

# A Recombinant Tail-Less Integrin $\beta_4$ Subunit Disrupts Hemidesmosomes, but Does Not Suppress $\alpha_6\beta_4$ -mediated Cell Adhesion to Laminins

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**Abstract.** To examine the function of the  $\alpha_6\beta_4$  integrin we have determined its ligand-binding ability and overexpressed two potentially dominant negative mutant  $\beta_4$  subunits, lacking either the cytoplasmic or extracellular domain, in bladder epithelial 804G cells. The results of cell adhesion and radioligand-binding assays showed that  $\alpha_6\beta_4$  is a receptor for several laminin isoforms, including laminin 1, 2, 4, and 5. Overexpression of the tail-less or head-less mutant  $\beta_4$  subunit did not suppress  $\alpha_6\beta_4$ -mediated adhesion to laminins, as both types of transfectants adhered to these ligands in the presence of blocking anti- $\beta_1$  antibodies as well as the controls. However, immunofluorescence experiments indicated that the endogenous  $\alpha_6\beta_4$  integrin and other hemidesmosomal markers were not concentrated in hemidesmosomes in cells overexpressing tail-less  $\beta_4$ , 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  $\beta_4$  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  $\beta_4$  subunit leads to a dominant negative effect on hemidesmosome assembly without suppressing initial adhesion to laminins. We conclude that the  $\alpha_6\beta_4$  integrin binds to several laminins and plays an essential role in the assembly and/or stability of hemidesmosomes, that  $\alpha_6\beta_4$ -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.

THE study of laminin binding integrins is of particular relevance because many of the effects of basement membranes on the proliferation and differentiation of cells can be recapitulated in vitro by laminins (Manthorpe et al., 1983; Grant et al., 1989; Panayotou et al., 1989; von der Mark and Ocalan, 1989; Caron, 1990) or blocked by anti-laminin antibodies in cell and organ culture systems (Grover and Adamson, 1985; Klein et al., 1988; Streuli et al., 1991). Recent results indicate the existence of several laminin isoforms, expressed in a tissue-specific fashion (Engvall et al., 1990; Sanes et al., 1990; Rousselle et al., 1991; Marinkovich et al., 1992b). In addition, at least six integrins have been implicated in binding to laminins and, in several cases, their binding specificity appears to be overlapping (for review see Mercurio, 1990; Hynes, 1992). The hypothesis that individual laminin-binding integrins have distinct cytoskeletal and signaling functions may help to explain the apparent redundancy of the laminin recognition system.

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There are several reasons for believing that the  $\alpha_6\beta_4$  integrin, which has been implicated in binding to laminin 1 (subunit composition:  $\alpha_1\text{-}\beta_1\text{-}\gamma_1$ ) and laminin 5 (kalinin, epiligrin, nicein;  $\alpha_3\text{-}\beta_3\text{-}\gamma_2$ ) (Lee et al., 1992; Niessen et al., 1994), is characterized by unique intracellular interactions. The cytoplasmic domain of the  $\beta_4$  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  $\beta_1$  and  $\beta_3$  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  $\alpha_6\beta_4$  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  $\beta_4$

subunit in hemidesmosome forming cells, we have shown 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)<sup>1</sup> (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 (Sonnenberg 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  $\alpha_6$  and  $\beta_4$  (Giancotti et al., 1992),  $\alpha_1$  (Tarone et al., 1993),  $\alpha_2$  (Defilippi et al., 1992),  $\alpha_3$  (Klein et al., 1993),  $\alpha_5$  and  $\beta_1$  (Giancotti and Ruoslahti, 1990), and  $\alpha_4$  (Vogel et al., 1993) were previously described. The antibody to  $\alpha_4$  was generated and characterized in the laboratory of Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). The adhesion-blocking rabbit antiserum against 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 Pem-

phigoid 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  $\alpha_1$  and  $\beta_1$  laminin subunits, respectively (Engvall et al., 1986). The monoclonal antibody C4 is directed to the  $\beta_2$  laminin chain (Hunter et al., 1989). The rabbit polyclonal antibody R-1301 was raised by immunization with a synthetic peptide from the  $\alpha_2$  laminin subunit (Paulsson et al., 1991). The monoclonal antibody 5H2 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  $\alpha_3$  and  $\beta_3$  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 Ehrig et al. (1990). Electrophoretic and immunoblotting analyses conducted in our laboratory have shown that it contains the  $\alpha_2$ ,  $\beta_2$ , and  $\gamma_1$  chains, corresponding to laminin 4. The same conclusion was reached in a recently published study (Sonnenberg et al., 1993). Immunoblotting with monoclonal antibodies to the human  $\alpha_1$ ,  $\beta_1$ ,  $\alpha_3$ , and  $\beta_3$  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 (Paulsson 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 (Sonnenberg 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 (Sonnenberg et al., 1993). Our own electrophoretic analysis indicated that the 165-kD  $\alpha_3$  chain, the 155-kD  $\gamma_2$  chain, the 140-kD  $\beta_3$  chain, and the 105-kD  $\gamma_2$  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 ~190 kD and possibly represents the precursor of the  $\alpha_3$  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- $\beta_4$   $\Delta$  854-1752 directs the expression of a  $\beta_4$  molecule lacking almost the entire cytoplasmic domain. The plasmid pCMV- $\beta_4$   $\Delta$  70-660 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- $\beta_4$   $\Delta$  854-1752 and pCMV- $\beta_4$   $\Delta$  70-660, respectively (Spinardi et al., 1993). Transfection protocols were as previously described (Giancotti et al., 1994). Clones with high level expression of recombinant  $\beta_4$  molecules were selected by performing fluorescence-activated cell sorting, immunofluorescence, and immunoprecipitation analyses, as previously described (Spinardi et al., 1993). The clone expressing a full-length human  $\beta_4$  subunit (clone A12) 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  $\mu$ g/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 <sup>125</sup>I by the lactoperoxidase-H<sub>2</sub>O<sub>2</sub> 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 Pepstatin 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<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and no EDTA. The immunoprecipitations were performed as previously described (Giancotti and Ruoslahti, 1990) and analyzed by SDS-PAGE. All

1. Abbreviations used in this paper: BPAG2, Bullous Pemphigoid Antigen 2.

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  $\beta_4$  cytoplasmic peptide antibody. Samples containing 100  $\mu$ g total proteins were precipitated with 6 vol of acetone. 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  $^{125}$ I-Protein A (ICN Biomedicals, Irvine, CA) and autoradiography or by 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 Phosphorimager (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  $\mu$ M cycloheximide (Sigma) for 1 h and with 1  $\mu$ M monensin (Sigma) for 5 min before the assay. Cells were detached by incubation in 0.25% trypsin (GIBCO BRL) for 5 min. After blocking with Soybean Trypsin Inhibitor (Sigma), the cells were washed and resuspended in serum free DMEM containing 20  $\mu$ M cycloheximide. For antibody inhibition experiments, the cells were preincubated with the indicated concentrations of antibodies for 15 min at 4°C before plating. Cells were added at  $1-2 \times 10^5$  per well and incubated for the indicated times at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Quantitation of the results was as previously described (Giancotti et al., 1986b).

