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

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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 of 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 $\beta_4$ 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-$\beta_4$ 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 $\beta_4$ 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 $\beta_4$ 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 $\beta_4$ cytoplasmic tail and the availability of specific $\alpha_6\beta_4$ ligands regulate receptor localization to these structures.

Key words: $\alpha_6\beta_4$, $\beta$ 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 $\alpha/\beta$ heterodimers, and the specificity of integrins for particular extracellular ligands depends upon which $\alpha$ and $\beta$ 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 $\beta_1$, $\beta_3$, and $\beta_5$ 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 α6β4 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 α6β4 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 epithelial cells (Uematsu et al., 1994). Type II hemidesmosomes are defined as structures having α6β4 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, α6β4 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 α6β4 (Sanchez-Aparicio et al., 1997; Niessen et al., 1997a).

The function of α6β4 in endothelial cells is currently unknown. However, since endothelial cells express α6β4 and HD1/plectin in situ (Hieda et al., 1992), α6β4 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 α6β4 in culture (Hieda et al., 1992), we expressed recombinant α6β4 in endothelial cells and asked whether recombinant α6β4 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 α6β4 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 β4 cytoplasmic domain. Additionally, we demonstrate that the β4 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 α6β4 from the fibrillar structures to type I hemidesmosome-like structures, which are also associated with vimentin intermediate filaments. Thus, the localization of α6β4 in distinct vimentin-associated structures in endothelial cells can be regulated by the availability of specific ligands for α6β4.

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 β6 subunit intracellular domain (IL-2R-β6 chimera), the oligonucleotides 5'-AGCTTGCCTGCTACTACGTCGGGCGG-3' and 5'-TCAGACCCACGGTAGAGGGCA-3', encoding an AccI were hybridized and then inserted at the HindIII/XhoI sites of the previously described plasmid encoding the extracellular and transmembrane domains of the IL-2 receptor (LaFlamme et al., 1992). The cDNA, β6-1, a kind gift of Dr Shintaro Suzuki (University of Southern California School of Medicine) was subcloned into the EcoRI site of pUC19, generating the plasmid pUC19-β6-1. The AccI/XhoI fragment of pUC19-β6-1, encoding amino acids 854-1752 (Suzuki and Naitoh, 1990), was then inserted into the newly generated AccI site and existing XhoI site, generating the plasmid CMV-IL2-β6. To construct β6-D1315-1752, 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'-CGCGGACCGGGGCGGGC-TAGGGGCGTACATCATCTAGATGTAGT-3' and 5'-CTAGACTATCAGATGTAGTACCTGGCCTCGGCCCAGGC-3'. Ligation into the plasmid CMV-IL-2R-β6 in place of the BssHII/XbaI fragment of the full-length β6 tail. To construct β6-D1488-1752, containing a truncation after the connecting segment (CS), a stop codon was inserted at amino acid 1488 by hybridizing the oligonucleotides 5'-GGCGGCTGTAGATCAGCCTGGTG-3' and 5'-CTAGACACCGGCTACACGC-3' and then ligating them into plasmid CMV-IL-2R-β6 in place of the NolI/XhoI fragment of the full-length β6 tail. β6-D1315-1456, lacking the CS, was generated utilizing the polymerase chain reaction (PCR). Initially, two PCR products were generated using the plasmid CMV-IL-2R-β6 and the following primers: Reaction (1) NH2-terminal primer 1, 5'-TGAATACACGCTCA-3', which hybridized upstream of the BssHI site, and COOH-terminal primer 2, 5'-CGGTGCGGATAGTTGCGCTCCCCTGCTCAG-3'. Reaction (2) NH2-terminal primer 3, 5'-CTAGACACCGGCTACACG-3' and COOH-terminal primer 4, 5'-GGTGGACGAGGTC-3', which hybridized downstream of the NotI 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 BssHII and NotI and then ligated into CMV-IL2-β6 in place of the BssHII/NotI fragment of the full-length β6 tail. β6-D1315-1456 mutant, encoding only the CS, and β6-D1218-1456 mutant encoding the second FN Type III repeat and β6 were also generated using PCR. The plasmid CMV-IL2-β6 and the primer 5'-GAGTCTCTCCTCTAGATCCGAACTAGTCCAGCC-3', were used for both reactions and the primer 5'-AGGGACAAGCTTACTGAGTGGCCTTGCT-3' was used to generate β6-D1218-1456. Each product was then digested with XhoI and HindIII and was ligated into the CMV-IL2 plasmid as a HindIII/XhoI 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 α6A and β6A 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 TECHNOS (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 β1 subunit from Gibco-BRL (Gaithersburg, MD), rat mAb GoH3 against the α6 subunit from Pharmingen (San Diego, CA), fluorescein (FITC)-conjugated, as well as unconjugated, mouse mAb K20 against the human α3 and α5 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 β1 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 α3 and β2, respectively, were a kind gift from Dr William G. Carter (Carter et al., 1994). Rabbit polyclonal antiserum to a C-terminal peptide of integrin β3 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 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 MgCl2, 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 α6β4 and β1 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 α6A and β4A 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 NaPO4, 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-β4 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 α6β4 concentrates in fibrillar adhesion-like structures in HMEC-1 cells
Expression of the α6β4 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 α6β4 (Hieda et al., 1992). Consistent with this finding, primary dermal microvascular endothelial cells and the human dermal microvascular endothelial cell line, HMEC-1, express α6β4 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 α6β4 in adhesion structures in endothelial cells is due to its low expression level, or to the absence of other components required to assemble α6β4 into distinct adhesive structures, we expressed the recombinant human α6A and β4A subunits in HMEC-1 cells and examined their localization pattern using immunofluorescence microscopy. In transiently transfected HMEC-1 cells, we observed recombinant α6β4 localized in novel fibrillar structures on the basal cell surface (Fig. 1). The same fibrillar localization pattern was observed for recombinant α6β4 when expressed in early passage primary dermal microvascular endothelial cells (data not shown). This α6β4-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 α6β4 concentrates in fibrillar structures where vinculin is not detected (data not shown). In the previously described structures, α6β4 was observed to flank vinculin-containing focal adhesions.

