacted with endogenous wild-type β6 in total extracts and immunoprecipitates from both control clone Z10 and clone F28. Fainter bands at ~70 kD, possibly representing proteolytic fragments of β6, were also detected (Fig. 2 B). Phosphorimager analysis indicated that the head-less recombinant molecule is expressed at levels 5.3 times higher than endogenous β6 in clone F28. From this experiment, we concluded that the recombinant head-less subunit is overexpressed in clone F28. Although the ratios of recombinant to endogenous β6 subunits in clones F28 and B13 were determined by different methods, the results suggest that the extent of overexpression of head-less β6 in clone F28 is greater than that of tail-less β6 in clone B13.

The αβ6 Integrin Is a Receptor for Laminins 1, 2, 4, and 5

To examine the effects of the truncated β6 subunits on αβ6-mediated cell adhesion, we sought to define the ligands of αβ6, and determine which integrins with an overlapping ligand-binding specificity were expressed by 804G cells. The repertoire of integrins expressed by 804G cells was examined by immunoprecipitation. After labeling of the cell surface with 125I, the 804G cells were extracted and immunoprecipitated with antibodies to synthetic peptides modeled after various integrin cytoplasmic domains. As shown in Fig. 3, the results indicated that 804G cells express high levels of the α6β6, and α6β6 integrins and lower levels of α6β1. A prolonged exposure of the gel (lane to the far right in Fig. 3) revealed that they also express minor levels of α6β1. No β6 subunit could be detected in association with α6, even after prolonged exposure of the gel, indicating that 804G cells do not express the α6β6 integrin.

Cell adhesion assays showed that the 804G cells adhere well to fibronectin, type IV collagen, and laminin 4, but interact more weakly with laminin 1 and 2 (Fig. 4 A). In addition, time course experiments indicated that 804G cells adhere well to the laminin 5 matrix deposited by RAC-11/PD cells (Fig. 4 B). Although the matrix form of laminin 5 cannot be directly compared to the other purified soluble monomeric ligands tested, these results clearly show that the 804G cells can interact well with laminin 4 and 5, and less well with laminin 1 and 2. Since the adhesion of 804G cells to the laminin 5 matrix and to laminin 4 was not affected by antibodies reacting with rat β1 (Fig. 4 C), we concluded that adhesion to laminin 4 and 5 could involve α6β6.

To directly test the ligand-binding ability of the α6β6 integrin in the absence of potentially confounding influences of other integrins or cellular regulatory factors, radioligand-binding assays were performed. A partially recombinant form of the α6β6 integrin was purified from clone A12 cells by immunoaffinity chromatography on the 3E1 monoclonal antibody, as described in Materials and Methods. Microradiographs were coated with 25 ng of purified integrin, and then incubated with various concentrations of iodinated laminin 1, 2, 4, and 5 or fibronectin. The laminin 5 used in this experiment was immunopurified to homogeneity and did not contain detectable amounts of laminin 6, the laminin isoform to which laminin 5 is covalently associated in tissues. The
The hypothesis that a recombinant tail-less form of αβ₄ can suppress the function of endogenous αβ₄ was based on the assumption that the tail-less integrin would be able to bind effectively to extracellular ligand. To test this assumption, we purified the receptor containing a tail-less human β₄ subunit from clone B13 and the receptor containing a full-length human β₄ subunit from clone A12, and compared their binding properties by using a radioligand-binding assay. Microtiter wells were coated with 25 ng of the two receptors and incubated with 100 ng of radiiodinated laminin 4 in the presence of various concentrations of cold ligand. As shown in Fig. 5, A and B, the binding of radioactive laminin 4 to both receptors was effectively competed by excess cold ligand. In both cases complete inhibition of binding was observed with ~3.5 pmol, corresponding to a 250-fold excess of cold over radioactive laminin 4. The displacement curves generated by the tail-less and wild-type receptor were very similar, suggesting that the two receptors bind to ligand with similar kinetics. Scatchard analysis of the results indicated that the two receptors display a very similar affinity for laminin 4 (Fig. 5, C and D). Indeed, the estimated Kₛ of wild-type receptor was 8.45 × 10⁻⁸ mol/liter and that of tail-less receptor was 7.04 × 10⁻⁸ mol/liter. These results demonstrate that the truncated tail-less αβ₄ integrin retains an intact ligand-binding ability in vitro and indicate that deletion