### Radioligand-binding Assay

The partially recombinant wild-type and tail-less  $\alpha_6\beta_4$  integrins used in radioligand-binding experiments were purified from the transfected 804G clones A12 and B13, respectively. Approximately  $1.5 \times 10^9$  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- $\beta$ -D-Glucopyranoside (Calbiochem, San Diego, CA), 5 mM EDTA, 0.01% Aprotinin, 4  $\mu$ g/ml Pepstatin A, 10  $\mu$ g/ml Leupeptin, 1 mM PMSF, and 1 mM MnCl<sub>2</sub> 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 mg of purified 3E1 monoclonal antibody to 1 ml of Sepharose-Protein G (Pharmacia LKB Biotechnology, Piscataway, NJ). After 4 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- $\beta$ -D-Glucopyranoside. The integrins were eluted with 5 bed volumes of 50 mM Triethylamine, pH 10, 150 mM NaCl, 50 mM *n*-Octyl- $\beta$ -D-Glucopyranoside into tubes containing neutralizing buffer (10% by volume 1 M 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 was consistently higher than 95%.

Laminins 1, 2, 4, and 5 and fibronectin were radioiodinated 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 the various preparations was determined by counting in a gamma counter a TCA-precipitated aliquot of the peak fractions. Specific activities were  $1.3 \times 10^6$  CPMs/ $\mu$ g for laminin 1,  $1.9 \times 10^6$  CPMs/ $\mu$ g for laminin 2,  $3.3 \times 10^6$  CPMs/ $\mu$ g for laminin 4,  $0.5 \times 10^6$  CPMs/ $\mu$ g for laminin 5, and  $9.9 \times 10^6$  CPMs/ $\mu$ g for fibronectin.

Purified integrins were diluted to 0.25  $\mu$ g/ml with PBS containing 1 mM MnCl<sub>2</sub>. Removable microtiter wells (Microtest III, Falcon) were coated with 100  $\mu$ l 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 MnCl<sub>2</sub> 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 Mm MnCl<sub>2</sub> and counted in a gamma counter. Nonspecific 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/0.1 ml) was added to each well in presence of increasing concentrations of cold ligand (0–2.5  $\mu$ g/0.1 ml). The results were subjected to Scatchard analysis.

### Immunofluorescence

The 804G transfectants 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  $\beta_4$  3E1 and anti-*c-myc* Ab-1 monoclonal antibodies were used at 5  $\mu$ g/ml. The anti-BPAG1 human serum and the  $\alpha_6$  cytoplasmic domain rabbit serum were diluted 1:200. The anti-BPAG2 fusion protein IgGs were used at 25  $\mu$ g/ml. The affinity-purified  $\beta_4$  cytoplasmic peptide antibodies were used at 5  $\mu$ g/ml. After extensive washing, the cells were incubated for 45 min with 0.5–1  $\mu$ g/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 Citi-Fluor (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 Axiophot Fluorescence Microscope.

### Electron Microscopy

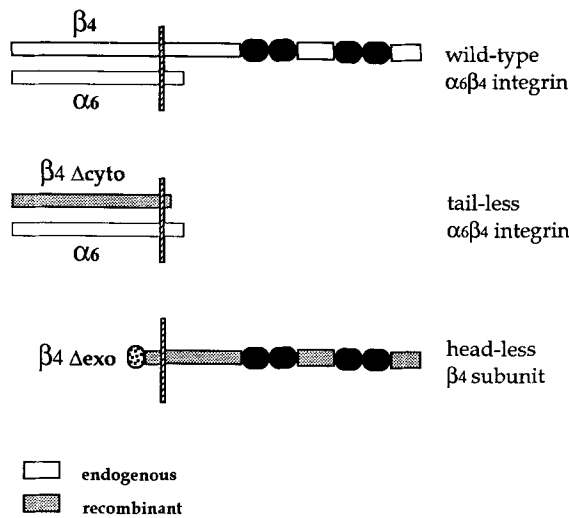
Cells grown on laminin 4-coated Aclar plastic coverslips were rinsed in PBS and fixed overnight at 4°C in 0.05 M Sodium Phosphate buffer, pH 7.0, containing 2% Glutaraldehyde and 0.1 M Sucrose. After washing in 0.1 M Phosphate buffer, the coverslips were incubated in 2% Osmium Tetroxide in 0.1 M Phosphate buffer for 1 h and embedded in Epon (Milner 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 $\beta_4$ Integrin Subunits

Previous results indicated that a truncated tail-less human  $\beta_4$  subunit ( $\Delta$  cyto 874-1752) combines with endogenous  $\alpha_6$  and reaches the cell surface, but is not incorporated in hemidesmosomes. In contrast, a truncated head-less  $\beta_4$  subunit ( $\Delta$  exo 70-660) does not associate with endogenous  $\alpha_6$ , 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  $\beta_4$  subunit could exert a dominant negative effect by competing with endogenous wild-type  $\alpha_6\beta_4$  for adhesive ligands. Conversely, the head-less recombinant  $\beta_4$  subunit could compete with the endogenous  $\alpha_6\beta_4$  receptor for binding to cytoskeletal elements or regulatory factors (Fig. 1):

To test the potential dominant negative effect of truncated  $\beta_4$  subunits in hemidesmosome-forming cells, we selected 804G clones with potential for high level expression of either the tail-less ( $\Delta$  cyto 874-1752) or the head-less ( $\Delta$  exo 70-660) human  $\beta_4$  subunit, as described in Materials and

