Binding of Integrin \(\alpha 6\beta 4\) to Plectin Prevents Plectin Association with F-Actin but Does Not Interfere with Intermediate Filament Binding

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Abstract. Hemidesmosomes are stable adhesion complexes in basal epithelial cells that provide a link between the intermediate filament network and the extracellular matrix. We have investigated the recruitment of plectin into hemidesmosomes by the \(\alpha 6\beta 4\) integrin and have shown that the cytoplasmic domain of the \(\beta 4\) subunit associates with an NH \(_2\)-terminal fragment of plectin that contains the actin-binding domain (ABD). When expressed in immortalized plectin-deficient keratinocytes from human patients with epidermolysis bullosa (EB) simplex with muscular dystrophy (MD-EBS), this fragment is colocalized with \(\alpha 6\beta 4\) in basal hemidesmosome-like clusters or associated with F-actin in stress fibers or focal contacts. We used a yeast two-hybrid binding assay in combination with an in vitro dot blot overlay assay to demonstrate that \(\beta 4\) interacts directly with plectin, and identified a major plectin-binding site on the second fibronectin type III repeat of the \(\beta 4\) cytoplasmic domain. Mapping of the \(\beta 4\) and actin-binding sites on plectin showed that the binding sites overlap and are both located in the plectin ABD. Using an in vitro competition assay, we could show that \(\beta 4\) can compete out the plectin ABD fragment from its association with F-actin. The ability of \(\beta 4\) to prevent binding of F-actin to plectin explains why F-actin has never been found in association with hemidesmosomes, and provides a molecular mechanism for a switch in plectin localization from actin filaments to basal intermediate filament-anchoring hemidesmosomes when \(\beta 4\) is expressed. Finally, by mapping of the COOH-terminally located binding site for several different intermediate filament proteins on plectin using yeast two-hybrid assays and cell transfection experiments with MD-EBS keratinocytes, we confirm that plectin interacts with different cytoskeletal networks.

Key words: actin • epidermolysis bullosa • hemidesmosome • \(\alpha 6\beta 4\) integrin • plectin

H emidesmosomes are junctional protein complexes that stably bind basal epithelial cells to the basement membrane in (pseudo)stratified and some complex epithelia. They are essential for a tight link between the intracellular intermediate filament system and proteins of the extracellular matrix. Hemidesmosomes consist of at least four distinct proteins. Two of these, the integrin \(\alpha 6\beta 4\) and the bullous pemphigoid antigen 180 (BP180)

1. Abbreviations used in this paper: ABD, actin-binding domain; A BS, actin-binding sites; AD, activation domain; A GB, actin-G buffer; BD, binding domain; BP, bullous pemphigoid antigen; CS, connecting segment; EB, epidermolysis bullosa; HA, hemagglutinin; IFBD, intermediate filament binding site; MD-EBS, muscular dystrophy associated with EB simplex; FNIII, fibronectin type III; GAL4, galactose metabolism regulatory gene 4; GFAP, glial fibrillary acidic protein; GST, glutathione S-transferase; MBP, maltose-binding protein; NHK, normal human keratinocytes; PA-J EB, pyloric atresia associated with junctional EB; SC, synthetic complete medium.

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The α6β4 integrin binds to several laminins, particularly the major basement membrane component laminin-5 (Niessen et al., 1994). It is prominently expressed in stratified and complex epithelia, in which it is present only in hemidesmosomes (Kajii et al., 1989; Stepp et al., 1990; Sonnenberg et al., 1991). The α6β4 integrin has a central role in the assembly and maintenance of hemidesmosomes, as demonstrated by the severe nature of the disease pyloric atresia associated with junctional epidermolysis bullosa (EB) (PA-JEB), which is caused by the absence of α6 or β4. PA-JEB patients suffer from extensive blistering of the skin due to the absence of functional hemidesmosomes (Vidal et al., 1995; Ruzzi et al., 1997). A similar skin phenotype occurs in mice in which the gene encoding α6 or β4 had been disrupted by homologous recombination (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996).

Essential for the role of α6β4 in hemidesmosome assembly is the unique cytoplasmic domain of the β4 subunit. It is over 1,000 amino acid residues in length, and contains two pairs of fibronectin type III (FNIII) repeats separated by a connecting segment (CS) (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). Specifically, sequences within the first and second FNIII repeats and in the CS are important for the formation of a complex with plectin and its recruitment into hemidesmosomes at the basal surface of the cell (Spinardi et al., 1993; Niessen et al., 1997a; Nievers et al., 1998). Fusion proteins containing the first two FNIII repeats and part of the CS of β4 form a complex with plectin in vitro (Niessen et al., 1997b), and recombinant β4 protein fragments containing these sequences were found to bind plectin in in vitro assays (Reznicek et al., 1998). The CS and third FNIII repeat are essential for association with the BP180 protein (Borradori et al., 1997; Aho and Uitto, 1998; Schaapveld et al., 1998). Therefore, the α6β4 integrin clearly has a structural role, but it is also involved in the transduction of cell adhesion-modulating signals (for reviews see Giancotti, 1996; Borradori and Sonnenberg, 1999; Nievers et al., 1999).

The BP180 protein (BPA G2), a type II transmembrane protein, is only expressed in epithelial tissues (Nishizawa et al., 1993; Aho and Uitto, 1999), and is probably also a receptor for laminin-5 (Reddy et al., 1998). BP180 is recruited into hemidesmosomes by direct binding to the β4 cytoplasmic domain (Borradori et al., 1997, 1998; Schaapveld et al., 1998; see also Hopkinson et al., 1995, 1998), and there is data suggesting that it is also involved in the recruitment of BP230 to hemidesmosomes (Borradori et al., 1998).

The two hemidesmosomal plaque proteins, BP230 and plectin, belong to the plakin family, which is widely expressed cytoskeleton-associated proteins (Rohrbarg and Watt, 1997). BP230 (BPA G1) is predominantly expressed in stratified and some complex epithelia. The COOH terminus of BP230 was found to decorate intermediate filament networks in vivo and to bind intermediate filament proteins in vitro (Yang et al., 1996; Leung et al., 1999), but the function of its NH$_2$ terminus remains unknown. Various BPA G1 proteins (dystonins) exist that are only expressed in neuronal tissue. These contain an NH$_2$-terminal actin-binding domain (ABD) as a result of alternative mR N A splicing (Brown et al., 1995; Guo et al., 1995; Yang et al., 1996).

