1998-08-27

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Journal of Cell Science 111, 2717-2728 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS4526

Endothelial cells assemble two distinct $\alpha_6\beta_4$-containing vimentin-associated structures: roles for ligand binding and the β_4 cytoplasmic tail

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Accepted 7 July; published on WWW 27 August 1998

SUMMARY

The $\alpha_6\beta_4$ laminin binding integrin functions in the assembly of type I hemidesmosomes, which are specialized cell-matrix adhesion sites found in stratified epithelial cells. Although endothelial cells do not express all the components of type I hemidesmosomes, endothelial cells can express the $\alpha_6\beta_4$ integrin. Because endothelial cells lose expression of $\alpha_6\beta_4$ in culture, we expressed recombinant $\alpha_6\beta_4$ in the dermal microvascular endothelial cell line, HMEC-1, to test whether endothelial cells can assemble adhesion structures containing $\alpha_6\beta_4$. Using immunofluorescence microscopy, we found that recombinant $\alpha_6\beta_4$ concentrates specifically in a novel fibrillar structure on the basal surface of endothelial cells in the absence of an exogenous laminin substrate. This localization is regulated by an intracellular mechanism, because the β_4 cytoplasmic domain is sufficient to direct a reporter domain (IL-2R) to the fibrillar structures independently of recombinant $\alpha_6\beta_4$. In addition, this IL-2R-β_4 chimera is sufficient to recruit the intermediate filament-associated protein HD1/plectin to these fibrillar structures and this also occurs in the absence of recombinant $\alpha_6\beta_4$. The fibrillar localization pattern, as well as the recruitment of HD1/plectin, requires the first and second fibronectin type III repeats and the connecting segment of the β_4 tail. In addition, when endothelial cells are provided a laminin 5-rich matrix, recombinant $\alpha_6\beta_4$ redistributes from the fibrillar structure to type I hemidesmosome-like structures. The β_4 cytoplasmic domain can also direct a reporter domain to these type I hemidesmosome-like structures; however, this process is dependent upon the expression of recombinant $\alpha_6\beta_4$. Biochemical analysis indicates that both the fibrillar and the type I hemidesmosome-like structures are associated with the vimentin intermediate filament cytoskeleton. Thus, the results illustrate that endothelial cells have the essential components necessary to assemble at least two distinct $\alpha_6\beta_4$-containing and vimentin-associated structures on their basal surface and that the β_4 cytoplasmic tail and the availability of specific $\alpha_6\beta_4$ ligands regulate receptor localization to these structures.

Key words: $\alpha_6\beta_4$, β cytoplasmic domain, HD1/plectin, Endothelial cell, Vimentin

INTRODUCTION

Integrins are a major family of transmembrane cell-surface receptors used primarily in cell-matrix interactions important for many diverse biological processes, such as embryonic development, wound healing, tumor cell metastasis and tissue differentiation (Adams and Watt, 1993). Integrins are α/β heterodimers, and the specificity of integrins for particular extracellular ligands depends upon which α and β subunits comprise the integrin heterodimer (Hynes, 1992). Integrins generally concentrate in multi-protein, cell-matrix adhesion complexes, where integrin extracellular domains directly interact with matrix proteins such as fibronectin (FN), laminin and collagen, and their intracellular domains interact with the cell's cytoskeletal and signal transduction apparatus (Clark and Brugge, 1995; Yamada and Miyamoto, 1995).

Integrins containing β_1, β_3, and β_5 subunits interact with the microfilament system and localize to adhesion complexes known as focal adhesions (Sastry and Horwitz, 1993). In contrast, the $\alpha_6\beta_4$ integrin is thought to interact primarily with the keratin intermediate filament system because of its localization in type I hemidesmosomes in stratified epithelial cells (Stepp et al., 1990), although it can also interact with the actin cytoskeleton in migrating cells (Rabinovitz and Mercurio, 1997; Shaw et al., 1997). Type I hemidesmosomes are specialized cell-substrate junctions that mediate the adhesion of stratified epithelial cells to the underlying laminin 5-rich basement membrane. Ultrastructurally, type I hemidesmosomes are described as regions of the plasma membrane in close contact with the laminin 5-containing basement membrane, characterized by distinct electron-dense cytoplasmic plaques where keratin intermediate filaments are anchored (Green and Jones, 1996; Jones et al., 1994). In addition to $\alpha_6\beta_4$, several other components of type I hemidesmosomes have been identified, including the transmembrane protein BP180, and several intracellular plaque

proteins, including IFAP300, HD1, plectin and BP230. Although the role of these proteins in hemidesmosome formation is not yet known, IFAP300, HD1, plectin and BP230 are related proteins (Green and Jones, 1996), and are all likely to have the ability to bind intermediate filaments (Skalli et al., 1994; Foisner et al., 1988; Guo et al., 1995). Since current evidence indicates that HD1 and plectin are the same protein, we will refer to them as HD1/plectin (McLean et al., 1996; Smith et al., 1996; Gache et al., 1996).

Although type I hemidesmosomes are primarily limited to basal stratified epithelial cells, HD1/plectin and $\alpha_6\beta_4$ are found in cell types such as endothelial cells, that do not form ultrastructurally defined type I hemidesmosomes. In these cells, it has been suggested that HD1/plectin and $\alpha_6\beta_4$ are assembled into less organized multiprotein complexes, for which the term type II hemidesmosomes has been given to distinguish them from the 'classical' type I hemidesmosome of basal epidermal cells (Uematsu et al., 1994). Type II hemidesmosomes are defined as structures having $\alpha_6\beta_4$ and HD1/plectin, but lacking BP180 and BP230, as well as the electron-dense intracellular plaques (Hieda et al., 1992). In the mammary gland epithelial cell line, BMGE-H, $\alpha_6\beta_4$ and HD1/plectin have been observed in structures which flank vinculin-containing focal adhesions (Uematsu et al., 1994). These structures have also been referred to as type II hemidesmosomes. The extracellular matrix protein associated with these structures remains unidentified. Interestingly, similar structures can be identified in fibroblastic cells expressing recombinant $\alpha_6\beta_4$ (Sanchez-Aparicio et al., 1997; Niessen et al., 1997a).

The function of $\alpha_6\beta_4$ in endothelial cells is currently unknown. However, since endothelial cells express $\alpha_6\beta_4$ and HD1/plectin in situ (Hieda et al., 1992), $\alpha_6\beta_4$ may function in endothelial cells by connecting the vimentin intermediate filament cytoskeleton to the plasma membrane at sites of cell-matrix adhesion. Because endothelial cells lose expression of $\alpha_6\beta_4$ in culture (Hieda et al., 1992), we expressed recombinant $\alpha_6\beta_4$ in endothelial cells and asked whether recombinant $\alpha_6\beta_4$ can localize to adhesion structures and whether these structures are distinct from those observed in epithelial and fibroblastic cells in culture. We found that recombinant $\alpha_6\beta_4$ concentrates specifically in a novel fibrillar adhesion-like structure in the absence of exogenous ligand, and that receptor localization to this structure occurs by an intracellular mechanism dependent on the β_4 cytoplasmic domain. Additionally, we demonstrate that the β_4 cytoplasmic domain is sufficient to recruit HD1/plectin to these fibrillar structures and that these structures are associated with vimentin intermediate filaments. We also show that laminin 5 causes the redistribution of $\alpha_6\beta_4$ from the fibrillar structures to type I hemidesmosome-like structures, which are also associated with vimentin intermediate filaments. Thus, the localization of $\alpha_6\beta_4$ in distinct vimentin-associated structures in endothelial cells can be regulated by the availability of specific ligands for $\alpha_6\beta_4$.