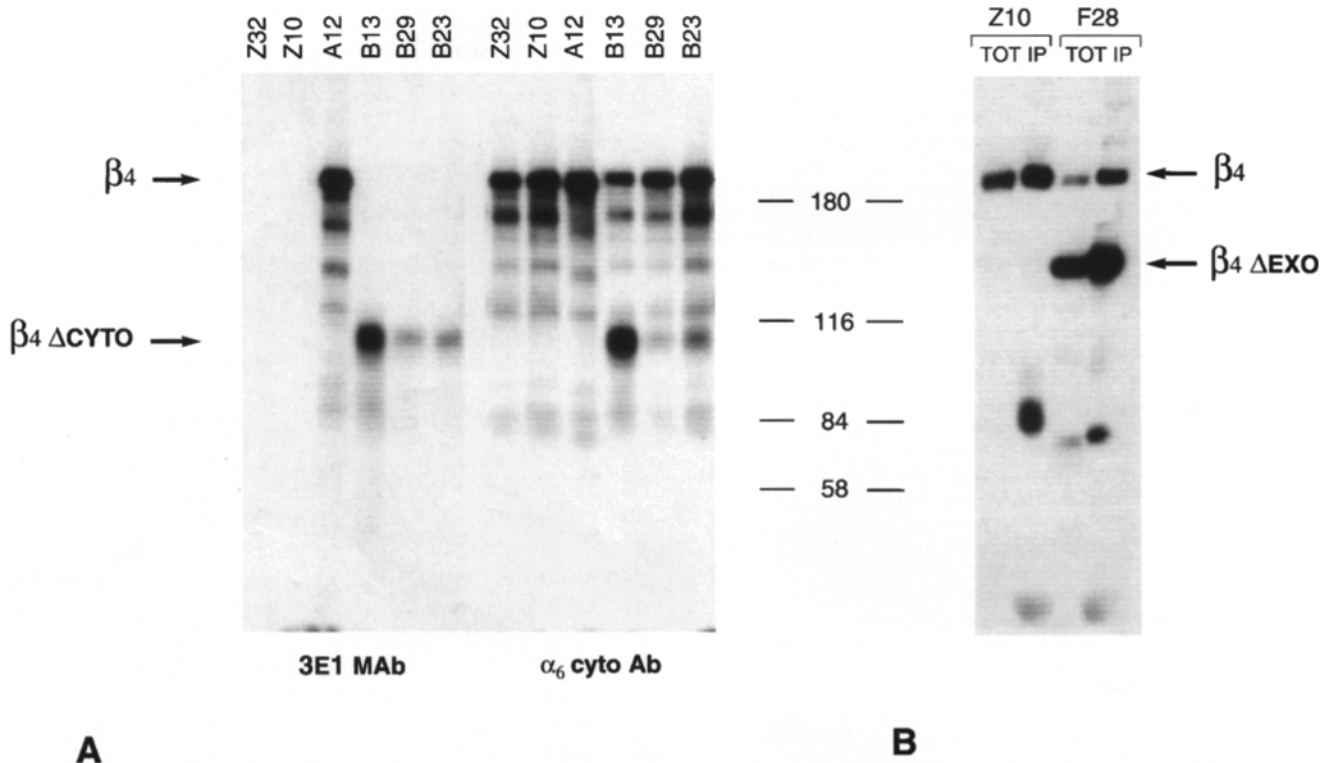


**Figure 1.** Schematic representation of wild-type and recombinant mutant  $\alpha_6\beta_4$  integrins. (*Top*) Wild-type endogenous  $\alpha_6\beta_4$  integrin. (*Middle*) Hybrid heterodimer consisting of the recombinant tail-less human  $\beta_4$  subunit ( $\Delta$ cyto) and associated endogenous  $\alpha_6$  subunit. (*Bottom*) Head-less human  $\beta_4$  subunit ( $\Delta$ exo), which does not combine with endogenous  $\alpha_6$ . The heterodimer containing a tail-less  $\beta_4$  subunit is expected to compete with the endogenous integrin for binding to extracellular ligand. The head-less  $\beta_4$  polypeptide may instead compete with endogenous wild-type  $\alpha_6\beta_4$  for interaction with intracellular molecules.

**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  $^{125}\text{I}$  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-



**Figure 2.** Immunoprecipitation analysis of recombinant  $\beta_4$  molecules expressed in 804G cells. (*A*) The indicated clones were labeled with  $^{125}\text{I}$  and extracted with lysis buffer. Aliquots containing the same amount of TCA precipitable radioactivity were immunoprecipitated with an excess amount of the 3E1 monoclonal antibody or the  $\alpha_6$  cytoplasmic domain antiserum. Samples were separated by SDS-PAGE under reducing conditions and subjected to autoradiography. (*B*) The indicated clones were either directly extracted in sample buffer (*TOT*) or immunoprecipitated with the  $\beta_4$  cytoplasmic peptide antibody before electrophoresis (*IP*). The lanes marked *TOT* contain 100  $\mu\text{g}$  of total proteins, while those labeled *IP* contain the material immunoprecipitated from 6 mg of total proteins. Both types of samples were separated by SDS-PAGE under reducing conditions and probed by immunoblotting with affinity-purified  $\beta_4$  cytoplasmic peptide antibodies. Bound antibodies were detected by incubation with  $^{125}\text{I}$ -Protein A followed by autoradiography.

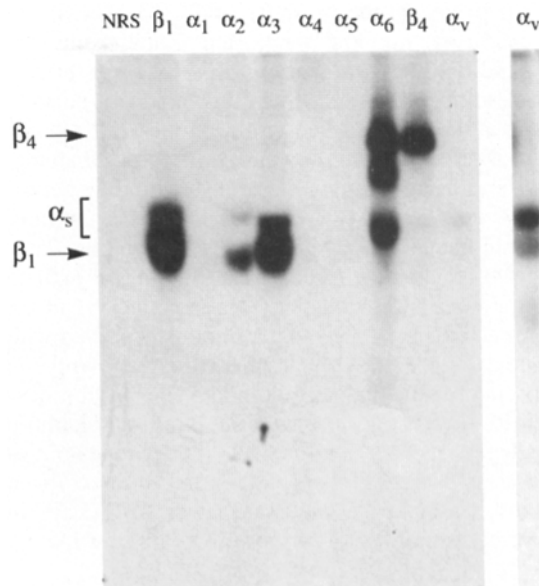
ing the cytoplasmic domain of  $\alpha_{6A}$ . The anti- $\alpha_{6A}$  antiserum was selected because  $\alpha_{6A}$  is the only  $\alpha_6$  subunit isoform expressed in 804G cells (unpublished results). As shown in Fig. 2 A (right), the antiserum immunoprecipitated two types of  $\alpha_6$  heterodimers from the B clones: those containing the 100-kD recombinant tail-less  $\beta_4$  molecule and those containing the 200-kD wild-type endogenous  $\beta_4$  subunit. Phosphorimager analysis indicated that the recombinant tail-less  $\beta_4$  subunit was 1.6 times more abundant than endogenous  $\beta_4$ , but endogenous  $\beta_4$  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  $\beta_4$  is overexpressed in clone B13, but not in clones B23 and B29.

The level of recombinant  $\beta_4$  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  $\beta_4$ . This  $\beta_4$  cytoplasmic domain antibody was expected to react well with both the human head-less  $\beta_4$  molecule and the endogenous rat wild-type  $\beta_4$  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  $\beta_4$  expressing clone F28 were either directly extracted in sample buffer or immunoprecipitated with excess amount of the  $\beta_4$  cytoplasmic domain antibody before immunoblotting. The  $\beta_4$  cytoplasmic domain antibodies bound to the recombinant head-less  $\beta_4$  subunit in clone F28 and reacted with endogenous wild-type  $\beta_4$  in total extracts and immunoprecipitates from both control clone Z10 and clone F28. Fainter bands at  $\sim 70$  kD, possibly representing proteolytic fragments of  $\beta_4$ , 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  $\beta_4$  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  $\beta_4$  subunits in clones F28 and B13 were determined by different methods, the results suggest that the extent of overexpression of head-less  $\beta_4$  in clone F28 is greater than that of tail-less  $\beta_4$  in clone B13.

### The $\alpha_6\beta_4$ Integrin Is a Receptor for Laminins 1, 2, 4, and 5

To examine the effects of the truncated  $\beta_4$  subunits on  $\alpha_6\beta_4$ -mediated cell adhesion, we sought to define the ligands of  $\alpha_6\beta_4$  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  $^{125}\text{I}$ , 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  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$  integrins and lower levels of  $\alpha_2\beta_1$ . A prolonged exposure of the gel (lane to the far right in Fig. 3) revealed that they also express minor levels of  $\alpha_v\beta_1$ . No  $\beta_1$  subunit could be detected in association with  $\alpha_6$ , even after prolonged exposure of the gel, indicating that 804G cells do not express the  $\alpha_6\beta_1$  integrin.