The large plectin protein (~500 kD) is the most widely expressed member of the plakin family (for review see Wiche, 1998). It belongs to the spectrin superfamily of actin-binding proteins (like dystonin) that share a conserved ABD, itself a tandem duplication of the calponin homology domain (Hartwig, 1994; Van Troys et al., 1999). Three conserved actin-binding sequences (AB S 1–3) have been identified within the ABD (Hemmings et al., 1992; Fabbrizio et al., 1993; Corrado et al., 1994). The PLEC1 gene contains alternative first coding exons, and the resulting plectin splice variants show a characteristic tissue expression. The sequence encoding the ABD is present in all plectin mRN A s (Li et al., 1996; M Lund et al., 1996; Elliott et al., 1997). Indeed, plectin is found in association with actin stress fibers and focal contacts (Seifert et al., 1992; Sanchez-Aparicio et al., 1997). Recently, it was shown that NH$_2$-terminal plectin fragments containing the ABD associate with F-actin stress fibers in vivo in transfected plectin-deficient fibroblasts and bind monomeric (G-)actin in vitro. Furthermore, plectin might be involved in the regulation of actin filament dynamics via phosphatidylinositol 4,5-bisphosphate (PIP2) (Andra et al., 1998). The COOH-terminal part of plectin contains six tandem repeat domains (R 1–6) that bind to, or can codistribute with, several types of intermediate filament proteins; epithelial keratins, vimentin, desmin and GFAP (glial fibrillary acidic protein), the neurofilament triplet proteins, and lamin B (for review see Wiche, 1998). The intermediate filament binding site (IFBD) on plectin was mapped to a region of ~50 amino acids at the end of the fifth repeat, R 5 (Nikolic et al., 1996). In addition to actin and intermediate filaments, there is evidence for an interaction of plectin with microtubules (Herrmann and Wiche, 1987; Svitkina et al., 1996), suggesting that it can interlink all three major protein cytoskeletal systems (Wiche, 1998). The functional importance of plectin is demonstrated by the fact that mutations in the PLEC1 gene cause muscular dystrophy associated with EB simplex (MD-EBS), a disease characterized by skin blistering and skeletal muscle myopathies (Gache et al., 1996; McLaren et al., 1996; Smith et al., 1996). A similar phenotype was seen in null-mutant mice in which the PLEC1 gene had been disrupted (Andre et al., 1997). There is ample evidence for an association of plectin with the integrin β4 subunit within the hemidesmosome (see references above), and there are some indications that plectin can associate with BP180 and thereby influence BP180 localization (Gache et al., 1996; Aho and Uitto, 1997; Schaapveld et al., 1998).

In this study, we have investigated the role of plectin in the formation of hemidesmosomes. Firstly, we wished to understand why F-actin has not been found in hemidesmosomes, even though plectin contains a spectrin-like ABD, and why β4, which can form a complex with plectin, is never found in association with the actin cytoskeleton. Secondly, we endeavored to show that the recruitment of plectin into hemidesmosomes by β4 is the result of a direct interaction between plectin and the β4 cytoplasmic domain, and if this could be established, the location of the binding sites on these proteins could be determined. Our
third aim was to investigate the relation between the binding of plectin to β4 and intermediate filaments. The recruitment of plectin into hemidesmosomes, and the function of its NH2-terminal ABD and COOH-terminal IFBD sequences was, for the first time, studied in the absence of endogenous plectin in MD-EBS keratinocytes. We demonstrate that plectin molecules associate in a mutually exclusive manner with either actin filaments or β4 in hemidesmosomes as a result of competitive binding. In this paper, we show which intermediate filament proteins are capable of directly binding to the plectin-IFBD and thereby present further evidence for the versatility of plectin as a cytoskeletal linker protein.

Materials and Methods

Generation of Immortalized MD-EBS Keratinocytes

Skin punch biopsies (4 mm in diameter) were removed from the trunk of two unrelated patients diagnosed with MD-EBS and transported to the laboratory in transport medium (DMEM containing the following antibiotics: 0.01% (wt/vol) streptomycin, 0.01% (wt/vol) penicillin, and 2.5 U/ml fungisone). The tissue was cut into several pieces and incubated in 0.5% (wt/vol) trypsin, 0.1% (wt/vol) EDTA for 90 min at 37°C to separate the epidermis from the dermis. The epidermal sheets were reincubated in fresh trypsin/EDTA at 37°C for 5 min, pipetted vigorously to encourage basal cell detachment, after which trypsinization was arrested by the addition of DMEM containing 10% FCS, and the resulting cell suspensions were centrifuged. The keratinocyte suspension was plated into two T25 flasks (G reiner) together with 5 × 105 irradiated 3T3 feeder cells and keratinocyte medium (Rheinwald and Green, 1975) without EGF. Cells were checked and medium (keratinocyte medium with EGF) changed twice weekly. At 80% confluence, cells were trypsinized, split at 1:5, and replated with fresh 3T3 feeder cells.

At passage two, cells were plated in T25 flasks with irradiated 3T3 feeder cells and allowed to grow to 40–50% confluence. The medium was changed to keratinocyte growth medium, a low calcium medium (0.15 mM Ca2+), with 10 ng/ml EGF, 5 μg/ml insulin, 0.5 μl/ml hydrocortisone, and 0.4% (vol/vol) bovine pituitary extract (Clonetics Corp.) for 48 h, thus allowing cells to spread out as a monolayer. The human papillomavirus 16 expression plasmid pJ4196 (Storey et al., 1988) was used for the immortalization of MD-EBS cells. The cell line that grew from single cells was cloned using cloning rings (C-1059; Greiner) together with 5 × 105 irradiated 3T3 feeder cells and keratinocyte medium (Rheinwald and Green, 1975) without EGF. Cells were checked and medium (keratinocyte medium with EGF) changed twice weekly. At 80% confluence, cells were trypsinized, split at 1:5, and replated with fresh 3T3 feeder cells.

A fter transfection, keratinocytes grew for one or two passages and then entered a growth crisis for a period of up to 12 wk. A fter this crisis, colonies growing from single cells were cloned using cloning rings (C-1059; Sigma Chemical Co.), and then passaged using the same medium. Two MD-EBS cell lines (PEB-1 and 2) were used in this study.

Cell Lines and Antisera

Immortalized normal human keratinocytes (NHK) from foreskin and the immortalized β4-deficient PA-JEB keratinocyte cell line were described previously (Steinbergen et al., 1996; Schaapveld et al., 1998). Immortalized NHK and MD-EBS keratinocytes were grown in keratinocyte serum-free medium (GIBCO BRL) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were grown at 37°C in a humidified, 5% CO2 atmosphere. The MD-EBS and PA-JEB keratinocytes were transiently transfected with cDNA constructs using Lipofectin (GIBCO BRL) according to the manufacturer's procedure.

Rabbit polyclonal antisera against α6 and β4 have been described previously (Hogervorst et al., 1993; Niessen et al., 1994). M ouse mab 58B4 against β4 was prepared by Dr. A.M. Martinez de Velasco and Mr. D. Kramer in our laboratory. M ouse mabs 450-11A (K ennel et al., 1990) and 43E1 (H eslie et al., 1984) against β4 were kind gifts of Dr. S.J. Ken nel (Oak Ridge National Laboratory, Oak Ridge, TN) and Dr. E. Engvall (The Burnnh Institute, L a Jolla, CA), respectively. M ouse mab 233 (Nishiwa et al., 1995) against BP180 and mouse mab 121 (Hieda et al., 1992) against plectinHD1 were kind gifts of Dr. K. Ow arie (University of Nagoya, Nagoya, Japan). A fter 1 h incubation, the antibodies were puriﬁed by afﬁnity chromatography on a plectin (1-339)/maltose-binding protein (MBP) Sepharose column. M ouse mab 12F12, also known as BM-140 (Marinkovich et al., 1992), against laminin-5, was a kind gift from Dr. K. Bor degen (Cutane ous Biology Research Center, Charlestown, MA). M ouse mab 7A8 against plectin was purchased from Sigma Chemical Co. M ouse mabs V9 against vimentin and K1L1 recognizing a broad spectrum of keratins, were purchased from Coulter/Immunotech. M ouse mab SPK-14 against keratin 14 was a kind gift from Dr. D. I vanly (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). M ouse mab 12C05 and rabbit polyclonal antisera HA-11 against the hemagglutinin (HA) epitope (PYYDPD) were purchased from Santa Cruz Biotechnology. Sheep anti-mouse and donkey anti-rabbit HRP-coupled secondary antisera, and donkey anti-rabbit Texas Red conjugated antisera were purchased from A mersham Pharmacia Biotech, and FITC-conjugated goat anti-mouse antisemur from Rockland.

