Two Different Mutations in the Cytoplasmic Domain of the Integrin β4 Subunit in Nonlethal Forms of Epidermolysis Bullosa Prevent Interaction of β4 with Plectin

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The integrin α6β4 plays a crucial role in the assembly and maintenance of hemidesmosomes. Previous work has shown that the recruitment of plectin into hemidesmosomes is dependent on β4 and involves a region of the β4 cytoplasmic domain, which contains the first two fibronectin (FNIII) repeats and a short region of the connecting segment. Two missense mutations (R1225H and R1281W) in β4 that are responsible for nonlethal forms of epidermolysis bullosa are located in the second FNIII repeat. One of them is confined to a loop region that connects two β strands (EC′) whereas the other is located at the N-terminal end of the second FNIII repeat. We here report that these mutations render β4 unable to interact with plectin and prevent the localization of plectin in hemidesmosomes. Substitution of a lysine residue (K1279W) that forms part of the same loop as R1281 had no effect on the ability of β4 to recruit plectin. Furthermore, we show that an extended loop structure in β4, composed of the amino acids DDN (1262–1264), which resembles the RGD integrin-binding loop in fibronectin, is not involved in the binding to plectin. These results further demonstrate that binding of β4 to plectin is essential for the proper formation and function of hemidesmosomes and that loss of the interaction between β4 and plectin is associated with a mild form of epidermolysis bullosa. ***Key words: cytoskeleton/epidermolysis bullosa/hemidesmosome/integrin/plectin. J Invest Dermatol 117:1405–1411, 2001***

Hemidesmosomes are stable adhesion complexes that facilitate the linkage between the keratin intermediate filament system and the extracellular matrix in stratified and pseudo-stratified epithelia (Borradori and Sonnenberg, 1999). They consist of at least five components of which three are transmembrane spanning proteins: the integrin α6β4, which serves as a receptor for laminin-5 (Stepp *et al*, 1990; Sonnenberg *et al*, 1991; Niessen *et al*, 1994), the collagenous bullous pemphigoid antigen BP180 (Giudice *et al*, 1992), and the tetraspanin CD151 (Sterk *et al*, 2000). The two cytoplasmic constituents of hemidesmosomes include the plakin family members plectin (Hieda *et al*, 1992; Gache *et al*, 1996) and the bullous pemphigoid antigen BP230 (Stanley *et al*, 1981). Abnormalities in hemidesmosomal components or structures have been associated with tissue fragility and blistering disorders of the skin, demonstrating the importance of hemidesmosomes for the adhesion between the dermis and the epidermis, as well as for tissue integrity (Stanley, 1995; Christiano and Uitto, 1996). For example, patients who lack α6 or β4, due to mutations in the genes encoding these subunits, have rudimentary hemidesmosomes or completely lack these structures (Vidal *et al*, 1995; Niessen *et al*, 1996; Pulkkinen *et al*, 1997; Ruzzi *et al*, 1997). These patients are characterized by pyloric atresia associated with junctional epidermolysis bullosa (PA-JEB). They suffer from severe blistering of the skin and often do not survive beyond the age of 1 y. Similarly, α6 and β4 null mice exhibit severe skin blistering and die perinatally (Dowling *et al*, 1996; Georges-Labouesse *et al*, 1996; van der Neut *et al*, 1996). Most of the mutations identified in PA-JEB patients concern nonsense mutations or mutations at splice sites that result in the early termination of translation of the β4 protein. Missense mutations resulting in the substitution of a single amino acid have also been demonstrated. Two of these point mutations (R1225H and R1281W) have been disclosed in patients with a nonlethal form of epidermolysis bullosa (EB) (Pulkkinen *et al*, 1998; Nakano *et al*, 2001). Mutations in the plectin gene are responsible for a variant form of EB with a late onset of muscular dystrophy. They have been associated with a reduced anchorage of intermediate filaments to hemidesmosomes (McLean *et al*, 1996; Smith *et al*, 1996). As a result, keratinocytes are more fragile and rupture easily upon application of stress, resulting in a split through the basal keratinocytes. Mice that are deficient in plectin recapitulate most of the features of these patients, but die much earlier around birth probably because essential functions of plectin are lost in other tissues, e.g., the heart and skeletal muscle (Andrä *et al*, 1997).

The integrin β4 subunit is characterized by an unusually long cytoplasmic domain that harbors four fibronectin type III (FNIII) repeats, residing in two pairs separated by a connecting segment (Hogervorst *et al*, 1990; Suzuki and Naitoh, 1990). Previous studies have shown that a fragment of β4 containing the first pair of FNIII repeats and the first 36 amino acids (1320–1355) of the connecting segment are crucial for the recruitment of plectin in hemidesmosomes (Niessen *et al*, 1997a; Nievers *et al*, 1998). Furthermore, it was shown that this fragment contains a binding site for plectin and

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Abbreviations: ABD, actin binding domain; FNIII, fibronectin type III; GAL4, galactose metabolism regulatory gene 4; PA-JEB, pyloric atresia associated with junctional epidermolysis bullosa.

