

# 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<sub>2</sub>-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 bind-

ing 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)<sup>1</sup>, are transmembrane proteins that are involved in anchoring the basal epithelial cells to the underlying basement membrane. The hemidesmosomal plaque proteins, BP230 and plectin, are localized in the cytoplasm, and play a major role in the binding of intermediate filaments to

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1. *Abbreviations used in this paper:* ABD, actin-binding domain; ABS, actin-binding site; AD, activation domain; AGB, actin-G buffer; APB, actin polymerization 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-JEB, pyloric atresia associated with junctional EB; SC, synthetic complete medium.

hemidesmosomes (Borradori and Sonnenberg, 1996; Green and Jones, 1996; Burgeson and Christiano, 1997).

The  $\alpha 6 \beta 4$  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 (Kajiji et al., 1989; Stepp et al., 1990; Sonnenberg et al., 1991). The  $\alpha 6 \beta 4$  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  $\alpha 6$  or  $\beta 4$ . 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  $\alpha 6$  or  $\beta 4$  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  $\alpha 6 \beta 4$  in hemidesmosome assembly is the unique cytoplasmic domain of the  $\beta 4$  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  $\beta 4$  form a complex with plectin in vitro (Niessen et al., 1997b), and recombinant  $\beta 4$  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  $\alpha 6 \beta 4$  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 (BPAG2), 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  $\beta 4$  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 (Ruhrberg and Watt, 1997). BP230 (BPAG1) 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<sub>2</sub> terminus remains unknown. Variant BPAG1 proteins (dystonins) exist that are only expressed in neuronal tissue. These contain an NH<sub>2</sub>-terminal

actin-binding domain (ABD) as a result of alternative mRNA 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 (ABS 1-3) have been identified within the ABD (Hemmings et al., 1992; Fabrizio 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 mRNAs (Liu et al., 1996; McLean et al., 1996; Elliott et al., 1997). Indeed, plectin is found in association with actin stress fibers and focal contacts (Seifert et al., 1992; Sánchez-Aparicio et al., 1997). Recently, it was shown that NH<sub>2</sub>-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) (Andrä et al., 1998). The COOH-terminal part of plectin contains six tandem repeat domains (R1-R6) that bind to, or can codistribute with, several types of intermediate filament proteins; epidermal 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, R5 (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; McLean et al., 1996; Smith et al., 1996). A similar phenotype was seen in null-mutant mice in which the *PLEC1* gene had been disrupted (Andrä et al., 1997). There is ample evidence for an association of plectin with the integrin  $\beta 4$  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  $\beta 4$ , 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  $\beta 4$  is the result of a direct interaction between plectin and the  $\beta 4$  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  $\beta 4$  and intermediate filaments. The recruitment of plectin into hemidesmosomes, and the function of its NH<sub>2</sub>-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  $\beta 4$  in hemidesmosomes as a result of competitive binding. In addition, 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 (Greiner) together with  $5 \times 10^5$  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 Ca<sup>2+</sup>), with 10 ng/ml EGF, 5  $\mu$ g/ml insulin, 0.5 U/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 pJ4 $\Omega$ 16 (Storey et al., 1988) used for the immortalization was a gift from Dr. A. Storey (Molecular Virology Laboratory, ICRF, London, United Kingdom). For transfection, keratinocytes were washed once with OPTI-MEM (GIBCO BRL) and 4 ml OPTI-MEM was added to each flask and incubated at 37°C for 4 h. For each T25 flask, the following solutions were prepared and incubated for 30 min at room temperature before mixing: solution A, 10  $\mu$ g DNA (human papillomavirus 16 plasmid) in 0.5 ml OPTI-MEM; and solution B, 30  $\mu$ l Lipofectin (18292-011; GIBCO BRL) in 1.5 ml OPTI-MEM. The two solutions were combined, mixed gently, and incubated for a further 10–15 min. All medium was removed from the flasks and 2 ml of transfection mixture was added. The cells were incubated overnight at 37°C, and the next day 4 ml keratinocyte medium and fresh irradiated 3T3 feeder cells were added. Medium was changed twice a week, and cultures were split at 80% confluence.

