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The β_4 Subunit Cytoplasmic Domain Mediates the Interaction of $\alpha_6\beta_4$ Integrin with the Cytoskeleton of Hemidesmosomes

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The $\alpha_6\beta_4$ integrin is structurally distinct from all the other known integrins because the cytoplasmic domain of β_4 is unusually large and contains four type III fibronectin-like modules toward its C-terminus. To examine the function of the β_4 cytoplasmic tail, we have expressed full-length and truncated human β_4 cDNAs in rat bladder epithelial 804G cells, which form hemidesmosome-like adhesions in vitro. The cDNA encoded wild-type β_4 subunit associated with endogenous α_6 and was recruited at the cell surface within hemidesmosome-like adhesions. A recombinant form of β_4, lacking almost the entire cytoplasmic domain associated with α_6, reached the cell surface but remained diffusely distributed. A β_4 molecule lacking almost the entire extracellular portion did not associate with α_6 but was correctly targeted to the hemidesmosome-like adhesions. Thus, the cytoplasmic portion of β_4 contains sequences that are required and may be sufficient for the assembly of the $\alpha_6\beta_4$ integrin into hemidesmosomes. To localize these sequences we examined the properties of additional mutant forms of β_4. A truncated β_4 subunit, lacking the most C-terminal pair of type III fibronectin homology domains, was incorporated into hemidesmosome-like adhesions, but another recombinant β_4 molecule, lacking both pairs of type III fibronectin repeats, was not. Finally a recombinant β_4 molecule, which was created by adjoining the region of the cytoplasmic domain including all type III repeats to the transmembrane segment, was efficiently recruited in hemidesmosome-like adhesions. Taken together these results suggest that the assembly of the $\alpha_6\beta_4$ integrin into hemidesmosomes is mediated by a 303–amino acid region of β_4 tail that comprises the first pair of type III fibronectin repeats and the segment between the second and third repeats. These data imply a function of a specific segment of the β_4 cytoplasmic domain in interaction with cytoskeletal components of hemidesmosomes.

INTRODUCTION

The integrins comprise a large family of homologous heterodimeric receptors that mediate the adhesion of cells to extracellular matrices and other cells (reviewed in Buck and Horwitz, 1987; Hynes, 1987, 1992; Ruoslahti and Pierschbacher, 1987; Ginsberg *et al.*, 1988; Hemler, 1990; Springer, 1990). Both the α and the β subunits of integrins have a large extracellular portion, a transmembrane segment, and generally a short cytoplasmic domain. The cytoplasmic domains of integrins interact with the cytoskeleton and possibly with signaling molecules, but the molecular mechanisms of these interactions are not well known.

In fibroblasts, upon binding to the proper extracellular ligand, most β_1 and β_3 integrins are recruited into adhesion plaques (Chen *et al.*, 1985; Damsky *et al.*, 1985; Giancotti *et al.*, 1986; Dejana *et al.*, 1988; Singer *et al.*, 1988; Carter *et al.*, 1990b). These structures contain cytoskeletal molecules connecting to the actin filament system and molecules potentially involved in signaling (reviewed in Burridge *et al.*, 1988, 1992). The analysis of recombinant truncated and chimeric integrins has indicated that sequences within the cytoplasmic domain

of the β_1 and β_3 subunit are required for incorporation into adhesion plaques (Solowska *et al.*, 1989, 1991; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992) suggesting that the intracellular portion of β_1 and β_3 interacts with the cytoskeleton. In accordance with this hypothesis in vitro binding studies have shown that the cytoplasmic segment of β_1 is able to bind to α-actinin (Otey *et al.*, 1990) and talin (Horwitz *et al.*, 1986).

Integrins lacking the cytoplasmic domain of their β subunit bind well to extracellular ligand in vitro (Solowska *et al.*, 1989) but do not mediate cell adhesion as efficiently as their wild-type counterparts in vivo (Hayashi *et al.*, 1990; Hibbs *et al.*, 1991). This suggests that association with the cytoskeleton may stabilize the binding of integrins to extracellular ligand perhaps by lowering the k_{off} rate of individual receptors. Recent results suggest that the intracellular portion of α subunit may modulate the interaction of the cytoplasmic tail of the β subunit with cytoskeletal elements (Chan *et al.*, 1992; La Flamme *et al.*, 1992; Takada *et al.*, 1992). The data suggests a model in which integrins, upon binding to extracellular ligand, cluster within the cell membrane and undergo a conformational change that facilitates the interaction of the β subunit cytoplasmic tail with the cytoskeleton. Once adhesion has been initiated, various cytoskeletal and signaling elements assemble at the cytoplasmic face of adhesion plaques. This assembly may require an intracellular signal perhaps mediated by protein kinase C (Burn *et al.*, 1988; Woods and Couchman, 1992).

The $\alpha_6\beta_4$ integrin is a basement membrane receptor (Kajiji *et al.*, 1989; De Luca *et al.*, 1990; Sonnenberg *et al.*, 1990a,b; Lee *et al.*, 1992) that is likely to have cytoplasmic interactions distinct from those of all the other known integrins. Instead of being concentrated in adhesion plaques this receptor is found in hemidesmosomes (Carter *et al.*, 1990a; Stepp *et al.*, 1990; Jones *et al.*, 1991; Sonnenberg *et al.*, 1991). The hemidesmosomes are punctuate junctions that mediate the stable attachment of stratified and transitional epithelia to basement membranes (reviewed in Schwarz *et al.*, 1990; Legan *et al.*, 1992). At the electron microscopic level they appear as tripartite structures resembling half desmosomes with their innermost plaque linked to keratin filaments. Although the association of $\alpha_6\beta_4$ with hemidesmosomes is not obligatory because the integrin is also expressed in tissues that do not form these structures, all the available information suggests that $\alpha_6\beta_4$ interacts with the intermediate filament system and not with the actin filament system.

The intracellular interactions of $\alpha_6\beta_4$ may be determined by the cytoplasmic domain of the β_4 subunit. The intracellular portion of β_4 is much larger (~1000 amino acids) than that of all the other known β subunits (~50 amino acids) and bears no apparent homology with them (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990). It contains, toward its C-terminus, two pairs of type III fibronectin homology domains separated by a region that shows variations, possibly as a result of alternative splicing of mRNA.

To examine the hypothesis that the large cytoplasmic domain of β_4 interacts with the cytoskeleton at hemidesmosomes, wild-type and truncated human β_4 subunits were expressed in cells that form hemidesmosome-like adhesions in culture. The results indicate that the cytoplasmic portion of β_4 is both required and sufficient for incorporation of the integrin into hemidesmosome-like adhesions. Targeting to these adhesive structures is mediated by a 303–amino acid region of β_4 tail, which includes the first two type III repeats and the sequences between the second and third repeat. These findings suggest that a specific portion of the β_4 subunit cytoplasmic domain may interact with cytoskeletal component(s) of hemidesmosomes.

MATERIALS AND METHODS

Antibodies

The α_6 subunit-specific polyclonal antibody was elicited by immunizing a rabbit with a synthetic peptide reproducing the C-terminal 29–amino acid portion of the human α_6 subunit (Giancotti *et al.*, 1992). The β_4 subunit-specific rabbit polyclonal antiserum was raised by immunization with a synthetic peptide modeled after the C-terminal 31–amino acid segment of human β_4 as previously described (Giancotti *et al.*, 1992). Both the anti-α_6 and the anti-β_4 peptide antibodies cross-react with rodent subunits. In contrast, the monoclonal antibody 3E1 recognizes the extracellular portion of human, but not rodent, β_4 subunits (Giancotti *et al.*, 1992). The monoclonal antibody AP-1, which binds to an epitope of c-myc comprising the amino acid sequence EQKLISEEDL (residues 410–419), was purchased from Oncogene Science (Uniondale, NY). Human autoantibodies directed to the Bullous Pemphigoid Antigen 230 kDa were provided by Dr. J.-D. Fine (University of North Carolina, Chapel Hill, NC).