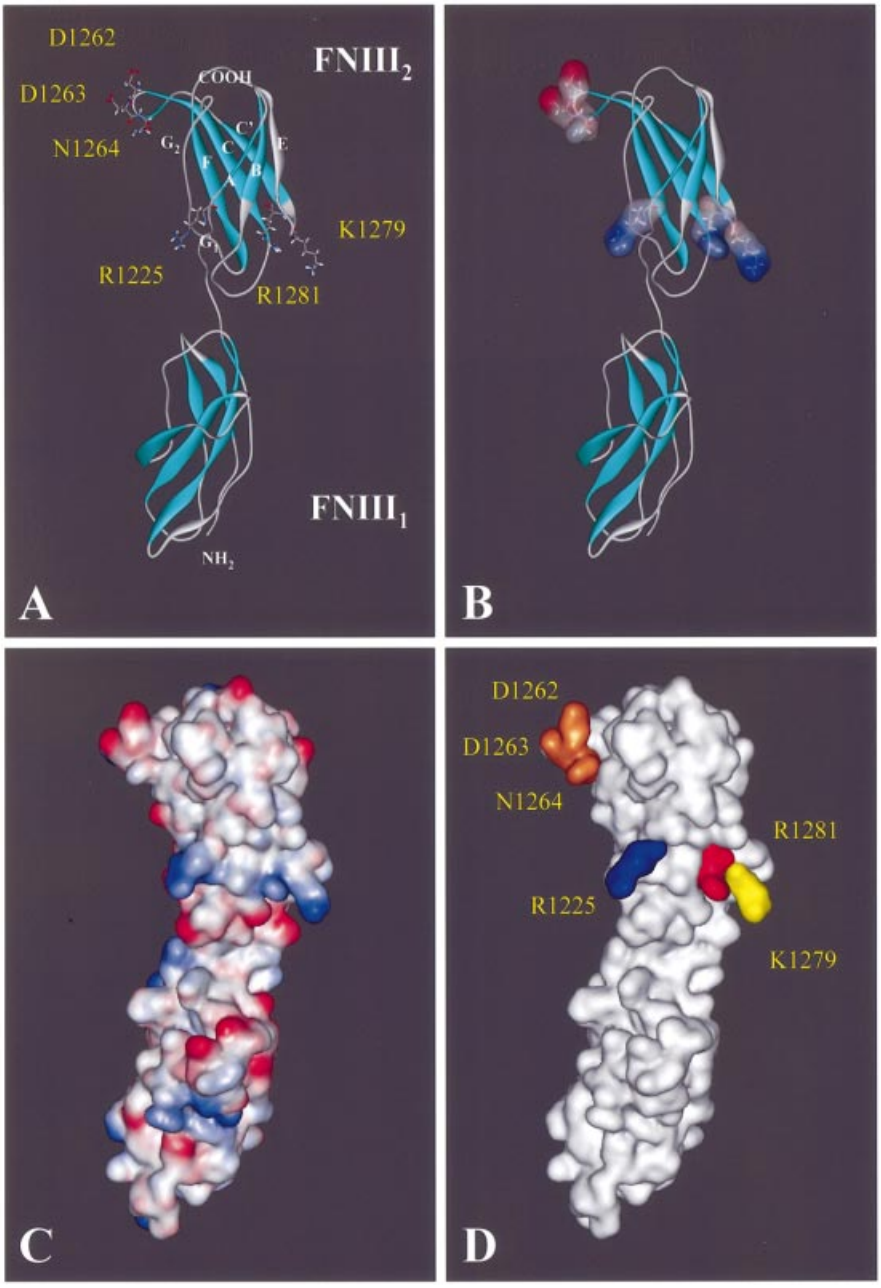


Figure 1. Crystal structure of the first pair of FNIII repeats of β4. (*A*) Ribbon diagram showing the amino acids R1225, K1279, and R1281 and the residues D1262, D1263, and N1264 of the DDN loop. (*B*) Same as (*A*) with the indicated residues as surface fills. (*C*) Space-filling model according to surface charge distribution with red representing residues with acidic side chains, white representing neutral side chains, and blue basic side chains. (*D*) Same as (*C*), with the residues R1225, K1279, and R1281 being highlighted in blue, red, and yellow and the residues DDN (1262–1264) in orange. Images prepared with WebLab using the coordinates from 1qg3.

that this interaction depends on the presence of the actin binding domain (ABD) of plectin (Geerts *et al*, 1999). The mutations that have been described in patients with nonlethal forms of EB localized at the same region that we identified to be essential for the recruitment of plectin in hemidesmosomes. They therefore may affect the binding of β4 to plectin. The determination of the crystal structure of the first pair of FNIII repeats (de Pereda *et al*, 1999) allowed us to map the location of these mutations on the three-dimensional structure. It thus became clear that the mutations are present in close proximity to each other and that they are located on the same side of the structure. Interestingly, both mutations concern an arginine residue, which is considered to be strongly basic. The mutations are separated from the extended C–C′ loop in the second FNIII repeat consisting of the residues DDN (1262–1264) that, in analogy to an RGD loop region in fibronectin, has been suggested to function as a potential protein binding site.

Previous studies have indicated that the R1281W mutation abolishes the binding of β4 to plectin (Geerts *et al*, 1999; Nievers *et al*, 2000). In this study, we confirm this finding and show that the mutation R1225H has the same effect. The mutation K1279W that is part of the loop structure as is R1281W did not abrogate binding. Finally, we show that the DDN loop plays no role in the binding of β4 to the ABD of plectin and thus may constitute a binding site for an as yet unidentified protein.