The β4 intracellular domain is sufficient to target a reporter to α6β4-containing fibrillar structures
We next tested whether the β4 intracellular domain was sufficient to direct the localization of a reporter to the fibrillar
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 β4 intracellular domain (IL-2R-β4 chimera, see Fig. 2). When the chimeric receptor was cotransfected with the recombinant α6β4 receptor into HMEC-1 cells, the chimeric receptor colocalized with the recombinant α6β4 receptor in fibrillar structures on the cell surface (Fig. 3). These results indicate that the β4 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 α6β4 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 α6β4 at these fibrillar structures. However, we could not detect laminin containing the β1, β2, α3 or γ chains at these sites by immunofluorescence microscopy (data not shown). This result suggests that α6β4 may be targeted to these structures by an intracellular mechanism rather than an extracellular receptor-ligand interaction. To examine whether the localization of the β4 chimera was dependent upon the presence of the recombinant α6β4 receptor, we transfected endothelial cells with only the wild-type IL-2R-β4 chimera and analyzed its distribution on the cell surface. We found that it localized to the same fibrillar structures in the

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**Fig. 1.** Recombinant α6β4 concentrates in fibrillar structures on the surface of HMEC-1 cells. HMEC-1 cells were transiently transfected with the α6A and β3A 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 β4 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 β4 intracellular domain and various β4 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 β4 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 β1 intracellular domain.

**Fig. 3.** The β4 intracellular domain is sufficient to direct a reporter domain to fibrillar structures on the basal surface of endothelial cells where the recombinant α6β4 integrin concentrates. HMEC-1 cells were cotransfected with recombinant α6β4 and the IL-2R-β4 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 α6β4 integrin, mAb 3E1 (B). The overlayed image of A and B is shown in (C), illustrating the colocalization (yellow) between the chimera and recombinant α6β4. The images shown are optical sections collected from a confocal z-series scan corresponding to the basal surface of the cell.
Fig. 4. The β4 intracellular domain is sufficient to direct a reporter domain to fibrillar structures in the absence of recombinant α6β4. HMEC-1 cells transiently expressing either the wild-type β4 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 β4 chimera to target to type I hemidesmosomes, a human keratinocyte cell line (HaCat) transiently expressing the wild-type β4 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.

