A minimal region on the integrin β4 subunit that is critical to its localization in hemidesmosomes regulates the distribution of HD1/plectin in COS-7 cells

Carien M. Niessen, Esther H. M. Hulsman, Lauran C. J. M. Oomen, I. Kuikman and Arnoud Sonnenberg
Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands
*Author for correspondence (e-mail: asonn@nki.nl)

SUMMARY

The integrin α6β4 is a major component of hemidesmosomes, in which it mediates firm adhesion to laminin 5. Previous studies have shown that the incorporation of α6β4 into hemidesmosomes requires a 303 amino acid stretch of the cytoplasmic domain of β4, comprising part of the first fibronectin type III (FNIII) repeat, the second FNIII repeat and the segment that connects the second to the third FNIII repeat (connecting segment). Now, we have further defined sequences within β4 that are critical for its localization in hemidesmosomes and we demonstrate that these sequences also induce the redistribution of HD1/plectin into junctional complexes containing the integrin α6β4 in COS-7 cells, transfected with cDNAs encoding α6A and β4. Truncation of the cytoplasmic domain of β4 after amino acids 1,382 or 1,355 in the connecting segment, by which a potential tyrosine activation motif (TAM) is removed, does not prevent the localization of α6β4 in hemidesmosomes in the rat bladder carcinoma cell line 804G and neither did it eliminate the ability of α6β4 to change the subcellular distribution of HD1/plectin in COS-7 cells. In contrast, β4 subunits in which the entire connecting segment had been deleted or which were truncated after amino acid 1,328, which removes almost the complete segment, had lost both of these functions. Furthermore, when β4 subunits with either a deletion of the second FNIII repeat or a small deletion in this repeat were co-expressed with α6, the integrins were not localized in hemidesmosomes and did not induce the redistribution of HD1/plectin in COS-7 cells. Finally, the fourth FNIII repeat of β4 could not replace the second in either of these activities. These findings establish that a region in β4, which encompasses the second FNIII repeat and a stretch of 27 amino acids (1,329-1,355) of the connecting segment, is critical for the localization of α6β4 in hemidesmosomes and that it regulates the distribution of HD1/plectin.

Key words: Integrin α6β4, Hemidesmosome, Laminin 5, HD1/plectin

INTRODUCTION

Integrins are transmembrane glycoproteins, consisting of αβ heterodimers, which serve as receptors for extracellular matrix components and which are involved in the organization of the cytoskeleton (Hynes, 1992). Integrins also participate in the transduction of signals that affect proliferation, migration and differentiation of cells (Schwartz et al., 1995; Clark and Brugge, 1995; Yamada and Miyamoto, 1995). Many integrins are clustered at sites of focal contacts, which are specialized domains of the plasma membrane to which the actin microfilament network is anchored. Cytoplasmic sequences of the β subunits are essential for this function (Sastry and Horwitz, 1993).

The integrin α6β4 is unusual in that it is concentrated in hemidesmosomes (Stepp et al., 1990; Sonnenberg et al., 1991; Jones et al., 1991), which serve as specific cell surface attachment sites for the keratin filament network in stratified and complex epithelia (Borradori and Sonnenberg, 1996; Green and Jones, 1996). The integrin α6β4 is a receptor for several laminin isoforms (Lee et al., 1992; Sonnenberg et al., 1993; Niessen et al., 1994b), one of which is laminin 5, a major component of epidermal basement membranes (Rousselle et al., 1991; Carter et al., 1991). The importance of α6β4 for cell adhesion and the formation of hemidesmosomes is supported by several observations. (1) Antibodies directed against α6β4 prevent hemidesmosome formation and induce detachment of the epidermis in vitro (Jones et al., 1991; Kurpakus et al., 1991). (2) In a group of hereditary subepidermal blistering disorders of the skin, junctional epidermolysis bullosa (JEB), laminin 5 (Pulkkinen et al., 1994; Aberdam et al., 1994) or the integrin α6β4 (Vidal et al., 1995; Niessen et al., 1996; Brown et al., 1996) are either functionally abnormal or absent. Deficiency of either of these proteins is associated with an impaired formation of hemidesmosomes and dermo-epidermal separation through the lamina lucida of the basement membrane. (3) Null mutant mice for the α6 or β4 integrin subunits show widespread dermo-epidermal separation and lack hemidesmosomes (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996).

The cytoplasmic domain of β4, which is more than 1,000 amino acids long (Suzuki and Naitoh, 1990; Hogervorst et al., 1990) and which contains two pairs of fibronectin (FNIII) repeats separated from each other by a connecting segment, appears to be required for the localization of α6β4 in hemidesmosomes. Specifically, it has been reported that a 303...
amino acid stretch, encompassing the C-terminal part of the first FNIII repeat, the second FNIII repeat and the connecting segment, contains sequences critical for the incorporation of α6β4 into hemidesmosomes (Spinardi et al., 1993). In addition, mutagenesis experiments indicate that phosphorylation of a tyrosine activation motif (TAM) in the connecting segment is critical for the localization of α6β4 in hemidesmosomes (Mainiero et al., 1995).