After transfection, keratinocytes grew for one or two passages and then entered a growth crisis for a period of up to 12 wk. After 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  $\beta 4$ -deficient PA-JEB keratinocyte cell line were described previously (Steenbergen et al., 1996; Schaapveld et al., 1998). Immortalized NHK, PA-JEB, and MD-EBS keratinocytes were grown in keratinocyte serum-free medium (GIBCO BRL) supplemented with 50  $\mu$ g/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% CO<sub>2</sub> 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  $\alpha 6$  and  $\beta 4$  have been described previously (Hogervorst et al., 1993; Niessen et al., 1994). Mouse mAb 58XB4 against  $\beta 4$  was prepared by Dr. A.M. Martínez de Velasco and Mr. D. Kramer in our laboratory. Mouse mAbs 450-11A (Kennel et al., 1990) and 4.3E1 (Hessle et al., 1984) against  $\beta 4$  were kind gifts of Dr. S.J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN) and Dr. E. Engvall (The Burnham Institute, La Jolla, CA), respectively. Mouse mAb 233 (Nishizawa et al., 1993) against BP180 and mouse mAb 121 (Hieda et al., 1992) against plectin/HD1 were kind gifts of Dr. K. Owaribe (University of Nagoya, Nagoya, Japan). Rabbit polyclonal antiserum against BP180 was kindly provided by Dr. L. Bruckner-Tuderman (University of Münster, Münster, Germany). The rabbit polyclonal antiserum recognizing BP230 (Tanaka et al., 1990) was a kind gift from Dr. J.R. Stanley (University of Pennsylvania, Philadelphia, PA). Polyclonal antibodies against plectin were generated by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein containing the NH<sub>2</sub>-terminal domain of plectin (residues 1–339). The antibodies were purified by affinity chromatography on a plectin (1–339)/maltose-binding protein (MBP) Sepharose column. Mouse mAb 6F12, also known as BM-140 (Marinkovich et al., 1992), against laminin-5, was a kind gift from Dr. R.E. Burgeson (Cutaneous Biology Research Center, Charlestown, MA). Mouse mAb 7A8 against plectin was purchased from Sigma Chemical Co. Mouse mAbs V9 against vimentin and KLI, recognizing a broad spectrum of keratins, were purchased from Coulter/Immunotech. Mouse mAb SPK-14 against keratin 14 was a kind gift from Dr. D. Ivanyi (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Mouse mAb 12C05 and rabbit polyclonal antiserum HA-11 against the hemagglutinin (HA) epitope (YPYDVPDYA) 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 Amersham Pharmacia Biotech, and FITC-conjugated goat anti-mouse antiserum from Rockland.

### Western Blotting

MD-EBS keratinocytes were plated in 6-well tissue culture plates and lysed in 80  $\mu$ l sample buffer. Lysates were boiled for 5 min at 95°C, and 40  $\mu$ l samples analyzed by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blots were blocked in 2% (wt/vol) baby milk powder in TBST (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 TBST for 1 h at room temperature. After 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 antiserum incubation. Proteins were detected using the chemiluminescence procedure (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Immunofluorescence

MD-EBS and PA-JEB keratinocytes were switched to HAMF12/DMEM (1:3) containing 10% (vol/vol) FCS, 2 mM L-glutamine, 0.4  $\mu$ g/ml hydrocortisone (Sigma Chemical Co.), and 1  $\mu$ M isoproterenol (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 phalloidin in PBS containing 2% BSA for 30 min at 37°C. After washing with PBS, cells were incubated with the secondary antiserum, goat anti-mouse-FITC or donkey anti-rabbit-Texas red diluted 1:100 in PBS containing 2% BSA. After rinsing in PBS, the preparations were mounted in Vectashield (Vector Laboratories Inc.) and viewed under a BioRad MRC-600 confocal laser scanning microscope.

### cDNA Constructs

All nucleotide and amino acid positions are numbered with the ATG initiation 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 <sup>17</sup>Sequencing kit (Amersham Pharmacia Biotech).

Plectin-ABD, a clone containing alternative exon 1c, 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 kind gift from Dr. E. Sander, Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands), a derivative of the eukaryotic expression vector pcDNA3 (Invitrogen Corp.) that contains an extra sequence 5' of the multiple cloning site encoding the HA tag.