### Figure 4. Ligand-binding specificity of the αβ₄ integrin.

(A) Parental 804G cells were plated for 30 min at 37°C on microtiter wells coated with increasing concentrations of the indicated purified matrix proteins. The extent of cellular adhesion was determined by measuring the absorbance generated by the attached cells, as described in Materials and Methods. (B) The 804G cells were plated on wells coated with the laminin 5 matrix deposited by the RAC-11/PD cells, with 20 μg/ml of fibronectin, or 20 μg/ml of laminin 4 and incubated at 37°C for the indicated varying times. (C) The 804G cells were plated for 1 h at 37°C on wells coated with the laminin 5 matrix, 2 μg/ml of fibronectin or 6 μg/ml of laminin 4 in presence of the indicated dilutions of anti-beta 1 antiserum. These concentrations of fibronectin and laminin 4 were chosen for coating because preliminary experiments had indicated that they were able to promote, in a 1-h assay conducted in the absence of anti-beta 1 antibodies, an extent of cell adhesion comparable to that observed on the laminin 5 matrix. The 0 point on the abscissa corresponds to the values measured in the absence of blocking antibodies. All the adhesion assays were conducted in triplicate and standard deviations, and did not exceed 22% of each mean value. (D) Microtiter wells were coated with purified, partially recombinant αβ₄ integrin (25 ng/well) and incubated with the indicated concentrations of radiolabeled ligands in presence of 1 mM MnCl₂ for 4 h at room temperature. Bound ligand was measured in a gamma counter. Each point represents the mean of duplicates from a representative experiment. Nonspecific binding did not exceed 25% of total binding and was subtracted for each dose.

The Tail-Less αβ₄ Integrin Displays an Intact Affinity for Extracellular Ligand In Vitro

The hypothesis that a recombinant tail-less form of β₄ can
of the $\beta_4$, cytoplasmic domain does not result in a gross conformational change in the extracellular domain of the integrin.

**High-Level Expression of Head-Less or Tail-Less Recombinant $\beta_4$ Does Not Suppress $\alpha_\beta_4$-dependent Adhesion and Spreading**

To test the effects of the two recombinant truncated $\beta_4$ subunits on cell adhesion, adhesion assays were performed with the control clone Z10, the head-less $\beta_4$ expressing clone F28, and the tail-less $\beta_4$ expressing clones B13, B23, and B29. To selectively analyze the function of $\alpha_\beta_4$, the cells were plated on laminin 4 and 5 in the presence of antibodies capable of blocking endogenous $\beta_4$ integrins. Fig. 6 A shows that the clones B13, F28, and B29 adhered to the laminin 5 matrix deposited by the RAC-11/PD cells with kinetics and to an extent similar to that of control clone Z10. In addition, the clones B13, F28, and B29 adhered to wells

![Figure 5](https://example.com/image) Scatchard analysis of laminin 4 binding to partially recombinant, wild-type and tail-less $\alpha_\beta_4$ integrin. A constant concentration of radiolabelled laminin 4 was incubated in wells coated with 25 ng/0.1 ml of the partially recombinant, purified wild-type or tail-less integrin. Displacement of radiolabeled laminin 4 was measured as a function of increasing concentrations of cold ligand added (0–2.5 $\mu$g/0.1 ml). Nonspecific binding was calculated by measuring binding to wells coated with BSA alone and was subtracted from each point. Results shown represent the mean of duplicates. (A) Displacement curve of wild-type integrin; (B) displacement curve of tail-less integrin; (C) Scatchard plot of wild-type integrin; (D) Scatchard plot of tail-less integrin.

Figure 6. Measurement of $\alpha_\beta_4$-dependent adhesion in cells expressing the head-less or tail-less recombinant $\beta_4$ subunit. The indicated clones were incubated in presence of a 1:50 dilution of the anti-beta 1 antiserum for varying times on wells coated with the laminin 5 matrix (A), in presence of a 1:50 dilution of the anti-beta 1 antiserum for 30 min on wells coated with varying amounts of laminin 4 (B), or in the absence of anti-beta 1 antiserum for 30 min on wells coated with varying amounts of fibronectin (C). The assays were conducted in triplicate and standard deviations did not exceed 18% of each mean value. The lower maximal adhesion observed in C reflects a lower input of cells in this particular experiment.