Western Blotting

M D-EBS keratinocytes were plated in 6-well tissue culture plates and lysed in 80 μl sample buffer. Lysates were boiled for 5 min at 95°C, and 40 μl samples analyzed by SD S-PA GE. A fter electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blots were blocked in 2% (wt/vol) bovine milk powder in T B S T (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.01% [vol/vol] TWEEN 20) for 1 h at 37°C. Subsequently, blots were incubated with primary antisera in 0.2% (wt/vol) baby milk powder in T B S T for 1 h at room temperature. A fter extensive washing, blots were incubated for 1 h at room temperature with secondary HRP-coupled antisera in the same buffer as used for the primary antisemur incubation. Proteins were detected using the chemiluminescence procedure (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunofluorescence

M D-EBS and PA-JEB keratinocytes were switched to H A M 12/F D E M (1:3) containing 10% (vol/vol) FCS, 2 mM L-glutamine, 0.4 μg/ml hydrocortisone (Sigma Chemical Co.), and 1 μg isoprotenerol (Sigma Chemical Co.) 24 h before the immunolabeling procedure was started. Keratinocytes grown on glass coverslips were washed twice with PBS (pH 7.2), and fixed for 10 min at room temperature in freshly prepared 1% (wt/vol) paraformaldehyde in PBS. Fixed cells were washed twice with PBS and permeabilized in 0.5% (vol/vol) Triton X-100 in PBS for 5 min at room temperature. Cells were rinsed and incubated in 2% (wt/vol) BSA in PBS for 30 min at room temperature, washed with PBS, and incubated with primary antisera or TRITC-conjugated phalladin in PBS containing 2% BSA for 30 min at 37°C. A fter washing with PBS, cells were incubated with the secondary antisemur, goat anti-mouse-FITC or donkey anti-rabbit-bit-Texas red diluted 1:100 in PBS containing 2% BSA. A fter rinsing in PBS, the preparations were mounted in V ectashield (Vector Laboratories Inc.) and viewed under a Biorad M R C-600 confocal laser scanning microscope.

cDNA Constructs

A ll nucleotide and amino acid positions are numbered with the α T G initia tion codon at position one. Plasmid inserts were generated by PCR using the proofreading Pwo DNA polymerase (Boehringer Mannheim) and gene-specific sense and antisense primers containing restriction site tags.
All plasmid inserts were confirmed by sequence analysis using the 7-Sequencing kit (A merkham Pharma cia Biotech). cDNA for the alternatively exonic exon 1e, exons 2–8, and almost the complete exon 9 of human epithelial plectin cDNA (position 1–1018), and plectin-IFBD (see below) were inserted into pcDNA3HA (a derivative of the eu-
1–1018), and plectin-IFBD (see below) were inserted into pcDNA3HA (a derivative of the eu-
karyotic expression vector pcDNA 3 (Invitrogen Corp.) that contains an extra- mers were inserted into pcDNA3HA (a derivative of the eu-
karyotic expression vector pcDNA 3 (Invitrogen Corp.) that contains an extra-
ary sequence overhang extension PCR. The mutagenesis primers 5
GCGCGTCTTGAGCTGTGACCACTGGAAG (sense) and 5
GCGCGTCTTGAGCTGTGACCACTGGAAG (sense) and 5
GAGTTCATGGCTTTAGGTCGTGCGCTCTTC-3 (antisense), representing positions 3826–3856 of the human β4 coding sequence, were used with normal human β4 cDNA as a template. The resulting DNA fragments were first cloned into the pActCT2 vector, verified by DNA sequencing, and used in yeast two-hybrid analysis (see Fig. 5 B). For cell transfection experiments, the mutation was subsequently introduced into full-length β4 cDNA in pRC/ CMV by exchanging of the appropriate DNA restriction fragments (see Fig. 6). DNA fragments encoding different fragments of the β4 cytoplasmic domain were isolated from pcActCT2-β4 plasmids (described above) and cloned into the bacterial GST fusion protein expression vector pPl126, a derivative of the pGEX-3X vector (A mad Corp. Ltd.). That contains a slightly modified multiple cloning site encoding the HA tag, for the production of recombinant GST fusion proteins (see Fig. 8). Plectin-A BD was isolated from pcDNA 3HA-A-plectin A BD (described above) and inserted into the bacterial MBP fusion protein expression vector pMAL-c2X (New England Biolabs Inc.), for the production of MBP fusion proteins (see Fig. 8). β4 cDNA expression constructs used for the experiments in Fig. 11 have been described previously (Niessen et al., 1997a,b).

**Yeast Two-Hybrid Assay**

Yeast strain Saccharomyces cerevisiae/PJ 69–4A (a gift from Dr. P. J. ames, Department of Biomedical Chemistry, University of Wisconsin, Madison, WI), which contains the genetic markers trpl-901, leu2-3, his3-200, gal4A, gal80A, LYS2:G ALL1 H153, G AL2 A D E 2 (J. ames et al., 1996), was used as the host for the two-hybrid assay. It contains two tightly regulated reporter genes, His and A de, which makes it suitable for the sensitive de-
tection of protein interactions. The use of PJ 69–4A was essentially as de-
scribed in Schaapveld et al. (1998). In brief, PJ 69–4A cells were cotrans-
fected with a pActCT2 (derivated) as well as a pActS2-1-derived plasmid, and aliquots of the same transformation mixture were spread on plates containing SC-L medium, yeast synthetic complete medium (SC) lacking only the vector markers Leu (for pActCT2 and derivatives) and Trp (for pActS2-1 and derivatives), as well as on SC-L THA plates, lacking Leu, Trp, as well as the interaction markers His and A de. Plates were scored after 6 and 12 d of growth, and the number of colonies on SC-L THA plate compared with that on the SC-L CT plate. Positive and negative controls were used as in Schaapveld et al. (1998). Cotransformation efficiencies (on nonselective SC-L CT plates) for all plasmid combinations were always at least 106 cfu/μg plasmid DNA, and the difference between the various plasmid combinations tested was never greater than twofold. Cotransfor-
mation plasmids used were pActCT2-1 with an empty pActS2-1 and an empty pActCT2 vec-
tor, with a derived pActS2 plasmid and an empty pActCT2 vector, or with an empty pActS2 vector and a derived pActCT2 plasmid never resulted in the growth of colonies on selective SC-L THA plates, showing that none of the GAL4 fusion proteins encoded by the recombinant plasmids used could cause activation of the His and A de reporter genes by themselves. For pActCT2-cytoplasmic actin (Actin 5, and keratin 8 constructs, a slight auto-