The yeast galactose metabolism regulatory gene 4 (GAL4) plasmids containing human  $\beta 4$  (sequence data available from EMBL/GenBank/DBJ under accession nos. X51841 and X52186), human epithelial plectin (U53204), and mouse plectin (rat accession number X59601) cDNA subclones and full-length cDNA encoding human  $\alpha$  skeletal muscle actin (J00068), human  $\beta$  cytoplasmic actin (AB004047), human  $\gamma$  cytoplasmic actin (M19283), human keratin 5 (M28496), human keratin 8 (M34225), human keratin 14 (J00124), human keratin 18 (M26326), human GFAP (S40719), and mouse vimentin (M26251) are described in Figs. 5, 7, and 9. Numbers in superscript correspond to the amino acid residues of subclones encoded within the GAL4 activation domain (AD) or binding domain (BD) fusion proteins. Vectors used were the yeast GAL4 AD or BD expression vectors pACT2 or pAS2-1 (Clontech). The template DNAs used for PCR were full-length cDNAs encoding human  $\beta 4$ , human  $\alpha$  skeletal muscle actin, mouse vimentin, human plectin (kind gifts from Dr. T. Magin, Institut für Genetik, University of Bonn, Bonn, Germany), human keratins 5, 8, 14, and 18, and cDNA subclones containing bps 1–1018, 2377–3156, and 13054–13725 of human epithelial plectin. Other templates used were EST clones obtained from the IMAGE cDNA clone collection at the Resource Center/Primary Database of the German Human Genome Project (RZPD, Berlin, Germany): 975524 and 1064756 for mouse and 52195 for human plectin 3' clones; 361338, 364065, and 381924 for human GFAP; 611141 and 613287 for human  $\beta$  cytoplasmic actin; and 41909 and 362511 for human  $\gamma$  cytoplasmic actin.

The full-length  $\beta 4$  cDNA construct in the eukaryotic expression vector pRc/CMV (Invitrogen Corp.) has been described previously (Niessen et al., 1997a,b). The CGG to TGG mutation in codon 1281 of  $\beta 4$ , a human patient mutation that results in an R to W amino acid substitution at position 1281 (Pulkinen et al., 1998), was introduced into human  $\beta 4$  cDNA by sequence overlap extension PCR. The mutagenesis primers 5'-GACAAC-CCTAAGAACTGGATGCTGCTTATTG-3' (sense) and 5'-CAATAA-GCAGCATCCAGTTCTTAGGGTTGTC-3' (antisense), representing positions 3826–3856 of the human  $\beta 4$  coding sequence, were used with normal human  $\beta 4$  cDNA as a template. The resulting DNA fragments were first cloned into the pACT2 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  $\beta 4$  cDNA in pRc/CMV by exchanging of the appropriate DNA restriction fragments (see Fig. 6).

DNA fragments encoding different fragments of the  $\beta 4$  cytoplasmic domain were isolated from pACT2- $\beta 4$  plasmids (described above) and cloned into the bacterial GST fusion protein expression vector pRP261, a derivative of the pGEX-3X vector (Amrad Corp. Ltd.) that contains a slightly modified multiple cloning site, for the production of recombinant GST fusion proteins (see Fig. 8).

Plectin-ABD was isolated from pcDNA3HA-plectin ABD (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).

The  $\beta 4$  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* PJ69-4A (a gift from Dr. P. James, Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI), which contains the genetic markers *trp1-901*, *leu2-3*, *his3-200*, *gal4 $\Delta$* , *gal80 $\Delta$* , *LYS2::GAL1-HIS3*, *GAL2-ADE2* (James et al., 1996), was used as the host for the two-hybrid assay. It contains two tightly regulated reporter genes, *His* and *Ade*, which makes it suitable for the sensitive detection of protein interactions. The use of PJ69-4A was essentially as described in Schaapveld et al. (1998). In brief, PJ69-4A cells were cotransformed with a pACT2(-derived) as well as a pAS2-1(-derived) plasmid, and aliquots of the same transformation mixture were spread on plates containing SC-LT medium, yeast synthetic complete medium (SC) lacking only the vector markers *Leu* (for pACT2 and derivatives) and *Trp* (for