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coated with increasing amounts of laminin 4 (Fig. 6 B) or fibronectin (Fig. 6 C) to an extent similar to that of control clone Z10. Thus, neither the head-less nor the tail-less βa subunit can suppress α6β4-dependent adhesion to laminin 4 or 5.

**Overexpression of a Mutant βa Subunit Lacking Extracellular Sequences Does Not Affect Hemidesmosome Assembly**

To determine if high level expression of the head-less βa integrin subunit affects the assembly and/or stability of

![Figure 7. Immunofluorescent detection of recombinant full-length and head-less βa subunits in hemidesmosomes.](image-url)

Cells of the control clone Z10, the full-length βa expressing clone A12, and the head-less βa expressing clone F28 were cultured on glass coverslips for 48 h, and then were either fixed directly with cold methanol (A and B) or extracted with 0.2% Triton X-100 before fixation in methanol (C-H). Clone A12 cells were stained with the anti-human βa monoclonal antibody 3E1 (A) and clone Z10 with the anti-c-myc monoclonal antibody Ab-1 (B) followed by FITC-conjugated goat anti-mouse IgGs. Clone A12 cells were doubly stained with the 3E1 monoclonal antibody and the rabbit anti-BPAG2 antibody followed by Texas red-conjugated goat anti-mouse IgGs and FITC-labeled goat anti-rabbit IgGs (3E1 staining in C, and BPAG2 staining in E). Clone F28 cells were doubly stained with the monoclonal antibody Ab-1 and with the rabbit anti-BPAG2 antibody followed by Texas red-conjugated goat anti-mouse IgGs and FITC-labeled goat anti-rabbit IgGs (Ab-1 staining in D, and BPAG2 staining in F). Clone A12 and F28 cells were also stained with anti-BPAG1 human antibodies followed by FITC-labeled goat anti-human IgGs (G and H, respectively).
hemidesmosomes, we compared the subcellular localization of the head-less $\beta_4$ and that of two cytoskeletal markers of hemidesmosomes, BPAG1 and 2, in clone F28 cells with the distribution of recombinant full-length $\beta_4$ and BPAG1 and 2 in clone A12 cells. As shown in Fig. 7 A, immunofluorescent staining with the 3El monoclonal antibody indicated that the recombinant full-length $\beta_4$ subunit is concentrated at the basal surface of clone A12 cells within granular structures, possibly representing individual hemidesmosomes. These structures often merged into patches, but were excluded from circular areas thus generating a distinctive "Swiss-cheese"-like pattern. Extraction of clone A12 cells with a buffer containing 0.2% Triton X-100, before fixation and incubation with the 3El monoclonal antibody, did not affect the intensity of the staining associated with hemidesmosomes, but eliminated the immunofluorescence originating from the cytoplasm or the plasma membrane outside hemidesmosomes (Fig. 7 C), indicating that the recombinant full-length $\beta_4$ subunit within hemidesmosomes is largely resistant to extraction with nonionic detergents. Double immunofluorescent staining with the 3El monoclonal antibody and with rabbit polyclonal antibodies to BPAG2 revealed a precise colocalization of the recombinant $\beta_4$ molecule and the hemidesmosomal marker in the Triton X-100 resistant structures (Fig. 7, C and E). In addition, immunofluorescence with antibodies to BPAG1 resulted in a staining pattern similar to that generated by the 3El and BPAG2 antibodies (Fig. 7 G). These results indicate that the recombinant full-length $\beta_4$ subunit and BPAG1 and 2 colocalize in hemidesmosomes in clone A12 cells and that within these structures these mol-

Figure 8. Lack of detection of recombinant tail-less $\beta_4$ subunit and endogenous $\alpha_6$ and $\beta_4$ subunits in hemidesmosomes in clone B13. Cells of the tail-less $\beta_4$ expressing clone B13 were cultured on glass coverslips for 48 h, and then were either fixed with cold methanol (A) or extracted with 0.2% Triton X-100 before fixation in methanol (B, D, and E). Cells of the control clone Z10 were extracted with 0.2% Triton X-100 before fixation in methanol (C and F). Clone B13 cells were stained with the anti-human $\beta_4$ 3El monoclonal antibody followed by FITC-conjugated goat anti-mouse IgGs (A and D). Cells of the B13 and Z10 clones were stained with the $\alpha_6$ (B and C) or the $\beta_4$ cytoplasmic domain antibody (E and F) followed by FITC-labeled goat anti-rabbit IgGs.
ecules are largely resistant to extraction with 0.2% Triton X-100.