Expression Constructs

Expression constructs encoding wild-type and mutant-truncated human β_4 subunits were assembled in the eukaryotic expression vector pRc-CMV, which contains a cytomegalovirus promotor and a neomycin resistance gene (Invitrogen, San Diego, CA). The expression constructs pCMV-β_4, encoding a full-length β_4 subunit, and pCMV-$\beta_4\Delta$854-1752, encoding a β_4 molecule lacking almost the entire cytoplasmic domain, were previously described (Giancotti *et al.*, 1992). The construct pCMV-β_4 Δ1183-1752 directs the expression of a β_4 polypeptide lacking both pairs of type III Fn repeats. To generate this plasmid, pCMV-β_4 was digested with *Sac* I, polished with mung bean exonuclease, and ligated to a 12-mer *Xba* I linker containing an in frame stop codon (Promega, Madison, WI). This DNA was digested with *Bsp*EI and *Xba* I, and the resulting 4.9-kilobase (kb) fragment was ligated to the 6.3-kb *Bsp*EI-*Xba* I fragment of pCMV-β_4. The expression construct pCMV-$\beta_4\Delta$1486-1752, encoding a β_4 subunit lacking the C-terminal pair of type III Fn modules, was created from pCMV-β_4 by engineering a stop codon at amino acid position 1486. pCMV-β_4 was digested with *Not* I and *Xba* I. The resulting 10-kb fragment was then ligated to an oligonucleotide cassette containing an in frame stop codon (5′-GGCCGTAGGAATTCCTAGCTAGCTAG-3′). The expression construct pCMV-$\beta_4\Delta$734-1160 encodes a mutant β_4 subunit with an internal deletion of the N-terminal half of the cytoplasmic domain. To obtain this plasmid, pCMV-β_4 was digested with *Sca* I. The two largest fragments resulting from this digestion were then ligated together. The plasmid pCMV-$\beta_4\Delta$70-660 encodes a β_4 subunit in which most of the extracellular sequences were replaced

by a *c-myc* epitope tag. To generate this construct we first used the polymerase chain reaction to synthesize a 110-base pair DNA fragment that comprises the epitope of the monoclonal antibody (mAb) AP-1 and is flanked by appropriate restriction sites. The synthesis reaction included the target DNA pCMV-c-myc containing the entire human *c-myc* cDNA (Prendergast *et al.*, 1991) and the oligonucleotide primers 5′-TCCCCGCGGAGCAAAAGCTC-3′ and 5′-GCTCTAGACTTA-AGCGCACAAGAGTT-3′. The first oligonucleotide hybridizes to the upper cDNA strand and adds a *Ksp* I site on the 5′ end of the DNA synthesized. The second oligonucleotide is complementary to the lower cDNA strand and adds a *Bfr* I site immediately followed by a *Xba* I site on the 3′ end of the fragment synthesized. The product of this polymerase chain reaction was digested with *Ksp* I and *Xba* I and then ligated to the large 4.8-kb *Ksp* I-*Xba* I fragment of pCMV-β_4. The resulting plasmid was finally linearized with *Bfr* I and *Xba* I and ligated to the 3.6-kb *Bfr* I-*Xba* I fragment of pCMV-β_4. Correctness of all the constructs was verified by sequencing.

Sequencing was performed by the dideoxy chain termination method using sequenase (United States Biochemical, Cleveland, OH). Restriction enzymes were from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), and Boehringer Mannheim (Indianapolis, IN). T4 DNA ligase, the large fragment of Klenow polymerase, and nucleotides were from Boehringer Mannheim. Mung bean exonuclease and shrimp alkaline phosphatase were from United States Biochemical.

Cells and Transfection

Before transfection, the rat bladder carcinoma 804G cells (Izumi *et al.*, 1981) were cultured in Dulbecco's modified minimal essential medium (DMEM) with 10% bovine calf serum. The cDNA constructs encoding wild-type and mutant human β_4 subunits were transfected into the 804G cells by the calcium phosphate precipitation method. Primary clones expressing the neomycin resistance gene were selected in DMEM containing 400 μg/ml of geneticin (G418, GIBCO, Grand Island, NY). Clones expressing the recombinant β_4 molecules were then identified by performing immunofluorescence and immunoprecipitation analyses. Fluorescence-activated cell sorting was used to select, among these clones, those characterized by comparable levels of expression of each recombinant β_4 polypeptide (clones A, B, C, D, E, and F). The 3E1 monoclonal antibody was used to detect the recombinant molecules expressed by clones A, B, C, D, and E; the anti-c-myc Ab-1 antibody was used to detect the recombinant polypeptide expressed by clone F. The control cell line (clone Z) was generated by transfection of pRC-CMV alone and subsequent selection in DMEM containing 400 μg/ml of G418. The transfected cell lines were maintained in DMEM supplemented with 400 μg/ml of G418.

Immunoprecipitation

Cells were labeled in suspension with ^{125}I by the lactoperoxidase-H_2O_2 method or labeled metabolically with 100 μCi/ml of ^{35}S-methionine/cysteine (Translabel, ICN, Irvine, CA; >1000 Ci/mmol) for 10 h and immunoprecipitated as previously described (Giancotti and Ruoslahti, 1990). After washing, the cells were extracted with lysis buffer containing 50 mM tris(hydroxymethyl)aminomethane, pH 7.5, 150 mM NaCl, 0.5 % Triton X-100, 0.01 % aprotinin (Sigma), 4 μg/ml pepstatin A (Sigma), 10 μg/ml leupeptin (Sigma), 1 mM phenylmethanesulfonyl fluoride, and 10 mM EDTA. Cell extracts were clarified by centrifugation at 15 000 rpm and preincubated with normal rabbit IgG-agarose or normal mouse IgG-agarose (Sigma). Samples were then incubated with an excess of the primary antibody followed by Protein A-Sepharose or goat anti-mouse IgG-agarose (Sigma). The affinity beads were washed with lysis buffer containing 0.1 % sodium dodecyl sulfate (SDS). The immunoprecipitations were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Laemmli, 1970) and autoradiography with X-Omat AR films (Eastman Kodak, Rochester, NY).

Immunofluorescence

The 804G transfectants were cultured at low density for ~24 h on glass coverslips. To examine the subcellular localization of recombinant β_4 molecules, the transfected cells were fixed with cold methanol for 2 min, incubated briefly with phosphate-buffered saline containing 0.02% bovine serum albumin (PBS-0.02% BSA) and then stained for 45 min with the purified anti-human β_4 3E1 mAb or the anti-c-myc AP-1 mAb both at 0.5 μg/ml. After extensive washing with PBS-0.02% BSA, the cells were incubated for 45 min with 0.5–1 μg/ml of affinity purified fluorescein isothiocyanate (FITC)-conjugated or Texas Red-conjugated goat anti-mouse IgGs (Molecular Probes, Eugene, Oregon). After washing, the coverslips were mounted in Citi-Fluor (Chemical Laboratory of the University of Kent, Canterbury, UK). To determine if the subcellular localization of recombinant β_4 molecules coincided with that of the hemidesmosomal marker Bullous Pemphigoid Antigen 230 kDa (BPA 230), the transfected cells were subjected to double staining with the 3E1 mAb and the Bullous Pemphigoid (BP) patient serum. To increase the accessibility of BPA 230 to antibody staining, the transfectants were briefly permeabilized with 0.2% Triton X-100. After fixation with PBS-3.7% paraformaldehyde, the samples were incubated first with the 3E1 mAb followed by FITC-labeled anti-mouse IgGs and then with the BP serum diluted 1:100 followed by TR-labeled anti-human IgGs (Molecular Probes). The stained samples were examined with a Zeiss Axiophot Fluorescent Microscope (Thornwood, NY) and with a Molecular Dynamics Confocal Laser Scanning Microscope. Confocal microscope analysis was performed by using the Sarastro 2000 program. Fluorescence-activated cell sorter (FACS) analysis was performed by using excess amount of purified 3E1 mAb followed by FITC-labeled goat anti mouse IgGs, essentially as previously described (Giancotti *et al.*, 1986; Giancotti and Ruoslahti, 1990; Giancotti *et al.*, 1992). Samples were analyzed with a Becton & Dickinson (Mountainview, CA) FACSCAN Flow Cytometer.