pAS2-1 and derivatives), as well as on SC-LTHA plates, lacking *Leu*, *Trp*, as well as the interaction markers *His* and *Ade*. Plates were scored after 6 and 12 d of growth, and the number of colonies on the SC-LTHA plate compared with that on the SC-LT plate. Positive and negative controls used were as in Schaapveld et al. (1998). Cotransformation efficiencies (on nonselective SC-LT plates) for all plasmid combinations were always at least  $10^4$  cfu/ $\mu$ g plasmid DNA, and the difference between the various plasmid combinations tested was never greater than twofold. Cotransformation of yeast PJ69-4A with an empty pAS2-1 and an empty pACT2 vector, with a derived pAS2 plasmid and an empty pACT2 vector, or with an empty pAS2 vector and a derived pACT2 plasmid never resulted in the growth of colonies on selective SC-LTHA plates, showing that none of the GAL4 fusion proteins encoded by the recombinant plasmids used could cause activation of the *His* and *Ade* reporter genes by themselves. For pACT2- $\beta 4^{1457-1752}$ , -keratin 5, and -keratin 8 constructs, a slight autonomous activation of the reporter genes was found, but this could be repressed by the addition of 2 mM 3-amino-1,2,4-triazole (a *His* antagonist) (A8506; Sigma Chemical Co.) to the medium.

### Purification of Recombinant Fusion Proteins

*Escherichia coli* strain BL21(DE3), genotype F<sup>-</sup> *ompT gal [dcm] [lon] hsdS<sub>B</sub>* carrying DE3  $\lambda$  prophage with the T7 RNA polymerase gene (Novagen), was transformed with recombinant pRP261 plasmids. Colonies obtained were used to inoculate Luria-Bertani medium containing 100  $\mu$ g/ml ampicillin, and cultures were grown overnight at 37°C and 250 rpm. Cultures were then diluted 1:20 in fresh medium, grown to an OD<sub>600</sub> of 0.7 at 30°C and 200 rpm, and induced by the addition of isopropyl  $\beta$ -D-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. Lysates 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 8.0). Bound proteins were eluted in 50 mM Tris-HCl (pH 8.0), containing 10 mM reduced glutathione.

Recombinant MBP-plectin ABD fusion protein was expressed and purified as described above, except that amylose resin (800-21; New England Biolabs) was used for the affinity purification, and that equilibration and elution of the resin was in 20 mM Tris-HCl (pH 7.4), 1 mM  $\beta$ -mercaptoethanol without or with 10 mM maltose, respectively. Buffers containing the eluted fusion proteins were exchanged by ultrafiltration using Centricon 10 filters (Amicon; Millipore).

### Dot Blot Assay

Purified recombinant GST fusion proteins containing different fragments of the  $\beta 4$  cytoplasmic domain (0.4 mg/ml) dissolved in actin polymerization buffer (APB) (10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 100 mM KCl, 0.5 mM ATP, 0.1 mM  $\beta$ -mercaptoethanol, pH 7.5) or actin-G buffer (AGB) (10 mM Tris-HCl, 0.2 mM CaCl<sub>2</sub>, 0.1 mM  $\beta$ -mercaptoethanol, pH 7.5) were pre-cleared by centrifugation at 100,000 *g* for 30 minutes at 4°C in a TLA 100.3 rotor (Beckman). Subsequently, protein concentration of the cleared lysates was determined using Bradford protein assay. Bovine  $\alpha$  skeletal muscle actin (A3653; Sigma Chemical Co.) in AGB was polymerized at 4.5  $\mu$ M for 1 h at room temperature. Samples were diluted to 10–30  $\mu$ g/ml in the appropriate buffer and spotted, 100  $\mu$ l per well, on nitrocellulose membrane using a Hoeffer 96 wells dot blot system (Amersham Pharmacia Biotech). Membrane strips were subsequently incubated in blocking buffer (APB or AGB supplemented with 0.2% [wt/vol] heat-inactivated BSA, and 10  $\mu$ g/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin). [<sup>35</sup>S]methionine/cysteine-labeled plectin-ABD protein was obtained by coupled in vitro transcription/translation of pcDNA3HA-plectin ABD DNA using the TnT<sup>®</sup> coupled reticulocyte lysate system (Promega). Nonincorporated radiolabeled amino acids were removed from the in vitro translation mixture using Centricon 10 filters. Purified translation mixture (25  $\mu$ l) was diluted into 3 ml of blocking buffer and incubated for 3 h at room temperature with the nitrocellulose strips. The strips were then washed three times in blocking buffer, air dried, and exposed using Kodak X-Omat AR film.