We next analyzed the subcellular distribution of the over-expressed recombinant head-less β subunit and BPAG1 and 2 in clone F28 cells. The transfectants were extracted with Triton X-100 and stained with the monoclonal antibody Ab-1 reacting with the c-myc epitope tag included in this recombinant truncated β molecule. The antibody generated negligible staining in control cells of the Z10 clone (Fig. 7 B), but reacted prominently with hemidesmosomal structures in clone F28 cells (Fig. 7 D). Double immunostaining experiments demonstrated a precise colocalization of the recombinant head-less β, with BPAG2 (Fig. 7, D and F), BPAG1 (Fig. 7 H, BPAG1 staining only is shown), and the endogenous α subunit (not shown). These results suggest that the overexpressed recombinant head-less β subunit accumulates in hemidesmosomes without causing any apparent redistribution of the endogenous αβ integrin and the BPAG1 and 2 antigens normally associated with hemidesmosomes.

Electron microscopic analyses were conducted to examine the structural integrity of hemidesmosomes in clone F28 cells. Cells of the control clone Z10 and the head-less β expressing clone F28 were cultured for 48 h on laminin 4-coated Aclar coverslips, and then fixed. Vertical sections, cut perpendicularly to the substratum, were examined with the electron microscope. The results showed that control cells had a number of submembranous densities associated with the basal cell surface in correspondence of substratum attachment sites (see Fig. 11 A). The appearance of these structures was similar to that of the previously described hemidesmosomes in 804G cells in that they contained a relatively well defined inner plaque (Ridelle et al., 1991). A double-blind analysis indicated that the clone Z10 had an average of 66 hemidesmosomes per cell section. Analysis of clone F28 cells indicated that these structures were neither significantly diminished (6.2 per cell per vertical section) nor altered (Fig. 11 B). Thus, Z10 and F28 cells appear to have similar numbers of normally appearing hemidesmosomes. From the immunofluorescence and electron microscopy observations, we concluded that expression of a head-less β subunit, at levels 5.3-fold higher than those of endogenous wild-type β, does not affect hemidesmosome assembly and/or stability.

Overexpression of a Mutant β Subunit Lacking the Cytoplasmic Domain Disrupts Hemidesmosomes

Immunofluorescence experiments were performed to determine if the tail-less recombinant β subunit has a dominant
negative effect on hemidesmosome assembly and/or stability. Staining of clone B13 cells with the 3El monoclonal antibody indicated that the tail-less recombinant β₄ subunit was diffusely distributed at the surface of these cells (Fig. 8 A). Extraction with 0.2% Triton X-100 before fixing and antibody incubation resulted in an almost-complete loss of staining (Fig. 8 D). Thus, in contrast to the recombinant wild-type β₄, which is largely insoluble in Triton X-100, the tail-less β₄ subunit is soluble in nonionic detergent, presumably because it cannot establish proper cytoskeletal connections.

We next wondered if expression of the tail-less mutant subunit could affect the incorporation in hemidesmosomes of wild-type endogenous α₅β₅. Cells of the control clone Z10 and the tail-less β₄ expressing clone B13 were treated with Triton X-100 and stained with affinity-purified antibodies to synthetic peptides reproducing the cytoplasmic domain of either α₅ or β₅. As shown in Fig. 8, B and E, although some punctuate staining was occasionally observed (arrows), neither antibody detected significant amounts of α₅β₅ at the basal surface of clone B13 cells. In contrast, both antibodies generated a Swiss-cheese-like staining in clone Z10 cells (Fig. 8, C and F). This finding suggests that expression of tail-less β₄ prevents the incorporation of endogenous α₅β₅ integrin in hemidesmosomes.