**Purification of Recombinant Fusion Proteins**

E. coli strain BL21 (DE3), genotype F–ompT gal [lacI] (on) hsdS20 carrying DE3 λ prophage with the T7 RNA polymerase gene (Novagen), was transformed with recombinant plasmid. Colonies were obtained by inoculate Luria-Bertani medium containing 100–μg/ml ampicillin, and cultures were grown overnight at 37°C and 250 rpm. Bacterial cultures were then diluted 1:200 in fresh medium, grown to an OD 600 of 0.7 at 30°C and 200 rpm, and induced by the addition of isopropyl β-
thiogalactopyranoside (IPTG) to 0.4 mM for an additional 3 h. Bacteria were harvested by centrifugation at 4,000 g, resuspended in PBS containing 1 mM EDTA and 1% (vol/vol) Triton X-100, and lysed by sonication. Ly-
sates were cleared by centrifugation for 10 min at 10,000 g and 4°C, and the resulting supernatants were incubated with glutathione agarose beads (G 4510; Sigma Chemical Co.). Beads with affinity-bound proteins were washed three times with PBS containing 1% (vol/vol) Triton X-100, and equilibrated in 50 mM Tris-HCl (pH 7.0). Bound proteins were eluted in 50 mM Tris-HCl (pH 7.0), containing 10 mM reduced glutathione.

Recombinant MBP-plectin A BD fusion protein was expressed and puri-
fied as described above, except that amyocelein resin (800–20; New England Biolabs) was used for the affinity purification, and that elution of the resin was in 20 mM Tris-HCl (pH 7.4), 1 mM β-mecapto-
etanol without or with 10 mM mals, respectively. Buffers containing the eluted fusion proteins were exchanged by ultrafiltration using Centri-
con 10 filters (A micon; Microlop).
tion of 0.1 vol of 10× initiation mix (20 mM MgCl₂, 1 M KCl, 5 mM ATP) for 1 h at room temperature. Polymerized actin filaments complexed with plectin were pelleted by centrifugation for 1 h at 100,000 g and 20°C, and resuspended in APB. A ctn-plectin complexes were then incubated with 10 μM of purified, precleared GST-β4 fusion proteins (as described above) for 1 h at room temperature. A ctn filaments with bound protein were pelleted as described above, and corresponding amounts of pellet and supernatant were analyzed by SDS-PAGE.

Results

Expression and Distribution of Hemidesmosomal Proteins in Immortalized MD-EBS Keratinocytes

Two immortalized keratinocyte cell lines were derived from unrelated MD-EBS patients, and analyzed by Western blot using specific antisera for the different hemidesmosome proteins (Fig. 1). Expression of the hemidesmosome proteins was similar in the two MD-EBS cell lines, but different from that in NHK cells because the MD-EBS keratinocytes expressed β4, BP230, and BP180, but not plectin.

Next, we analyzed the distribution of hemidesmosome proteins in MD-EBS cells using confocal immunofluorescence microscopy (Fig. 2). As expected, no reaction was found with plectin antiserum (Fig. 2 E). The integrin α6β4, as well as BP180 and BP230 were concentrated at cell-substrate contact sites in patch-like structures (Fig. 2, A–C and F). This staining pattern is typical for hemidesmosome-like structures in cultured keratinocytes (Borradori et al., 1998; Schaapveld et al., 1998). The extracellular ligand of α6β4, laminin-5, was concentrated underneath the hemidesmosome-like structures (Fig. 2 D). These observations indicate that at least on a laminin-5 matrix, keratinocytes can form hemidesmosomes in the absence of plectin.

Expression of β4 in PA-JEB Keratinocytes Alters the Distribution of Plectin Compared with that of F-Actin

The above results prompted us to investigate whether the intact plectin molecule is also capable of associating with F-actin. Therefore, we compared its distribution with that of F-actin in PA-JEB keratinocytes that endogenously express full-length plectin. In addition, we studied the distribution of these two proteins in PA-JEB keratinocytes that stably express the integrin α6β4 at their cell surface. In both cell types, the staining pattern of plectin with the mAb 121 against plectin/HD1 and an affinity-purified polyclonal antiserum against the plectin-ABD was similar. In the PA-JEB keratinocytes, both antibodies stained plectin diffusely throughout the cytoplasm, with some enrichment of the protein in regions where F-actin was also found to be concentrated (Fig. 4, A–C). In general, the staining pattern of plectin seen with the mAb 121 appeared more in spots, whereas the staining produced by the polyclonal plectin antiserum was often continuous and clearly associated with cytoskeletal elements (Fig. 4, G–I). Plectin was only occasionally found to be associated with F-actin at the cell periphery, where microtubules and/or intermediate filaments may be running parallel to F-actin and be linked to it by plectin (Fig. 4, J–L) (Svitkina et al., 1996). A dissociation of plectin with F-actin probably also occurs at other sites in the cell, but it may not be as easily recognized there because the cytoskeletal fibers it links do not run parallel to one another but are intertwined. Furthermore, it should be realized that the distribution of F-actin may be different from that of plectin, because plectin can also cross-link other cytoskeletal systems, i.e., intermediate filament with microtubules (Svitkina et al., 1996). In PA-JEB cells that stably express the integrin α6β4, both

Expression of the Plectin-ABD in MD-EBS Keratinocytes Results in Its Association with both Actin Stress Fibers and Hemidesmosomes

Plectin contains a highly conserved NH₂-terminal ABD, and is therefore potentially capable of binding actin; yet F-actin has not been found in association with hemidesmosomes. We speculated that binding of α6β4 to plectin might prevent it from binding to F-actin, possibly by competing with actin for overlapping binding sites on plectin. To examine the subcellular distribution of the NH₂-terminal ABD of plectin, an expression vector carrying plectin-ABD cDNA, a clone encoding the first 339 amino acid residues of human epithelial plectin encompassing its ABD fused to an HA tag, was transiently transfected in MD-EBS cells (Fig. 3).

In the majority of cells, the plectin fragment was efficiently expressed, and its staining pattern overlapped with that of phalloidin-stained F-actin (Fig. 3, A–C). However, in a population of ~10–20% of the cells, it was also present in basally located hemidesmosome-like clusters not associated with F-actin (Fig. 3, D–F). Only very few cells exclusively express the plectin fragment in hemidesmosome-like clusters (Fig. 3, G–I).

These data suggest that the NH₂ terminus of plectin is not only capable of associating with F-actin, but also with α6β4-containing hemidesmosome-like structures.
antibodies stained hemidesmosome-like structures, which were devoid of F-actin (Fig. 4, D–F).

These results demonstrate that plectin is associated with the cytoskeleton, including F-actin, or with hemidesmosome-like structures, and that the localization of plectin with the latter is dependent on the expression of a6β4.