MATERIALS AND METHODS

Cell lines and antisera African green monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 U per ml penicillin, and 100 μg per ml streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. The β4-deficient PA-JEB keratinocyte cell line has been described previously (Schaapveld *et al*, 1998). PA-JEB cells were cultured in keratinocyte serum-free medium (SFM; Gibco-BRL) supplemented with 50 μg per ml bovine pituitary extract, 5 ng per ml epidermal growth factor, 100 U per ml penicillin, and 100 μg per ml streptomycin. The mouse monoclonal antibody (MoAb) 121 (Hieda *et al*, 1992; Okumura *et al*, 1999) against HD1/plectin was a kind gift of Dr. K. Owaribe (University of Nagoya, Japan)

and the rat MoAb 439-9B (Kennel *et al*, 1989) against β4 was obtained from Dr. S.J. Kennel (Oak Ridge Laboratory, Oak Ridge, TN). Secondary antibodies were purchased from Rockland (Gilbertsville, PA) [fluorescein isothiocyanate (FITC) conjugated goat antimouse IgG] and Molecular Probes (Eugene, OR) (Texas-Red-conjugated goat antirat IgG).

Construction of expression plasmid of β4 mutants The construction of the pRc/CMV-β4 expression vector has been described previously (Niessen *et al*, 1997a). The polymerase chain reaction (PCR) overlap extension method was used to introduce mutations (R1225H, K1279W, R1281W, and D1263E) and a three amino acid deletion (ΔDDN, positions 1262–1264) in the β4 cytoplasmic domain. Sense and corresponding antisense oligonucleotides containing the appropriate mutations or deletion were used together with 5′ (CGTAGAAC-GTCATCGCTG) and 3′ (CCTGCTGAAGCCTGACACTC) primers. The resulting PCR fragments of 1.5 kb were digested with *Bgl*II and *Bss*HII and used to replace the corresponding fragment of the wild-type β4 cDNA in the plasmid pUC-β4 (Niessen *et al*, 1997a). Subsequently, the full-length β4 cDNAs carrying the various mutations were released by *Eco*RI digestion and ligated into the eukaryotic expression vector pRc/CMV (InVitrogen, San Diego, CA). The correctness of all constructs was verified by sequencing.

DNA transfections PA-JEB cells were grown to 40% confluency in 12-well tissue culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ). Transient transfections were performed with 0.8 μg cDNA using lipofectin (Gibco-BRL) according to the manufacturer's instructions. Transfection mixtures were replaced by SFM after 6 h and incubated in this medium overnight. Subsequently, the SFM was replaced with HAMF12/DMEM (1:3) for an additional 24 h after which the cells were assayed for gene expression. COS-7 cells were transiently transfected using the DEAE-dextran method (Cullen, 1987) with 2 μg of cDNA per construct and assayed for gene expression after 48 h.

Immunoblot analysis Lysates were prepared from transfected COS-7 cells by suspending the cells in sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE), transferred onto PVDF membranes (Immobilon-P; Millipore), blocked for 1 h with 2% baby milk powder in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.01% Tween-20) and probed with the rat MoAb 439-9B. The membrane was washed with TBST and then incubated with horseradish peroxidase conjugated sheep antimouse antibody (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at room temperature. After washing, the membrane was subjected to enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to film.

Immunofluorescence microscopy PA-JEB cells grown on glass coverslips were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After rinsing in PBS and blocking with 2% bovine serum albumin in PBS for 60 min at room temperature, the cells were incubated with primary antibodies for 45 min at room temperature and then washed three times with PBS. Cells were subsequently incubated with FITC-labeled antimouse IgG and Texas-Red-labeled antirat IgG for 45 min at room temperature. Coverslips were washed three times, mounted in Mowiol/DAPCO overnight, and viewed under a Leica confocal scanning laser microscope.

Yeast two-hybrid assay The GAL4 fusion plasmids used in this study are depicted in **Fig 4**. Superscript numbers correspond to the β4 amino acid residues (according to Niessen *et al*, 1997a) that were fused in-frame to the GAL4 activation domain (AD). The β4 fragments (1115–1457) containing different mutations were generated by PCR amplification of the corresponding full-length mutant β4A cDNAs using sense and antisense primers encoding *Bam*HI restriction sites at their 5′ ends. The amplified fragments were gel-purified, digested with *Bam*HI, and cloned in the corresponding site of the yeast GAL4(AD) expression vector pACT2 (Clontech Laboratory). The construction of the plectin ABD, fused in-frame to the GAL4 DNA-binding domain (BD) of the pAS2.1 vector, has been described previously (Geerts *et al*, 1999).