MATERIALS AND METHODS

Construction of chimeric receptors

To generate a plasmid encoding a chimeric receptor containing the interleukin-2 receptor extracellular and transmembrane reporter domains connected to the β_4 subunit intracellular domain (IL-2R-β_4 chimera), the oligonucleotides 5′-AGCTTGCC**GTCTAC**GCTGGGC-3′ and 5′-TCGAGCCCAGC**GTAGAC**GGCA-3′, encoding an *Acc*I, site were hybridized and then inserted at the *Hin*dIII/*Xho*I sites of the previously described plasmid encoding the extracellular and transmembrane domains of the IL-2 receptor (LaFlamme et al., 1992). The cDNA, β_4-1, a kind gift of Dr Shintaro Suzuki (University of Southern California School of Medicine) was subcloned into the *Eco*RI site of pUC19, generating the plasmid pUC19-β_4-1. The *Acc*I/*Xba*I fragment of pUC19-β_4-1, encoding amino acids 854-1752 (Suzuki and Naitoh, 1990), was then inserted into the newly generated *Acc*I site and existing *Xba*I site, generating the plasmid CMV-IL2R-β_4. To construct β_4-Δ1315-1752, containing a truncation after the second FN Type III repeat, a stop codon was inserted at amino acid 1315 by hybridizing the following oligonucleotides: 5′-CGCGCAACGGGGCCGGC-TGGGGGCCTGAGCGGGAGGCCATCATC**TGA**TAGT-3′ and 5′-CTAGACTA**TCA**GATGATGGCCTCCCGCTCAGGCCCCCAGCC-GGCCCCGTTG-3′ and ligating them into the plasmid CMV-IL-2R-β_4 in place of the *Bss*HII/*Xba*I fragment of the full-length β_4 tail. To construct β_4-Δ1488-1752, containing a truncation after the connecting segment (CS), a stop codon was inserted at amino acid 1488 by hybridizing the oligonucleotides 5′-GGCCGCTG**TGA**TAGCCCG-TGT-3′ and 5′-CTAGACACGGGCTA**TCA**CAGC-3′ and then ligating them into plasmid CMV-IL2R-β_4 in place of the *Not*I/*Xba*I fragment of the full-length β_4 tail. β_4-Δ1315-1456, lacking the CS, was generated using the polymerase chain reaction (PCR). Initially, two PCR products were generated using the plasmid CMV-IL-2R-β_4 as a template and the following primers: Reaction (1) NH_2-terminal primer 1, 5′-TGAGATCACAGCCTA-3′, which hybridized upstream of the *Bss*HII site, and COOH-terminal primer 2, 5′-CGGGTGGGC-GTGTCGGGCACGATGATGGCCTCCCGCTCAG-3′. Reaction (2) NH_2-terminal primer 3, 5′-CTGAGCGGGAGGCCATCATCGT-GCCCGACACGCCCACCCG-3′ and COOH-terminal primer 4, 5′-GTTGGGCAGGAGGTC-3′, which hybridized downstream of the *Not*I site. The products of these reactions were combined and used as a template for PCR using the primers 1 and 4 listed above. The product of this reaction was digested with *Bss*HII and *Not*I and then ligated into CMV-IL-2R-β_4 in place of the *Bss*HII/*Not*I fragment of the full-length β_4 tail. The β_4-1315-1456 mutant, encoding only the CS, and β_4-1218-1456 mutant encoding the second FN type III repeat and the CS, were also generated using PCR. The plasmid CMV-IL-2R-β_4 and the primer 5′-GAGTCTCTCGAG**TCA**ACCAGCAGTCAGGCG-3′, were used for both reactions and the primer 5′-AGGGACA-AGCTTACCCTGGCCACCCAGCCC-3′ was used to generate β_4-1315-1456 and the primer 5′-AGGGACAAGCTTGTGCCCAG-CGAGCCAGGG-3′ was used to generate β_4 1218-1456. Each product was then digested with *Xho*I and *Hin*dIII and was ligated to the CMV-IL-2R plasmid as a *Hin*dIII/*Xho*I fragment. The construction of each mutant was confirmed by DNA sequence analysis (Sambrook et al., 1989). The construction of cDNA clones encoding the full-length α_6A and β_4A subunits were described previously (Clarke et al., 1995; Shaw et al., 1993).

Cells

The human dermal microvascular endothelial cell line, HMEC-1, was a generous gift from Drs Thomas Lawley (Emory University School of Medicine) and Edwin Ades (Centers for Disease Control) (Ades et al., 1992). Early passage primary human dermal microvascular endothelial cells were purchased from VEC Technologies (Rensselaer, NY) and were cultured as recommended by the provider. Normal human foreskin fibroblasts and the adult human skin epithelial cell line, HaCat, were cultured as previously described (LaFlamme et al., 1992; Boukamp et al., 1988). Rat bladder carcinoma 804G cells (Izumi et al., 1981) were a kind gift from Dr Ryoichi Oyasu (Northwestern University).

Immunological reagents

Several commercial antibodies were used: mouse mAb clone VIN-11-5 against vinculin and mouse mAb clone VIM 13.2 against vimentin from Sigma Chemical Co. (St Louis, MO), mouse mAb clone VIM 3B4 against vimentin from Boehringer-Mannheim (Indianapolis, IN), mouse mAb 3E1 against the β_4 subunit from Gibco-BRL (Gaithersburg, MD), rat mAb GoH3 against the α_6 subunit from Pharmingen (San Diego, CA), fluorescein (FITC)-conjugated, as well as unconjugated, mouse mAb K20 against the β_1 subunit from Immuntech (Westbrook, ME), mouse mAbs P1B5 and P1E6 against the human α_3 and α_2 subunits, respectively, from Becton Dickinson (Bedford, MA), FITC-conjugated mouse mAb against the human IL-2 receptor from Accurate Chemical and Scientific Corporation (Westbury, NY), mouse mAb 4E10 specific for the laminin β_1 chain from Gibco BRL, and mouse mAb P3E4 against epiligrin from Chemicon (Temecula, CA). Mouse mAbs C2-9 and B4-6, specific for laminin chains α_3 and γ_2 respectively, were a kind gift from Dr William G. Carter (Carter et al., 1994). Rabbit polyclonal antiserum to a C-terminal peptide of integrin β_4 was a kind gift from Dr Filippo G. Giancotti (Giancotti et al., 1992). Mouse mAb-121 to HD1/plectin was a kind gift from Dr Katsushi Owaribe (Nagoya University, Nagoya, Japan) (Hieda et al., 1992). Rhodamine-conjugated goat anti-mouse IgG was purchased from Boehringer-Mannheim (Indianapolis, IN). Texas Red-conjugated donkey anti-rat IgG and FITC-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch Labs (West Grove, PA).

Transfections, immunofluorescence and flow cytometry

Cells were transiently transfected using electroporation as previously described (LaFlamme et al., 1992). Transfected cells were plated onto glass coverslips in serum-containing medium or onto coverslips coated with a laminin 5-rich matrix, purified as previously described (Langhofer et al., 1993), or with purified laminin 1 (Becton Dickinson) in serum-free medium for 2 hours, and then in medium containing 10% fetal bovine serum overnight. Cells were fixed and immunostained as previously described (LaFlamme et al., 1992). In some cases, cells were extracted for 3 minutes at room temperature with a 'cytoskeletal' extraction buffer containing 1% Tween-40, 0.5% sodium deoxycholate, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF and 20 mM Tris-HCl, pH 7.4, prior to fixation. For dual-staining with two mouse mAbs, the cells were first incubated with the unconjugated mAb followed by rhodamine-conjugated goat anti-mouse IgG. To block sites that may bind mouse IgG, coverslips were blocked for 30 minutes in 100 μg/ml normal mouse IgG (Cappel, Durham NC), washed briefly and then incubated with the second mAb, which was FITC-conjugated. For dual staining with the rat GoH3 mAb and mouse mAb VIM 3B4, a Texas Red-conjugated anti-rat antibody pre-absorbed with mouse IgG and a FITC-conjugated anti-mouse antibody pre-absorbed with rat IgG (Jackson Immunoresearch Labs) were used. The samples were analyzed using a 100× oil immersion lens on an Olympus Model BX60 microscope equipped with epifluorescence. Confocal microscope images were collected using a Noran-Oz confocal laser scanning imaging system on a Nikon Diaphot microscope using a 100× oil immersion lens. Two dimensional views of the *z*-series volume along the *x*-*z* and *y*-*z* planes were produced using the reslice tool in the InterVision 3D software (Noran Instruments, Madison, WI). Expression levels of the chimeric receptors and the levels of endogenous expression of $\alpha_6\beta_4$ and β_1 integrin subunits on HMEC-1 and HaCat cells were analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer as previously described (LaFlamme et al., 1994).

Cell fractionation and western blotting

An equal number of HMEC-1 cells transiently transfected with recombinant human α_6A and β_4A subunits were plated in serum containing medium onto two 100 mm dishes previously coated with a laminin 5-rich matrix. After 36 hours, the cells were washed several times in PBS. To generate a whole cell lysate, cells from one plate were solubilized directly in 500 μl SDS-PAGE sample buffer (40 mM $NaPO_4$, 2% SDS, 10 mM EDTA) at 95°C, the DNA present was sheared by passage through a 25-gauge needle, and the lysate was clarified by centrifugation. Cells from the second plate were extracted for 5 minutes in 500 μl of Tween-40/deoxycholate extraction buffer described above, with the addition of 100 μM calpain inhibitor (Boehringer-Mannheim). The soluble fraction was collected and clarified by centrifugation. After several washes in PBS, the fraction resistant to extraction (insoluble fraction) was solubilized with 350 μl SDS-PAGE sample buffer at 95°C as described above. The protein concentration in each fraction was determined using the Micro-BCA protein assay from Pierce (Rockford, IL). 10 μg of protein from each sample were resolved by 7.5% SDS-PAGE and then analyzed by western blot with the anti-β_4 integrin polyclonal antibody. The filter was then stripped for 1 hour at 70°C in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.7, and reprobed with the anti-vimentin mAb clone VIM 13.2. The stripping procedure was repeated and the filter was probed a third time with the anti-vinculin mAb clone VIN-11-5.