BP180 is another transmembrane component of hemidesmosomes (Giudice et al., 1992). Defects in the gene for BP180 have been described in a distinct subset of JEB, called generalized atrophic benign EB (Jonkman et al., 1995; McGrath et al., 1995). The mechanisms by which BP180 contributes to hemidesmosome formation and stability is not well understood. Evidence has been provided suggesting that BP180 associates with the α6 subunit (Hopkinson et al., 1995). This interaction may represent a means by which BP180 is localized in hemidesmosomes. However, recent transfection experiments indicate that the distribution of BP180 into hemidesmosomes is regulated by the integrin α6β4 and is dependent on sequences in the cytoplasmic domain of β4 (Borradori et al., 1997).

At least two hemidesmosomal plaque proteins, BP230 and plectin, have been identified and characterized using cDNA cloning (Sawamura et al., 1991; Tanaka et al., 1991; Wiche et al., 1991). These two molecules belong to a family of intermediate filament binding proteins (Green et al., 1992) and have been shown to associate with intermediate filament proteins in vitro (Foisner et al., 1988; Wiche, 1989; Yang et al., 1996). Furthermore, in the epidermis of BP230-null mice, the association of keratin filaments with hemidesmosomes is strongly reduced (Guo et al., 1995). IFAP300 and HD1 are two other components associated with hemidesmosomes (Skalli et al., 1994; Hieda et al., 1992) and probably related or even identical to plectin, because of their similar molecular masses and tissue distribution (Wiche et al., 1984; Foisner and Wiche, 1991; Jones et al., 1994; Skalli et al., 1994; Hieda et al., 1992). The identity between these molecules is further supported by the observation that mutations in the plectin gene, found in a subset of patients with epidermolysis bullosa simplex associated with muscular dystrophy, are associated with a loss of immunoreactivity with anti-plectin and anti-HD1 antibodies (Chavanas et al., 1996; McLean et al., 1996; Smith et al., 1996).

The hemidesmosomal plaque proteins plectin, HD1 and IFAP300 are not found exclusively in hemidesmosomes. Plectin and HD1 are constituents of focal contacts and the cytoskeleton in several cell types (Seifert et al., 1992; Foisner et al., 1994; Hieda et al., 1992; Sánchez-Aparicio et al., 1997) while IFAP300 is found in desmosomes (Skalli et al., 1994). Similarly, α6β4 is present in different simple epithelial tissues (Sonenberg et al., 1990), immature thymocytes (Wadsworth et al., 1992), subsets of endothelial cells (Kennel et al., 1992) and Schwann cells and perineurial fibroblasts in peripheral nerves (Sonenberg et al., 1990; Niessen et al., 1994a; Feltrì et al., 1994). In some of these tissues, cytoplasmic variants of β4, which arise by alternative splicing of mRNA, have been detected. For example, in peripheral nerves, a variant has been described in which 53 amino acids (β4B; Niessen et al., 1994a) have been inserted in the connecting segment. In another variant (β4C) found in placenta, 70 amino acids are inserted in another part of the connecting segment (β4C; Tamura et al., 1990). Both variants are expressed at relatively low levels compared to β4A.

The interaction of α6β4 with the cytoskeleton in cells that do not form hemidesmosomes has not yet been defined. In these cells, the association of α6β4 with the cytoskeleton may occur via HD1/plectin. It has already been found that HD1 and α6β4 are colocalized at the basal side of certain epithelial cells, in the absence of the hemidesmosome-specific proteins BP230 and BP180 (Uematsu et al., 1994; Kemperman et al., 1994). Furthermore, transfection of the β4 cDNA in β1-deficient GD25 cells (Fässler et al., 1995) results in an overlapping distribution of HD1 and α6β4, which is different from the normal localization of HD1 in focal contacts (Sánchez-Aparicio et al., 1997). Recently, we have shown that a recombinant fragment of the β4 cytoplasmic domain (the two pairs of FNIII repeats and the connecting segment) forms a complex with HD1 and that the same region of β4 is responsible for the redistribution of HD1 in COS-7 cells after transfection with cDNAs for α6 and β4 (Niessen et al., 1997). Whether β4 directly associates with HD1 or via other connecting molecules is not clear.

In this study, we have analyzed the potential of mutant forms of the β4 subunit, lacking parts of the cytoplasmic domain, to be incorporated into hemidesmosomes of 804G rat bladder carcinoma cells and assessed their ability to recruit HD1 to the basal side in junctions containing the α6β4 integrin in COS-7 cells.

MATERIALS AND METHODS

Cells and antibodies

The rat cell line 804G (Izumi et al., 1981) was grown in ISCOVE’s medium containing 10% FCS, penicillin and streptomycin, and for the stable transfectants, with G418 (400 μg/ml). The African monkey kidney cell line COS-7 was cultured in DMEM with 10% FCS, penicillin and streptomycin.