Cell adhesion assays showed that the 804G cells adhere



**Figure 3.** Immunoprecipitation analysis of integrins expressed by 804G cells. Parental 804G cells were labeled with  $^{125}\text{I}$  and extracted with lysis buffer. Aliquots containing the same amount of TCA precipitable radioactivity were immunoprecipitated with the indicated subunit-specific anti-integrin antibodies. Samples were separated by SDS-PAGE under nonreducing conditions and subjected to autoradiography. Immunoprecipitation with the anti- $\alpha_6$  antibody yielded, in addition to intact  $\beta_4$ , two proteolytic fragments of 170 and 140 apparent mol wt. In agreement with previous results (Giancotti et al., 1992), these fragments were not recognized by the  $\beta_4$  cytoplasmic domain antibody because they lack the corresponding epitope. The results of an 8-h exposure are shown except for the lane to the far right which represents a 36-h exposure of the  $\alpha_v$  immunoprecipitation.

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  $\beta_1$  (Fig. 4 C), we concluded that adhesion to laminin 4 and 5 could involve  $\alpha_6\beta_4$ .

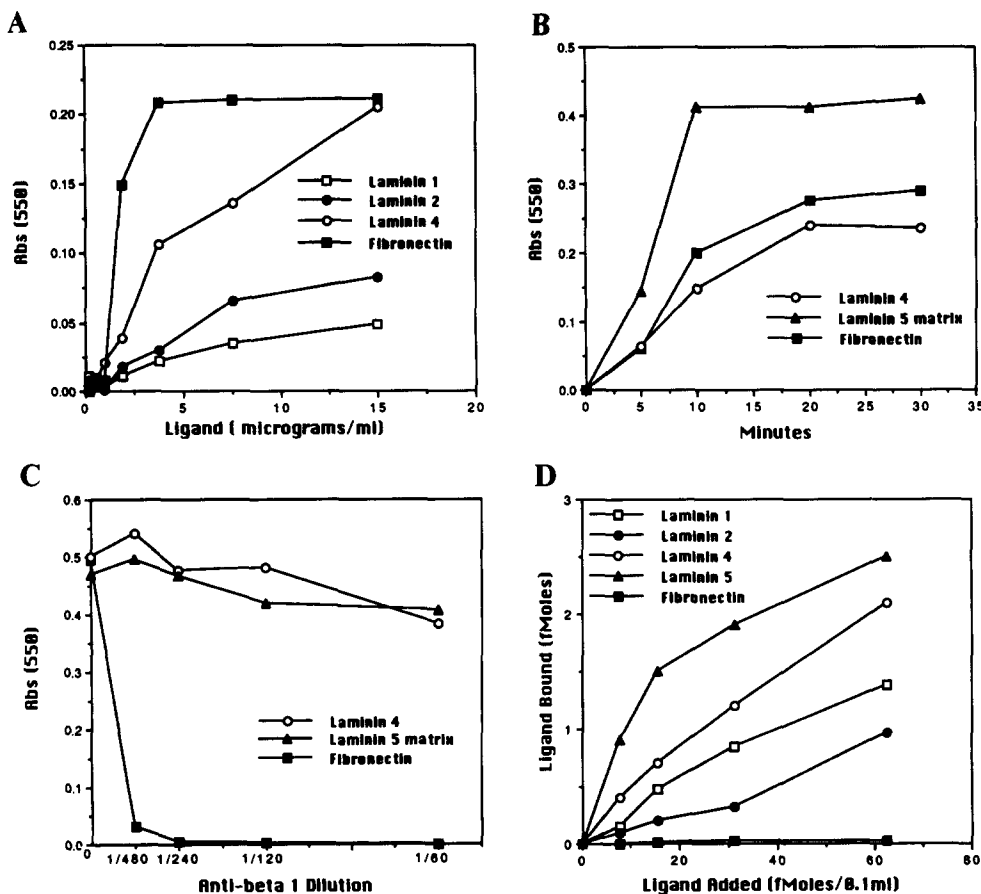
To directly test the ligand-binding ability of the  $\alpha_6\beta_4$  integrin in the absence of potentially confounding influences of other integrins or cellular regulatory factors, radioligand-binding assays were performed. A partially recombinant form of the  $\alpha_6\beta_4$  integrin was purified from clone A12 cells by immunoaffinity chromatography on the 3E1 monoclonal antibody, as described in Materials and Methods. Microtiter wells 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

assay was performed in the presence of 1 mM MnCl<sub>2</sub>, because this cation was previously reported to effectively sustain the ligand-binding function of  $\alpha_6\beta_4$  (Niessen et al., 1994). The results of this experiment indicated that purified  $\alpha_6\beta_4$  binds in a dose dependent way to laminin 1, 2, 4, and 5 (Fig. 4 D). Binding to laminin 4 and 5 was larger than that to laminin 1 and 2 suggesting a hierarchy of binding activities between the various  $\alpha_6\beta_4$  ligands. However, affinity constants could not be directly extrapolated from these data, since no curve reached saturation. The binding of  $\alpha_6\beta_4$  to laminin 1, 2, 4, and 5 was specific, since the integrin did not bind to fibronectin (Fig. 4 D) and the binding observed with laminins could be prevented by 1 mM EDTA. A synthetic peptide reproducing the major cell binding sites in fibronectin (GRGDSP) did not interfere with the binding of laminin 4 to  $\alpha_6\beta_4$  suggesting that this integrin, like other laminin-binding integrins, is not RGD dependent (not shown). Taken together, these observations indicate that the  $\alpha_6\beta_4$  integrin binds to several laminin isoforms, including laminin 1, 2, 4, and 5.