RESULTS

Recombinant $\alpha_6\beta_4$ concentrates in fibrillar adhesion-like structures in HMEC-1 cells

Expression of the $\alpha_6\beta_4$ integrin has been observed in a variety of endothelial cells in situ including dermal microvascular endothelial cells (Enenstein and Kramer, 1994; Hieda et al., 1992; Kennel et al., 1992; Koukoulis et al., 1991). However, when endothelial cells are placed in culture they lose expression of $\alpha_6\beta_4$ (Hieda et al., 1992). Consistent with this finding, primary dermal microvascular endothelial cells and the human dermal microvascular endothelial cell line, HMEC-1, express $\alpha_6\beta_4$ at low levels (Xu et al., 1994), which are not detected in any distinct subcellular localization pattern (data not shown). In order to evaluate whether the inability to detect $\alpha_6\beta_4$ in adhesion structures in endothelial cells is due to its low expression level, or to the absence of other components required to assemble $\alpha_6\beta_4$ into distinct adhesive structures, we expressed the recombinant human α_6A and β_4A subunits in HMEC-1 cells and examined their localization pattern using immunofluorescence microscopy. In transiently transfected HMEC-1 cells, we observed recombinant $\alpha_6\beta_4$ localized in novel fibrillar structures on the basal cell surface (Fig. 1). The same fibrillar localization pattern was observed for recombinant $\alpha_6\beta_4$ when expressed in early passage primary dermal microvascular endothelial cells (data not shown). This $\alpha_6\beta_4$-containing structure appears to be distinct from the previously described type II hemidesmosomes in epithelial cells and type II-like hemidesmosomes in fibroblasts (Uematsu et al., 1994; Niessen et al., 1997a). The majority of recombinant $\alpha_6\beta_4$ concentrates in fibrillar structures where vinculin is not detected (data not shown). In the previously described structures, $\alpha_6\beta_4$ was observed to flank vinculin-containing focal adhesions.

The β_4 intracellular domain is sufficient to target a reporter to $\alpha_6\beta_4$-containing fibrillar structures

We next tested whether the β_4 intracellular domain was sufficient to direct the localization of a reporter to the fibrillar

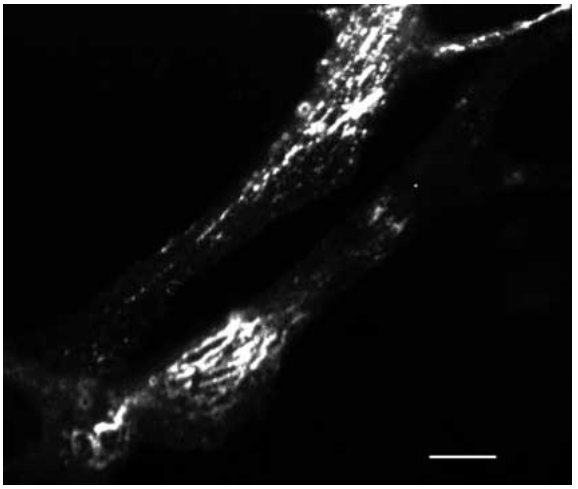

Fig. 1. Recombinant $\alpha_6\beta_4$ concentrates in fibrillar structures on the surface of HMEC-1 cells. HMEC-1 cells were transiently transfected with the α_6A and β_4A integrin subunits and seeded on glass coverslips for 14-18 hours in serum-containing medium. The cells were fixed and stained with mAb 3E1 specific for the β_4 subunit. The samples were then analyzed on an Olympus Model BX60 microscope equipped with epifluorescence. Bar, 10 μm.

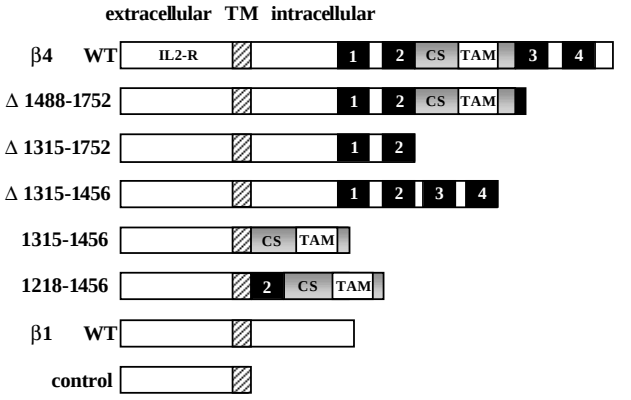

Fig. 2. Schematic representation of chimeric receptors containing the wild-type β_4 intracellular domain and various β_4 intracellular domain deletion mutants. Chimeric receptors were constructed containing the IL-2 receptor (IL2-R) extracellular and transmembrane (TM) domains connected to either the wild-type or mutant β_4 cytoplasmic tails. The four fibronectin type III repeats are numbered and represented by the black boxes, and the tyrosine activation motif (TAM)-containing connecting segment (CS) is represented by the shaded region. Also shown are the control receptor containing only the extracellular and transmembrane reporter domains, and the chimeric receptor containing the β_1 intracellular domain.

structures in endothelial cells. To do so, we constructed a chimeric receptor containing an extracellular reporter domain consisting of the IL-2 receptor extracellular and transmembrane domain connected to the wild-type β_4 subunit intracellular domain (IL-2R-β_4 chimera, see Fig. 2). When the chimeric receptor was cotransfected with the recombinant $\alpha_6\beta_4$ receptor into HMEC-1 cells, the chimeric receptor colocalized with the recombinant $\alpha_6\beta_4$ receptor in fibrillar structures on the cell surface (Fig. 3). These results indicate that the β_4 cytoplasmic domain connected to an extracellular reporter can interact with cytoplasmic components localized at these fibrillar structures. The confocal images shown in Fig. 3 represent the optical plane corresponding to the basal cell surface, suggesting that the fibrillar structures are concentrated on the basal surface of the cell and therefore may function in adhesion.

An intracellular mechanism regulates receptor localization at these fibrillar structures

Since $\alpha_6\beta_4$ is a receptor for the laminin isoforms 1, 2, 4 and 5 (Spinardi et al., 1995; Lee et al., 1992), we tested whether laminin colocalized with $\alpha_6\beta_4$ at these fibrillar structures. However, we could not detect laminin containing the β_1, β_2, α_3 or γ_2 chains at these sites by immunofluorescence microscopy (data not shown). This result suggests that $\alpha_6\beta_4$ may be targeted to these structures by an intracellular mechanism rather than an extracellular receptor-ligand interaction. To examine whether the localization of the β_4 chimera was dependent upon the presence of the recombinant $\alpha_6\beta_4$ receptor, we transfected endothelial cells with only the wild-type IL-2R-β_4 chimera and analyzed its distribution on the cell surface. We found that it localized to the same fibrillar structures in the

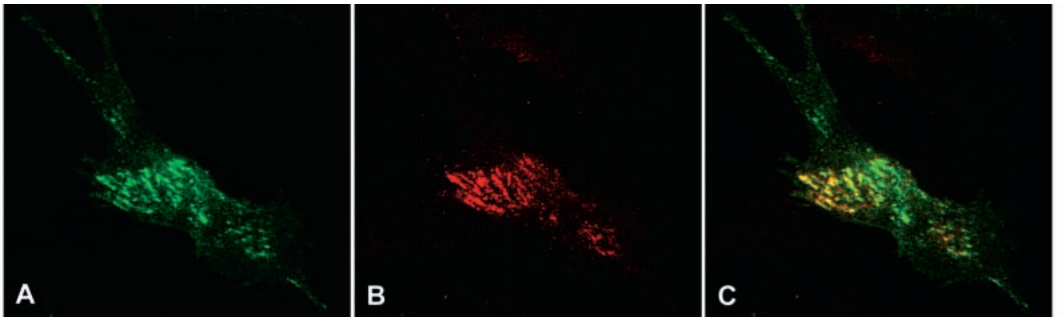

Fig. 3. The β_4 intracellular domain is sufficient to direct a reporter domain to fibrillar structures on the basal surface of endothelial cells where the recombinant $\alpha_6\beta_4$ integrin concentrates. HMEC-1 cells were cotransfected with recombinant $\alpha_6\beta_4$ and the IL-2R-β_4 chimeric receptor and seeded on glass coverslips in serum-containing medium as described in Fig. 1. The cells were fixed and dual-stained with antibodies specific for the IL-2 receptor (A) or the $\alpha_6\beta_4$ integrin, mAb 3E1 (B). The overlayed image of A and B is shown in (C), illustrating the colocalization (yellow) between the chimera and recombinant $\alpha_6\beta_4$. The images shown are optical sections collected from a confocal *z*-series scan corresponding to the basal surface of the cell.