Direct Interaction between the NH2 Terminus of Plectin and the Cytoplasmic Domain of β4

To investigate whether the NH2-terminal ABD of plectin can interact directly with the cytoplasmic domain of the β4 integrin subunit, and to determine the binding site on β4, a yeast two-hybrid assay was performed. The plectin-A BD fragment was expressed as a GAL4 BD fusion, together with one of a set of overlapping fragments of the β4 cytoplasmic domain (as GAL4 AD fusions) in the yeast strain PJ69-4A. Interactions were detected by the growth of yeast colonies on selective SC-LTHA plates (Fig. 5 A).

A high plating efficiency, indicating that the reporter genes were efficiently expressed as a result of a strong interaction between the plectin-A BD and β4, was observed with the β41115–1457, β41115–1382, and β41115–1355 constructs. The site of interaction on the β4 cytoplasmic domain could be mapped to the first and second FNIII repeat and 27 residues of the CS, as present in the β41115–1355 construct. The β41115–1328 construct with the last 27 amino acids deleted interacted only weakly with the plectin-A BD. The first FNIII repeat itself does not bind plectin-A BD, but is nevertheless essential for this binding, since its deletion in the β41217–1328 construct results in a complete abrogation of binding. No interaction could be found with the third or fourth FNIII repeat, alone or when present as a pair. Therefore, the interaction of the NH2 terminus of plectin with β4 is specific for the first pair of FNIII repeats and 27 amino acids of the CS (amino acids 1115–1355).

The R1281W β4 Mutation Abrogates Binding to Plectin without Interfering with Other Protein Interactions of β4

Recently, two patients with nonlethal PA-JEB have been described who were either homozygous or heterozygous for a missense mutation, i.e., a single amino acid substitution from R to W at residue 1281 of β4 (Pulkkinen et al., 1998). Since the R to W mutation was within the binding site for plectin mentioned above, we introduced it into wild-type β4 cDNA to study its effect on the binding of β4 to plectin. This was first tested in a yeast two-hybrid assay (Fig. 5 B). The results show that β4-plectin binding was completely abrogated by this mutation, confirming that the second FNIII repeat of β4 is essential for binding to plectin. We then tested whether the intramolecular interaction within the wild-type β4 cytoplasmic domain, as described previously (Schaapveld et al., 1998), was affected in the β4R1281W mutant. Wild-type (β41115–1457) and R1281W (β41115–1457*) constructs were expressed in yeast as GAL4 AD fusions together with a GAL4 BD fusion of β41457–1752. Both wild-type (β41115–1457) and mutant (β41115–1457*) bound strongly to the β41457–1752 fusion protein, indicating that intramolecular binding could still occur in the β4R1281W mutant protein.

Next, we tested the effect of the R1281W mutation on the function of β4 in hemidesmosome formation in im-
mortalized keratinocytes isolated from a PA-JEB patient who completely lacked β4 (Schaapveld et al., 1998). PA-JEB keratinocytes were transiently transfected with full-length wild-type β4 or β4R1281W cDNA and analyzed by confocal immunofluorescence microscopy (Fig. 6). In transfected PA-JEB cells, both the wild-type β4 and the β4R1281W protein were found at the basal side of the cell in hemidesmosome-like clusters. Whereas wild-type β4 was colocalized with plectin, BP180, and BP230 in hemidesmosome-like structures (Fig. 6, A, B, and C, respectively), the β4R1281W protein was only colocalized with BP180 and BP230 (Fig. 6, D–F).

Thus, the data show that plectin can directly bind β4. The recruitment of BP180 into hemidesmosomes does not seem to require plectin; BP180 (and BP230) and plectin can be independently recruited by β4.

Direct Binding of both β4 and Actin to the Plectin-ABD and Mapping of Overlapping Sites of Interaction

The cell transfection studies described in Fig. 3 suggest that both F-actin and β4 can bind to the plectin-ABD. Binding of β4 to an NH2-terminal part of plectin (residues 1–1128) has been shown previously by Rezniczek et al. (1998) using in vitro binding assays, but this interaction appeared not to require the presence of the plectin-ABD, since its deletion from a smaller plectin fragment (residues 546–1128) did not result in the abrogation of binding to β4. To examine the binding of β4 to the plectin-ABD and the presence of additional binding sites COOH-terminal of the ABD, a set of overlapping fragments of the entire plectin NH2-terminus (residues 1–1155) was generated. Each of these was expressed in yeast as a GAL4 BD fusion, together with β41115–1457 as a GAL4 AD fusion (Fig. 7 A). Binding of β41115–1457 to the plectin NH2 terminus could only be observed for the plectin-ABD fragment that contains residues 1–339 used in the experiments described above.

The plectin-ABD clone contains the first 9 exons of the plectin PLEC1 gene, including the variable exon 1c (encoding residues 1–65), which is specific for epithelial plectin and encodes a protein sequence with no known homologies. Exons 2–8 encode the plectin-ABD (amino acid residues 66–302), and exon 9 (residues 303–343) encodes a unique stretch of amino acids. For further mapping of the β4-binding site and for comparing it with that of actin, plectin-ABD subclones were constructed and tested in a yeast two-hybrid assay with β41115–1457 or with full-length actin. As shown in Fig. 7 B, the plectin-ABD binds not only β4, but also actin. The sequences encoded by exons
1c and 9 are not involved in binding to \( \beta 4 \). Deletion of NH\(_2\)-terminal sequences within the ABD, even of only the first four residues, disrupted binding to \( \beta 4 \) completely, suggesting that the first part of the ABD is essential. COOH-terminal deletions showed that deletions extending into the ABD abrogate binding even when all three ABS sequences (residues 72–83, 144–170, and 182–196) were intact, showing that the last part of the ABD is also required. Therefore, the minimal region in plectin required for binding to \( \beta 4 \) comprises residues 65–302, i.e., the complete ABD.

Investigation of the binding site for actin on the same panel of plectin fragments showed that deletion of the first 35 residues in plectin did not affect its interaction with actin, but that removal of all 64 residues encoded by exon 1c abolished it, in contrast to the binding of \( \beta 4 \). This could be due to steric hindrance of the ABD in juxtaposition to the GAL4 BD moiety present in the GAL4-plectin fusion protein, since data in the literature do not reveal that amino acids NH\(_2\)-terminal of the ABD are required for binding to actin in other actin-binding proteins (Fabbrizio et al., 1993; Corrado et al., 1994). COOH-terminal deletions showed that no residues COOH-terminal of the plectin-ABD are required for binding of actin either. However, in contrast to \( \beta 4 \), actin could still bind when COOH-terminal parts of the ABD were deleted up to and including ABS3. Only deletion of ABS2 resulted in complete loss of actin binding. Therefore, ABS1 and ABS2 are sufficient to bind actin. Plectin is versatile in its binding of actin; three different actin isoforms were tested for inter-
action with plectin^1–339^; α skeletal muscle actin, β cytoplasmic actin, and γ cytoplasmic actin, and all effectively bind plectin (data not shown).

In conclusion, the binding sites for β4 and actin both reside in the plectin-ABD. Both sites start at the beginning of the ABD; actin binding only requires A BS1 and A BS2; the binding of β4 also requires A BS3 and more COOH-terminal sequences. β4 and actin do not bind to each other; they do not interact in the yeast two-hybrid assays (data not shown). Together, these results suggest that the binding site for β4 overlaps with that for actin. Therefore, only one of these proteins can bind to a single plectin molecule, binding thus being mutually exclusive.