The mouse mAbs 450-9D (Kennel et al., 1990) and 4.3E1 (Hessle et al., 1984) and the rat mAb 439-9B (Kennel et al., 1989) are all directed against the extracellular domain of human β4. Mouse mAbs 450-10D and 450-11A recognize the cytoplasmic domain of β4 (Kennel et al., 1990). The following rabbit sera against the cytoplasmic domains of integrin subunits were used: anti-α3A (Dipersio et al., 1995), anti-β1 (De Filippi et al., 1991), anti-α6A (Hogervorst et al., 1993) and anti-β4 (Niessen et al., 1994a). Dr K. Owaribe (Nagoya University, Nagoya, Japan) kindly provided us with the mouse mAb 121 directed against the HD1 molecule (Owaribe et al., 1991).

Labeling and immunoprecipitation of integrins

Cells were surface-labeled with 125I using the lactoperoxidase procedure, and then 1% Nonidet P-40 cell lysates were used for immunoprecipitation as described (Sonnenberg et al., 1990). Immune complexes were collected with Protein A-Sepharose (Pharmacia, Uppsala, Sweden). Affinity-purified rabbit antiserum against mouse IgG was included in immunoprecipitations, involving the mouse mAbs 4.3E1, 450-9D, 450-10D and 450-11A. The immunoprecipitates were analyzed on SDS-5% polyacrylamide gels under non-reducing conditions.

cDNA constructs

The β4A cDNA (in normal case) and protein sequences (in superscript) are numbered according to Suzuki and Naitoh (1990). The β4A, β4B and β4C cDNA constructs have been described previously (Niessen et al., 1994b, 1997). To obtain the β4A-187 cDNA, the full-length β4A cDNA in the eukaryotic expression vector pRc-CMV
was digested with NcoI at position 4,586 of the β4 sequence and in the polyclinker 3′ of the β4 sequence and religated to remove the sequences 4,586-6,562 of the β4 cDNA. Other β4 truncations were obtained as follows: a β4 cDNA sequence up to the EcoRV site at position 4,922 was prepared by insertion of an EcoRI fragment isolated from plasmid 586 containing full-length β4A cDNA into the EcoRI site of pBluescript II KS+ (pBSKII+, Stratagene, La Jolla, CA), digestion with EcoRV (in the β4 sequence and in the polyclinker 3′ of the β4 sequence) and religation. An Xhol linker, which contained stop-codons in all three reading frames, was inserted into the Xhol site in the polyclinker 3′ of the β4 sequence after blunting of the vector using Klenow DNA polymerase. This resulted in construct A. β4 cDNA sequences containing the truncations were obtained by PCR and subsequently exchanged with wild-type β4 sequences of construct A. PCR was performed using native βf1 DNA polymerase (Stratagene, La Jolla, CA) and β4A cDNA as a template. For the βA1436, βA1436, βA4135, βA4132 and βA4128 constructs, PCRs were performed with primer 3 in combination with primers 13, 27, 426, 120 or 173, respectively. The PCR products were digested with BglII (at position 2580 of the β4 sequence) and inserted into the BglII and EcoRV sites (at positions 2,580 and 4,922 of the β4A sequence respectively) of plasmid A. The cDNAs encoding the mutant β4 constructs were subcloned into the Xhol site of the eukaryotic expression vector pRC-CMV. For the cDNAs encoding the βA41219-1319, βA41224-1456, βA41229-1265 truncations and the βA1529IV replacement mutant, PCR was performed with primers 3/173, primers 3/343, primers 234/15, primers 235/15, primers 299/300 or primers SO1/SO2, respectively. Primers 173, 343, 234, 235, 299 and 300 contain a HindIII site which is not included in the β4 sequence.

The PCR product obtained with primers 234/15, encoding the connecting segment and the last two FNIII repeats of β4s, was digested with HindIII (in primer 234) and EcoRV (at position 4,922 of the β4 sequence) and inserted into pBSKII+ previously digested with HindIII and EcoRV, resulting in construct B. Product 3-173 was digested with HindIII (at position 2,825 of the β4A sequence), and inserted in the HindIII site of construct B to obtain construct C, which encodes the Δ1219-1319 deletion. Construct C was partially digested with HindIII to insert the cDNA encoding the fourth repeat (product 299-300) into the HindIII site between the cDNAs encoding the first FNIII repeat and the connecting segment, resulting in construct D. Similarly, construct E, which encodes the Δ1322-1456 deletion was obtained, using product 235-15, digested with HindIII and EcoRV, insertion into the HindIII and EcoRV sites of pBSKII+, and subsequent insertion of product 3-343 previously digested with HindIII. Constructs C, D and E and PCR product SO1/SO2 (Δ1249-1265) were digested with SacI and NotI (at positions 3,674 and 4,586 of the β4 sequence, respectively) and exchanged for the SacI-NotI fragment of full-length β4A cDNA inserted in the EcoRI site of pUC18, to obtain the cDNAs encoding the βA41219-1319, βA41529IV, βA41322-1456 and βA41249-1265 mutants, respectively. The cDNAs encoding the mutant β4 subunits were inserted in the EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen Corp., San Diego, CA). All constructs were verified by dideoxy sequencing.