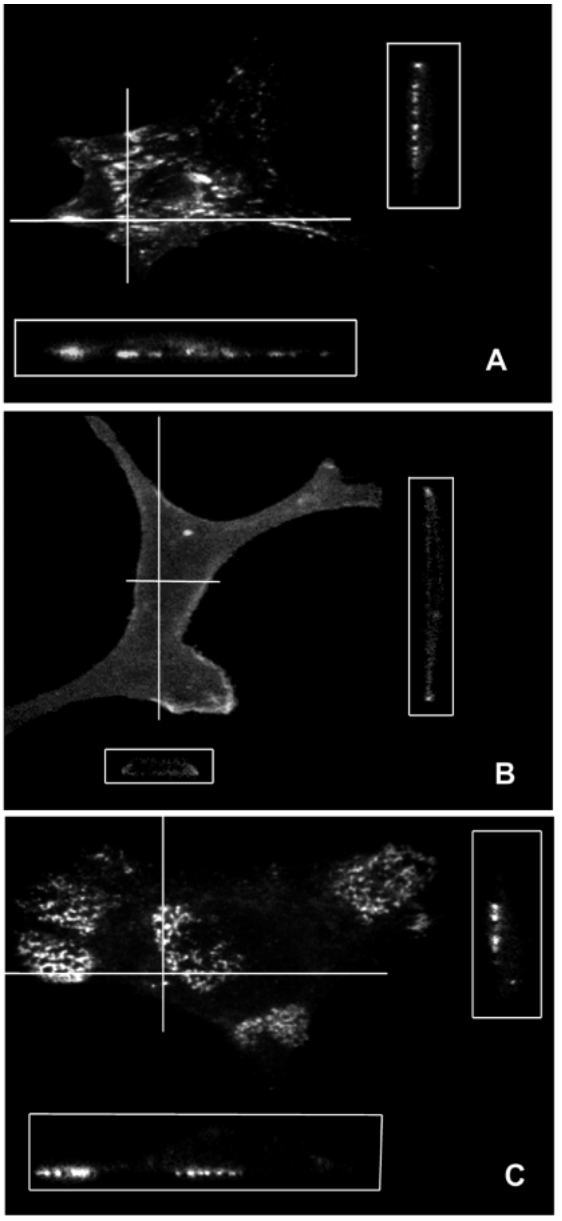

Fig. 4. The β_4 intracellular domain is sufficient to direct a reporter domain to fibrillar structures in the absence of recombinant $\alpha_6\beta_4$. HMEC-1 cells transiently expressing either the wild-type β_4 chimeric receptor (A) or the control receptor (B) were seeded on glass coverslips in serum-containing medium as previously described. As a positive control for the ability of the β_4 chimera to target to type I hemidesmosomes, a human keratinocyte cell line (HaCat) transiently expressing the wild-type β_4 chimera was seeded on a laminin 5-rich matrix deposited by 804G rat bladder carcinoma cells (C). The cells were fixed, permeabilized, and stained with the antibody specific for the IL-2 receptor. The localization of the chimeric receptors on the basal cell surface was confirmed by confocal microscopy. The insets show the vertical sections of the three-dimensional image sectioned in the *x-z* and *y-z* planes. The perpendicular lines indicate the positions from which the vertical sections were taken.

absence of recombinant $\alpha_6\beta_4$ (Fig. 4A) as it did in the presence of recombinant $\alpha_6\beta_4$ (Fig. 3). Therefore, it is possible that this structure is assembled independently of $\alpha_6\beta_4$-ligand interactions. In addition, confocal microscopic analysis of vertical sections indicated the staining was concentrated specifically at the basal cell surface (Fig. 4A), whereas a control chimera lacking an intracellular domain stained diffusely on the cell surface (Fig. 4B). As an additional control, we tested the ability of the β_4 chimera to target previously characterized $\alpha_6\beta_4$-containing type I hemidesmosomes in culture (Hormia et al., 1995; Langhofer et al., 1993). We were able to detect the β_4 chimera along with endogenous $\alpha_6\beta_4$ at hemidesmosomes in HaCat cells plated on a laminin 5-rich matrix as indicated by the well-characterized hemidesmosomal 'Swiss-cheese'-like staining pattern localized specifically on the basal surface of the cell (Fig. 4C). These results indicate that in the absence of recombinant $\alpha_6\beta_4$, the β_4 intracellular domain is sufficient to direct the chimeric receptor to distinct fibrillar structures on the basal surface of endothelial cells. This suggests that the β_4 intracellular domain interacts with cellular protein(s) concentrated at these structures, which direct its localization.

The first pair of FN type III repeats and the connecting segment are required for the localization to fibrillar adhesion-like structures

The β_4 cytoplasmic domain contains conserved amino acid motifs found to mediate protein-protein interactions in other proteins (Campbell and Spitzfaden, 1994). These motifs include two pairs of FN type III structural repeats separated by a connecting segment (CS), which contains a tyrosine-activation motif that has been shown to function in signal transduction in other receptors (Timson-Gauen et al., 1996). It has previously been shown that a region of the β_4 intracellular domain contained within the first pair of FN type III repeats and the CS is involved in regulating the localization of $\alpha_6\beta_4$ to type I hemidesmosomes in rat bladder carcinoma 804G cells and type II hemidesmosome-like structures in fibroblastic cells (Niessen et al., 1997a; Spinardi et al., 1993). To determine whether the same or distinct regions of the β_4 cytoplasmic domain are important for mediating the interactions directing the chimera to the fibrillar structures in endothelial cells, we constructed chimeric receptors containing various deletions in the β_4 intracellular domain, focusing on the region of the β_4 tail containing the FN type III repeats and the CS (Fig. 2). Similar levels of cell surface expression were observed for each of these chimeric receptors by flow cytometry (data not shown). The localization of each mutant was analyzed on transiently transfected HMEC-1 cells by immunofluorescence microscopy and the results from three independent experiments were compared (Table 1). When we analyzed the cell surface distribution of the chimeric receptors, we found that the second pair of FN type III repeats is not required for the fibrillar localization, since the chimeric receptor, β_4-Δ1488-1752, containing the β_4 cytoplasmic domain truncated after the CS, showed the same fibrillar localization pattern as the wild-type IL-2R-β_4 chimera (Fig. 5A,B). However, the CS is required for the fibrillar localization pattern, since the chimeric receptors β_4-Δ1315-1752 and β_4-Δ1315-1456, which lack the CS, stained diffusely on the cell membrane (Fig. 5C,D). Although the CS is required for the fibrillar localization, the CS is not sufficient to direct the receptor to the fibrillar structures, since the chimeric receptor β_4-1315-1456, containing only the CS, was diffusely distributed on the cell surface (Fig. 5E). The

Table 1. Fibrillar localization of the β_4 chimera and the recruitment of HD1/plectin

	Transfected chimera					
	β_4 chimera	β_4 Δ1315-1456	β_4 Δ1315-1752	β_4 Δ1488-1752	β_3 chimera	Control chimera
Fibrillar localization*	57.5±2.4	7.2±3.6	0.7±0.5	54.8±4.3	54.2±6.0‡	0
HD1 colocalization§	86.4±0.6	0	0	84.2±1.4	0	0

*The percentage of transfected cells in which the various chimeric receptors were detected in a fibrillar localization pattern, or ‡were detected in focal adhesions.