Biochemical Evidence for Competitive Binding of β4 and F-Actin to the Plectin-ABD

To confirm the yeast two-hybrid results described above, an in vitro binding assay was performed. GST-β4 fusion proteins or F-actin were spotted on nitrocellulose using a dot blot system and the immobilized proteins were overlaid with in vitro translated, radio-labeled plectin-ABD protein (Fig. 8, A and B). Two different buffers were used in the overlay assay: A PB, a high-salt buffer which maintains F-actin in a polymerized state, and A GB, a low-salt buffer in which F-actin can depolymerize to monomeric G-actin. The plectin-A BD strongly bound F-actin, GST-β4^1115–1382^, but not GST-β4^1115–1382^* (R1281W) or GST-β4^1320–1668^, which is in accordance with the yeast two-hybrid assay results (Figs. 5 and 7). No significant binding was found to GST-β4^1115–1328^, although this mutant protein interacted weakly with the plectin-A BD in the yeast two-hybrid assay. These different results may reflect a difference in the sensitivity of the two assays, with the yeast two-hybrid assay being more sensitive than the dot blot assay. Consistent with the absence of binding of the β4^1115–1328^ mutant to plectin in the dot blot assay, the mutant protein was also unable to induce a redistribution of plectin in cell transfection experiments (Niessen et al., 1997b; Schaapveld et al., 1998). Binding of the plectin-ABD to the GST-β4^1115–1382^ fusion protein was stronger in the presence of low salt (A GB). Thus, binding is probably ionic in nature, explaining why charged residues, i.e., R1281, are important for β4-plectin interactions. In contrast, binding of the plectin-A BD to actin was significantly weaker in the presence of low salt.

To obtain evidence that β4 and F-actin indeed compete for binding to the plectin-A BD, we performed an in vitro competition assay. F-actin, polymerized in the presence of MBP-plectin-A BD fusion protein, was incubated with a 10-fold molar excess of soluble GST fusion proteins. Then the F-actin complexes were precipitated by centrifugation and the supernatant and pellet were collected separately (Fig. 8 C). In the absence of GST-β4 fusion protein, or in the presence of GST-β4^1115–1308^ or GST-β4^1115–1308^*, all MBP-plectin protein was found in the pellet together with actin. However, in the presence of GST-β4^1115–1382^, about half of the MBP-plectin protein appeared in the supernatant due to binding to the soluble GST-β4 fusion protein. The results show that β4 can efficiently compete with F-actin for binding to the plectin-A BD.

The Plectin COOH Terminus Interacts with Intermediate Filament Proteins, but Not with β4

The results presented so far provide evidence for a role of the NH2 terminus of plectin in the binding of both F-actin and β4. To rule out a role for the COOH terminus of plectin more precisely of its globular repeat domains, we also tested cDNA clones encoding this part of the plectin molecule. Since earlier studies (Wiche et al., 1993; Nikolic et al., 1996) had indicated a role for sequences at the end of the R5 domain in binding to intermediate filament proteins, plectin fragments containing this putative IFBD were also tested for interaction with the keratins 5, 8, 14, and 18, and with vimentin and GFA P. Two plectin cDNA clones were tested in the yeast two-hybrid assay. One encoded most of R5, the complete R6, and the COOH tail, and the other encoded most of R6 and the COOH tail. They were expressed in yeast as GAL4 BD fusion proteins together with GAL4 AD fusion proteins of β4^1115–1457^, β4^1320–1668^, or β4^1457–1752^, or of different intermediate filament proteins (Fig. 9). No interaction was found between the plectin fragments and any of the β4 fragments tested. However, specific interaction of the larger plectin fragment, which contained the IFBD at the end of R5, could be detected with different types of intermediate filament proteins.
Binding was observed with keratins 14 and 18, but not with keratins 5 or 8, and with both vimentin and GFAP. Thus, the results show that the plectin COOH terminus has no function in the binding to the \( \beta_4 \) cytoplasmic domain, but confirm the presence of a distinct intermediate filament protein–binding site in the plectin domain R5, which we have shown to bind the type I and III, but not type II intermediate filament proteins.

**The Plectin-IFBD Associates with the Intermediate Filament Cytoskeleton in Plectin-deficient MD-EBS Keratinocytes**

To investigate whether the plectin-IFBD fragment that interacts with intermediate filament proteins in a yeast two-hybrid assay also associates with intermediate filaments in keratinocytes, we transiently transfected MD-EBS cells with an expression vector containing the plectin-IFBD fused to an HA tag, and analyzed the results by confocal immunofluorescence microscopy (Fig. 10). In contrast to normal keratinocytes in vivo, which do not express vimentin but only keratins, the EBS-MD keratinocytes coexpressed vimentin and keratin intermediate filaments. In cells expressing plectin-IFBD, the HA-tagged protein was found to be distributed together with keratins in delicate filamentous structures at sites of cell–substrate contact, in which \( \alpha_6\beta_4 \) is concentrated, as shown by staining for \( \alpha_6 \). Similar results were obtained when instead of the anti-\( \alpha_6 \) antiserum, a polyclonal antiserum against \( \beta_4 \) was used. The wild-type integrin \( \alpha_6\beta_4 \) (A–C) is colocalized with plectin, BP180, and BP230. The mutant integrin \( \alpha_6\beta_4^{R1281W} \) is not colocalized with plectin (D), but is colocalized with BP180 (E) and BP230 (F). Sections were focused at the cell–substrate interface. Bar, 10 \( \mu \)m.
The Integrin \( \alpha 6 \beta 4 \) Organizes the Vimentin Intermediate Filament Network through Plectin upon Expression in PA-JEB Keratinocytes

To study a possible indirect role for the integrin \( \alpha 6 \beta 4 \) (via plectin) in the organization of the vimentin intermediate filament network, immortalized \( \beta 4 \)-deficient PA-JEB keratinocytes were transiently transfected with cDNAs encoding wild-type or mutant \( \beta 4 \) and used in confocal immunofluorescence microscopy (Fig. 11). In nontransfected cells, vimentin is present in rather loose filamentous networks throughout the cytoplasm, but in cells expressing \( \beta 4 \) it was also detected in dense basally located clusters colocalized with \( \alpha 6 \beta 4 \) (Fig. 11, A-C). These results show that expression of \( \beta 4 \) leads to a reorganization of the vimentin intermediate filament network. This most likely occurs by binding of \( \beta 4 \) to the ABD in the plectin NH\(_2\) terminus, accompanied by binding of vimentin to the IFBD in its COOH terminus. The effect of \( \beta 4 \) on the vimentin network would then be indirect and dependent on the binding of \( \beta 4 \) to plectin.

Further evidence for this was obtained by transfection of one mutant \( \beta 4 \) protein, \( \beta 4^{1355} \), that can efficiently bind plectin, and one \( \beta 4 \) mutant, \( \beta 4^{1328} \), that binds plectin in yeast two-hybrid assays, but much less efficiently. In cells transfected with \( \beta 4^{1355} \) cDNA, the \( \beta 4 \) protein fragment was present in basal hemidesmosome-like clusters colocalized with vimentin (Fig. 11, D-F). The \( \beta 4^{1328} \) mutant was also found in basal clusters, but it only occasionally colocalized with vimentin (Fig. 11, G-I).