### The Tail-Less $\alpha_6\beta_4$ Integrin Displays an Intact Affinity for Extracellular Ligand In Vitro

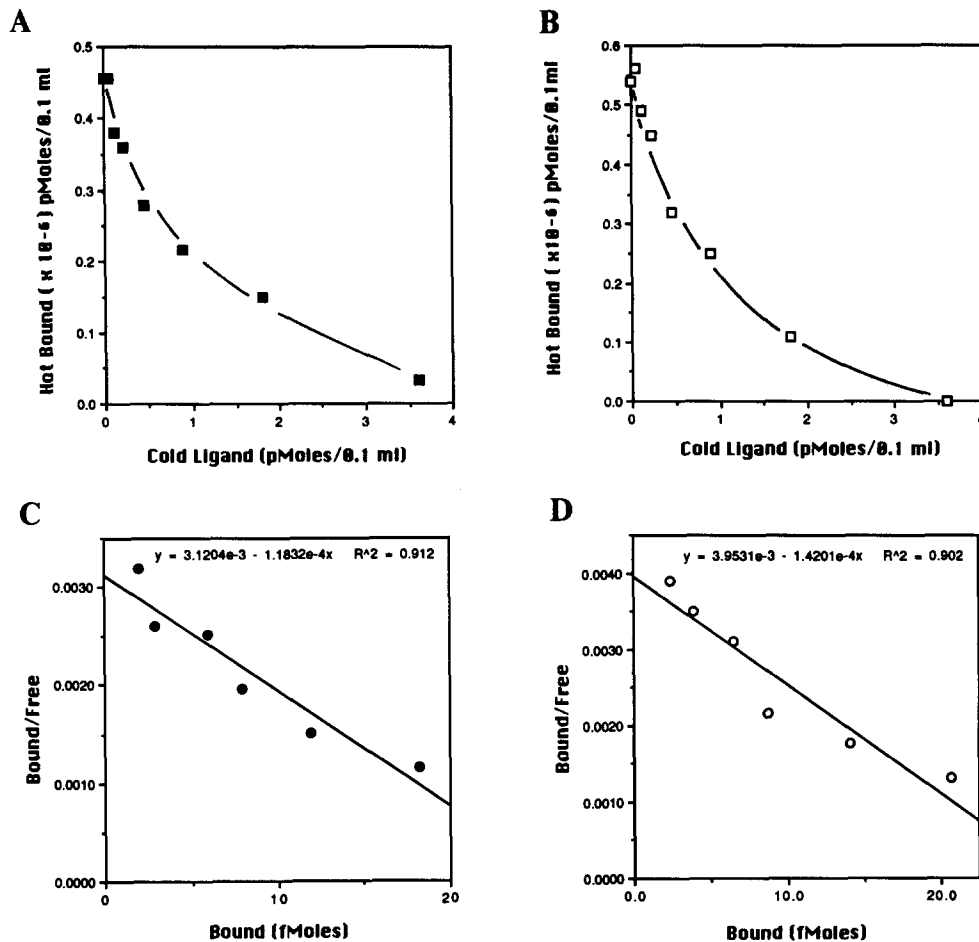
The hypothesis that a recombinant tail-less form of  $\beta_4$  can

suppress the function of endogenous  $\alpha_6\beta_4$  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  $\beta_4$  subunit from clone B13 and the receptor containing a full-length human  $\beta_4$  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  $\sim 3.5$  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  $K_d$  of wild-type receptor was  $8.45 \times 10^{-8}$  mol/liter and that of tail-less receptor was  $7.04 \times 10^{-8}$  mol/liter. These results demonstrate that the truncated tail-less  $\alpha_6\beta_4$  integrin retains an intact ligand-binding ability in vitro and indicate that deletion



**Figure 4.** Ligand-binding specificity of the  $\alpha_6\beta_4$  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  $\mu$ g/ml of fibronectin, or 20  $\mu$ g/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  $\mu$ g/ml of fibronectin or 6  $\mu$ g/ml of laminin 4 in presence of the indicated dilutions of anti- $\beta_4$  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- $\beta_4$

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  $\alpha_6\beta_4$  integrin (25 ng/well) and incubated with the indicated concentrations of radiolabeled ligands in presence of 1 mM MnCl<sub>2</sub> 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.



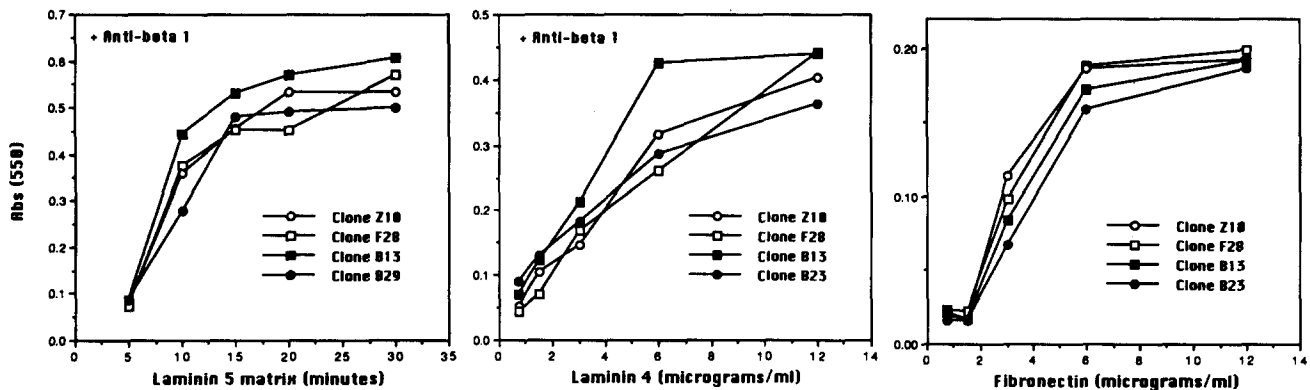
**Figure 5.** Scatchard analysis of laminin 4 binding to partially recombinant, wild-type and tail-less  $\alpha_6\beta_4$  integrin. A constant concentration of radiolabeled laminin 4 was incubated in wells coated with 25 ng/0.1 ml of the partially recombinant, purified wild-type or tail-less integrin. Displacement of radiolabeled laminin 4 was measured as a function of increasing concentrations of cold ligand added (0–2.5  $\mu\text{g}/0.1$  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.

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 $\alpha_6\beta_4$ -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  $\alpha_6\beta_4$ , the cells were plated on laminin 4 and 5 in the presence of antibodies capable of blocking endogenous  $\beta_1$  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