RESULTS

Expression of Human Integrin β_4 Subunits

The function of the large cytoplasmic domain of β_4 was examined by introducing wild-type and truncated human β_4 subunits into epithelial cells. 804G cells were chosen for the following reasons. First, 804G cells assemble structures with the typical electron microscopic appearance of hemidesmosomes when maintained on uncoated substrate in vitro (Ridelle *et al.*, 1991). Second, immunoelectron microscopic studies have shown that the hemidesmosomes formed by 804G cells are immunochemically similar to those seen in tissues, in that they contain an endogenous $\alpha_6\beta_4$ integrin and the hemidesmosomal marker BPA 230 (Jones *et al.*, 1991). Third, immunofluorescence experiments have demonstrated that the $\alpha_6\beta_4$ integrin and BPA 230 colocalize at the basal surface of 804G cells in punctuate structures possibly representing individual hemidesmosomes. These often merge generating a distinctive "swiss-cheese"-like pattern (Jones *et al.*, 1991). Finally, 804G cells are of rat origin and previous results have shown that recombinant human β_4 subunits can efficiently combine with endogenous rodent α_6 polypeptides. The resulting hybrid heterodimers are selectively recognized by the anti-human β_4 3E1 mAb (Giancotti *et al.*, 1992). Taken together, these results suggest that 804G cells have the intracellular machinery needed for proper expression and localization of recombinant β_4 subunits. Preliminary immunoprecipitation experiments indicated that these cells, in addition to substantial levels of $\alpha_6\beta_4$,

express $\alpha_3\beta_1$ and minor levels of $\alpha_1\beta_1$ and an α_v-containing complex (unpublished data).

Figure 1 illustrates the structure of the wild-type and mutant β_4 cDNA generated by recombinant DNA techniques. These include a cDNA encoding the wild-type β_4 subunit (A), various cDNAs directing the expression of β_4 molecules carrying cytoplasmic domain deletions (B, C, D, and E), and a cDNA encoding a β_4 polypeptide in which most of the extracellular sequences were replaced by a c-myc epitope tag (F). The cDNAs were ligated in pRcCMV, a eukaryotic expression vector carrying the promoter and enhancer sequences of the immediate early gene of human cytomegalovirus. The resulting expression contructs were transfected into 804G cells, and primary clones were selected with G418. To identify those clones that expressed substantial levels of recombinant β_4 subunits, we performed immunofluorescence and immunoprecipitation experiments. Cells transfected with constructs A, B, C, D, and E were analyzed with the 3E1 mAb reacting with the extracellular domain of human, but not rat, β_4 subunit. The Ab-1 mAb, which binds to the amino acid sequence EQKLISEEDL (residues 410–419) of c-myc, was used to examine cells transfected with construct F. Finally, FACS analysis was used to select among the β_4 expressors those with comparable cell surface levels of the different recombinant β_4 molecules.

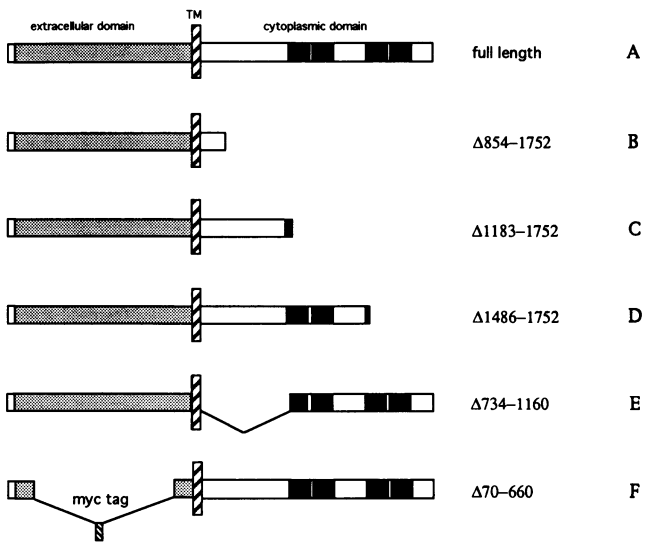

Figure 1. Schematic diagram of the cDNA-encoded full-length and truncated human β_4 molecules. The salient features of wild-type and truncated β_4 subunits encoded by the cDNAs used for transfection are shown. From the left: leader sequence (□), extracellular domain (□), c-myc epitope tag (□), transmembrane domain (▨), intracellular (□), type III fibronectin-like repeats (■). C-terminal truncations and deletions are indicated by the stretch of amino acids that were removed. Each construct, corresponding recombinant β_4 polypeptide and corresponding transfected cell line, is indicated by the same letter code (A, B, C, D, E, and F).

The Cytoplasmic Domain of β_4 Mediates the Incorporation of $\alpha_6\beta_4$ into Hemidesmosomes

To study the role of the large cytoplasmic domain of β_4 in the assembly of $\alpha_6\beta_4$ into hemidesmosomes, we examined 804G cells expressing the wild-type human β_4 subunit (clone A), a truncated β_4 molecule lacking the cytoplasmic domain (clone B), and a mutant form of β_4 in which most of the extracellular sequences were replaced by a c-myc epitope tag (clone F). Immunoprecipitation experiments were performed to determine the ability of these recombinant human β_4 polypeptides to form heterodimers with endogenous α_6. The control clone Z and the β_4 expressing clones A, B, and F were metabolically labeled with ^{35}S-methionine/cysteine and extracted in detergent buffer. The extracts were immunoprecipitated with several different antibodies. Two mAbs were used to immunoprecipitate the recombinant human β_4 subunits: 3E1, reacting with the extracellular portion of human β_4, and Ab-1, reacting with the c-myc epitope tag included in construct F. The β_4 and α_6 cytoplasmic peptide antibodies, which were previously shown to crossreact with rodent subunits (Giancotti *et al.*, 1992), were used to detect the endogenous $\alpha_6\beta_4$ integrins of 804G cells.

Figure 2 shows the SDS-PAGE analysis of the immunoprecipitates. As expected, the 3E1 mAb did not immunoprecipitate any material from control cells Z, although these cells express an endogenous rat $\alpha_6\beta_4$ integrin recognized by the β_4 cytoplasmic peptide antibody. In contrast, immunoprecipitation with the 3E1 antibody yielded a complex of the intact human β_4 subunit with endogenous α_6 from clone A cells and a complex of the 95-kDa tail-less β_4 with endogenous α_6 from clone B cells. The data suggests that the both these recombinant β_4 polypeptides are able to form hybrid heterodimers with endogenous rat α_6. As predicted, the β_4 cytoplasmic peptide serum did not recognize the tail-less β_4 subunit expressed by clone B cells, because this molecule does not contain the relevant C-terminal epitope. Immunoprecipitation analysis of clone F indicated that these cells express a 140-kDa molecule reacting with both the anti-c-myc Ab-1 antibody and the β_4 cytoplasmic peptide serum. No endogenous α_6 subunit could be precipitated in association with this recombinant polypeptide by using the Ab-1 antibody and, conversely, the 140-kDa protein could not be coimmunoprecipitated with endogenous α_6 by the anti-α_6 cytoplasmic peptide antibody. This result indicates that this recombinant β_4 molecule, lacking almost the entire extracellular domain, can not form stable heterodimers with endogenous α_6. Thus, the data suggests that the full-length and the tail-less human β_4 subunits associate with endogenous α_6, but the head-less molecule does not.