The PJ69-4 A strain (a kind gift of Dr. P. James, University of Winsconsin, Madison, WI), containing the genetic markers trp1-901, leu2-3, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, and GAL2-ADE2 (James *et al*, 1996), was used as host for all two-hybrid assays presented in this study. This strain contains two tightly regulated selectable GAL4 driven reporter genes, His and Ade, allowing sensitive detection of protein–protein interactions containing GAL4 fusion proteins. The GAL4(AD) and GAL4(DB) fusion plasmids were cotransformed into PJ69-4 A, as described previously (Clontech manual; James *et al*, 1996), and equal aliquots of transformed cells were spread out on plates containing yeast synthetic complete medium lacking leu and trp (vector markers) (SC-LT) or leu, trp, his, and ade (vector and interaction markers) (SC-LTHA). Plates were incubated at 30°C and growth of colonies was scored after 6 and 10 d. The plating efficiencies on -leu, -trp,-his,-ade plates, compared with the plating efficiency on -leu,-trp, was used as a measure of the strength of the signal generated by the two-hybrid interaction. Expression of the fusion proteins was analyzed by immunoblotting with antibodies against the GAL AD or BD (sc-1663 and sc-510, respectively; Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Mapping of β4 mutations on the three-dimensional structure of the second FNIII (Fig. 1) repeat reveals close proximity of the amino acids In patients suffering from nonlethal forms of EB, two missense mutations (R1225H and R1281W) have been identified in the gene for the integrin β4 subunit. Mapping of the amino acid substitutions caused by these mutations on the three-dimensional structure of the first pair of β4 FNIII repeats revealed that one (R1281W) is localized in the loop region that connects two β-strands (EC′), whereas the other (R1225H) is present at the N-terminal end of the second FNIII repeat (**Fig 1**). Both residues are positioned in close proximity to each other and together with K1279 may form a binding sequence of basic residues for a negatively charged protein domain. The basic residues are clearly separated from the hairpin loop that connects the β strands C and C′. This loop, which is composed of the residues DDN (1262–1264), may constitute another important binding site, because like the RGD loop in fibronectin, which serves as a binding site for integrins, it is well exposed (de Pereda *et al*, 1999).

Introduction of R1281W or R1225H mutations in full-length β4 cDNA prevents the recruitment of plectin into hemidesmosomes To investigate the role of the above three positively charged amino acids and that of the negatively charged DDN loop in the assembly of hemidesmosomes, we generated β4 cDNA constructs containing the mutations R1225H, K1279W, R1281W, D1263E, or ΔDDN. First, we verified that the different β4 mutants were of the correct size by expressing the different mutant β4 constructs in COS-7 cells, followed by immunoblotting with antibodies against β4. As shown in **Fig 2**, all mutant β4 constructs were expressed and the size of the produced polypeptides corresponded to that of wild-type β4. Subsequently, the constructs were transfected into PA-JEB cells, a cell line obtained from a

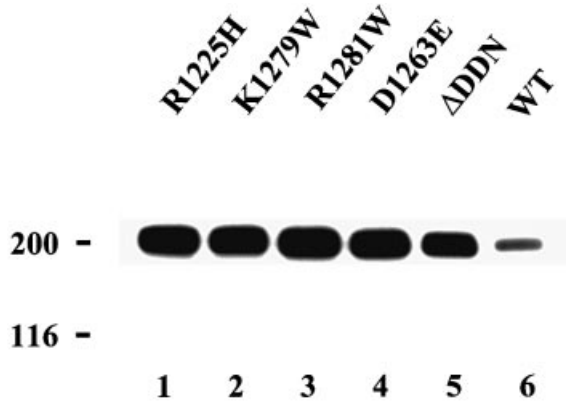
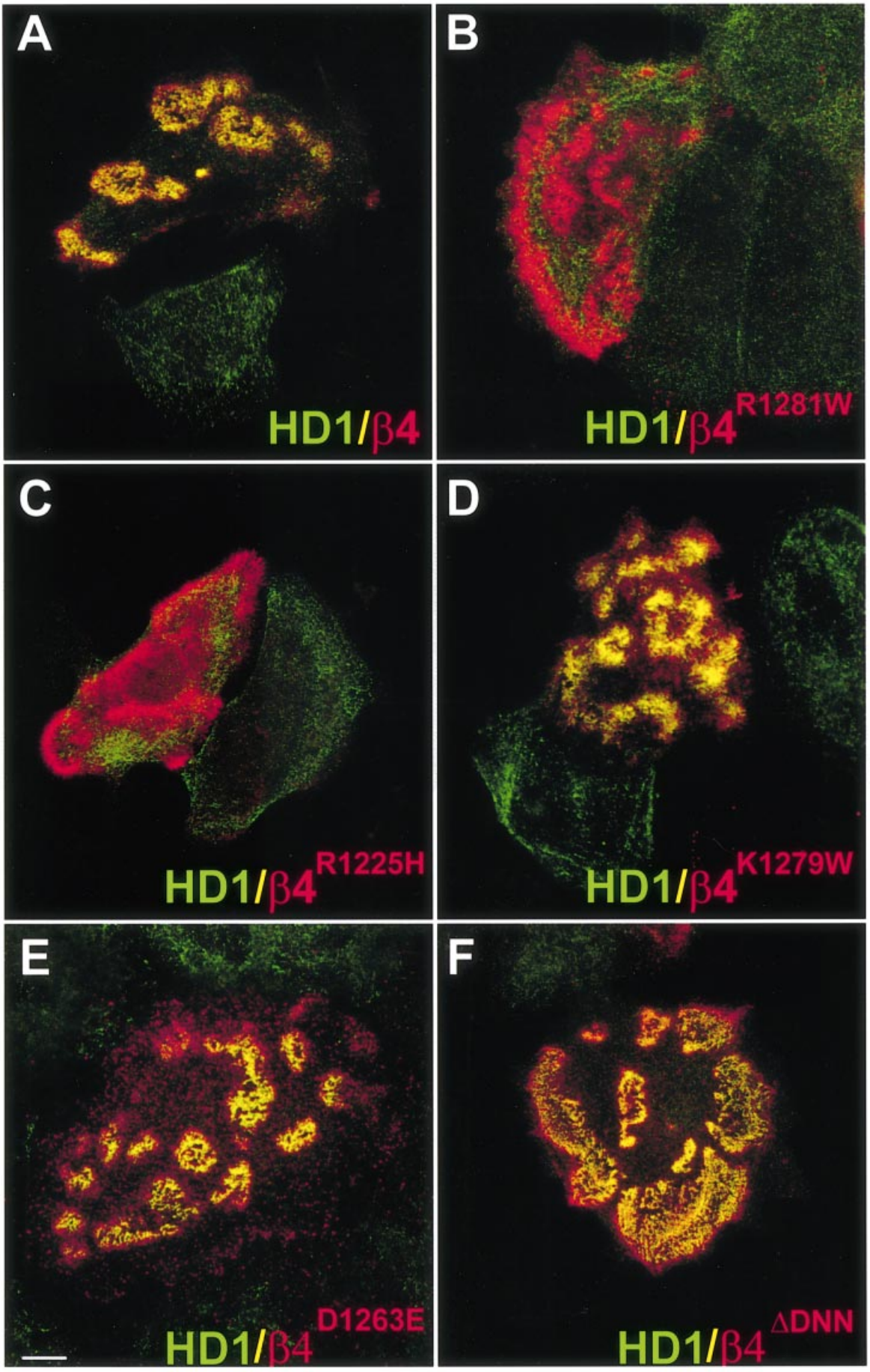