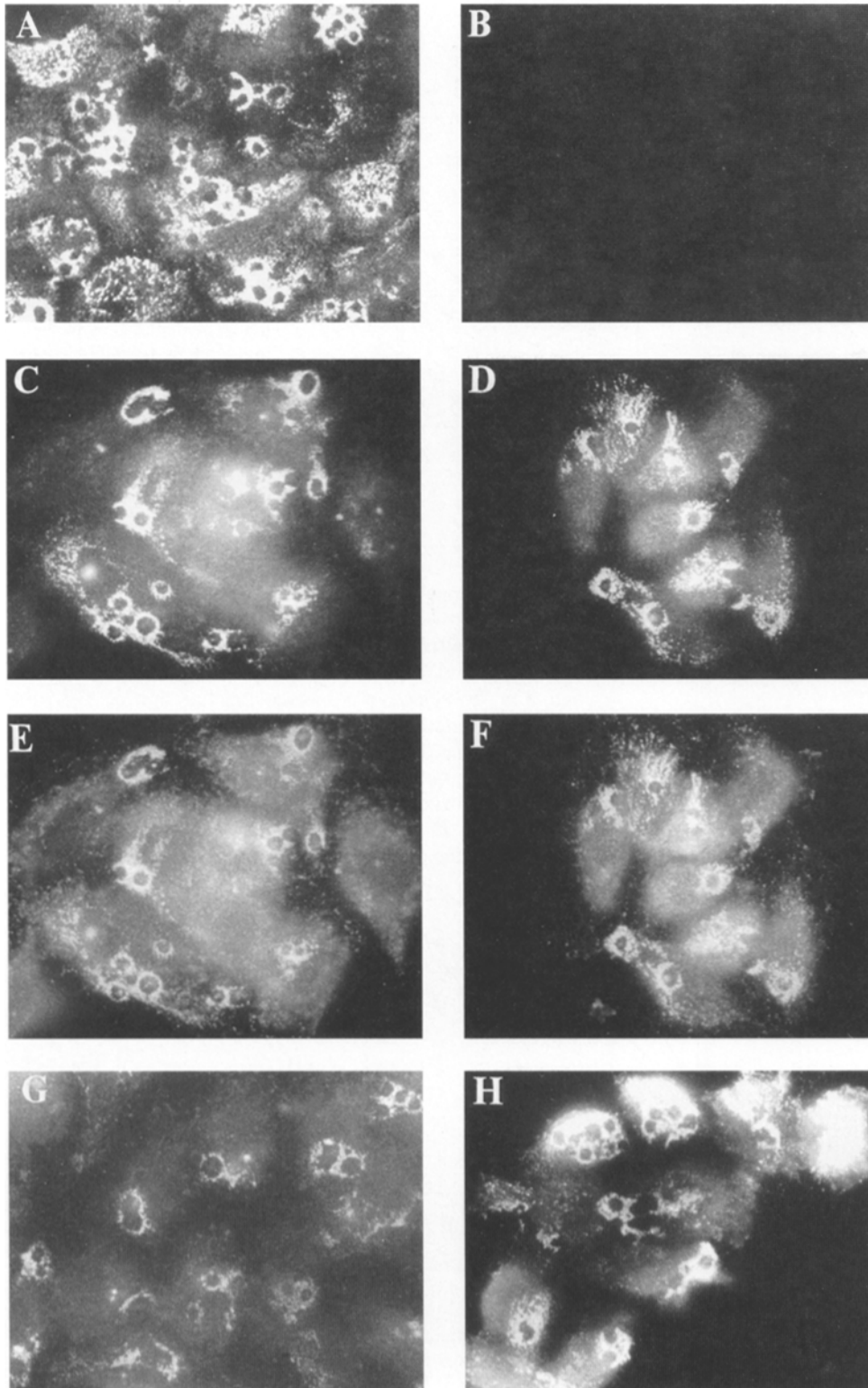


**Figure 6.** Measurement of  $\alpha_6\beta_4$ -dependent adhesion in cells expressing the head-less or tail-less recombinant  $\beta_4$  subunit. The indicated clones were incubated in presence of a 1:50 dilution of the anti-beta 1 antiserum for varying times on wells coated with the laminin 5 matrix (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.

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  $\beta_4$  subunit can suppress  $\alpha_6\beta_4$ -dependent adhesion to laminin 4 or 5.

**Overexpression of a Mutant  $\beta_4$  Subunit Lacking Extracellular Sequences Does Not Affect Hemidesmosome Assembly**

To determine if high level expression of the head-less  $\beta_4$  integrin subunit affects the assembly and/or stability of

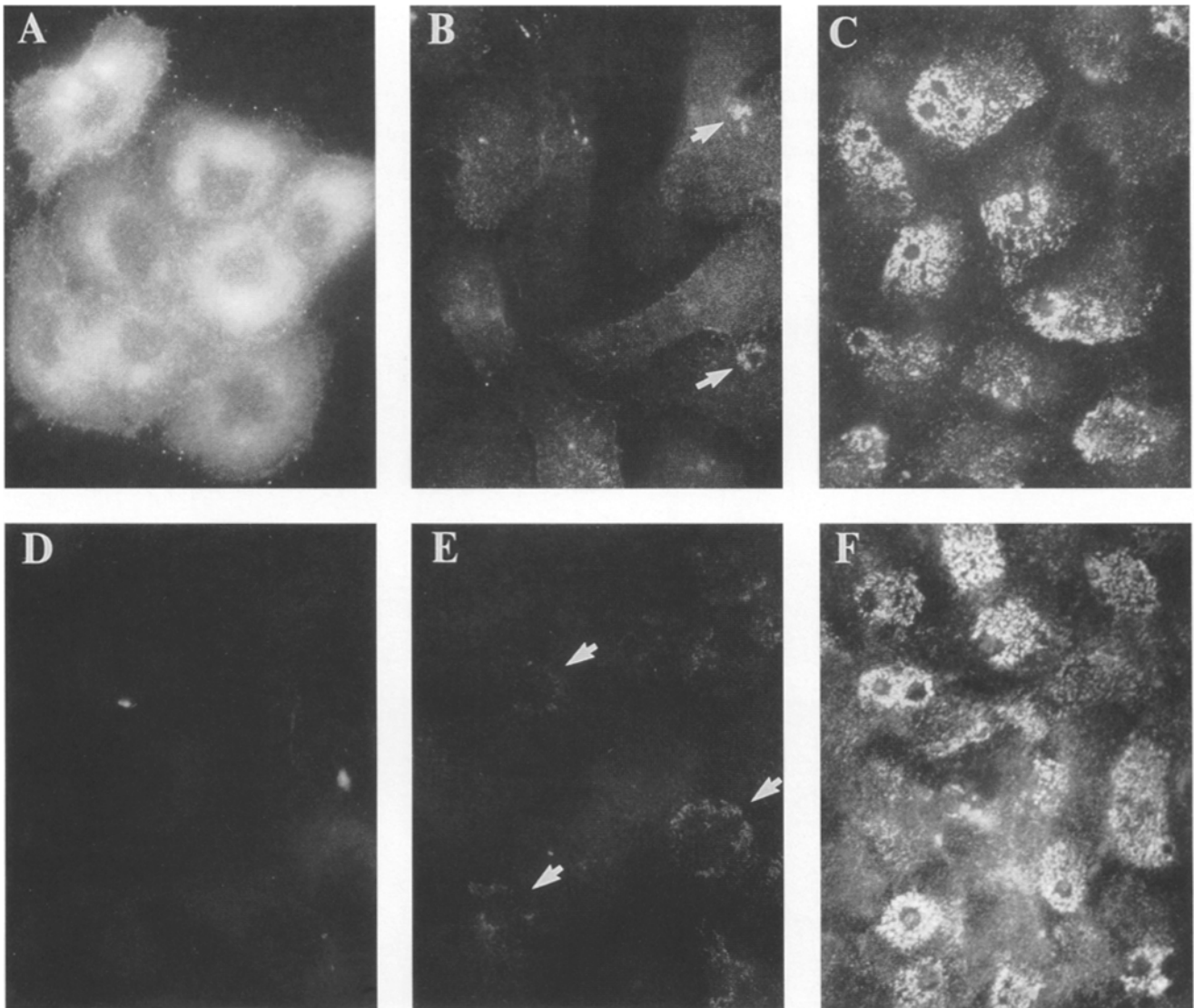


**Figure 7.** Immunofluorescent detection of recombinant full-length and head-less  $\beta_4$  subunits in hemidesmosomes. Cells of the control clone Z10, the full-length  $\beta_4$  expressing clone A12, and the head-less  $\beta_4$  expressing clone F28 were cultured on glass coverslips for 48 h, and 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  $\beta_4$  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 3E1 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 3E1 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 3E1 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 3E1 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  $\beta_4$  3E1 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.