These results show that \( \beta 4 \) is capable of organizing the vimentin intermediate filament cytoskeleton. It does so indirectly, via plectin, since only \( \beta 4 \) mutants that contained the plectin binding site (like \( \beta 4^{1355} \)) were capable of organizing vimentin filaments into hemidesmosome-like basal clusters. Since this \( \beta 4 \) mutant cannot recruit BP180 or BP230 (Schaapveld et al., 1998), the BP proteins do not seem to play a role in this process.

Discussion

Plectin-deficient MD-EBS Keratinocytes Form Hemidesmosome-like Structures

We have established immortalized keratinocytes from a human MD-EBS patient. In these cell lines, hemidesmosome-like clusters containing \( \alpha 6 \beta 4 \), BP180, and BP230 (but not plectin) were present at the basal side of the MD-EBS cells at sites of cell–substratum contact, as was the ligand for \( \alpha 6 \beta 4 \) laminin-5. Except for the absence of plectin, these hemidesmosome-like clusters are indistinguishable from hemidesmosomes in NHK cells (Borradori et al., 1998; Schaapveld et al., 1998). FACS® analysis of the MD-EBS cells gave expression levels of the \( \alpha 6 \) and \( \beta 4 \) subunits that are about half of those in NHK cells (data not shown). In plectin-deficient null-mutant mice, hemidesmosomes were also present, but their number as well as the amount of \( \beta 4 \) at the basal side of the epidermal cells was reduced, indicating an important role for plectin in maintaining the
The integrity of hemidesmosomes (Andrà et al., 1997). Evidently, normal basal localization of α6β4 into hemidesmosome-like clusters can occur in MD-EBS keratinocytes, but plectin might have a role in retaining α6β4 at the cell surface. It should be noted that hemidesmosome stability and/or the localization of hemidesmosome proteins is probably influenced by mechanical stress in the epidermis of the null-mutant mice. The resistance to mechanical stress of the hemidesmosomes in the MD-EBS keratinocytes has not yet been tested.

**Competitive Binding of β4 and Actin to a Single Binding Site on the Plectin-ABD**

Expression of a plectin fragment containing the ABD in MD-EBS keratinocytes resulted in two different types of subcellular distribution: colocalization with actin stress fibers or a patch-like organization which is typical for hemidesmosomes. The decoration of actin stress fibers was also found in similar experiments with plectin in fibroblasts (Andrà et al., 1998) and with other proteins of the spectrin family (Hemmings et al., 1992; Winder et al., 1995; Yang et al., 1996; Hanein et al., 1997). However, a unique feature of the ABD of plectin, is that in keratinocytes it can also dictate the localization of plectin into hemidesmosomes, together with α6β4. Direct binding of β4 to the plectin-ABD was demonstrated using yeast two-hybrid and dot blot overlay assays. In addition, we showed that β4 can effectively compete plectin-ABD out of an F-actin–plectin complex in an in vitro actin cosedimentation assay. This suggests that competition between β4 and actin for overlapping binding sites on the plectin-ABD is the molecular basis for the relocalization of plectin from actin-containing cytoskeletal complexes (like actin stress fibers or focal contacts) to β4-containing hemidesmosomes at the basal side of keratinocytes when β4 is expressed. It is not yet clear whether these processes are also regulated at a different level, i.e., by protein phosphorylation, or by a regulation of actin filament dynamics via phosphatidylinositol 4,5-bisphosphate (PIP2), for example (Andrà et al., 1998).

Since hemidesmosomes are generally seen as stable adh-
interaction site between the COOH terminus of plectin and the cytoplasmic domain of β4 and of a full-length intermediate filament proteins by yeast two-hybrid analysis. Yeast host strain PJ6-4A was cotransformed with pA S2-plectin and one each of the listed pACT2-β4 subclone constructs, or with empty pACT2 vector to determine the interaction sites between plectin and β4 or intermediate filament proteins. The plectin R5-R6 clone encodes amino acid residues 4068–4687 of mouse plectin (containing most of repeat R5, repeat R6, and the COOH tail), the plectin R6 clone encodes residues 4351–4574 of human plectin (most of repeat R6 and the COOH tail). Similar results as for the human plectin R6 clone were obtained with a mouse plectin clone encoding residues 4206–4687 (part of repeat R5, repeat R6, and the COOH tail; data not shown). Details are as for Fig. 5.

The interaction site between the COOH terminus of plectin and the cytoplasmic domain of β4 and of full-length intermediate filament proteins by yeast two-hybrid analysis. Yeast host strain PJ6-4A was cotransformed with pA S2-plectin and one each of the listed pACT2-β4 subclone constructs, or with empty pACT2 vector to determine the interaction sites between plectin and β4 or intermediate filament proteins. The plectin R5-R6 clone encodes amino acid residues 4068–4687 of mouse plectin (containing most of repeat R5, repeat R6, and the COOH tail), the plectin R6 clone encodes residues 4351–4574 of human plectin (most of repeat R6 and the COOH tail). Similar results as for the human plectin R6 clone were obtained with a mouse plectin clone encoding residues 4206–4687 (part of repeat R5, repeat R6, and the COOH tail; data not shown). Details are as for Fig. 5.

### β4 Contains a Single Binding Site for Plectin

In a recent study, two distinct plectin-binding sites were identified on the β4 cytoplasmic domain: one that encompasses the first two FNIII repeats and the CS (residues 1126–1457), and another that contains the second pair of FNIII repeats and the COOH terminus (residues 1457–1752). NH2-terminal fragments of epithelial rat plectin were shown to interact with both binding sites on β4 in vitro in overlay assays, as well as in vivo by transient transfection of rat kangaroo Ptk2 cells and hemidesmosome-forming 804G rat bladder carcinoma cells, in which β4 and plectin protein fragments were shown to be colocalized mainly in dense perinuclear structures in the cytoplasm (Resniczek et al., 1998).

Previously, we have identified a region on the β4 cytoplasmic domain, in the first pair of FNIII repeats and the beginning of the CS (within residues 1115–1355) that is involved in the formation of a complex with plectin and its recruitment to hemidesmosomes (Niessen et al., 1997a,b; Schaapveld et al., 1998). In this study, we confirmed the presence of the NH2-terminal plectin-binding site on the β4 protein, and mapped it to residues 1217–1355. No evidence was found for the more COOH-terminal binding site reported by Resniczek et al. (1998). Full-length β4 containing the amino acid mutation R1281W, in marked contrast to wild-type β4 (Schaapveld et al., 1998), no longer recruited plectin into basal hemidesmosome-like clusters after being expressed in PA-JEB keratinocytes. This strongly suggests that only the NH2-terminal plectin-binding site on β4 is required for the proper localization of plectin into hemidesmosomes.

A notable difference with the results obtained by Resniczek et al. (1998) is that we located the binding site for β4 in the plectin-ABD and were not able to demonstrate interaction of β4 with NH2-terminal fragments of plectin from which the ABD is absent. Nevertheless, it remains possible that sequences downstream of the ABD, although not able to mediate binding by themselves, could enhance the binding of the ABD to β4. Such a scenario of cooperative binding may offer an explanation for the finding that in the transfected MD-EBS cells, the plectin-ABD alone is found to be primarily colocalized with F-actin.