### Actin Cosedimentation Assay

Bovine  $\alpha$  skeletal muscle actin (5  $\mu$ M in AGB) was allowed to polymerize in the presence of MBP-plectin ABD fusion protein (1  $\mu$ M) by the addi-

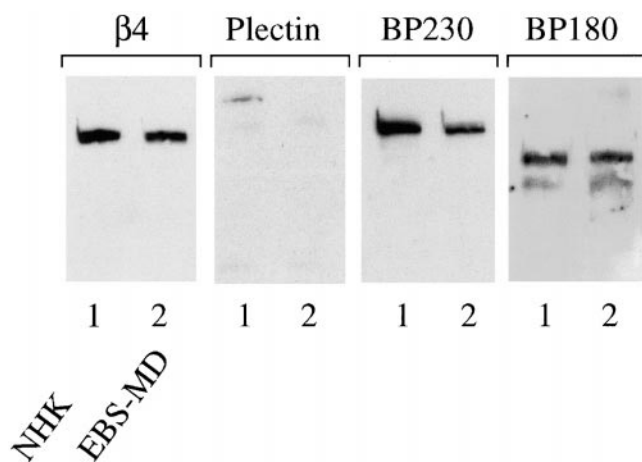
tion of 0.1 vol of 10× initiation mix (20 mM MgCl<sub>2</sub>, 1 M KCl, 5 mM ATP) for 1 h at room temperature. Polymerized actin filaments complexed with plectin were pelleted by centrifugation for 1 h at 100,000 *g* and 20°C, and resuspended in APB. Actin-plectin complexes were then incubated with 10 μM of purified, precleared GST-β4 fusion proteins (as described above) for 1 h at room temperature. Actin 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.



**Figure 1.** Expression of hemidesmosomal proteins in immortalized MD-EBS keratinocytes. Western blot of lysates of normal (NHK; lanes 1) and plectin-deficient (MD-EBS; lanes 2) human keratinocytes, probed with mAb 450-11A against integrin β4 (200 kD), mAb 7A8 against plectin (>500 kD), polyclonal antiserum against BP230 (230 kD), or polyclonal antiserum against BP180 (180 kD). All hemidesmosome proteins tested, except plectin, were present in MD-EBS keratinocytes.