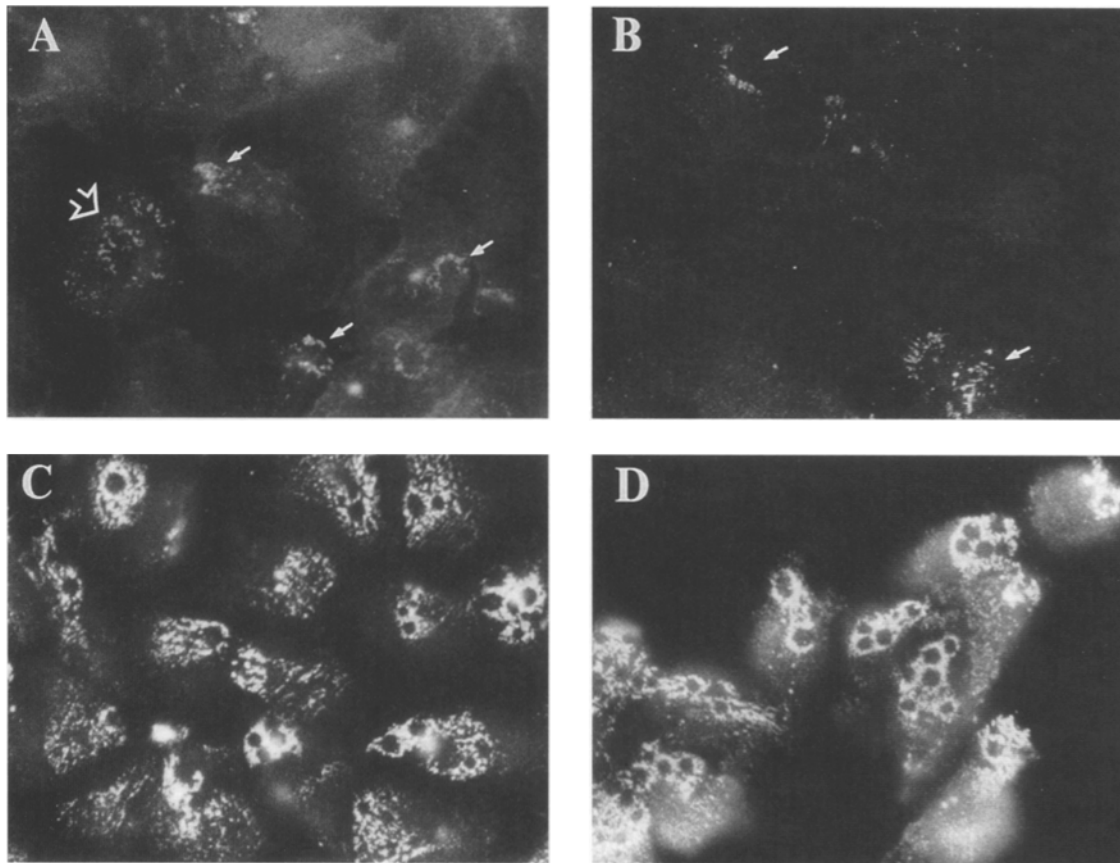
We next analyzed the subcellular distribution of the overexpressed recombinant head-less  $\beta_4$  subunit and BPAG1 and 2 in clone F28 cells. The transfectants were extracted with Triton X-100 and stained with the monoclonal antibody Ab-1 reacting with the *c-myc* epitope tag included in this recombinant truncated  $\beta_4$  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  $\beta_4$  with BPAG2 (Fig. 7, D and F), BPAG1 (Fig. 7 H, BPAG1 staining only is shown), and the endogenous  $\alpha_6$  subunit (not shown). These results suggest that the overexpressed recombinant head-less  $\beta_4$  subunit accumulates in hemidesmosomes without causing any apparent redistribution of the endogenous  $\alpha_6\beta_4$  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  $\beta_4$  expressing clone F28 were cultured for 48 h on laminin 4-coated Aclar coverslips, and then fixed. Vertical sections,

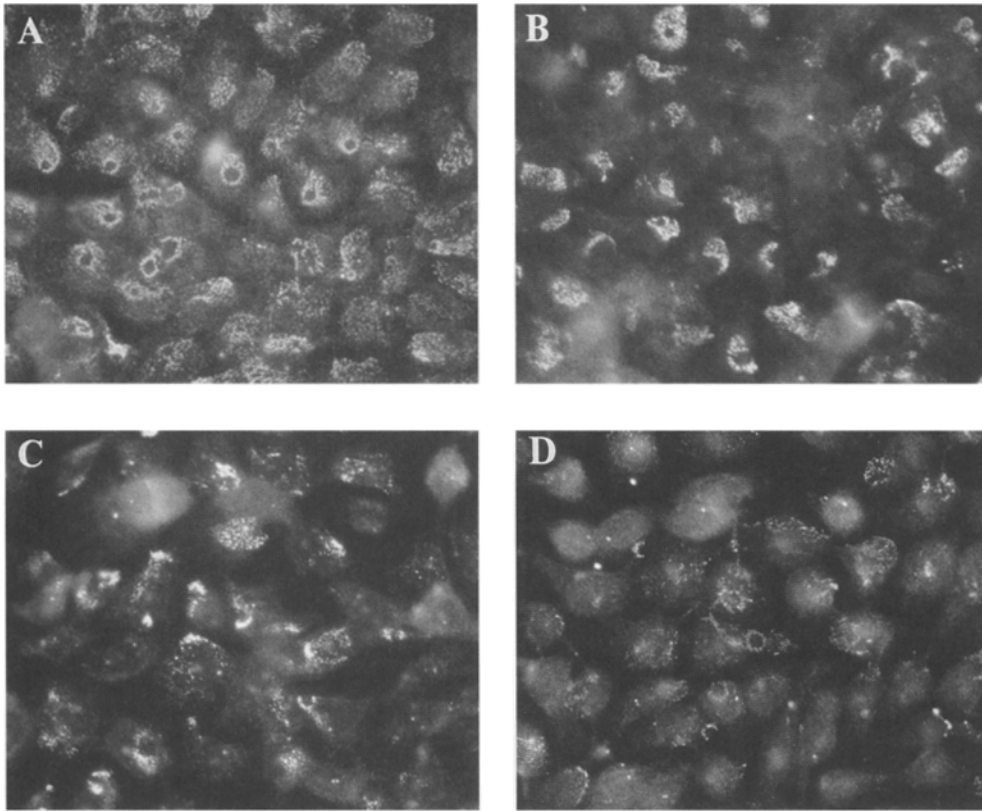
cut perpendicularly to the substratum, were examined with the electron microscope. The results showed that control cells had a number of submembranous densities associated with the basal cell surface in correspondence of substratum attachment sites (see Fig. 11 A). The appearance of these structures 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 6.6 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  $\beta_4$  subunit, at levels 5.3-fold higher than those of endogenous wild-type  $\beta_4$ , does not affect hemidesmosome assembly and/or stability.

#### ***Overexpression of a Mutant $\beta_4$ Subunit Lacking the Cytoplasmic Domain Disrupts Hemidesmosomes***

Immunofluorescence experiments were performed to determine if the tail-less recombinant  $\beta_4$  subunit has a dominant



**Figure 9.** Lack of detection of BPAG1 and BPAG2 in hemidesmosomes in clone B13. Cells of the tail-less  $\beta_4$  expressing clone B13 (A and B) or the control clone Z10 (C and D) were cultured on glass coverslips for 48 h, extracted with 0.2% Triton X-100, and then fixed in methanol. The cells were stained with the rabbit anti-BPAG2 antibody followed by FITC-labeled goat anti-rabbit IgGs (A and C) or with human antibodies to BPAG1 followed by FITC-labeled goat anti-human IgG (B and D).