Surface labeling and immunoprecipitation were used to study expression of the recombinant β_4 molecules at the cell surface. As shown in Figure 3, the wild-type

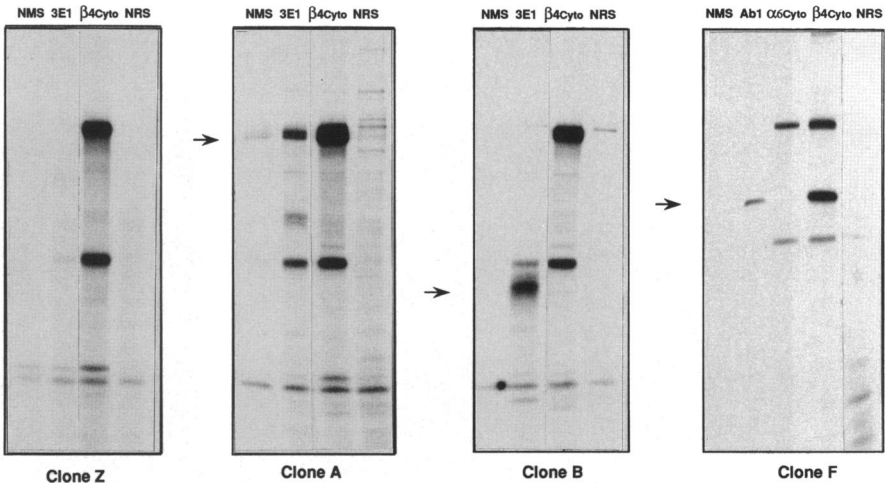

Figure 2. Immunoprecipitation analysis of metabolically labeled clones Z, A, B, and F. Cells from the indicated clones were labeled with ^{35}S-methionine/cysteine and extracted with detergent buffer. The extracts of clones Z, A, and B were immunoprecipitated with normal mouse serum (NMS), the 3E1 mAb (3E1), the β_4 cytoplasmic peptide antiserum (β_4 cyto), and normal rabbit serum (NRS). The extract of clone F was immunoprecipitated with normal mouse serum, the Ab-1 mAb (Ab-1), the α_6 cytoplasmic peptide antibody (α_6 cyto), the β_4 cytoplasmic peptide antiserum, and normal rabbit serum. Samples were separated on a 7% SDS-PAGE gel under reducing conditions. Arrows point to the bands corresponding to each one of the immunoprecipitated recombinant β_4 polypeptides.

and tail-less human β_4 subunits recognized by the 3E1 antibody (clones A and B) and the truncated molecule lacking the extracellular domain reacting with the anti-c-myc Ab-1 antibody (clone F) could be labeled at the cell surface and recovered by immunoprecipitation. Iodination at the cell surface of the recombinant β_4 molecule containing the c-myc epitope tag was less efficient than that of the other recombinant β_4 molecules. This is probably because of the paucity of iodinatable tyrosines in the extracellular segment of this molecule, because immunofluorescent staining of unpermeabilized cells indicated that it is expressed at the cell surface at a level similar to that of the other recombinant β_4 molecules (unpublished data). In accordance with previous results indicating that the α_6 molecule is poorly labeled by iodination at the cell surface (Sonnenberg *et al.*, 1990b), the α_6 subunits associated with endogenous β_4 and with the polypeptides encoded by constructs A and B could be clearly detected only upon a longer exposure of the gel. These results indicate that all the recombinant β_4 polypeptides reach the cell surface: the wild-type and the tail-less β_4 subunits, in association with endogenous α_6, and the truncated β_4 molecule lacking the extracellular domain, by itself.

We next examined the subcellular localization of the recombinant β_4 molecules by performing immunofluorescence experiments. The control clone Z and the β_4-expressing clone A were fixed with methanol and subjected to double staining with the 3E1 mAb and a BP patient serum that reacts with the hemidesmosomal marker BPA 230. The results were analyzed with confocal microscopy. As shown in Figure 4, the 3E1 antibody produced negligible labeling of control cells Z (Figure 4a) but reacted with distinct structures of clone A cells. These structures appeared as dots and patches and were confined to the optical plane corresponding to the basal cell surface (Figure 4c). A few small circular areas of the ventral membrane were excluded by the staining, so that the overall pattern had a swiss-cheese-like appearance. This pattern is typical of the hemidesmosome-like adhesions formed by 804G cells (Jones *et al.*, 1991). The BP serum produced a swiss cheese-like pattern of staining in both clone Z and A (Figure 4, b and d). In clone A, the staining generated by the 3E1 mAb and by the BP serum were identical and superimposable, indicating that the wild-type recombinant β_4 molecule colocalizes with BPA 230 in hemidesmosome-like adhesions (Figure 4, c and d). Furthermore, confocal microscopic analysis of vertical sections confirmed that the 3E1 staining of clone A was restricted

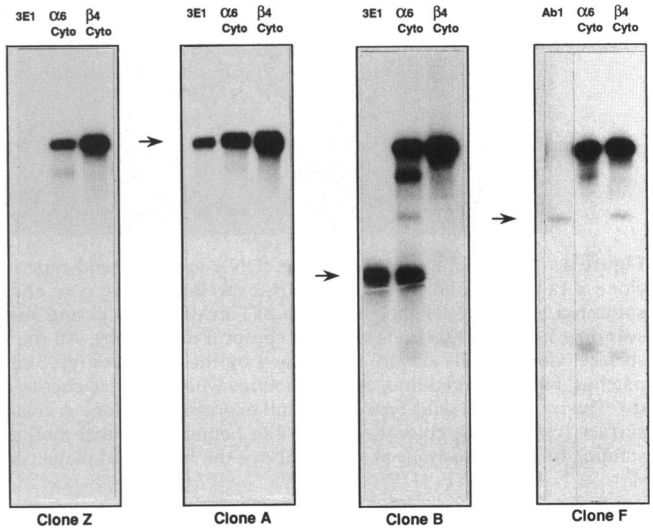

Figure 3. Immunoprecipitation analysis of surface-labeled clones Z, A, B, and F. Cells from the indicated clones were subjected to surface labeling with ^{125}I and extracted with detergent buffer. Cellular extracts were immunoprecipitated with the 3E1 mAb (3E1) or the Ab-1 mAb (Ab-1), the α_6 cytoplasmic peptide antibody (α_6 cyto), and the β_4 cytoplasmic peptide antiserum (β_4 cyto). Samples were separated on a 7% SDS-PAGE gel under reducing conditions. Arrows point to the bands corresponding to each one of the immunoprecipitated recombinant β_4 polypeptides.