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

The β4 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 β4 intracellular domain contained within the first pair of FN type III repeats and the CS is involved in regulating the localization of α6β4 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 β4 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 β4 intracellular domain, focusing on the region of the β4 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, β4Δ1488-1752, containing the β4 cytoplasmic domain truncated after the CS, showed the same fibrillar localization pattern as the wild-type IL-2R-β4 chimera (Fig. 5A,B). However, the CS is required for the fibrillar localization pattern, since the chimeric receptors β4Δ1315-1752 and β4Δ1315-1456, 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 β4-1315-1456, containing only the CS, was diffusely distributed on the cell surface (Fig. 5E). The interaction of the fibrillar structures, the CS is involved in regulating the localization of α6β4 to type I hemidesmosomes, which have previously been shown that a region of the hemidesmosomes, the human keratinocyte cell line (HaCat) transiently expressing the wild-type β4 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 α6β4 (Fig. 4A) as it did in the presence of recombinant α6β4 (Fig. 3). Therefore, it is possible that this structure is assembled independently of α6β4-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 β4 chimera to target previously characterized α6β4-containing type I hemidesmosomes in culture (Hormia et al., 1995; Langhofer et al., 1993). We were able to detect the β4 chimera along with endogenous α6β4 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 α6β4, the β4 intracellular domain is sufficient to direct the chimeric receptor to distinct fibrillar structures on the basal surface of endothelial cells. This suggests that the β4 intracellular domain interacts with cellular protein(s) concentrated at these structures, which direct its localization.
second FN type III repeat and the CS are also not sufficient to direct the reporter domain to fibrillar structures since the chimeric receptor $\beta_4$-1218-1456, containing only this portion of the $\beta_4$ tail, also stained diffusely on the cell surface (Fig. 5F). Therefore, the results indicate that the region of the $\beta_4$ 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 $\beta_4$ 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 $\beta_4$ 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-$\beta_4$ chimera in $\alpha_6$-deficient normal human fibroblasts. We found that the $\beta_4$ 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 $\beta_4$ intracellular domain and it is not a phenomenon of antibody exclusion, because the $\beta_1$ chimera directly colocalizes with vinculin in fibroblasts (Fig. 6D-F). This finding suggests that the $\beta_4$ 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 $\beta_4$ 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 $\beta_4$ chimera to these fibrillar structures, because the intermediate filament-associated protein, HD1/plectin, is expressed in endothelial cells (Hieda et al., 1995). We transiently expressed the wild-type IL-2R-$\beta_4$ chimera (A), or chimeric receptor mutants $\beta_4$-$\Delta1488$-$1752$ (B), $\beta_4$-$\Delta1315$-$1752$ (C), $\beta_4$-$\Delta1315$-$1456$ (D), $\beta_4$-$1315$-$1456$ (E) and $\beta_4$-$1218$-$1456$ (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.

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**Table 1. Fibrillar localization of the $\beta_4$ chimera and the recruitment of HD1/plectin**

<table>
<thead>
<tr>
<th>Transfected chimera</th>
<th>$\beta_4$ chimera</th>
<th>$\beta_4$-$\Delta1315$-$1456$</th>
<th>$\beta_4$-$\Delta1315$-$1752$</th>
<th>$\beta_4$-$\Delta1488$-$1752$</th>
<th>$\beta_3$ chimera</th>
<th>Control chimera</th>
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<td><strong>Fibrillar localization</strong></td>
<td>57.5±2.4</td>
<td>7.2±3.6</td>
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<td>54.8±4.3</td>
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<td><strong>HD1 colocalization§</strong></td>
<td>86.4±0.6</td>
<td>0</td>
<td>0</td>
<td>84.2±1.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*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.
et al., 1992) and is found colocalized with α6β4 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 β4 chimera, HD1/plectin consistently colocalized with the wild-type β4 chimera at fibrillar structures, as shown in Fig. 7A-C and Table 1. The recruitment of HD1/plectin is specific for the β4 intracellular domain because HD1/plectin was diffusely distributed in cells expressing the β1 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 β4 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 β4 mutants, which remained diffuse on the cell surface (Fig. 7F,G). Thus, these data demonstrate that the β4 intracellular domain, in the absence of the α6 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 β4 intracellular domain to direct the localization of HD1/plectin
Recombinant α6β4-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 α6β4 to a laminin 5-rich matrix to determine whether recombinant α6β4 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 α6β4 and plated on a laminin 5-rich matrix for 15 hours, the α6β4 integrin concentrated in a “Swiss-cheese”-like staining pattern, typical of type I hemidesmosomes (Fig. 8A). This same localization pattern was observed for recombinant α6β4 when expressed in early passage primary dermal microvascular endothelial cells plated on laminin 5 (data not shown). This result indicates that certain α6β4 ligands can regulate the subcellular localization of α6β4 in endothelial cells. However, even though α6β4 redistributed to what appears to be type I-like hemidesmosomes, the fibrillar structure also persisted. When cells transfected with only the IL-2R-β4 chimera were plated on a laminin 5-rich matrix, the β4 chimera concentrated in these fibrillar structures (Fig. 8B). In addition, in cells expressing both the recombinant α6β4 receptor and the IL-2R-β4 chimera, the β4 chimera was observed both at fibrillar structures lacking α6β4 and at type I hemidesmosome-like structures along with recombinant α6β4 (Fig. 8C,D). Therefore, the β4 tail is also sufficient to direct a reporter domain to type I hemidesmosome-like structures formed by recombinant α6β4 expressed in endothelial cells. This result suggests that cellular proteins, which can bind to the β4 tail, are recruited to these α6β4-mediated adhesions. The localization of the β4 chimera to type I-like hemidesmosomes is dependent upon the expression of recombinant α6β4.