**Figure 10.** Altered distribution of BPAG2 in clones expressing different levels of recombinant tail-less  $\beta_4$ . Control clone Z10 (A), clones B29 (B), and B23 (C) which express moderate levels of tail-less  $\beta_4$ , and clone B13 (D), which expresses high levels of tail-less  $\beta_4$ , were cultured on glass coverslips for 48 h, extracted with 0.2% Triton X-100, and then fixed in methanol. The cells were stained with the rabbit anti-BPAG2 antibody followed by FITC-labeled goat anti-rabbit IgGs.

negative effect on hemidesmosome assembly and/or stability. Staining of clone B13 cells with the 3E1 monoclonal antibody indicated that the tail-less recombinant  $\beta_4$  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  $\beta_4$  which is largely insoluble in Triton X-100, the tail-less  $\beta_4$  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  $\alpha_6\beta_4$ . Cells of the control clone Z10 and the tail-less  $\beta_4$  expressing clone B13 were treated with Triton X-100 and stained with affinity-purified antibodies to synthetic peptides reproducing the cytoplasmic domain of either  $\alpha_6$  or  $\beta_4$ . As shown in Fig. 8, B and E, although some punctuate staining was occasionally observed (arrows), neither antibody detected significant amounts of  $\alpha_6\beta_4$  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  $\beta_4$  prevents the incorporation of endogenous  $\alpha_6\beta_4$  integrin in hemidesmosomes.

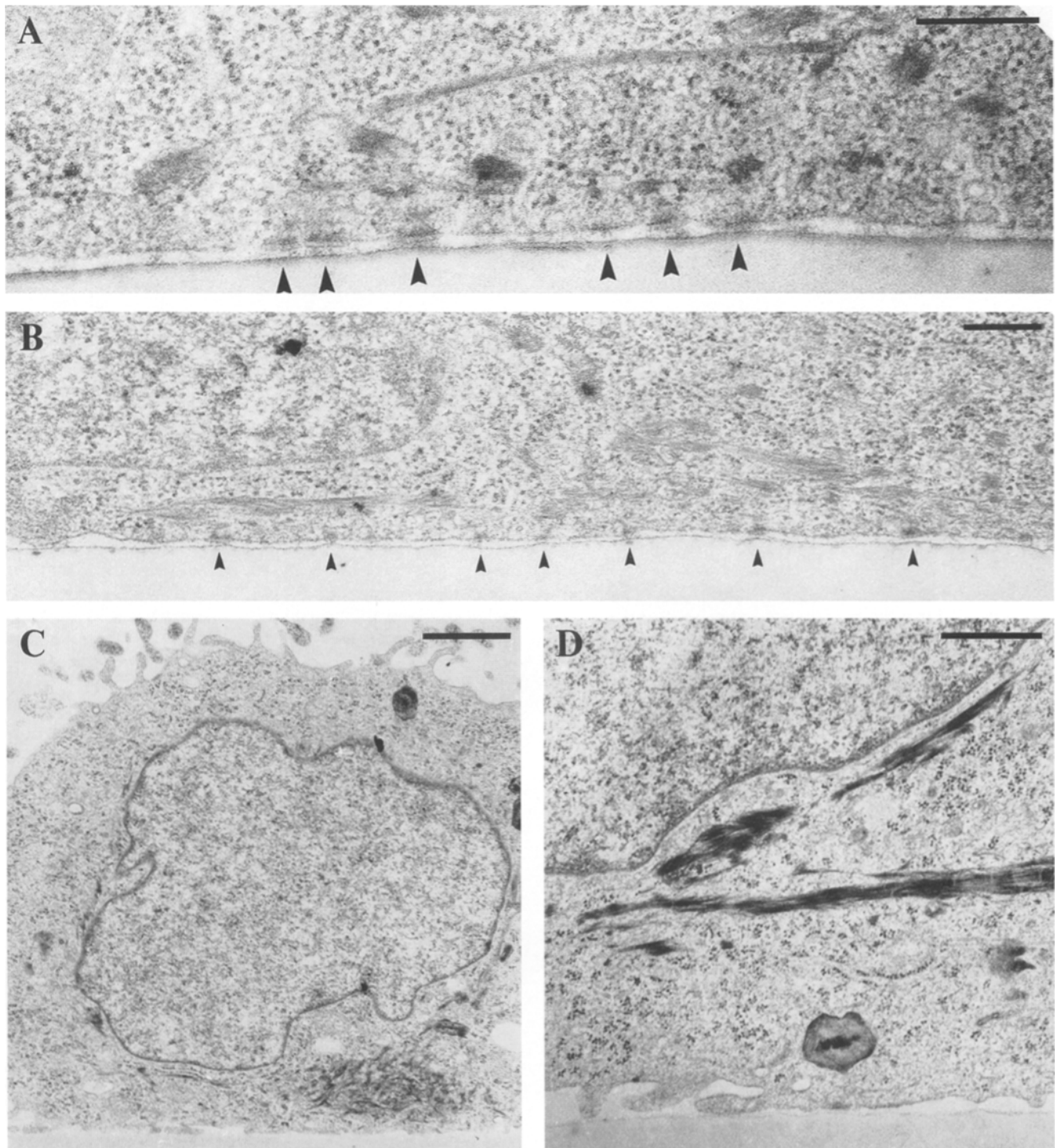
To examine the integrity of hemidesmosomes in cells overexpressing the tail-less  $\beta_4$  subunit, cells of the control clone Z10 and of the tail-less  $\beta_4$  expressing clone B13 were extracted with Triton X-100 and stained with antibodies to BPAG1 and 2. As shown in Fig. 9, A and B, the two 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  $\beta_4$  subunit was proportional to the level of expression, we analyzed clones B29 and B23, in which the ratio of recombinant tail-less  $\beta_4$  to endogenous  $\beta_4$  is 5 times lower than in clone B13. Cells of the control clone Z10 and tail-less  $\beta_4$  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  $\beta_4$  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



**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  $\mu\text{m}$ ; (C) 1  $\mu\text{m}$ ; (D) 0.5  $\mu\text{m}$ .

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  $\beta_1$  and  $\beta_2$  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,  $8.45 \times 10^{-8}$  mol/liter, is higher than that reported for the binding of the  $\alpha_5\beta_1$  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 base-

ment 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  $\alpha_2$  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 (Rousselle 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  $\sim 5$  times higher than the endogenous  $\alpha_6\beta_4$  integrin and did not interfere with  $\alpha_6\beta_4$ -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  $\beta_1$ ,  $\beta_3$ , or  $\beta_5$  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 p125 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  $\beta$  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 head-less  $\beta_4$  subunit suggest that the function of the  $\alpha_6\beta_4$  integrin may be regulated differently than that of other integrins. If intracellular regulators of  $\alpha_6\beta_4$  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 tail-less  $\beta_4$  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  $\alpha_6\beta_4$ . 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  $\beta_4$  subunit are significantly rounder and more detached from the culture substratum 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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