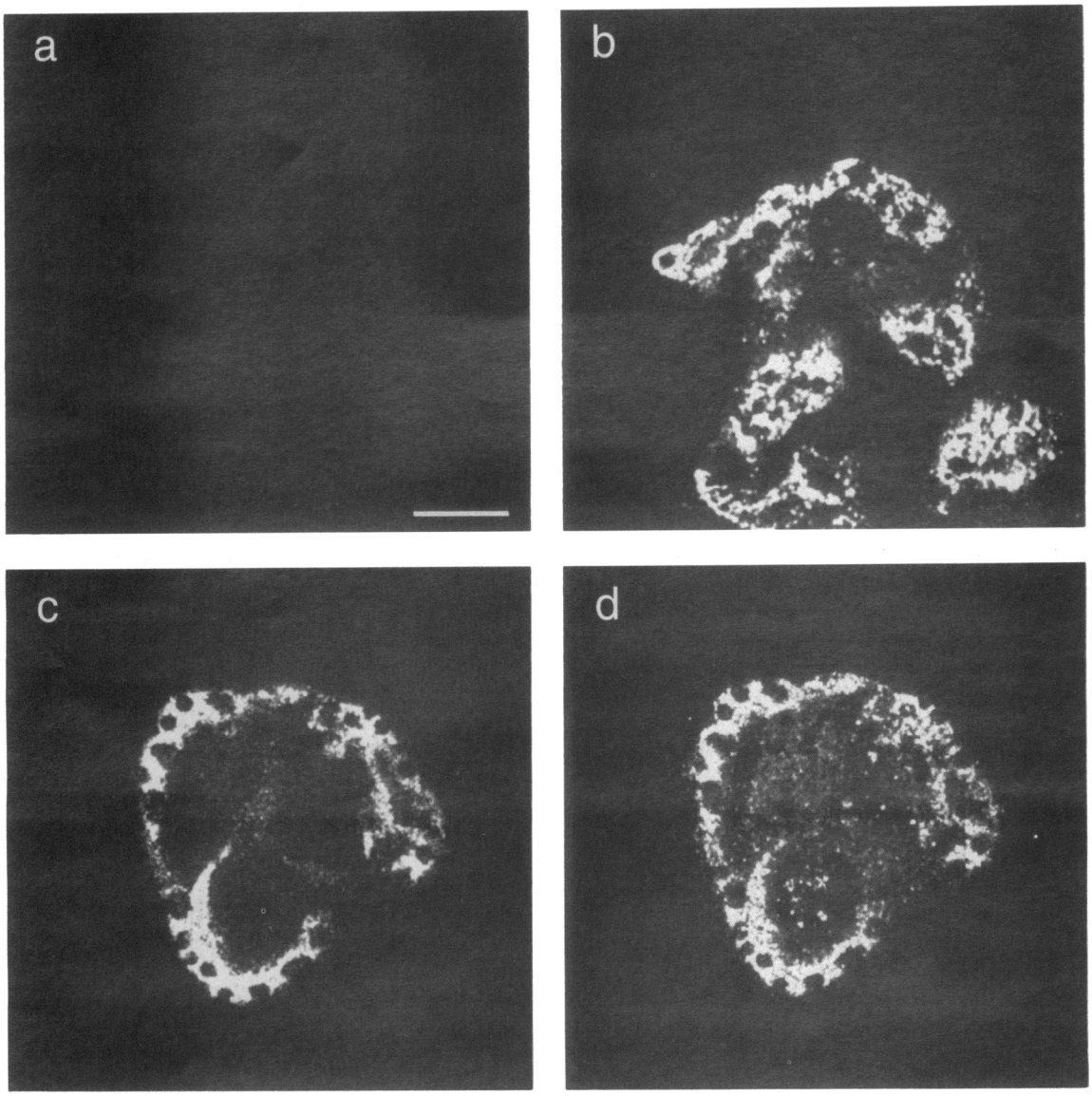

Figure 4. Immunolocalization of the cDNA-encoded wild-type human β_4 subunit in hemidesmosome-like adhesions. Cells of the control clone Z (a and b) and the wild-type β_4-expressing clone A (c and d) were cultured on glass coverslips for 24 h, permeabilized, fixed, and subjected to double labeling with the 3E1 mAb (a and c) and the BP patient serum (b and d). The fluorescent samples were analyzed by scanning horizontal sections with the confocal microscope. All the sections shown were taken at the optical plane corresponding to the basal surface. Control cells Z were not stained by the 3E1 mAb (a). Staining generated by the BP serum in clone Z was concentrated in dots and patches, which merged into larger structures with the swiss-cheese-like appearance typical of the hemidesmosome-like adhesions of 804G cells (b). The transfected wild-type β_4 subunit expressed by clone A cells was concentrated in swiss-cheese-like structures restricted to the basal cell surface (c), where it colocalized with the hemidesmosomal marker BP antigen (d). Both the 3E1 mAb and the BP serum did not give any staining in optical sections at >1 μm above the horizontal plane corresponding to the ventral cell surface. Bar, 7.5 μm.

to the basal cell surface (Figure 5a). Taken together, these results indicate that the recombinant human β_4 subunit is regularly incorporated into the hemidesmosome-like adhesions of 804G cells.

The clone B that expresses a truncated tail-less β_4 subunit was analyzed next. As shown in Figure 6b, the 3E1 antibody generated a diffuse staining of the plasma membrane and some intracellular vescicles in clone B cells. Confocal microscopic analysis of vertical sections indicated no concentration of the staining at the basal cell surface (Figure 5b). These findings indicate that the tail-less β_4 is not incorporated into hemidesmosomes and suggest that the cytoplasmic portion of β_4 is required for correct targeting of $\alpha_6\beta_4$ to these structures.

Although the 3E1 antibody generated some intracellular staining in clone B cells, it did not in clone A cells

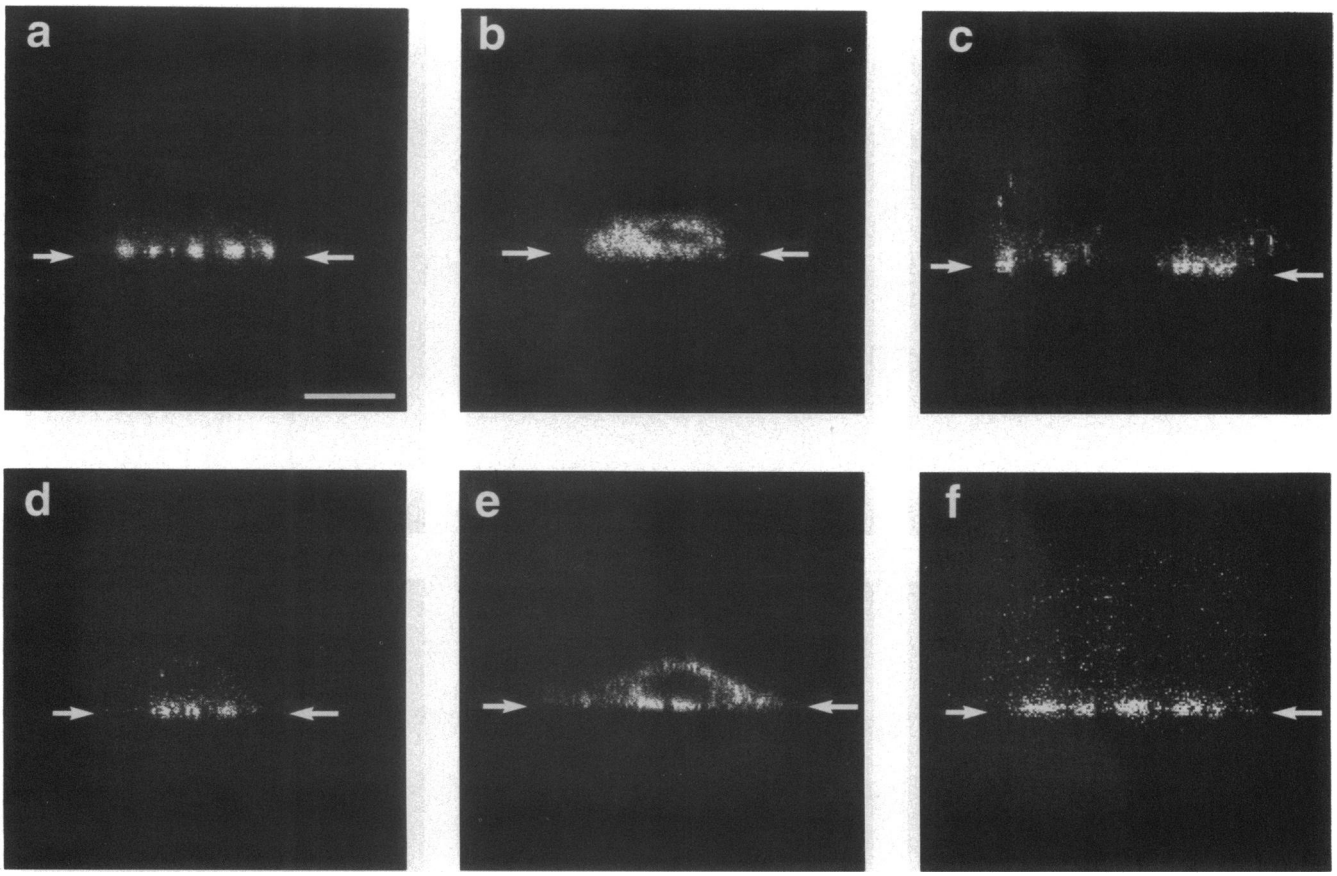

Figure 5. Analysis of the subcellular localization of recombinant β_4 molecules by confocal laser scanning microscopy of vertical sections. Cells of the clones A, B, F, D, C, and E were fixed and permeabilized with methanol and then stained with the 3E1 mAb. Vertical sections of individual cells are shown. The recombinant β_4 molecules expressed by cells of the clones A (a), F (c), D (d), and E (f) are concentrated in patches confined to the basal cell surface (indicated by the arrows), whereas the cDNA-encoded β_4 polypeptides expressed by cells of the clones B (b) and C (e) are diffusely distributed. Bar, 7.5 μm.

(Figures 5 and 6, a and b). This observation suggests that the tailless β_4 molecule is internalized at a faster rate than wild-type β_4, perhaps because the $\alpha_6\beta_4$ integrins that are not incorporated in hemidesmosomes are internalized at a higher rate. Alternatively, the tail-less β_4 subunit may be transported to the cell surface at a reduced rate, possibly because intracellular association with cytoskeletal elements is required for optimal export of $\alpha_6\beta_4$ integrins. FACS analysis, however, indicated that the clones expressing β_4 molecules with cytoplasmic domain deletions had similar levels of surface expression (Figure 7). The presence of similar amounts of recombinant β_4 molecules at the surface of clone A and B cells suggests that the inability of the tail-less β_4 subunit to be incorporated in hemidesmosome-like adhesions is because of defective cytoskeletal interactions rather than defective transport to the cell surface or an increased rate of internalization.