To examine the integrity of hemidesmosomes in cells over-expressing the tail-less β₄ subunit, cells of the control clone Z10 and of the tail-less β₄ expressing clone B13 were extracted with Triton X-100 and stained with antibodies to BPAG1 and 2. As shown in Fig. 9, A and B, the two hemidesmosomal proteins were largely absent from the basal surface of Triton X-100–treated cells of the B13 clone. Although some residual granular staining could be detected in a minor percentage of cells, the BPAG1 and 2 positive granules detected in clone B13 were limited to restricted areas of the basal surface (arrows in panels A and B) and rarely generated a Swiss-cheese-like pattern (open arrow in panel A). Moreover, the general distribution of BPAG2 was less altered than that of BPAG1. In contrast with the results obtained with clone B13, both the BPAG1 and the BPAG2 antibody generated a Swiss-cheese-like staining at the basal surface of control clone Z10 (Fig. 9, C and D). Control immunoprecipitation experiments from metabolically labeled cells indicated that the biosynthesis of BPAG1 and BPAG2 was not decreased in clone B13 as compared to control clones Z10 and Z32 (data not shown). These results suggest that expression of the tail-less mutant integrin subunit interferes with the assembly or stability of hemidesmosomes.

To determine if the extent of disruption of hemidesmosomal markers in cells expressing the tail-less β₄ subunit was proportional to the level of expression, we analyzed clones B29 and B23, in which the ratio of recombinant tail-less β₄ to endogenous β₄ is 5 times lower than in clone B13. Cells of the control clone Z10 and tail-less β₄ expressing clones B13, B29, and B23 were treated with Triton X-100 and stained with antibodies to BPAG2. As shown in Fig. 10, the altered distribution of BPAG2 was much less pronounced in clones B29 (panel B) and B23 (panel C) than in clone B13 (panel D), and in many instances the granular basal staining generated by the two antibodies merged at least partially into patches and occasionally into a Swiss-cheese-like pattern. Thus, the effect of tail-less β₄ on hemidesmosomes is proportional to its level of expression.
To obtain direct evidence of the effect of tail-less $\beta_4$ on hemidesmosomes, cells of the control clone Z10 and the tail-less $\beta_4$ expressing clone B13 were cultured for 48 h on laminin 4-coated Aclar coverslips and analyzed by electron microscopy. The result of these experiments indicated that clone B13 cells had a greatly diminished number of submembranous densities associated with the basal cell surface as compared with cells of the control clone Z10 (Fig. 11, C and D). A double-blind evaluation of the results indicated that clone B13 cells contain an average of 0.12 submembranous densities.

Figure 11. Electron microscopic analysis of cells expressing the recombinant head-less or tail-less $\beta_4$ subunit. The control clone Z10 (A), the head-less $\beta_4$ expressing clone F28 (B), and the tail-less $\beta_4$ expressing clone B13 (C and D) were grown for 48 h on laminin 4-coated Aclar plastic coverslips. Cross-sections of the cells were obtained and processed for electron microscopy as described in Materials and Methods. Large arrowheads in A point to hemidesmosomal structures at the basal surface of the control clone Z10. Small arrowheads in B point to similar structures at the basal surface of clone F28 cells. Bars: (A and B) 0.25 $\mu$m; (C) 1 $\mu$m; (D) 0.5 $\mu$m.
that the distribution of BPAG2 is less disrupted than that of densities per vertical section. These densities were not as well organized as the hemidesmosomes of clone Z10 an F28 and rarely contacted the substratum (see Fig. 11 D for one example). Since the immunofluorescence studies indicated that the distribution of BPAG2 is less disrupted than that of other hemidesmosomal components in clone B13, it is possible that the residual submembranous densities detected in this clone during the electron microscopic analysis represent small aggregates of BPAG2. Finally, it was evident from the electron microscopic analysis that clone B13 cells did not form an extended contact with the substratum and were rounder than control cells (Fig. 11 C). Thus, although clone B13 cells can adhere well after being plated on laminin 4 and 5 for the short incubation times of the adhesion assay (see for example Fig. 6 A and B), they acquire a less adhesive morphology after a more prolonged period of culture. Taken together, the results of the immunofluorescence and electron microscopic analyses indicate that expression of a mutant tail-less $\beta_4$ subunit disrupts the hemidesmosomes of 804G cells, and that this disruption is accompanied by the acquisition of a less adhesive morphology as compared to that of control cells.