The sequencing of the primers used are: primer 3 (2,517-2,541, sense): 5′GCTGTCGAGTTTGCCAAGCATGCA3′, primer 13 (posn 4,406-4,434, antisense): 5′CAGTTGTTGAGAGTGTTGAGATTTG-3′, primer 27 (posn 4,251-4,271, antisense): 5′GCAAGAGGGTATGGGAGGATGATG3′, primer 120 (posn 4,094-4,111, antisense): 5′GTCAAGGCTGATCCATCGG3′, primer 246 (posn 4,056-4,073, antisense): 5′CTAGTGAGCCTGCTCG3′, primer 173 (posn 3,763-3,780, antisense): 5′TTAACGGTCTCGTGTTGCGGCC3′, primer 234 (posn 4,084-4,101, sense): 5′TTAAAGCTTCTCGTGTTGCGGCC3′, primer 235 (posn 4,493-4,512, sense): 5′TTAAAGCTTCTCTGTTGCGGCC3′, primer 299 (posn 4,926-4,942, sense): 5′TTAAAGCTTCTCTGTTGCGGCC3′, primer 300 (posn 5,114-5,129, antisense): 5′GAAGAGCTTCTCAGGATGAG3′, primer 343 (posn 4,071-4,088, antisense): 5′TTAACGCTTCTGCGGGGAGG3′, primer 15 (posn 5,100-5,123, antisense): 5′GACTGCTAAGATGATGATG3′, primer SO1 (posn 3,639-3,657, sense): 5′CCACCTGTCGACAGCAAG3′, primer SO2 (posn 4,600-4,619, antisense): 5′TTCAGAGCTTGATGATG3′. HindIII restriction sites in the various primers are underlined.

Immunoblot analysis
Cells were lysed with 1% Nonidet P-40 (NP-40) in 25 mM Tris-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. Protein concentrations in the cell lysates were determined with the BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein were loaded on a 5% SDS-polyacrylamide gel under non-reducing conditions, separated and transferred to a nitrocellulose filter (Sommenberg et al., 1993). The blot was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 3% baby milk powder and 1% bovine serum albumin (BSA), washed and probed with specific antibodies (ascitic fluid, diluted 1:500 in blocking buffer) for 1 h at room temperature. Bound primary antibodies were visualized by chemiluminescence (ECL, Amersham Int., Buckinghamshire, UK), using horseradish peroxidase-conjugated secondary antibodies at a 1:5,000 dilution.

DNA transfections and immunofluorescence microscopy
The rat bladder carcinoma cell line 804G was transfected with the full-length and mutant β4 cDNAs using CaCl2 and HBS buffer (1 mM Na2HPO4, 6.5 mM glucose, 140 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.1). Cells were selected with 400 μg/ml G418 (Gibco-BRL, Gaithersburg, MD). To obtain cells that strongly express β4, bulk populations were sorted three times on the fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA) with the human β4-specific mAb 450-9D. For immunofluorescence microscopy, cells were plated on glass coverslips in culture medium. COS-7 cells were plated on glass coverslips and the next day transfected with the cDNA in combination with the various β4 cDNAs. After one or two days, cells were fixed with 1% formaldehyde in PBS for 10 minutes, permeabilized with 0.5% Triton X-100 for 5 minutes and blocked with 1% BSA for 20 minutes. All antibody incubation steps were performed for 30 minutes at 37°C, after which the coverslips were washed three times with PBS. For single labeling experiments, cells were first incubated with the anti-β4 mAb 450-9D and subsequently with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Zymed Lab. Inc., San Francisco). For double labeling studies, cells were first incubated with the mouse mAb 121 and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Subsequently, cells were incubated with the rat mAb 439-9B, followed by incubation with rabbit anti-rat-specific IgGs (Sommenberg et al., 1986) and stained with Texas Red-conjugated donkey anti-rabbit (Amersham). The coverslips were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) and viewed under a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Richmond, CA).

RESULTS
The integrin β4A and β4B subunits are localized in hemidesmosomes in 804G cells
Since the β4B variant is a minor variant in cells that assemble hemidesmosomes, we wondered whether the insertion of 53 amino acids in the connecting segment of this subunit affects its ability to be assembled into hemidesmosomes. To address this question, we transfected the rat bladder carcinoma cell line 804G, which forms hemidesmosomes in vitro (Riddelle et al., 1991) with human β4A or β4B cDNA. Immunoprecipitation
from lysates of cell surface-labeled transfectants revealed that the human β4A and β4B subunits are expressed on the cell surface complexed to rat α6 (Fig. 1). The human β4-specific mAbs 4.3E1, 450-9D and 450-11A precipitated the human β4A or β4B subunit, together with rat α6. Rabbit sera directed against α6 or β4 and the anti-β4 mAb 450-10D recognized both endogenous rat α6β4 and the rat/human chimeric α6β4 integrin. No α6β1 was precipitated by anti-α6. Anti-α3 antibodies precipitated α3β1, and rabbit anti-β1 serum precipitated β1, α3 and possibly also α2 (Fig. 1).