Clone F, expressing a β_4 molecule lacking most of the extracellular sequences, was examined next. Immunofluorescent staining with the anti-c-myc Ab-1 produced a swiss cheese-like pattern of staining (Figure 6c), and confocal microscopic analysis of vertical sections indicated that the labeling was restricted to the basal cell surface (Figure 5c). Double labeling experiments showed that the staining generated by the Ab-1 antibody coincided with that generated by BP serum (Figure 8, a and b). Therefore, the β_4 subunit lacking the extracellular domain, although unable to combine with endogenous α_6 and presumably unable to bind extracellular ligand, is correctly assembled into hemidesmosome-like adhesions. These results are consistent with the hypothesis that the cytoplasmic domain of β_4 contains sequence information that is sufficient for incorporation of the molecule into hemidesmosomes.

A 303-Amino Acid Region of β_4 Cytoplasmic Domain Is Involved in Cytoskeletal Interactions

To test the hypothesis that the incorporation of $\alpha_6\beta_4$ in hemidesmosomes is mediated by a specific region of β_4

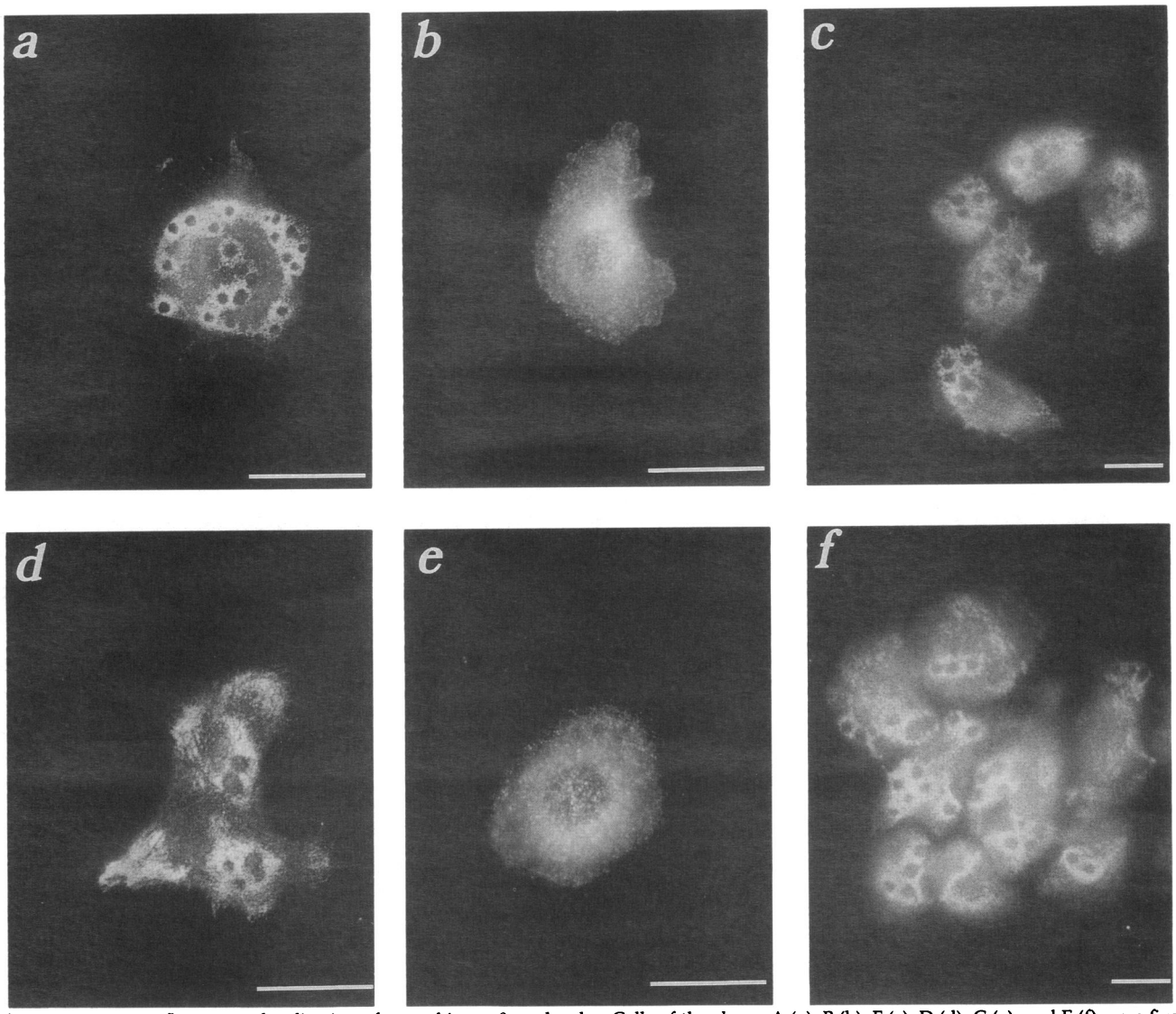

Figure 6. Immunofluorescent localization of recombinant β_4 molecules. Cells of the clones A (a), B (b), F (c), D (d), C (e), and E (f) were fixed and permeabilized with methanol and then stained with the 3E1 mAb. Samples were analyzed by epifluorescence microscopy. The recombinant β_4 molecules expressed by cells of the clones A (a), F (c), D (d), and E (f) are concentrated in swiss-cheese-like structures confined to the basal cell surface, whereas those expressed by cells of the clones B (b) and C (e) are diffusely distributed at the cell surface and in some intracellular vescicles. Bar, 10 μm.

cytoplasmic domain, we analyzed 804G cells expressing a recombinant β_4 molecule lacking the C-terminal pair of type III repeats (clone D), a truncated β_4 subunit lacking both pairs of type III repeats (clone C), and a mutant form of β_4 with an internal deletion of the N-terminal half of the cytoplasmic domain (clone E) (Figure 1). Detergent extracts of metabolically labeled cells were immunoprecipitated with various antibodies to determine if the recombinant β_4 molecules were able to combine with endogenous α_6 subunit (Figure 9). Immunoprecipitation with the 3E1 antibody yielded recombinant β_4 molecules of the expected size in association with endogenous α_6. As expected, the internally deleted molecule, which has an intact C-terminus, was precipitated by the β_4 cytoplasmic peptide antibodies (clone E), whereas the other two mutant forms were not (clones D and C). The results of these experiments indicate that the three recombinant β_4 molecules are expressed properly and can associate with endogenous α subunit.

Surface labeling and immunoprecipitation experiments demonstrated that the three mutant forms of β_4, expressed by clones D, C, and E, could be labeled at the cell surface and recovered by immunoprecipitation with the 3E1 mAb, indicating that they were exported to the cell membrane (Figure 10). FACS analysis with the 3E1 antibody indicated that the three recombinant

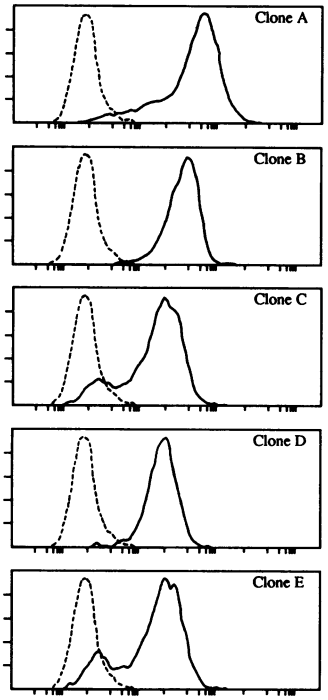

Figure 7. FACS analysis of 804G cells expressing wild-type and cytoplasmic domain mutant β_4 molecules. Intact live cells of the control clone Z and the β_4-expressing clones A, B, D, C, and E were incubated with the 3E1 mAb followed by fluorescein isothiocyanate-labeled goat anti mouse IgG. Intensity of fluorescence in individual cells was measured by flow cytometry. Relative cell numbers are plotted on the ordinate and the logarithm of fluorescence intensity on the abscissa. In each panel the intensity of 3E1 staining of the indicated clones (——) is compared to that of the control clone Z (– – –).

β_4 subunits were expressed at the cell surface at approximately equal levels (Figure 7, clones D, C, and E).