The α6β4 integrin associates with vimentin-type intermediate filaments in endothelial cells

Since the β4 tail is sufficient to recruit the intermediate filament-associated protein HD1/plectin, we tested whether the α6β4 integrin interacts with vimentin intermediate filaments in endothelial cells. HMEC-1 cells transiently transfected with recombinant α6β4, the wild-type IL-2R-β4 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, α6β4, β1 integrins or the β4 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, β1-containing focal contacts observed in unextracted cells (Fig. 9E) were entirely lost upon extraction (Fig. 9E,F). 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 β4 tail is sufficient to mediate association with the vimentin cytoskeleton, since the IL-2R-β4 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 α6β4 integrin is assembled into distinct intermediate filament-associated structures in endothelial cells.
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 a Tween-40/deoxycholate buffer for 3 minutes prior to fixation and were double-stained with either the rat mAb GoH3, specific for the $\alpha_6$ integrin subunit (A), the FITC-conjugated mAb specific for the IL-2 receptor (C), or the FITC-conjugated mAb K20 specific for the $\beta_1$ 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 $\mu$m.

**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 α6β4 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 β4 cytoplasmic domain connected to an extracellular reporter becomes concentrated in this structure, and is independent of the expression of recombinant α6β4. This suggests that this fibrillar structure can form in the absence of α6β4 and that the recruitment of receptors to this structure occurs by an intracellular mechanism involving the β4 tail. We demonstrate that the region of the β4 cytoplasmic tail required for its localization to these fibrillar structures in endothelial cells is the same region previously described to be required for α6β1 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 β4 tail are involved in regulating α6β4 localization at all three structures. We have also shown that the β4 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, α6β4 assembles into a type I hemidesmosome-like structure that is also associated with vimentin intermediate filaments. Thus, the presence of specific α6β4 ligands and the β4 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 α6β4, because the IL-2R-β4 chimera concentrates at these fibrillar structures on the basal surface of the cell in the absence of expression of recombinant α6β4. Consistent with this data, we have not been able to detect α6β4 ligands containing laminin chains β1, α3, α2 and β2 in these structures by immunofluorescence microscopy. It is possible that this structure is formed by integrin-matrix interactions other than those mediated by α6β4. However, we have also been unable to localize other integrins at this structure, including integrins containing α2, α5, α6, β1 and β3 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 β4 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 β4 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 β4 to the basal cell surface in epithelial, endothelial and fibroblastic cell types. Although the intracellular protein interactions directing the localization of α6β4 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 β4 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 β4 intracellular domain interacts with specific proteins in endothelial cells that can direct its localization to fibrillar structures, and the same region of the β4 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 α6β4 and the IL-2R-β4 chimera to these structures is not HD1/plectin, because HD1/plectin cannot be detected in any distinct staining pattern until the wild-type β4 chimera or chimeric mutant β4-D1488-1752 is expressed. This finding is in agreement with others whose data indicate that the cytoplasmic domain of β4 can directly interact with plectin and can determine the subcellular distribution of HD1/plectin (Sanchez–Aparicio et al., 1997; Reznicek et al., 1998; Niessen et al., 1997b). However, we and others have not been able to identify a separate region of the β4 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 α6β4 concentrates in fibrillar structures in the absence of exogenous ligand, the availability of specific α6β4 ligands can regulate the distribution of α6β4 in endothelial cells. When endothelial cells expressing recombinant α6β4 were plated on a laminin 5-rich matrix, the α6β4 integrin redistributed from the fibrillar structures to type I hemidesmosome-like structures. The fibrillar structure, however, persisted because in the absence of recombinant α6β4, the IL-2R-β4 chimera remained fibrillar on cells plated on a laminin 5 matrix. However, when cells expressing both the recombinant α6β4 receptor and the IL-2R-β4 chimera were plated on a laminin 5 matrix, the chimera partially redistributed to these α6β4-containing structures, suggesting that additional cytoplasmic proteins may also redistribute to this structure and may allow for the recruitment of the β4 chimera.

Although there is ultrastructural evidence suggesting the association of the cytotekatin-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 α6β4 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 α6β4-containing structures detected in endothelial cells are associated with vimentin intermediate filaments. Importantly, the β4 tail was shown to be sufficient to mediate association with the vimentin cytoskeleton. The association of the α6β4 integrin with vimentin was further confirmed by western blot analysis, which illustrated a specific enrichment of the β4 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 α6β4 into distinct vimentin-associated structures on their basal cell surface and that the subcellular localization of α6β4 and, ultimately, its contribution to endothelial function will depend upon the availability of specific α6β4 ligands.

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Note added in proof

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