To investigate the localization of the recombinant β4 subunits, we performed double immunofluorescence studies using the rat mAb 439-9B, specific for human β4, and the murine mAb 121 to HD1. As shown in Fig. 2A and C, both the β4A and β4B subunits were codistributed with HD1 within ‘Swiss-cheese’-like structures corresponding to hemidesmosomes in 804G cells (Riddelle et al., 1991). Computer-generated z-sections demonstrated that both HD1 and the recombinant β4 subunits were concentrated at the basal surface of the cells (Fig. 2B,D). These results indicate that the inserted 53 amino acids in the connecting segment of β4B do not interfere with its incorporation in hemidesmosome-like structures.

The N-terminal part of the connecting segment is involved in targeting α6β4 to hemidesmosomes

A previous study has shown that a region encompassing the complete second FNIII repeats and the connecting segment, as well as parts of the first and third FNIII repeats mediate the incorporation of α6β4 in hemidesmosomes (Spinardi et al., 1993). To define the minimal amino acid sequences in β4 required for this localization, cDNAs encoding mutant forms of β4 carrying C-terminal truncations (Fig. 3A) were constructed and transfected into 804G cells. Cell clones were obtained that stably expressed the truncated human β4 molecules on their surface, in association with rat α6, as assessed by FACS using mAbs directed against human β4 (not shown). To determine the expression of exogenous β4 relative to that of endogenous β4, we performed immunoblot analysis of cell extracts using mAb 450-10D, which recognizes both
Mapping of functional domains on the integrin β4 subunit

Fig. 2. The integrin β4A and β4B subunits are both incorporated into hemidesmosomes where they are co-localized with HD1/plectin. Rat 804G cells stably expressing β4A (A and B) or β4B (C and D) were double labeled for human β4 (red) and rat HD1 (green) and viewed under a confocal microscope. Sections A and C are focussed at the cell-substrate interface, whereas sections B and D are perpendicular to the substrate. In A and C, the arrowheads indicate the positions from which the perpendicular sections shown in B and D were taken. Micrographs with both transfected and untransfected cells are shown. The β4A and β4B molecules are present in a Swiss-cheese-like pattern confined to the basal cell surface, where they are colocalized with HD1 as indicated by the yellow color.

Fig. 3. Expression of full-length and C-terminally truncated mutant β4 molecules in 804G cells. (A) Schematic representation of full-length and mutant β4 molecules of which only the cytoplasmic domains are shown. The FNIII repeats are represented by boxes in which the number of the repeat is shown. The β4B-specific insert of 53 amino acids is shown by a triangle. The estimated molecular masses are indicated. A + means localization in hemidesmosomes in 804G cells and a distribution for HD1 at the basal side of the COS-7 cells, together with the human β4 subunit. A – indicates a diffuse distribution of β4 in the 804G cells and a distribution of HD1 in transfected COS-7 cells that is similar to that in untransfected COS-7 cells. (B) Immunoblot analysis of lysates of transfected 804G cells with mAb 450-9D, which is directed against human β4 and mAb 450-10D, which recognizes both the rat and human β4 subunits. Samples are 804G cells (lane 1) and 804G cells stably expressing βA (lane 2), β41487 (lane 3), β41436 (lane 4), β41382 (lane 5), β41355 (lane 6), β41328 (lane 7), β41218 (lane 8) or β41182 (lane 9). The 200 kDa band obtained with mAb 450-9D is also seen with a control antibody and thus represents a non-specific product. The positions of the molecular mass markers (in kDa) are shown at the right.

human and rat β4, as well as mAb 450-9D, which only reacts with human β4. Fig. 3B shows that the truncated molecules were of the expected size in all transfectants tested and that all of them were more weakly expressed than the endogenous β4 subunit.