The subcellular localization of the three β_4 mutants was examined by performing immunofluorescence experiments with the 3E1 antibody (Figure 6). The results of this analysis indicated that the recombinant molecule lacking the most C-terminal pair of type III repeats expressed by clone D and the mutant with the internal deletion expressed by clone E were concentrated in structures with a swiss cheese-like appearance (Figure 6, d and f), but the truncated form lacking both pairs of type III repeats expressed by clone C remained diffusely distributed at the cell surface and in some intracellular vescicles (Figure 6e). The intracellular staining observed in clone C cells was similar to that of clone B cells suggesting that the truncated β_4 molecule lacking both pairs of type III repeats may be transported to the cell surface less efficiently or may be internalized at a higher rate than wild-type β_4. Confocal microscopic analysis of vertical sections indicated that the staining produced by the 3E1 antibody was restricted to the ventral membrane in cells of the clones D and E (Figure 5, d and f) but was diffusely distributed in clone C cells (Figure 5e). Finally, double staining with the 3E1 mAb and BP serum showed that the recombinant β_4 polypeptides expressed by cells of the clones D and E codistributed with BPA 230 (Figure 8, c–f).

These results indicate that the recombinant β_4 molecule lacking the C-terminal pair of type III repeats and the internally deleted mutant β_4 polypeptide are recruited in the hemidesmosome-like adhesions of 804G cells, but the truncated β_4 subunit lacking both pairs of type III modules is not. Therefore, the deletion of the N-terminal half of the cytoplasmic domain or the truncation of the most C-terminal pair of type III repeats do not affect the ability of β_4 subunit to be recruited in the hemidesmosome-like adhesions. In contrast, the elimination of both pairs of type III repeats prevents the incorporation of the receptor in these structures. Taken together, these results suggest that assembly of the $\alpha_6\beta_4$ integrin in hemidesmosomes is mediated by a 303–amino acid region of β_4 comprising the first pair of type III repeats and the segment between the second and third repeats.

DISCUSSION

We have examined the function of the large cytoplasmic domain of the β_4 subunit, which distinguishes the $\alpha_6\beta_4$ integrin from all the other known integrins, by means of gene transfer techniques. The results indicate that a truncated β_4 molecule lacking almost the entire cytoplasmic domain combines with endogenous α_6 subunit and is transported to the cell surface but is not assembled in the hemidesmosome-like adhesions of 804G cells. In contrast, a mutant β_4 subunit lacking almost the entire extracellular domain does not associate with endogenous α_6 but is transported to the cell surface and is incorporated into hemidesmosome-like adhesions. The data suggests that the intracellular portion of β_4 interacts with cytoskeletal elements of hemidesmosomes and that this interaction is required and may be sufficient for incorporation of $\alpha_6\beta_4$ in hemidesmosomes. This finding offers a molecular basis to the observation that $\alpha_6\beta_4$ is found in vivo enriched at hemidesmosomes and not at adhesion plaques as most of the other integrins.

The observation that a mutant β_4 molecule lacking sequences implicated in ligand binding and in association with the α_6 subunit is correctly targeted to hemidesmosomes is not inconsistent with the hypothesis that binding to extracellular ligand is an important step in the assembly of adhesive junctions. The transfection experiments described in this study do not measure the ability of the recombinant β_4 polypeptides to mediate the de novo assembly of hemidesmosomes but rather their capacity to be incorporated, along with wild-type endogenous receptors, in these adhesive structures. Our results suggest that this process may be entirely mediated by an interaction of the cytoplasmic domain of β_4 with intracellular component(s) of hemidesmosomes. In this regard, the behavior of the recombinant β_4 molecule lacking most extracellular sequences is similar to that of two recently described mutant β_1 subunits that localize to adhesion plaques even if unable to bind to extracellular ligand (La Flamme *et al.*, 1992; Takada *et al.*, 1992).

Despite the ability of the recombinant β_4 polypeptide lacking most of the extracellular sequences to localize to hemidesmosome-like adhesions, it is likely that

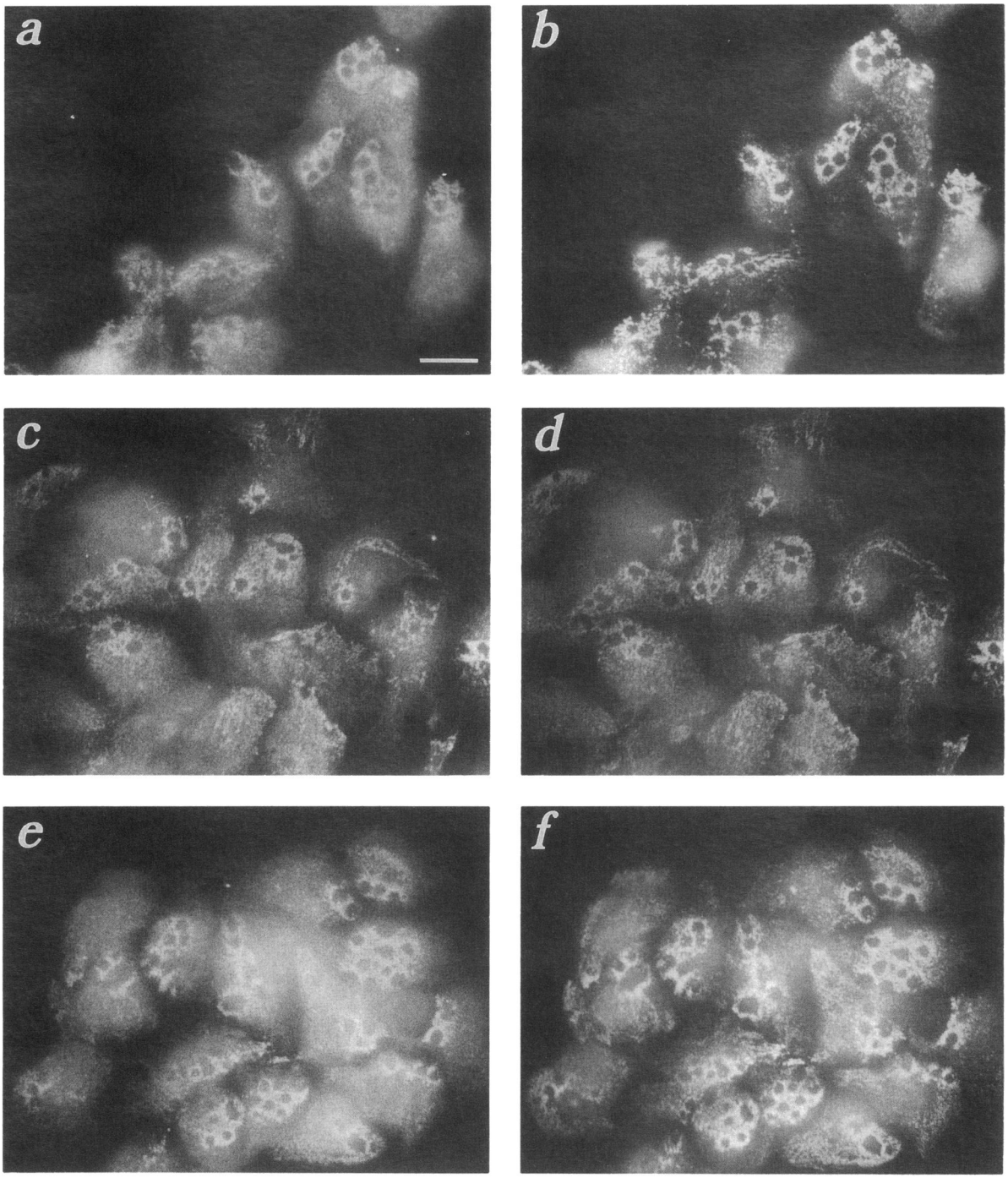

Figure 8. Colocalization of the recombinant β_4 molecules expressed by clones F, D, and E with the BP antigen. Cells of the clones F (a and b), D (c and d), and E (e and f) were cultured on glass coverslips for 24 h, permeabilized, fixed, and subjected to double labeling with the 3E1 mAb (a, c, and e) and BP patient serum (b, d, and f). Samples were analyzed by epifluorescence microscopy. Microphotographs were taken of the focal plane corresponding to the ventral cell surface. The two types of antibodies produced an identical and superimposable swiss-cheese-like pattern of staining at the basal surface of clone F, D, and E cells. Bar, 10 μm.