Figure 2. Detection of β4 mutants in lysates of transiently transfected COS-7 cells. Cells transfected with $\beta4^{R1225H}$ (*lane 1*), $\beta4^{K1279W}$ (*lane 2*), $\beta4^{R1281W}$ (*lane 3*), $\beta4^{D1263E}$ (*lane 4*), $\beta4^{\Delta DDN}$ (*lane 5*), and wild-type β4 (*lane 6*) were lyzed as described in *Materials and Methods*. Protein samples were separated on 6% SDS-PAGE under nonreducing conditions and analyzed by immunoblotting using MoAb 439-9B.


A
HD1/β4
B
HD1/β4R1281W
C
HD1/β4R1225H
D
HD1/β4K1279W
E
HD1/β4D1263E
F
HD1/β4ΔDNN

patient lacking β4 (Schaapveld *et al*, 1998), and the distribution of each mutant was analyzed by immunofluorescence microscopy. PA-JEB cells do not assemble hemidesmosomes, but their formation is readily induced upon expression of wild-type β4. Staining with antibodies against β4 or plectin identified these structures as dots and patches at the cell–substrate contact sites. **Fig 3** shows that all mutant β4 constructs were present at the cell basis in a pattern that suggests localization in hemidesmosomal structures. In contrast to wild-type β4, however, which was colocalized with plectin, in cells expressing the β4 mutants carrying the R1281W or R1225H mutations, the staining pattern of the plectin molecule was diffuse, as it was in untransfected PA-JEB cells. The recruitment of plectin by β4 into hemidesmosomes was not affected by the mutation K1279W, which occurs in the same loop as R1281W. Similarly, deletion of the DDN loop or the D1263E mutation did not abrogate the recruitment of plectin by β4 (**Fig 3**).

The mutations R1281W or R1225H in β4 abolish the interaction with plectin in yeast The finding that wild-type β4 and the β4 mutants K1279W, D1263E, and ΔDDN, but not β4 (R1281W) and β4 (R1225H), recruited plectin into hemidesmosome-like structures suggests that the R1281W and R1225H mutations prevent an interaction between β4 and plectin. We sought evidence for this using a two-hybrid assay in which a positive interaction confers adenine and histidine prototrophy, by allowing the expression of the *ADE2* and *His* genes. GAL4–β4 fusion constructs containing residues 1115–1457 of wild-type β4 or the various β4 mutants were created. The fusion constructs are stably expressed in yeast and their levels of expression are similar as determined by immunoblotting with antibodies against GAL4 (not shown).

As shown in **Fig 4**, wild-type β4 (1115–1457) interacts with the ABD of plectin (1–339), as measured by the growth of yeast on the selective plates. The mutation K1279W in β4 only slightly reduced the interaction between β4 (1115–1457) and the ABD of plectin, whereas deletion of the DDN (1262–1264) amino acids or the D1263E mutation had no detectable effect. The R1225H or R1281W mutations completely abolished the interaction with plectin, consistent with the inability of full-length β4 containing these mutations to recruit plectin. All constructs showed an