§The percentage of transfected cells with chimeric receptors in a fibrillar staining pattern in which HD1/plectin was colocalized with the chimera. It should be noted that in cells expressing mutants which stain diffusely, HD1/plectin could not be detected in any distinct structure. In each case, the mean ± s.d. is given for three independent experiments.

second FN type III repeat and the CS are also not sufficient to direct the reporter domain to fibrillar structures since the chimeric receptor β_4-1218-1456, containing only this portion of the β_4 tail, also stained diffusely on the cell surface (Fig. 5F). Therefore, the results indicate that the region of the β_4 intracellular domain required for its fibrillar localization in endothelial cells is contained within the first pair of Fn type III repeats and the CS. This is the same region of the β_4 intracellular domain determined to be required for the localization of $\alpha_6\beta_4$ to type I hemidesmosomes in 804G rat bladder carcinoma cells and type II hemidesmosome-like structures in fibroblastic cells (Spinardi et al., 1993; Niessen et al., 1997a). This result suggests that similar intracellular protein interactions with the β_4 cytoplasmic tail may be involved in regulating receptor localization to all three structures.

Receptor localization to type II hemidesmosome-like structures in fibroblasts is also regulated by an intracellular mechanism

In some epithelial cells that do not form type I hemidesmosomes in culture, the $\alpha_6\beta_4$ integrin has been found to localize in structures that have been referred to as type II hemidesmosomes. These structures have been described as $\alpha_6\beta_4$ concentrated in linear streaks flanking focal adhesions containing vinculin (Uematsu et al., 1994). When recombinant $\alpha_6\beta_4$ is expressed in fibroblasts, it also concentrates at sites resembling type II hemidesmosomes (Sanchez-Aparicio et al., 1997; Niessen et al., 1997a). For these reasons, it is possible that the localization of $\alpha_6\beta_4$ in fibroblasts might also be regulated by an intracellular mechanism. To test this possibility, we expressed the IL-2R-β_4 chimera in $\alpha_6\beta_4$-deficient normal human fibroblasts. We found that the β_4 chimera consistently localized to type II hemidesmosome-like structures in the absence of $\alpha_6\beta_4$ expression (Fig. 6A-C). This staining pattern is specific for the β_4 intracellular domain and it is not a phenomenon of antibody exclusion, because the β_1 chimera directly colocalizes with vinculin in fibroblasts (Fig. 6D-F). This finding suggests that the β_4 intracellular domain can also interact with intracellular proteins concentrated at these sites flanking focal adhesions, independent of the expression of recombinant $\alpha_6\beta_4$.

The β_4 intracellular domain directs the localization of HD1/plectin to fibrillar structures in endothelial cells

We evaluated HD1/plectin as a candidate intracellular protein required for the localization of the β_4 chimera to these fibrillar structures, because the intermediate filament-associated protein, HD1/plectin, is expressed in endothelial cells (Hieda

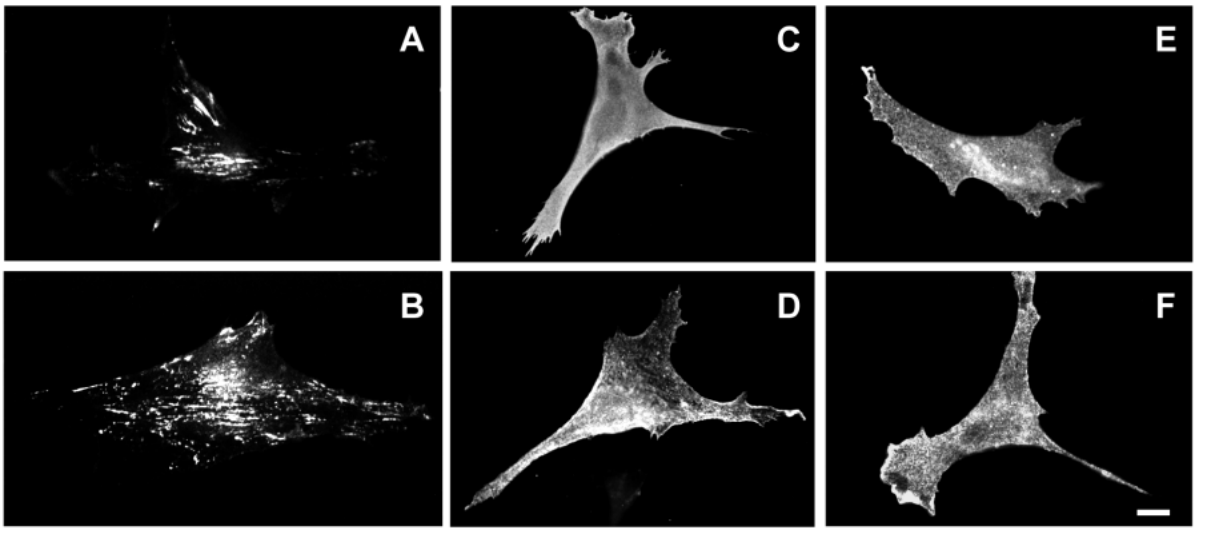

Fig. 5. The first pair of fibronectin type III repeats and the CS are required for receptor localization to fibrillar structures. HMEC-1 cells transiently expressing the wild-type IL-2R-β_4 chimera (A), or chimeric receptor mutants β_4-Δ1488-1752 (B), β_4-Δ1315-1752 (C), β_4-Δ1315-1456 (D), β_4-1315-1456 (E) and β_4-1218-1456 (F) were seeded onto glass coverslips and stained with the IL-2 receptor specific antibody. The samples were analyzed on an Olympus Model BX60 microscope equipped with epifluorescence. Bar, 10 μm.

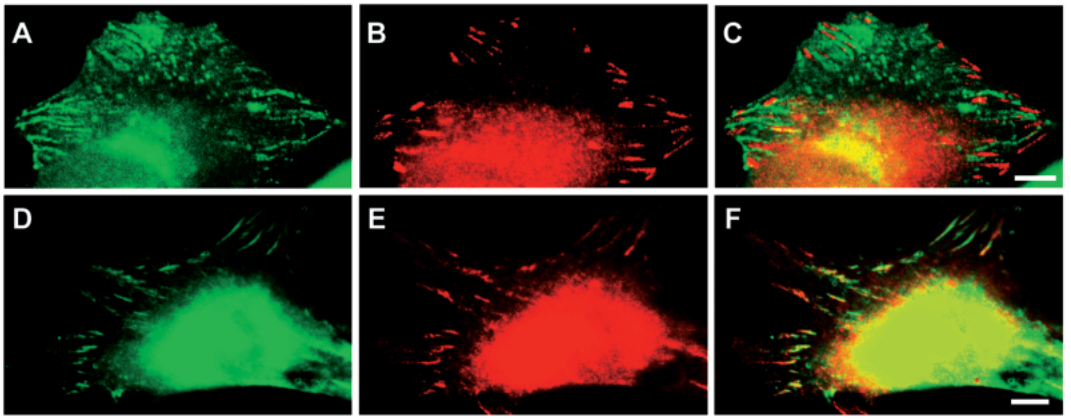

Fig. 6. Localization to type II hemidesmosomes-like structures in fibroblasts is regulated by an intracellular mechanism. Normal human fibroblasts transiently expressing the wild-type β_4 chimera (A-C) or the β_1 chimera (D-F) were seeded in serum-containing medium and then 15 hours later were fixed and double-stained with antibodies to the IL-2 receptor (A and D) and to vinculin (B and E). Samples were analyzed on an Olympus Model BX60 microscope equipped with epifluorescence. Overlayed images (C and F) were computer generated. Bars, 10 μm.

et al., 1992) and is found colocalized with $\alpha_6\beta_4$ in both type I and type II hemidesmosomes (Uematsu et al., 1994). When we examined the staining pattern of HD1/plectin in untransfected HMEC-1 cells, HD1/plectin consistently displayed a diffuse staining pattern and could not be detected in any distinct structures (data not shown). However, in cells transiently expressing the β_4 chimera, HD1/plectin consistently colocalized with the wild-type β_4 chimera at fibrillar structures, as shown in Fig. 7A-C and Table 1. The recruitment of HD1/plectin is specific for the β_4 intracellular domain because HD1/plectin was diffusely distributed in cells expressing the β_1 chimera, which is localized specifically in focal adhesions (Fig. 7D,E). When we examined the localization of HD1/plectin in endothelial cells expressing chimeric receptors with various deletions in the β_4 intracellular domain, we found that the chimeras that targeted to fibrillar structures were also able to consistently direct the localization of HD1/plectin (Fig. 7 and Table 1). HD1/plectin had no distinct localization pattern in cells expressing β_4 mutants, which remained diffuse on the cell surface (Fig. 7F,G). Thus, these data demonstrate that the β_4 intracellular domain, in the absence of the α_6 subunit and any extracellular interaction with the subendothelial matrix, is sufficient to direct the localization of HD1/plectin in endothelial cells. In addition, because HD1/plectin is known to bind to vimentin (Nikolic et al., 1996), the ability of the β_4 intracellular domain to direct the localization of HD1/plectin