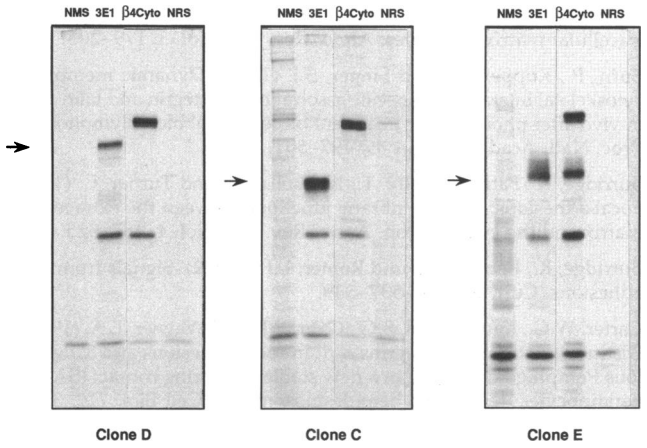

Figure 9. Immunoprecipitation analysis of metabolically labeled clones D, C, and E. Cells from the indicated clones were labeled with ^{35}S-methionine/cysteine and extracted with detergent buffer. Cellular extracts were immunoprecipitated with normal mouse serum (NMS), the 3E1 mAb (3E1), the β_4 cytoplasmic peptide antiserum (β_4 cyto), and normal rabbit serum (NRS). Samples were separated on a 7% SDS-PAGE gel under reducing conditions. Arrows point to the bands corresponding to each one of the immunoprecipitated recombinant β_4 polypeptides.

binding to extracellular ligand plays a role in the de novo assembly of hemidesmosomes. The results of previous experiments have indicated that antibodies directed to the extracellular portion of $\alpha_6\beta_4$ interferes with the assembly as well as the adhesive function of hemidesmosomes (Jones *et al.*, 1991; Kurpakus *et al.*, 1991b). Together with our current results, this suggests a model in which the $\alpha_6\beta_4$ integrin, upon binding to extracellular ligand, clusters within the plane of the plasma membrane and triggers the assembly of the hemidesmosomal plaque. If this model is correct, the mechanism of assembly of hemidesmosomes would resemble that of adhesion plaques.

The analysis of the subcellular localization of recombinant β_4 subunits carrying various cytoplasmic domain deletions indicate that the sequences responsible for the interaction of $\alpha_6\beta_4$ with hemidesmosomes reside in a 303–amino acid region of β_4. This region comprises the first two type III repeats and the segment between the second and third repeat. Type III fibronectin homology modules are found not only in extracellular matrix molecules and cell surface receptors (Patthy, 1990) but also in intracellular polypeptides, such as the cytoplasmic domain of β_4 and a number of myofibrillar elements (Benian *et al.*, 1989; Einheber and Fishman, 1990; Labeit *et al.*, 1990; Olson *et al.*, 1990). In fibronectin the tenth type III repeat contains the RGD sequence that interacts with the $\alpha_5\beta_1$ integrin (Pytela *et al.*, 1985), and the first repeat comprises a fibronectin self-assembly site (Morla and Ruoslahti, 1992). Twitchin and titin, two large muscle proteins almost entirely composed of type III repeats, form filaments in vivo (Benian *et al.*, 1989). Because at least some of the type III repeats are involved in protein-protein interactions, it is possible that the first pair of type III repeats of β_4 plays a similar role by associating with cytoskeletal elements. Alternatively, it is possible that this function is performed by the amino acid segment between the second and third type III repeats.

The region of β_4 tail identified in this study appears to be the target of multiple potential regulatory mechanisms. Two alternative splicing variant forms of β_4 are known that contain extra sequences of either 53 or 70 amino acids inserted in the segment between the second and third type III repeats (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990). Because these insertions interrupt the region of the β_4 cytoplasmic domain involved in interaction with the cytoskeleton, it is tempting to speculate that the two variant forms of β_4 differ from the canonical form in their ability to interact with cytoskeletal elements. In addition, we have previously reported that the cytoplasmic domain of β_4 may be cleaved in vivo by an intracellular protease, perhaps calpain, at a site that is 70 kDa away from the C-terminus of the molecule (Giancotti *et al.*, 1992). On the basis of our current results, it is predicted that such a cleavage would result in loss of interaction of the in-

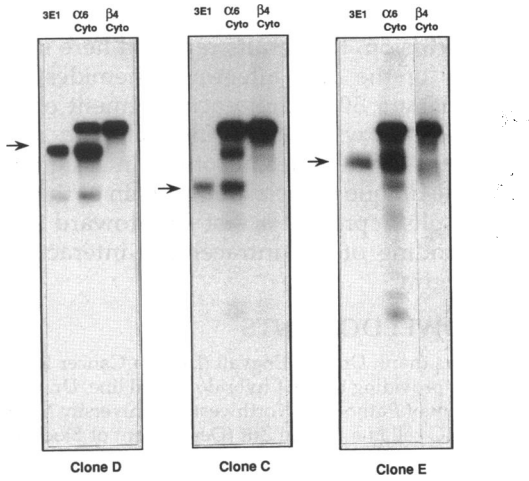

Figure 10. Immunoprecipitation analysis of surface-labeled clones D, C, and E. Cells from the indicated clones were labeled with ^{125}I at the cell surface and extracted with detergent buffer. Cellular extracts were immunoprecipitated with the 3E1 mAb (3E1), the α_6 cytoplasmic peptide antibody (α_6 cyto), and the β_4 cytoplasmic peptide antiserum (β_4 cyto). Samples were separated on a 7% SDS-PAGE gel under reducing conditions. Arrows point to the bands corresponding to each one of the immunoprecipitated recombinant β_4 polypeptides. The band below β_4 in the material immunoprecipitated by the α_6 cyto antibody from clone C cells is probably the largest of the proteolytic products of endogenous β_4 generated by calpain (Giancotti *et al.*, 1992). This interpretation is consistent with the fact that it is not detected in the material precipitated by the β_4 cyto serum. Note that a fragment of similar size is also visible, although less clearly, in the samples immunoprecipitated by the α_6 cyto antibody from the other clones (see Figure 3).

tegrin with the cytoskeleton. Thus, both alternative splicing and proteolytic processing may provide mechanisms for modulating the activities of $\alpha_6\beta_4$.

The identity of the intracellular molecule(s) interacting with the cytoplasmic domain of β_4 is not presently known. The ultrastructure of hemidesmosomes resemble that of one-half of a desmosome, yet hemidesmosomes and desmosomes do not have any known molecular component in common (Schwarz *et al.*, 1990; Legan *et al.*, 1992). In addition to $\alpha_6\beta_4$, the hemidesmosomes contain another transmembrane element, the Bullous Pemphigoid Antigen of 180 kDa. This protein has extracellular collagen-like sequences and overall organization similar to that of type II macrophage scavenger receptor (Giudice *et al.*, 1992). All the other known components of hemidesmosomes, BPA 230 (Westgate *et al.*, 1985; Klatte *et al.*, 1989; Sawamura *et al.*, 1991; Tanaka *et al.*, 1991), HD-1 (Hieda *et al.*, 1992), and 6A5 antigen (Kurpakus *et al.*, 1991a) are contained in the inner hemidesmosomal plaque at some distance from the plasma membrane. Electron microscopic analyses have shown that the inner hemidesmosomal plaque is connected to the plasma membrane by short filaments (Schwarz *et al.*, 1990; Legan *et al.*, 1992). Because no known hemidesmosomal component has been localized to these structures, it is likely that they contain novel molecular components and the cytoplasmic portion of β_4 subunit may interact with one or more of them.

In conclusion, the results reported here indicate that assembly of the $\alpha_6\beta_4$ integrin in hemidesmosomes is mediated by a 303–amino acid segment of the β_4 cytoplasmic tail, which may interact with intracellular components of hemidesmosomes. In assigning a function to the unique cytoplasmic domain of the β_4 subunit, these results represent a first step toward a molecular understanding of the intracellular interactions of the $\alpha_6\beta_4$ integrin.

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