We then determined the localization of the chimeric α6β4
molecules in the 804G transfectants by immunofluorescence microscopy using the mAb 450-9D. In line with previous findings (Spinardi et al., 1993), β41487, but not β41182, was incorporated in hemidesmosome-like structures (Fig. 4A,G). A mutant of β4 in which the remaining part of the third FNIII repeat present in β41487, as well as part of the connecting segment (β41436) were removed, and two mutants in which still larger parts of the connecting segment were deleted (β41382 and β41355) were, like β41487, localized in hemidesmosomes (Fig. 4B-D). The localization of these three mutants in hemidesmosomes, however, was unexpected because they contain only one (β41436) or none (β41382 and β41355) of the two tyrosine residues that were previously shown to be essential for the localization of α6β4 in hemidesmosomes (Mainiero et al., 1995). The distribution of β41328, which lacks almost the complete connecting segment and of β41218, also lacking the second FNIII repeat, was indistinguishable from that of β41182 (Fig. 4G). Thus, β41328 and β41218 were diffusely distributed at the cell surface and in a punctate pattern in the cytoplasm, rather than concentrated at the basal side of the cell (Fig. 4E,F and insets). The intracellular staining pattern has been seen previously with permeabilized 804G cells, and it probably represents stained Golgi and endoplasmic reticulum structures (Spinardi et al., 1993). These results suggest that crucial sequences responsible for the localization of α6β4 in hemidesmosomes are present within a short domain consisting of amino acids 1,329-1,355.

Expression of internal deletion mutants of β4

Since deletion of part of the first FNIII repeat does not interfere with the localization of α6β4 in hemidesmosomes, which suggests that this repeat is not involved (Spinardi et al., 1993), we investigated the contribution of the second FNIII repeat and the connecting segment to this localization in more detail. We
constructed a cDNA encoding a β4 subunit lacking the second FNIII repeat (β4<sup>A1219-1319</sup>), as well as a β4 mutant, in which only the connecting segment was deleted (β4<sup>A1322-1456</sup>), and transfected them into 804G cells (Fig. 5). Expression of the two β4 mutants was assessed by immunoblot analysis of cell extracts using mAbs 450-9D and 450-11A, the latter of which recognizes an epitope in the third FNIII repeat (C. M. Niessen, E. Rots and A. Sonnenberg, unpublished observations). As shown in Fig. 6A,B both β4 mutants were expressed and their size is in agreement with the predicted molecular mass of 180 kDa for β4<sup>A1219-1319</sup> and of 175 kDa for β4<sup>A1322-1456</sup>. An additional band of 120 kDa in the extracts of the β4<sup>A1219-1319</sup> transfected cells detected with mAb 450-9D, but not with 450-11A, probably represents a truncated β4 subunit lacking its C-terminus. Because the molecular masses of the human β4 mutants were close to that of the endogenous rat β4 subunit (190 kDa), these proteins could not be properly resolved by SDS-PAGE and in immunoblot analysis with mAb 450-10D they appeared as one broad band. Immunofluorescence analysis revealed a diffuse distribution of the β4<sup>A1219-1319</sup> mutant, which suggests that the second FNIII repeat contains crucial residues for the incorporation of α6β4 in hemidesmosomes (Fig. 7A). As expected, β4<sup>A1322-1456</sup>, lacking the connecting segment, was also not localized in hemidesmosomes (Fig. 7B). A small deletion of 17 amino acids in the second FNIII repeat of β4<sup>A1249-1256</sup> has recently been found in a patient with junctional EB associated with pyloric atresia (Vidal et al., 1995). As shown in Fig. 7C, this small deletion in the second FNIII repeat, like its total absence, prevents the incorporation of β4 into hemidesmosomes, although as determined by immunoblot analysis it was correctly synthesized (Fig. 6). The above results suggest that, besides amino acids within the stretch of residues 1,329-1,355 of the connecting segment, sequences within the second FNIII repeat are also involved in the localization of α6β4 in hemidesmosomes.

We have also replaced the second FNIII repeat by the fourth to examine whether the mere presence of a FNIII repeat at that position in β4 is sufficient for the hemidesmosomal localization of α6β4 (Fig. 5). The fourth FNIII repeat was chosen for this experiment because, in the presence of the second FNIII repeat, it is not essential for the localization of α6β4 in hemidesmosomes, and because its length and amino acid composition most closely resemble those of the second FNIII repeat (Fig. 9). The size of the β4<sup>IV</sup> mutant expressed in 804G cells was as expected (Fig. 6) and its localization was analyzed using the anti-human β4 mAb. As is shown in Fig. 7D, this mutant was present along the cell membrane and in a punctate pattern in the cytoplasm. Thus, the second FNIII repeat at that position cannot be replaced by the fourth to obtain localization of α6β4 in hemidesmosomes. The expression of the β4 deletion mutants did not interfere with the endogenous formation of hemidesmosomes in 804G cells, as is shown in the β4<sup>A1219-1319</sup> transfected cells, in which staining for BP230 and endogenous α6 revealed the typical ‘Swiss-cheese’-like pattern (Fig. 7E,F).