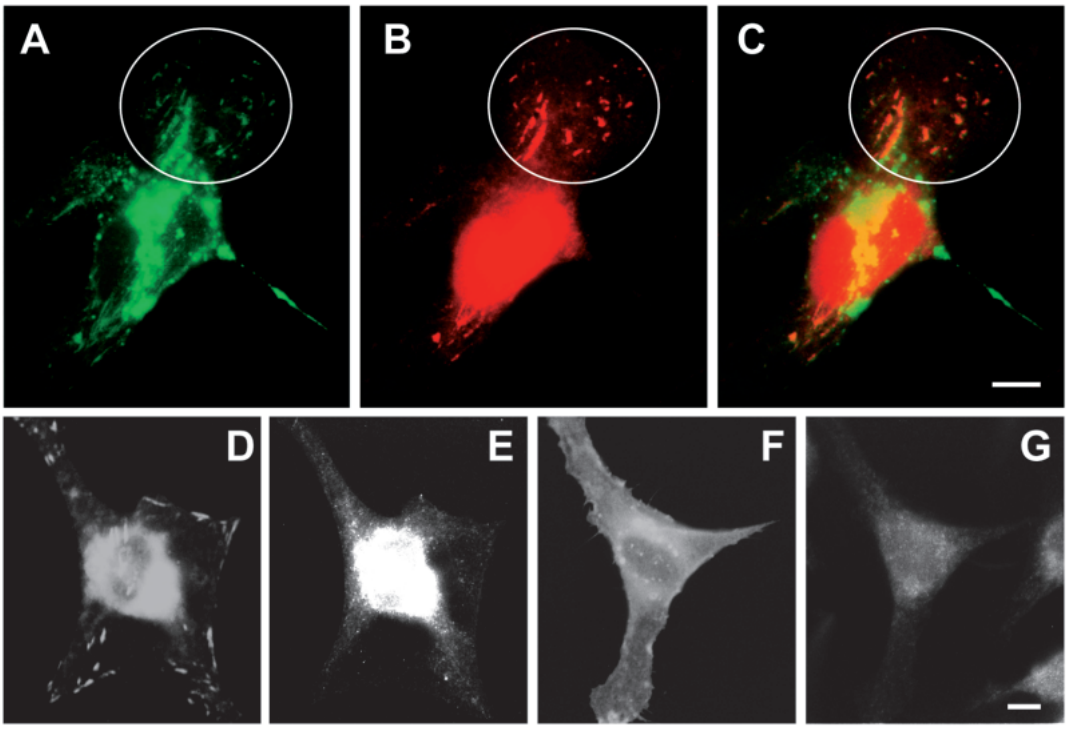

Fig. 7. The β_4 intracellular domain directs the localization of HD1/plectin to fibrillar structures in microvascular endothelial cells. HMEC-1 cells transiently expressing the wild-type β_4 chimera (A-C), the wild-type β_1 chimera (D,E) and β_4 chimera mutant β_4-Δ1315-1752 (F,G) were seeded as previously described in Fig. 1. Samples were dual-labeled with antibodies specific for the IL-2 receptor (A,D,F) and mAb-121 specific for HD1/plectin (B,E,G). The area of the cell showing the colocalization of HD1/plectin and the β_4 chimera is encircled. Samples were analyzed on an Olympus Model BX60 microscope equipped with epifluorescence and the overlayed image in C was computer generated. Bars,10 μm.

provides a potential mechanism by which $\alpha_6\beta_4$ may be linked to the vimentin intermediate filament system in endothelial cells.

Recombinant $\alpha_6\beta_4$-containing type I hemidesmosome-like structures form when transfected endothelial cells adhere to a laminin 5-rich matrix

The identity of the laminin isoforms expressed in subendothelial matrices in situ has not been fully characterized. However, EE laminin, which is related to laminin 5, has been observed in the basement membrane of microvascular endothelial cells (Carter et al., 1994). Therefore, we were interested in determining the response of endothelial cells expressing recombinant $\alpha_6\beta_4$ to a laminin 5-rich matrix to determine whether recombinant $\alpha_6\beta_4$ would assemble into an adhesion structure distinct from the fibrillar structures detected when cells were cultured on glass coverslips in the presence of serum. When endothelial cells were transiently transfected with recombinant $\alpha_6\beta_4$ and plated on a laminin 5-rich matrix for 15 hours, the $\alpha_6\beta_4$ integrin concentrated in a 'Swiss-cheese'-like staining pattern, typical of type I hemidesmosomes (Fig. 8A). This same localization pattern was observed for recombinant $\alpha_6\beta_4$ when expressed in early passage primary dermal microvascular endothelial cells plated on laminin 5 (data not shown). This result indicates that certain $\alpha_6\beta_4$ ligands can regulate the subcellular localization of $\alpha_6\beta_4$ in endothelial cells. However, even though $\alpha_6\beta_4$ redistributed to what appears to be type I-like hemidesmosomes, the fibrillar structure also persisted. When cells transfected with only the IL-2R-β_4 chimera were plated on a laminin 5-rich matrix, the β_4 chimera concentrated in these fibrillar structures (Fig. 8B). In addition, in cells expressing both the recombinant $\alpha_6\beta_4$ receptor and the IL-2R-β_4 chimera, the β_4 chimera was observed both at fibrillar structures lacking $\alpha_6\beta_4$ and at type I hemidesmosome-like structures along with recombinant $\alpha_6\beta_4$ (Fig. 8C,D). Therefore, the β_4 tail is also sufficient to direct a reporter domain to type I hemidesmosome-like structures formed by recombinant $\alpha_6\beta_4$ expressed in endothelial cells. This result suggests that cellular proteins, which can bind to the β_4 tail, are recruited to these $\alpha_6\beta_4$-mediated adhesions. The localization of the β_4 chimera to type I-like hemidesmosomes is dependent upon the expression of recombinant $\alpha_6\beta_4$.

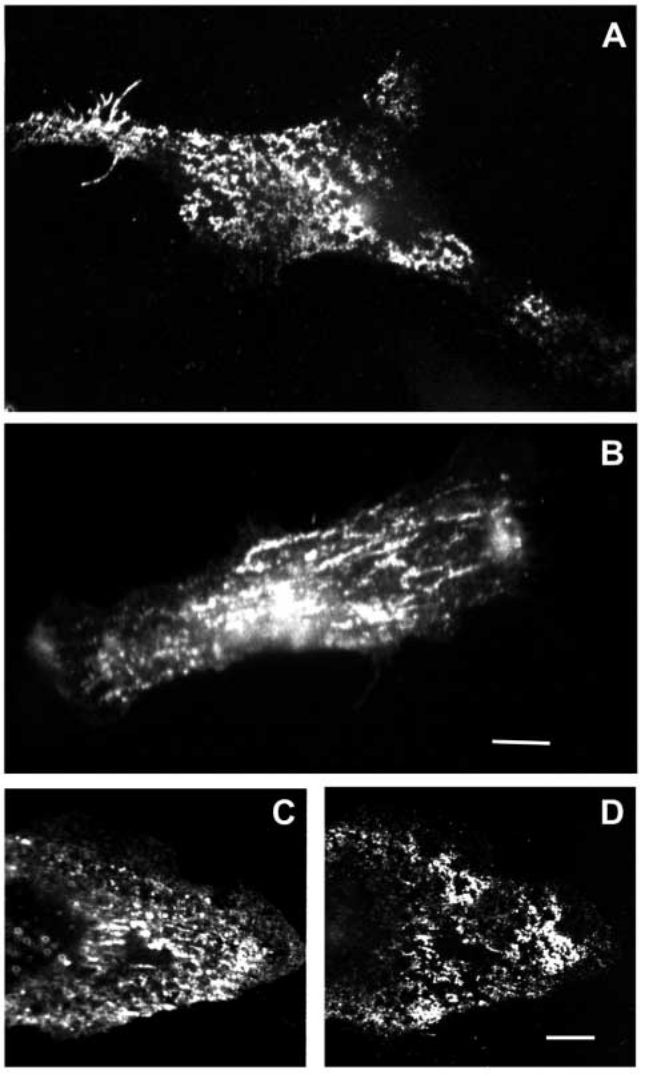

Fig. 8. Recombinant $\alpha_6\beta_4$-containing type I hemidesmosome-like structures form when transfected endothelial cells adhere to a laminin 5-rich matrix. HMEC-1 cells transiently expressing either the recombinant $\alpha_6\beta_4$ receptor (A), the wild-type IL-2R-β_4 chimera (B) or both recombinant $\alpha_6\beta_4$ and the wild-type β_4 chimera (C,D) were seeded on a laminin 5-rich matrix deposited by 804G cells. The cells were immunostained with mAb 3E1 specific for the recombinant $\alpha_6\beta_4$ receptor (A,D) or with an antibody specific for the IL-2 receptor (B,C). C and D show a single cell dual-labeled with the two antibodies. Bars, 10 μm.