However, the discrepant results could also stem from the fact that Resniczek et al. (1998) used denatured proteins for the in vitro overlay experiments, whereas the β4 cytoplasmic domain in vivo is most probably intrinsically folded (Resniczek et al., 1998; Schaapveld et al., 1998). Our concern with the cell transfection experiments presented by these authors is that overexpression of the β4 protein fragments could affect their localization and cause them to aggregate with other cytoplasmic structures, as has been described previously for proteins expressed at high levels (Johnston et al., 1998). In our studies, the β4 clones used for cell transfections contain the complete extracellular and transmembrane regions, and therefore the expression of β4 at the basal cell surface (the physiological location for α6β4) is limited by the amount of endogenous integrin α6 subunit.

### Plectin and BP180 Can Bind β4 in an Independent Manner

Reports of a decreased basal localization of BP180 in epidermal cells of a human MD-EBS patient (Gache et al., 1996) and of an interaction of the BP180 cytoplasmic domain with the plectin COOH terminus in a yeast two-hybrid experiment (Aho and Uitto, 1997) suggested a role for plectin in the recruitment of BP180 into hemidesmosomes. Previously, we provided evidence for this by reporting on the basal localization of BP180 in β4-deficient PA-JEB keratinocytes, in which mutant β4 proteins were transiently expressed. Two mutant β4 proteins that could not recruit plectin were also incapable of localizing BP180, whereas their recruitment of BP230 was severely impaired (Schaapveld et al., 1998).

However, results presented in this paper demonstrate that in MD-EBS keratinocytes, both BP180 and BP230 are colocalized with α6β4 in basal hemidesmosome-like clusters in the absence of plectin. Furthermore, we have found that when expressed in PA-JEB keratinocytes, the β4R1281W mutant was unable to bind plectin, but could recruit BP180 and BP230 into hemidesmosome-like clusters. Finally, no interaction could be detected between the COOH-terminal fragments of plectin used in this study and the complete BP180 cytoplasmic domain in a yeast two-hybrid assay (data not shown).
We reasoned that the two β4 mutant proteins used in our previous study, β4D1219–1319, in which the complete second FNIII repeat had been deleted, and β4D17, in which amino acids 1249–1265 of the second FNIII repeat are absent (Vidal et al., 1995), were incorrectly folded in such a way that the binding site for BP180 on β4 was rendered dysfunctional. There is evidence for an intramolecular folding in vitro of the β4 cytoplasmic domain (Rezniczek et al., 1998; Schaapveld et al., 1998), in which the COOH-terminal part of the β4 cytoplasmic domain could fold back and bind close to the first pair of FNIII repeats. If so, improper folding of the first pair of FNIII repeats could have an effect on the folding of the second pair of FNIII repeats, where the binding site for BP180 is localized. In the β4R1281W mutant, this intramolecular association is still intact. We conclude that binding of β4 to plectin and BP180 most probably occurs independently.

**An Arginine to Tryptophan Substitution in β4 Results in a JEB Rather than an MD-EBS Phenotype**

From our data it is expected that a patient who is homozygous for the R to W mutation at residue 1281 in β4, would have a phenotype similar to that of plectin-deficient patients, i.e., EBS. However, a β4 patient diagnosed to be homozygous for this mutation had a mild form of JEB (Pulkkinen et al., 1998). Possibly, the R1281W mutation is responsible for the JEB phenotype by preventing the proper expression of α6β4 at the cell surface, thereby compromising the adhesion function of the cell. A role for plectin in facilitating or maintaining high surface levels of α6β4 is suggested, because as mentioned previously, in the two MD-EBS cell lines used in this study a reduction in the levels of α6β4 has been observed. Indeed, the expression of α6β4 in the patient, although clearly detectable, was found to be diminished. In skeletal muscles, plectin is probably involved in connecting the actin filaments to intermediate filaments. These tissues do not express the integrin α6β4, and therefore a muscle phenotype is not expected in a patient homozygous for the R to W mutation. Our results from transfection experiments with the β4D17 (Schaapveld et al., 1998) and β4R1281W mutants (this study), which are both mutations identified in patients (Vidal et al., 1995; Pulkkinen et al., 1998), show that differ-
ences in the severity of the disease in different PA-JEB patients can also result from an impaired function of β4 to recruit BP180 and BP230 into hemidesmosomes. Both mutants are unable to recruit plectin, but only the β41317 protein had also lost its ability to interact with BP180. Experiments like those described in this paper could be valuable for providing insight into the molecular processes involved in EB disease.

The Plectin COOH Terminus Binds to the Intermediate Filament Cytoskeleton, Not to Hemidesmosome Proteins

We show that COOH-terminal plectin fragments can directly bind to various types of intermediate filament proteins in a yeast two-hybrid assay. Furthermore, upon expression in MD-EBS keratinocytes, the plectin-IFBD fragment is found localized along keratin intermediate filaments and induces a collapse of the vimentin network. This latter finding is of interest because it suggests a specific role for this domain of plectin in stabilizing the vimentin intermediate filament network and its association with hemidesmosomes. The significance of anchoring vimentin to hemidesmosomes (seen in the PA-JEB cells after transfection with β4 cDNA) is not clear, since keratinocytes normally do not express this intermediate filament protein, although it is sometimes seen in rapidly migrating cells. However, it may be more important for cells that are α6β4-positive and express type III intermediate filaments, such as certain types of fibroblasts and endothelial cells. In fact, it has been shown recently that vimentin is associated with the β4 subunit of the α6β4 integrin in endothelial cells (Homan et al., 1998). Coexpression of vimentin with keratins is observed in many epithelial tumors, and has been linked to metastatic disease (Fischer et al., 1989; Raymond and Leong, 1989). It is tempting to speculate that in transformed keratinocytes the expression of vimentin may alter the interaction between hemidesmosomes and keratins, and that this may play a role in the development of metastases.

In the yeast two-hybrid assay, the binding of plectin to vimentin depends on the presence of the plectin-IFBD (lo-
The different specificities of these plakins for the various intermediate filament proteins suggest slightly divergent functions in the organization of the intermediate filament network. In this respect, it is interesting to note that plectin cannot compensate for the loss of BP230, or vice versa, in human patients or null-mutant mice deficient for one of these proteins (Guo et al., 1995; Gach et al. 1996; A ndrã et al., 1997).

The data presented in this study conclusively prove that the NH$_2$ terminus of plectin is involved in interactions with actin filaments or with β4 and its COOH terminus in binding to intermediate filament proteins. A similar organization of binding sites has been found for desmoplakin: its NH$_2$ terminus binds to proteins in the adhesion complex (Bornslaeger et al., 1996; Kowalczyk et al., 1997; Smith and Fuchs, 1998), whereas its COOH terminus interacts with intermediate filament proteins (Stappenbeck et al., 1993; Kouklis et al., 1994; Meng et al., 1997). The assignment of specific and distinct protein-binding sites on the plectin molecule and the study of the regulation of plectin-binding events will allow a more detailed analysis of its function in interlinking adhesion complexes and the intracellular filament networks.

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