Discussion

In this study we report that high-level expression of a recombinant tail-less integrin $\beta_4$ subunit in 804G bladder epithelial cells disrupts hemidesmosomes without affecting $\alpha_6\beta_4$-mediated adhesion. Therefore, the tail-less $\beta_4$ subunit is a dominant negative mutant which selectively interferes with the association of the $\alpha_6\beta_4$ integrin with the hemidesmosomal cytoskeleton without perturbing its adhesive function. Two major conclusions can be drawn from this result. The first is that the $\alpha_6\beta_4$ integrin plays a crucial role in promoting the assembly or maintaining the stability of hemidesmosomes: indeed $\alpha_6\beta_4$ appears to be a necessary component of these structures, as its function cannot be replaced by the other transmembrane element of hemidesmosomes, BPAG2. The second major conclusion is that the ligand-binding function of the $\alpha_6\beta_4$ integrin, as measured by adhesion assay, does not require stable association with the hemidesmosomal cytoskeleton. Thus, the $\alpha_6\beta_4$ integrin appears to be regulated differently from $\beta_4$ and $\beta_1$ integrins which need to associate with the cytoskeleton to mediate efficient cell adhesion in vivo (Hayashi et al., 1990; Hibbs et al., 1991).

To analyze the consequences of dominant negative inhibition of $\alpha_6\beta_4$, we have conducted adhesion assays with the parental 804G cells and radioligand-binding studies with the purified partially recombinant $\alpha_6\beta_4$ integrin. A major conclusion resulting from these experiments is that, in addition to laminin 5 and 1, $\alpha_6\beta_4$ binds to laminin 2 and 4. Indeed, the affinity constant for binding to laminin 4 that we measured, $8.45 \times 10^{-4}$ mol/liter, is higher than that reported for the binding of the $\alpha_6\beta_1$ integrin to fibronectin (Hautanen et al., 1989). The observation that $\alpha_6\beta_4$ is a receptor for laminin 2 and laminin 4 may help to understand the function of this integrin in Schwann cells. It has been proposed that $\alpha_6\beta_4$-mediated adhesion to the basement membrane plays a crucial role during myelination, because the expression of $\alpha_6\beta_4$ is rapidly induced in Schwann cells at the onset of this process (Einheber et al., 1993). Since the Schwann cell basal membrane does not contain laminin 1 or 5, but laminin 2 and in a lesser amount laminin 4 (Sanes et al., 1990; Marinkovich et al., 1992b), our current results suggest that $\alpha_6\beta_4$ interacts with these latter ligands during myelination. In fact, it is possible that $\alpha_6\beta_4$ mediated recognition of laminin 2 and 4 provides the Schwann cells with a signal required for myelination since the $dy/dy$ mice, which lack the laminin $\alpha_1$ chain in both the muscle and the Schwann cell basement membranes (Sunada et al., 1994; Xu et al., 1994), develop a form of muscular dystrophy accompanied by peripheral nerve degeneration.

Our binding studies indicate also that the $\alpha_6\beta_4$ integrin binds to laminin 1 and laminin 5. The presence of laminin 5 in the anchoring filaments of hemidesmosomes (Roussel et al., 1991) and the ability of cell lines, which form hemidesmosomes in vitro, to deposit high amounts of this matrix molecule on the culture substratum (Langhofer et al., 1993; Sonnenberg et al., 1993) suggest that binding of $\alpha_6\beta_4$ to laminin 5 may be crucial for hemidesmosome assembly. In accordance with this hypothesis, it has been shown that antibodies which interfere with the adhesive function of $\alpha_6\beta_4$ can reduce hemidesmosomes in cultured cells and in vivo (Jones et al., 1991; Kurpakus et al., 1991). The selective inhibition of hemidesmosomes produced by the tail-less $\beta_4$ mutant described here demonstrates that $\alpha_6\beta_4$-mediated adhesion and hemidesmosome assembly can be separated experimentally, suggesting that the two phenomena have distinct requirements. Thus, although engagement of $\alpha_6\beta_4$ by extracellular ligand may be a prerequisite for hemidesmosome assembly, this process is likely to require a number of additional steps and the dominant negative tail-less $\beta_4$ subunit may interfere with one or more of these additional steps.