The $\alpha_6\beta_4$ integrin associates with vimentin-type intermediate filaments in endothelial cells

Since the β_4 tail is sufficient to recruit the intermediate filament-associated protein HD1/plectin, we tested whether the $\alpha_6\beta_4$ integrin interacts with vimentin intermediate filaments in endothelial cells. HMEC-1 cells transiently transfected with recombinant $\alpha_6\beta_4$, the wild-type IL2-R-β_4 chimera, or mock transfected, were seeded on a laminin 5-rich matrix. After 14-18 hours, the cells were extracted with a Tween-40/deoxycholate extraction buffer that removes the cytosol and the bulk of the actin cytoskeleton and associated proteins, but not the intermediate filament cytoskeleton and its associated proteins (Rabinovitz and Mercurio, 1997; Capco et al., 1982). Subsequent to extraction, the cells were fixed and the presence of vimentin, $\alpha_6\beta_4$, β_1 integrins or the β_4 chimera were determined by immunofluorescence microscopy. The fibrillar structures, as well as the type I hemidesmosome-like structures, were both resistant to extraction (Fig. 9A,C). In contrast, β_1-containing focal contacts observed in unextracted cells (Fig. 9E) were entirely lost upon extraction (Fig. 9F,G). Thus, the data suggests that both the type I hemidesmosome-like structures and the novel fibrillar structures are specifically associated with the vimentin-type intermediate filament cytoskeleton. Furthermore, the β_4 tail is sufficient to mediate association with the vimentin cytoskeleton, since the IL2-R-β_4 chimera resisted extraction and colocalized with the vimentin cytoskeleton in fibrillar structures in extracted cells. Taken together, these results provide further evidence in support of our hypothesis that the $\alpha_6\beta_4$ integrin is assembled into distinct intermediate filament-associated structures in endothelial cells.

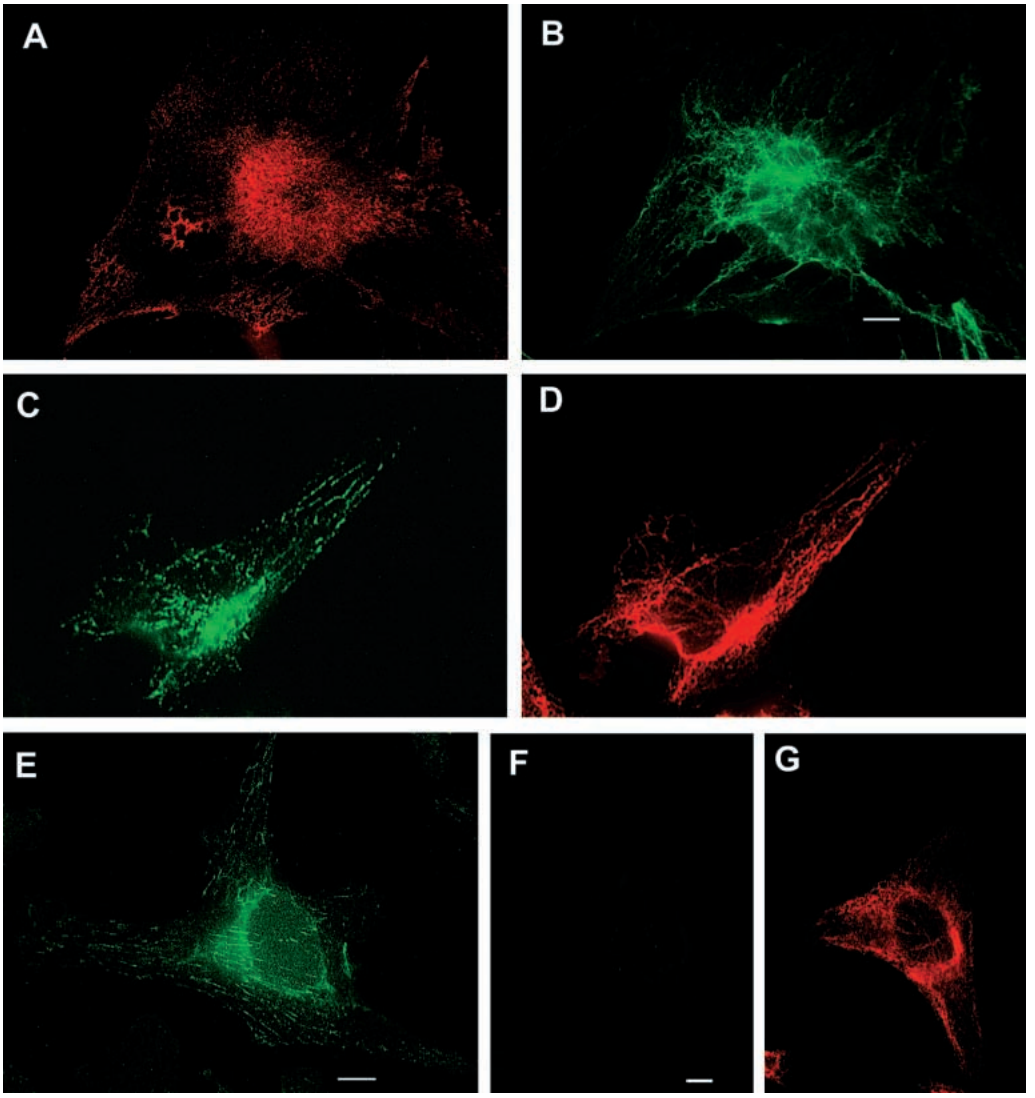

Fig. 9. $\alpha_6\beta_4$-containing structures are specifically associated with the vimentin cytoskeleton. HMEC-1 cells transiently expressing the recombinant $\alpha_6\beta_4$ receptor (A,B), the wild-type IL-2R-β_4 chimera (C,D) or mock-transfected HMEC-1 cells (E-G) were seeded on a laminin 5-rich matrix for 14-18 hours in serum-containing medium. The cells were extracted with a Tween-40/deoxycholate buffer for 3 minutes prior to fixation and were double-stained with either the rat mAb GoH3, specific for the α_6 integrin subunit (A), the FITC-conjugated mAb specific for the IL-2 receptor (C), or the FITC-conjugated mAb K20 specific for the β_1 integrin subunit (F), in combination with the mouse mAb VIM 3B4 specific for vimentin (B,D,G) as described in Materials and Methods. In E, cells were fixed, permeabilized with 0.4% Triton X-100, and then stained with mAb K20 as a control. Bars, 10 μm.

The association of the $\alpha_6\beta_4$ integrin with the vimentin cytoskeleton was further confirmed by biochemical analysis. HMEC-1 cells transiently transfected with recombinant $\alpha_6\beta_4$ were seeded onto a laminin 5-rich matrix for 36 hours. Cells were extracted with the Tween-40/deoxycholate extraction buffer. The presence of vimentin, the β_4 subunit and vinculin in the extraction buffer soluble and insoluble fractions were analyzed by western blot. As shown in Fig. 10, $\alpha_6\beta_4$ and vimentin were enriched in the insoluble fraction. In contrast, the actin-associated protein, vinculin, was present in the soluble fraction, but was undetectable in the insoluble fraction, further confirming that $\alpha_6\beta_4$ specifically associates with the vimentin-type intermediate filament cytoskeleton in endothelial cells.

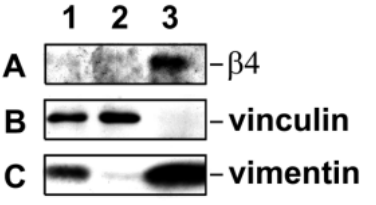

Fig. 10. $\alpha_6\beta_4$ is enriched in the cell fraction specifically containing intermediate filaments and associated proteins. HMEC-1 cells were transiently transfected with the α_6A and β_4A integrin subunits and plated onto a laminin 5-rich matrix. Cellular components were fractionated into soluble and insoluble pools based on their solubility in a 'cytoskeletal' buffer, which specifically solubilizes cellular contents that are associated with microfilaments but not associated with the intermediate filament cytoskeleton, as described in Materials and Methods. 10 μg of protein from a whole cell lysate (lane 1) and the soluble (lane 2) and insoluble (lane 3) fractions were analyzed by western blot with an anti-β_4 integrin polyclonal antibody (A), reprobed with the anti-vimentin antibody clone VIM 13.2 (C), and then with the anti-vinculin antibody clone VIN-11-5 (B).