The β4 domains crucial for hemidesmosome localization also determine colocalization with HD1 in COS-7 cells

The monkey kidney cell line COS-7 expresses HD1, associated with the cytoskeleton, but no other hemidesmosomal components, such as α6β4, BP230 or BP180. We have shown that, after transient transfection of α6A and β4 cDNAs in these cells, the distribution of HD1 changes. It is concentrated at the basal side of the cells, where it is colocalized with α6β4 (Niessen et al., 1997). We have tested the ability of all the
above mutant β4 subunits to recruit HD1 to the basal side of the cells. COS-7 cells, plated on glass coverslips, were transfected with full-length α6A cDNA in combination with the appropriate β4 constructs. After 2 days, the cells were fixed and double-stained with mAb 121 (anti-HD1) and 439-9B (anti-β4), to assess their localization. The results are summarized in Fig. 3A and shown in Fig. 8. Both β41382 (Fig. 8A,B) and β41355 (Fig. 8C,D) are colocalized with HD1 at the basal side of the COS-7 cells in a pattern that is similar to that observed for α6β4 and HD1 in RAC-11P cells (Sonnenberg et al., 1993). However, in COS-7 cells expressing the β41328 subunit, no colocalization of β4 and HD1 was observed. Staining of β41328 (Fig. 8E) was diffuse whereas the staining pattern for HD1 (Fig. 8F) was similar to that in untransfected cells, i.e. associated with the cytoskeleton (Niessen et al., 1997). Thus, residues within the stretch of amino acids 1329-1355 are not only involved in the localization of α6β4 in hemidesmosomes, but also in the localization of HD1 at the basal side of the cells. Analysis of the other β4 mutants showed that all constructs that are localized in hemidesmosome-like structures after transfection in 804G cells, were also colocalized with HD1 at the basal side in similarly transfected COS-7 cells. The β4 mutants, whose distribution in 804G cells was diffuse, were not colocalized with HD1 in COS-7 cells. In cells expressing these β4 mutants, HD1 was found to be associated with the cytoskeleton, as in untransfected COS-7 cells (not shown).

**DISCUSSION**

In the present study, it is demonstrated that sequences within the N-terminal 27 amino acids of the connecting segment (1,329-1,355) and the second FNIII repeat of the β4 cytoplasmic domain are critical and sufficient for the incorporation of α6β4 into hemidesmosomes. Deletion of either amino acids 1,329-1,355 or the second FNIII repeat resulted in β4 subunits that were associated with α6 and expressed at the cell surface, but which were not concentrated in hemidesmosomes in 804G cells. The same regions of β4 are also essential for the codistribution of α6β4 and the hemidesmosomal plaque protein HD1 into junctional complexes in transfected COS-7 cells.

FNIII repeats have been implicated in protein-protein interactions (Campbell and Spitzfaden, 1994). It is therefore conceivable that the second FNIII repeat provides a binding site for the interaction of β4 with one or more cytoskeleton-associated proteins. Alternatively, the binding site is located in the stretch of amino acids 1,329-1,355 and the second FNIII repeat may only be required for its proper presentation. Each FNIII repeat has a fold of seven β strands forming two closely packed
Mapping of functional domains on the integrin β4 subunit

A small deletion in the second FNIII repeat is likely to disrupt this conformation. The fact that a β4 mutant from a PA-JEB patient with a deletion of 17 amino acids in the second FNIII repeat (Fig. 9) was not localized in hemidesmosomes, when expressed in 804G cells, supports the supposition that an intact FNIII repeat is necessary for the localization of β4 in hemidesmosomes. However, our finding that the second FNIII repeat could not be replaced by the fourth repeat indicates that the mere presence of an FNIII repeat at the position of the second is not sufficient for incorporation of α6β4 in hemidesmosomes. Together, our results suggest that both the correct folding and specific sequences within the second repeat, together with the sequences within amino acids 1,329-1,355 of the connecting segment are required for the localization of α6β4 in hemidesmosomes. It still needs to be established whether the first FNIII repeat is also involved in the correct presentation of amino acids 1,329-1,355, but the finding by Spinardi et al. (1993) that deletion of the N-terminal part of the cytoplasmic domain of β4, including part of the first FNIII repeat, did not interfere with hemidesmosomal localization of the mutant β4 subunit, strongly suggests that the first repeat is not involved in the targeting of β4 to hemidesmosomes.

In the β4B variant, 53 amino acids have been inserted in the C-terminal part of the connecting segment, which we now have shown not to be involved in the localization of α6β4 in hemidesmosomes. The β4B variant is a minor variant in keratinocytes, which mainly express β4A, but it is more abundantly expressed in other tissues, such as peripheral nerves (Niessen et al., 1994). Perhaps, it is involved in additional or alternative protein-protein interactions in cell types that do not form hemidesmosomes.