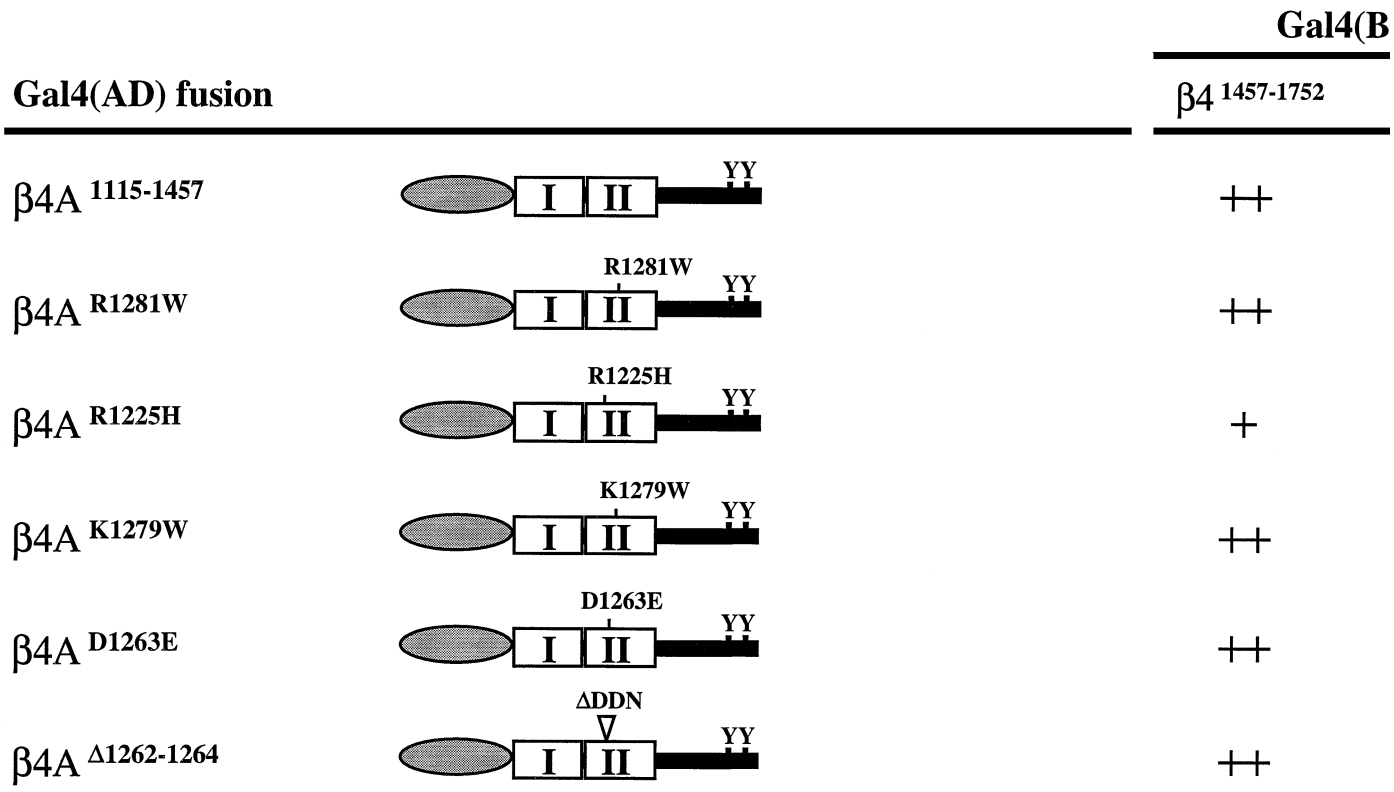


Figure 4. Yeast two-hybrid analysis of a direct interaction between β4 mutants and the ABD of plectin. Cotransformation of yeast host strain PJ69-4 A with β4 mutants in the pACT2 (AD) vector and plectin ABD or $\beta4^{1457–1752}$ in the pAS2.1 (BD) vector. Transformation mixtures were spread on SC-LT and SC-LTHA plates and grown at 30°C. Plating efficiency on selective SC-LTHA plates is expressed as a percentage of the plating efficiency on nonselective SC-LT plates from the same transformation. Plates were scored after 5 and 10 d. All efficiencies listed represent an average of multiple independent transformations. ++, plating efficiency on SC-LTHA is ⩾80% of the plating on SC-LT, colonies are fully developed on day 5; +, 40%–80% of the plating on SC-LT, colonies are small on day 5; –, no colonies on selective plates after 10 d of growth. Note that the interaction between the $\beta4^{1115–1457}$ mutants (K1279W, D1263E, and ΔDDN) and the plectin–ABD fusion protein, but not between $\beta4^{1115–1457}$ and $\beta4^{1457–1752}$, could also be shown by the growth of colonies on selective plates containing 2 mM 3-amino-1,2,4-triazole (a His antagonist).

Figure 3. Immunofluorescence analysis of plectin recruitment by β4 mutants. β4-deficient PA-JEB keratinocytes were transfected with cDNA encoding wild-type β4 and various β4 mutants. Cells were fixed, double-immunolabeled, and processed for immunofluorescence microscopy using mouse MoAb 121 against HD1 and rat MoAb 439-9B against β4. Composite images were generated by superimposition of the green (HD1/plectin) and red (β4) signals with areas of overlap appearing as yellow. Micrographs with both transfected and untransfected cells are shown. In cells expressing wild-type β4 or the β4 mutants, β4K1279W, β4D1263E or β4δDDN, but not β4R1281W or β4R1225H, HD1/plectin is colocalized with β4 in hemidesmosome-like structures.

interaction with a fragment containing the C-tail of β4 (1457–1752), an interaction that we previously have shown to involve the region 1382–1436 of the connecting segment (Nievers *et al*, 1999).

DISCUSSION

Using cell transfection experiments in combination with yeast two-hybrid assays, we demonstrated that the introduction of either of two separate point mutations in the second FNIII repeats of the integrin β4 subunit abrogates binding of the ABD of plectin to β4. These results help to explain why these mutations found in patients with nonlethal forms of EB result in a mild blistering phenotype. Because of an inability of β4 to recruit plectin into hemidesmosomes, the linkage of the intermediate filaments to hemidesmosomes is likely to be compromised, causing fragility of the cells and rupturing of the cell upon application of stress. Similar phenotypes are described in null mutant mice for the plectin gene, in which hemidesmosomes appeared to be unaffected although their mechanical stability was reduced (Andrä *et al*, 1997).