DISCUSSION

In this study, we expressed recombinant $\alpha_6\beta_4$ in the microvascular endothelial cell line HMEC-1 to determine whether $\alpha_6\beta_4$ can be assembled into adhesion structures by

endothelial cells. In the absence of exogenous laminin, we find that $\alpha_6\beta_4$ concentrates in fibrillar structures on the basal cell surface. Biochemical analysis indicates that this structure is specifically associated with the vimentin intermediate filament cytoskeleton. A chimeric receptor containing the β_4 cytoplasmic domain connected to an extracellular reporter becomes concentrated in this structure, and is independent of the expression of recombinant $\alpha_6\beta_4$. This suggests that this fibrillar structure can form in the absence of $\alpha_6\beta_4$ and that the recruitment of receptors to this structure occurs by an intracellular mechanism involving the β_4 tail. We demonstrate that the region of the β_4 cytoplasmic tail required for its localization to these fibrillar structures in endothelial cells is the same region previously described to be required for $\alpha_6\beta_4$ localization to both type I hemidesmosomes in epithelial cells and type II-like hemidesmosomes in fibroblastic cells (Spinardi et al., 1993; Niessen et al., 1997a). This suggests that similar protein interactions involving the β_4 tail are involved in regulating $\alpha_6\beta_4$ localization at all three structures. We have also shown that the β_4 cytoplasmic tail is sufficient to recruit HD1/plectin to these fibrillar structures, as well as to mediate association with the vimentin cytoskeleton. Lastly, we have demonstrated that in the presence of laminin 5, $\alpha_6\beta_4$ assembles into a type I hemidesmosome-like structure that is also associated with vimentin intermediate filaments. Thus, the presence of specific $\alpha_6\beta_4$ ligands and the β_4 tail can regulate the assembly of distinct vimentin-associated structures in endothelial cells.

Interestingly, the fibrillar structures on the basal surface of endothelial cells appear to be formed independently of $\alpha_6\beta_4$, because the IL-2R-β_4 chimera concentrates at these fibrillar structures on the basal surface of the cell in the absence of expression of recombinant $\alpha_6\beta_4$. Consistent with this data, we have not been able to detect $\alpha_6\beta_4$ ligands containing laminin chains β_1, α_3, α_2 and β_2 in these structures by immunofluorescence microscopy. It is possible that this structure is formed by integrin-matrix interactions other than those mediated by $\alpha_6\beta_4$. However, we have also been unable to localize other integrins at this structure, including integrins containing α_2, α_3, α_5, α_v, β_1 and β_3 subunits (data not shown), suggesting that non-integrin adhesion receptors may be localized at these structures. Although the mechanism regulating the formation of this fibrillar structure is not yet known, the β_4 chimera is detected in these structures within 6 hours of plating. This is the earliest time post-transfection that the expression of the chimera can be detected at the cell surface (data not shown).

Interestingly, the region of the β_4 cytoplasmic tail involved in regulating this fibrillar localization in endothelial cells has previously been shown to direct receptor localization to type I hemidesmosomes in epithelial cells and type II-like hemidesmosomes in fibroblasts (Spinardi et al., 1993; Niessen et al., 1997a), suggesting that the same cellular protein(s) is responsible for directing the localization of β_4 to the basal cell surface in epithelial, endothelial and fibroblastic cell types. Although the intracellular protein interactions directing the localization of $\alpha_6\beta_4$ appear to be the same in all three cell types examined, these interactions occur in distinct structures in each cell type. In epithelial cells, they occur at type I hemidesmosomes (Spinardi et al., 1993); in endothelial cells, interactions occur at a fibrillar structure on the basal cell surface (see Figs 1 and 4) and in fibroblasts, they occur at type II-like hemidesmosomal structures (Niessen et al., 1997a).

Interestingly, the same region of the β_4 intracellular domain required for its localization to fibrillar structures is also the region required for its ability to recruit HD1/plectin to these sites. Thus, the β_4 intracellular domain interacts with specific proteins in endothelial cells that can direct its localization to fibrillar structures, and the same region of the β_4 intracellular domain is also sufficient to direct the localization of the intermediate filament-associated protein HD1/plectin to these structures. Our results suggest that the cellular protein involved in directing the localization of $\alpha_6\beta_4$ and the IL-2R-β_4 chimera to these structures is not HD1/plectin, because HD1/plectin cannot be detected in any distinct staining pattern until the wild-type β_4 chimera or chimeric mutant β_4-Δ1488-1752 is expressed. This finding is in agreement with others whose data indicate that the cytoplasmic domain of β_4 can directly interact with plectin and can determine the subcellular distribution of HD1/plectin (Sanchez-Aparicio et al., 1997; Rezniczek et al., 1998; Niessen et al., 1997b). However, we and others have not been able to identify a separate region of the β_4 intracellular domain involved in regulating receptor localization to the basal cell surface from a region involved in targeting HD1/plectin to these structures. Both events appear to require amino acids contained within the first pair of FN type III repeats and the CS.

Although $\alpha_6\beta_4$ concentrates in fibrillar structures in the absence of exogenous ligand, the availability of specific $\alpha_6\beta_4$ ligands can regulate the distribution of $\alpha_6\beta_4$ in endothelial cells. When endothelial cells expressing recombinant $\alpha_6\beta_4$ were plated on a laminin 5-rich matrix, the $\alpha_6\beta_4$ integrin redistributed from the fibrillar structures to type I hemidesmosome-like structures. The fibrillar structure, however, persisted because in the absence of recombinant $\alpha_6\beta_4$, the IL-2R-β_4 chimera remained fibrillar on cells plated on a laminin 5 matrix. However, when cells expressing both the recombinant $\alpha_6\beta_4$ receptor and the IL-2R-β_4 chimera were plated on a laminin 5 matrix, the chimera partially redistributed to these $\alpha_6\beta_4$-containing structures, suggesting that additional cytoplasmic proteins may also redistribute to this structure and may allow for the recruitment of the β_4 chimera.

Although there is ultrastructural evidence suggesting the association of the cytokeratin-type intermediate filament cytoskeleton with both type I and type II hemidesmosomes (Stepp et al., 1990; Uematsu et al., 1994), there is no biochemical evidence to confirm a linkage between $\alpha_6\beta_4$ and the vimentin type intermediate filament cytoskeleton in any cellular structure. In this study, an in situ extraction technique was used to specifically solubilize all cellular components except for the intermediate filament cytoskeleton and its associated proteins. Using immunofluorescence microscopy, we show that both the fibrillar structure as well as the type I hemidesmosome-like structures remain organized in extracted cells, confirming that both $\alpha_6\beta_4$-containing structures detected in endothelial cells are associated with vimentin intermediate filaments. Importantly, the β_4 tail was shown to be sufficient to mediate association with the vimentin cytoskeleton. The association of the $\alpha_6\beta_4$ integrin with vimentin was further confirmed by western blot analysis, which illustrated a specific enrichment of the β_4 subunit and vimentin in the Tween-40/deoxycholate buffer-insoluble fraction. Actin-associated

proteins were present in the soluble fraction, yet were undetectable in the insoluble fraction. In conclusion, our data demonstrate that endothelial cells contain the components necessary to organize $\alpha_6\beta_4$ into distinct vimentin-associated structures on their basal cell surface and that the subcellular localization of $\alpha_6\beta_4$ and, ultimately, its contribution to endothelial function will depend upon the availability of specific $\alpha_6\beta_4$ ligands.

We thank Drs J. Sottile, A. Mastrangelo and A. Berrier for their helpful comments during the preparation of the manuscript. We thank Emma Samelson-Jones for the IL-2R-β_4-1218-1456 construct. We also thank Drs K. Owaribe, W. Carter and F. G. Giancotti for antibodies to HD1, laminin and the β_4 subunit respectively, Dr R. Oyasu for the 804G rat bladder carcinoma cell line and Drs T. Lawley and E. Ades for the HMEC-1 cell line. We thank Dr J. Mazurkiewicz for assistance with the confocal microscope. This work was supported by AHA-NYS affiliate grant 960148 to S. E. L. and in part by NIH grant GM-51540 to S. E. L. Suzanne Homan is supported by NIH-T32-HL-07194.

Note added in proof

A recent study has separated the localization of $\alpha_6\beta_4$ in type 1 hemidesmosomes from the recruitment of HD1/pectin. Schaapveld et al. (1998) J. *Cell Biol.* **142**, 271-284.

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