We predict that, upon binding to laminin 5, the $\alpha_6\beta_4$ integrin forms an orderly aggregate within the plane of the plasma membrane. Formation of the inner hemidesmosomal plaque would then be triggered either by a signal transmitted across the plasma membrane by the $\alpha_6\beta_4$ integrin or by a conformational change in the $\beta_4$ cytoplasmic domain. This model offers potential mechanisms by which the tail-less $\beta_4$ subunit can interfere with the assembly of hemidesmosomes. Since the radioligand-binding results indicate that the integrin containing the tail-less $\beta_4$ binds in vitro to extracellular ligand with an intact affinity and the adhesion assay results suggest that it contributes efficiently to cell adhesion, it is likely that the mutant integrin and the wild-type endogenous molecule bind simultaneously to extracellular ligand so as to come in close proximity or even cocluster within the plasma membrane. The tail-less $\beta_4$ may then interfere with hemidesmosome assembly by blocking the propagation of a conformational change across the membrane or the transmission of an intracellular signal by the wild-type integrin. In this model, tail-less $\beta_4$ behaves similarly to growth factor receptors deleted in their tyrosine kinase domain, which form signal transduction incompetent dimers with wild-type molecules (Schlessinger and Ullrich, 1992). These observations support the notion that integrins interact within the plasma membrane during outside-in signal transduction.

In this study we have also attempted to obtain a dominant negative effect by expressing a head-less $\beta_4$ subunit. However, this mutant molecule continued to accumulate in hemidesmosomes without causing any apparent disruption, even
when expressed at levels ~5 times higher than the endogenous αβ4 integrin and did not interfere with αβ1-mediated adhesion. The lack of effect of this mutant integrin subunit is, at first glance, surprising. Mutant cadherin molecules of similar design have been shown to disrupt cell--cell adhesion in developing Xenopus embryos (Kintner, 1992). In addition, it has been recently shown that single-subunit chimeric molecules containing the β1, β3, or β5 cytoplasmic domain interfere with the ability of endogenous integrins to localize to focal adhesions, to mediate cell adhesion, migration and matrix assembly (LaFlamme et al., 1994; Lukashev et al., 1994), and to respond to intracellular regulatory signals (Chen et al., 1994). Since these chimeras localize to focal adhesions when expressed at low levels (Geiger et al., 1992; LaFlamme et al., 1992) and are capable of stimulating the activation of the focal adhesion kinase pp125 FAK (Akiyama et al., 1994; Lukashev et al., 1994), the dominant negative effect consequent to their high level expression has been attributed to the titration of intracellular factors essential for integrin activity. At least some of these factors may be common to several integrins sharing the same β subunit, as the effects observed with single-subunit chimeras is trans-dominant (LaFlamme et al., 1994; Lukashev et al., 1994). Our negative results with the headless βs subunit suggest that the function of the αβ3 integrin may be regulated differently than that of other integrins. If intracellular regulators of αβ1 exist, they may be different from those regulating other integrins. Alternatively, they may not be present in 804G cells in quantities low enough to allow titration.

The ability of tailless β4 subunit to selectively interfere with hemidesmosome assembly in cultured cells suggests that it may be possible to examine the in vivo function of hemidesmosomes in transgenic mice, without disrupting initial cell adhesion to the basement membrane mediated by αβ4. It is likely that these junctions, which are formed in culture with relatively slow kinetics (our unpublished results), reinforce adhesion to the basement membrane and, indeed, our electron microscopy observations indicate that cells expressing the dominant negative β3 subunit are significantly rounder and more detached from the culture substrate than control cells. Finally, since a group of blistering skin diseases are caused by genetic or epigenetic factors acting on hemidesmosomal components (Uitto and Christiano, 1992), the introduction in transgenic mice of the dominant negative mutation described here may also provide information relevant to understanding the pathophysiology of this class of diseases.

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