The data further indicate that an important binding site for plectin is located in the second FNIII repeat of β4 and that mutations in this part of the molecule may lead to loss of recruitment of plectin by β4. Although our results are in perfect agreement with findings published previously (Niessen *et al*, 1997a; Geerts *et al*, 1999), they are in apparent contrast to findings reported by Rezniczek *et al* (1998), who showed the presence of a major binding site for β4 in plectin in the region C-terminal of the ABD, using *in vitro* binding assays. In addition, these authors presented evidence that there are two binding sites on β4 for plectin, one in the connecting segment and another in the extreme C-terminal part following the second pair of FNIII repeats of β4. We recently could confirm the presence of these binding sites in two-hybrid assays (Koster *et al*, manuscript in preparation). The importance of these sites for the recruitment of plectin by β4 is uncertain, however, because, despite the fact that these binding sites are intact in the β4 constructs carrying the R1225H or the R1281W mutation, these β4 mutants were unable to recruit plectin into hemidesmosomes. Nevertheless, it is possible that once plectin is recruited by binding of β4 to the plectin ABD, these additional plectin binding sites in the connecting segment and the extreme C-tail of β4 may help to strengthen the interaction between these two molecules. The importance of the binding site for plectin in the second FNIII repeat explains the phenotypes in the patients with nonlethal forms of EB (Pulkkinen *et al*, 1998; Nakano *et al*, 2001).

Interestingly, the mutation K1279W, which occurs in the same loop structure as R1281W, only slightly affected the interaction between plectin and β4. Because, like K1279, R1281 is expressed on the surface of the second FNIII repeat of β4, it is unlikely that an R1281W mutation abolishes the interaction with plectin by inducing a destabilization of the repeat. Rather it suggests that the R1281 residue, and perhaps also R1225, directly take part in interaction with plectin, possibly by forming a salt bridge with a negatively charged amino acid in the ABD of plectin. Currently, we are testing the effects of different amino acid substitutions in the ABD of plectin on the binding of this domain to β4.

Although the DDN sequence in the second FNIII repeat of the β4 molecule could well have been a binding site for plectin, we showed in both cell transfection experiments and yeast two-hybrid assays that this sequence is not involved in the binding to the ABD of plectin. This does not rule out, however, that this sequence could bind to another region of the plectin molecule, or even to another protein.

In conclusion, we have mapped two mutations (R1225H and R1281W), identified in patients with nonlethal EB, on the three-dimensional structure of the second FNIII repeat of β4 and have shown that these mutations are located close to each other. Mutation of these residues in a full-length β4 cDNA construct leads to an inability of the protein to recruit plectin, whereas three other mutations had no effect. Studies in yeast indicate that R1225 and R1281 are essential for binding plectin. We endeavor to clarify the exact site of interaction on the ABD of plectin in another study to fully understand the mechanism of this interaction.

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REFERENCES

Andrä K, Lassmann H, Bittner R, Shorny S, Fässler R, Propst F, Wiche G: Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev* 11:3143–3156, 1997

Borradori L, Sonnenberg A: Structure and function of hemidesmosomes: more than simple adhesion complexes. *J Invest Dermatol* 112:411–418, 1999

Christiano AM, Uitto J: Molecular complexity of the cutaneous basement membrane zone. Revelations from the paradigms of epidermolysis bullosa. *Exp Dermatol* 5:1–11, 1996

Cullen BR: Use of eukaryotic expression technology in the functional analysis of cloned genes. *Meth Enzymol* 152:684–704, 1987

Dowling J, Yu Q-C, Fuchs E: β4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J Cell Biol* 134:559–572, 1996

Gache Y, Chavanas S, Lacour JP, Wiche G, Owaribe K, Meneguzzi G, Ortonne JP: Defective expression of plectin/HD1 in epidermolysis bullosa simplex with muscular dystrophy. *J Clin Invest* 97:2289–2298, 1996

Geerts D, Fontao L, Nievers MG, et al: Binding of integrin α6β4 to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. *J Cell Biol* 147:417–434, 1999

Georges-Labouesse E, Messaddeq N, Yehia G, Cadalbert L, Dierich A, Le Meur M: Absence of integrin α6 leads to epidermolysis bullosa and neonatal death in mice. *Nat Genet* 13:370–373, 1996

Giudice GJ, Emery DJ, Diaz LA: Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 99:243–250, 1992

Hieda Y, Nishizawa Y, Uematsu J, Owaribe K: Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J Cell Biol* 116:1497–1506, 1992

Hogervorst F, von Kuikman I, dem Borne AEG Kr, Sonnenberg A: Cloning and sequence analysis of β4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J* 9:765–770, 1990

James P, Halladay J, Craig EA: Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144:1425–1436, 1996

Kennel SJ, Foote LJ, Falcioni R, Sonnenberg A, Stringer CD, Crouse C, Hemler ME: Analysis of the tumor-associated antigen TSP-180. Identity with α6β4 in the integrin superfamily. *J Biol Chem* 264:15515–15521, 1989