In a previous report, it has been shown that the tyrosine acti-

![Fig. 8. Distribution of HD1/plectin and β4 in COS-7 cells transfected with cDNAs for α6 and either β4\(^{1382}\), β4\(^{1355}\) or β4\(^{1328}\). COS-7 cells transiently transfected with β4\(^{1382}\) (A and B), β4\(^{1355}\) (C and D) or β4\(^{1328}\) (E and F) were stained with anti-HD1 antibody (mAb 121, A, C and E) and double labeled with anti-β4 antibody (mAb 439-9B, B, D and F). The β4\(^{1382}\) and β4\(^{1355}\) constructs, but not β4\(^{1328}\), are colocalized with HD1. Bars, 25 μm.](image)

![Fig. 9. Amino acid sequence alignment of the FNIII repeats of fibronectin (FN) and β4. Identical amino acids are indicated by asterisks and conservative exchanges by points. Horizontal bars represent gaps introduced to maximize alignment. A small insertion in the second FNIII repeat of β4 compared to the other FNIII repeats is shown in italic. The β-strand assignments (underlined) are indicated for the tenth FNIII repeat of fibronectin but are likely to be closely similar for other FN modules. The deletion of 17 amino acids, present in β4\(^{11249-1265}\), is boxed.](image)
evation motif (TAM), which is present in the connecting segment of the β4 cytoplasmic domain, is involved in the recruitment of α6β4 to hemidesmosomes (Mainiero et al., 1995). Substitution of the first tyrosine (Y1422) in the TAM sequence resulted in only partial incorporation of α6β4 into hemidesmosomes in 804G cells, whereas substitution of the second tyrosine or both tyrosine residues of the TAM with a phenylalanine residue (Y1440F) completely prevented the incorporation of α6β4 in hemidesmosomes. In contrast, our experiments show that β4 mutants in which either the second tyrosine in the TAM sequence (β41430) or the complete TAM sequence (β41382 and β41355) has been deleted normally incorporate into hemidesmosomes. Moreover, transfection of 804G cells with a cDNA encoding a β4 mutant in which both tyrosines are replaced by phenylalanines, resulted in an apparently normal localization of α6β4 in hemidesmosomes (results not shown). There is no obvious explanation for these contradictory results. However, recently, we found that the TAM sequence may coordinate the codistribution of BP180 with α6β4 and HD1/plectin in transfected COS-7 cells (Borradori et al., 1997).

Another major finding of our present study is that both the second FNIII repeat and the N-terminal stretch of 1,329-1,355 amino acids of the connecting segment are also essential for the recruitment of HD1 to the basal side of the COS-7 cells. These data are consistent with previous in vitro binding experiments, in which β4 and HD1 were found together in a complex (Niessen et al., 1997). In untransfected COS-7 cells, which do not express α6β4, HD1 is associated with the cytoskeleton but after expression of α6β4 it is preferentially colocalized with α6β4 at the basal side of the cell. Similarly, in G2D5 cells in which HD1 is predominantly found in focal contacts, after transfection with β4 cDNA, it is found in distinct spots at the basal side, together with α6β4 (Sánchez-Aparicio et al., 1997). These results suggest that the association of HD1 with the cytoplasmic domain of β4 prevails over its association with cytoskeletal components or with focal contact components. It is possible that HD1 links α6β4, either directly or indirectly, to the cytoskeleton in tissues or cell types that do not assemble genuine hemidesmosomes, such as Schwann cells or endothelial cells. The observation that the same region in β4 is required for both localization in hemidesmosomes in 804G cells and the ability of α6β4 to change the distribution of HD1 in COS-7 cells, suggests that the localization of α6β4 in hemidesmosomes is facilitated by the association of β4 with HD1.

It is likely that the proper assembly of hemidesmosomes and their connection with the cytoskeleton requires multiple interactions between the various hemidesmosomal proteins. The observation that, in junctional EB patients, who lack either BP180 (Jonkman et al., 1995; McGrath et al., 1995) or α6β4 (Vidal et al., 1995; Niessen et al., 1996), while expressing all other hemidesmosomal components, the formation of hemidesmosomes is frequently impaired, as assessed by electron microscopy, supports this hypothesis. Moreover, deficiency of the hemidesmosomal plaque proteins, HD1/plectin (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996) and BP230 (Guo et al., 1995), similarly affects the connection of keratin filaments with hemidesmosomes, as found in man and mouse, respectively. Based on our results, it is tempting to speculate that the formation of α6β4/HD1 complexes, as they occur in type II hemidesmosomes in several cell types (Umatsu et al., 1994; Kemperman et al., 1994) provides a core structure for the assembly of hemidesmosomes to which BP180 and BP230 associate in a later step.

The 804G cells already form hemidesmosomes in culture and express endogenous α6β4 on their cell surface. Thus, the β4 mutant cDNA transfection studies only allow investigation of the ability of mutant β4 subunits to localize in hemidesmosomes, but not their capacity to initiate the formation of hemidesmosomes. It will be interesting to know whether sequences within the stretch of amino acids 1329-1355 and the second FNIII repeat of β4 are also sufficient for its function in the assembly of hemidesmosomes, or that a larger region of β4 is required. Expression studies in the keratinocytes derived from a β4-deficient patient (Niessen et al., 1996) will allow us to study the initial events in hemidesmosome formation in more detail.

In conclusion, we have found that both the second FNIII repeat and a stretch of amino acids 1,329-1,355 of the connecting segment in β4 are crucial for the incorporation of α6β4 in hemidesmosomes in 804G cells and for the association of α6β4 with HD1 in COS-7 cells.

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