McLean WH, Pulkkinen L, Smith FJ, *et al*: Loss of plectin causes epidermolysis bullosa with muscular dystrophy: cDNA cloning and genomic organization. *Genes Dev* 10:1724–1735, 1996

Nakano A, Pulkkinen L, Murrel D, et al: Epidermolysis bullosa with congenital pyloric atresia: novel mutations in the β4 gene (ITGB4) and genotype/phenotype correlations. *Pediatr Res* 49:1–9, 2001

van der Neut R, Krimpenfort P, Calafat J, Niessen CM, Sonnenberg A: Epithelial detachment due to absence of hemidesmosomes in integrin β4 null mice. *Nat Genet* 13:366–369, 1996

Niessen CM, Hogervorst F, Jaspars LH, *et al*: The α6β4 integrin is a receptor for both laminin and kalinin. *Exp Cell Res* 211:360–367, 1994

Niessen CM, Raaij-Helmer LMH, Hulsman EHM, van der Neut R, Jonkman MF, Sonnenberg A: Deficiency of the integrin β4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties. *J Cell Sci* 109:1695–1706, 1996

Niessen CM, Hulsman EHM, Rots ES, Sánchez-Aparicio P, Sonnenberg A: Integrin α6β4 forms a complex with the cytoskeletal protein HD1 and induces its redistribution in transfected COS-7 cells. *Mol Biol Cell* 8:555–566, 1997a

Niessen CM, Hulsman EHM, Oomen LCJM, Kuikman I, Sonnenberg A: 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. *J Cell Sci* 110:1705–1716, 1997b

Nievers MG, Schaapveld RQJ, Oomen LCJM, Fontao L, Geerts D, Sonnenberg A: Ligand-independent role of the β4 integrin subunit in the formation of hemidesmosomes. *J Cell Sci* 111:1659–1672, 1998

Nievers MG, Schaapveld RQJ, Sonnenberg A: Biology and function of hemidesmosomes. *Matrix Biol* 18:5–17, 1999

Nievers MG, Kuikman I, Geerts D, Leigh IM, Sonnenberg A: Formation of hemidesmosome-like structures in the absence of ligand binding by the α6β4 integrin requires binding of HD1/plectin to the cytoplasmic domain of the β4 integrin subunit. *J Cell Sci* 113:963–973, 2000

Okamura M, Uematsu J, Hirako Y, Nishizawa Y, Shimizu H, Kido N, Owaribe K: Identification of the hemidesmosomal 500 kDa protein (HD1) as plectin. *J Biochem*, Tokyo, Japan 126:1144–1150, 1999

de Pereda JM, Wiche G, Liddington RC: Crystal structure of a tandem pair of fibronectin type III domains from the cytoplasmic tail of integrin α6β4. *EMBO J* 18:4087–4095, 1999

Pulkkinen L, Kimonis VE, Xu Y, Spanou EN, McLean WHI, Uitto J: Homozygous α6 integrin mutation in junctional epidermolysis bullosa with congenital duodenal atresia. *Hum Mol Genet* 6:669–674, 1997

Pulkkinen L, Rouan F, Bruckner-Tuderman L, et al: Novel ITGB4 mutations in lethal and nonlethal variants of epidermolysis bullosa with pyloric atresia: missense versus nonsense. *Am J Hum Genet* 63:1376–1387, 1998

Rezniczek GA, de Pereda JM, Reipert S, Wiche G: Linking integrin α6β4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the β4 subunit and plectin at multiple molecular sites. *J Cell Biol* 141:209–225, 1998

Ruzzi L, Gagnoux-Palacios L, Pinola M, Belli S, Meneguzzi G, D'Alessio M, Zambruno G: A homozygous mutation in the integrin α6 gene in junctional epidermolysis bullosa with pyloric atresia. *J Clin Invest* 99:2826–2831, 1997

Schaapveld RQJ, Borradori L, Geerts D, *et al*: Hemidesmosome formation is initiated by the β4 integrin subunit, requires complex formation of β4 and HD1/plectin, and involves a direct interaction between β4 and the bullous pemphigoid antigen 180. *J Cell Biol* 142:271–284, 1998

Smith FJD, Eady RAJ, Leigh IM, et al: Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nat Genet* 13:450–457, 1996

Sonnenberg A, Calafat J, Janssen H, et al: Integrin α6β4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell–basement membrane adhesion. *J Cell Biol* 113:907–917, 1991

Stanley JR: Autoantibodies against adhesion molecules and structures in blistering skin diseases. *J Exp Med* 181:1–4, 1995

Stanley JR, Hawley-Nelson P, Yuspa SH, Shevach EM, Katz SI: Characterization of bullous pemphigoid antigen: a unique basement membrane protein of stratified squamous epithelia. *Cell* 24:897–903, 1981

Stepp MA, Spurr-Michaud S, Tisdale A, Elwell J, Gipson IK: α6β4 integrin heterodimer is a component of hemidesmosomes. *Proc Natl Acad Sci USA* 87:8970–8974, 1990

Sterk LMTh, Geuijen CAW, Oomen LCJM, Calafat J, Janssen H, Sonnenberg A: The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin α6β4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol* 149:969–982, 2000

Suzuki S, Naitoh Y: Amino acid sequence of a novel integrin β4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J* 9:757–763, 1990

Vidal F, Aberdam D, Miquel C, *et al*: Integrin β4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nat Genet* 10:229–234, 1995