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

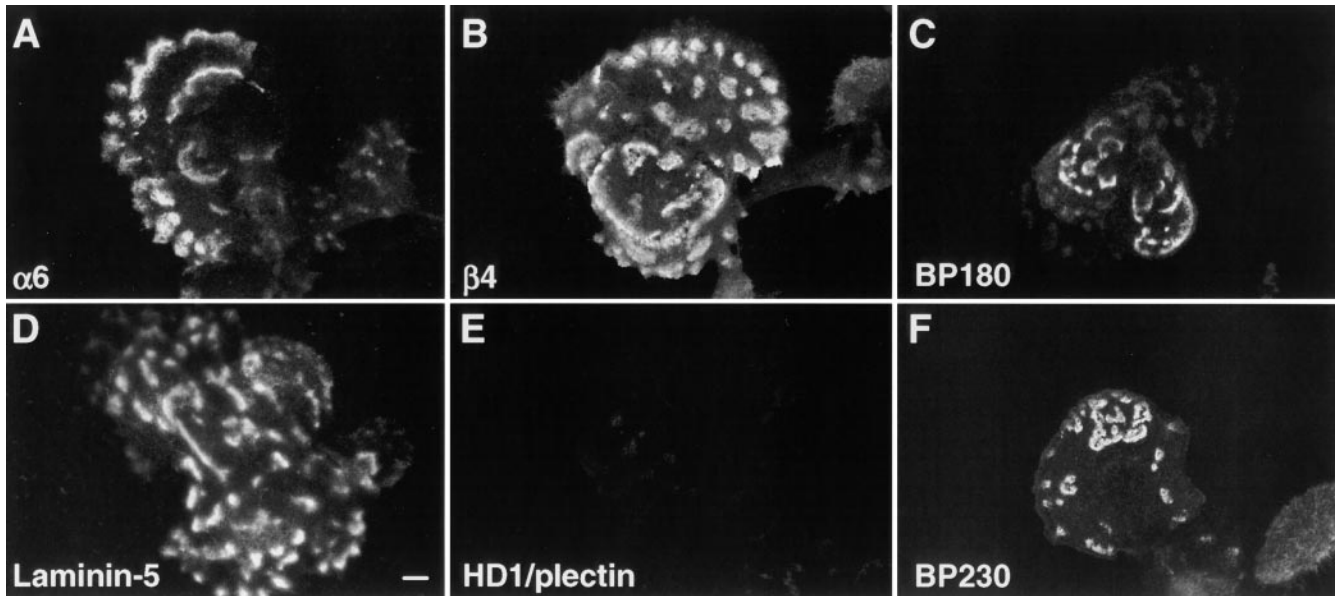
Plectin contains a highly conserved NH<sub>2</sub>-terminal ABD, and is therefore potentially capable of binding actin; yet F-actin has not been found in association with hemidesmosomes. We speculated that binding of α6β4 to plectin might prevent it from binding to F-actin, possibly by competing with actin for overlapping binding sites on plectin. To examine the subcellular distribution of the NH<sub>2</sub>-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 NH<sub>2</sub> terminus of plectin is not only capable of associating with F-actin, but also with α6β4-containing hemidesmosome-like structures.

### 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 cells 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). Association 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



**Figure 2.** Localization of hemidesmosome proteins in immortalized MD-EBS keratinocytes. Cells were fixed, immunolabeled, and subjected to confocal immunofluorescence microscopy using polyclonal antiserum against  $\alpha 6$  (A) or  $\beta 4$  (B), mAb 233 against BP180 (C), mAb 6F12 against laminin-5 (D), mAb 121 against plectin/HD1 (E), or polyclonal antiserum against BP230 (F). Results obtained for cell lines from two different, unrelated human patients were similar; shown here are the results for one patient. All hemidesmosome proteins tested, as well as the extracellular matrix protein laminin-5 (a ligand for the integrin  $\alpha 6\beta 4$ ), are concentrated at sites of cell-substrate contact in patches characteristic for hemidesmosome-like clusters, except plectin, which is not expressed. Sections were focused at the cell-substrate interface. Bar, 10  $\mu\text{m}$ .

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  $\alpha 6\beta 4$ .

#### **Direct Interaction between the NH<sub>2</sub> Terminus of Plectin and the Cytoplasmic Domain of $\beta 4$**

To investigate whether the NH<sub>2</sub>-terminal ABD of plectin can interact directly with the cytoplasmic domain of the  $\beta 4$  integrin subunit, and to determine the binding site on  $\beta 4$ , a yeast two-hybrid assay was performed. The plectin-ABD fragment was expressed as a GAL4 BD fusion, together with one of a set of overlapping fragments of the  $\beta 4$  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-ABD and  $\beta 4$ , was observed with the  $\beta 4^{1115-1457}$ ,  $\beta 4^{1115-1382}$ , and  $\beta 4^{1115-1355}$  constructs. The site of interaction on the  $\beta 4$  cytoplasmic domain could be mapped to the first and second FNIII repeat and 27 residues of the CS, as present in the  $\beta 4^{1115-1355}$  construct. The  $\beta 4^{1115-1328}$  construct with the last 27 amino acids deleted interacted only weakly with the plectin-ABD. The first FNIII repeat itself does not bind plectin-ABD, but is nevertheless essential for this binding, since its deletion in the  $\beta 4^{1217-1328}$  construct results in a complete abrogation of binding. No interaction could be found with the third or

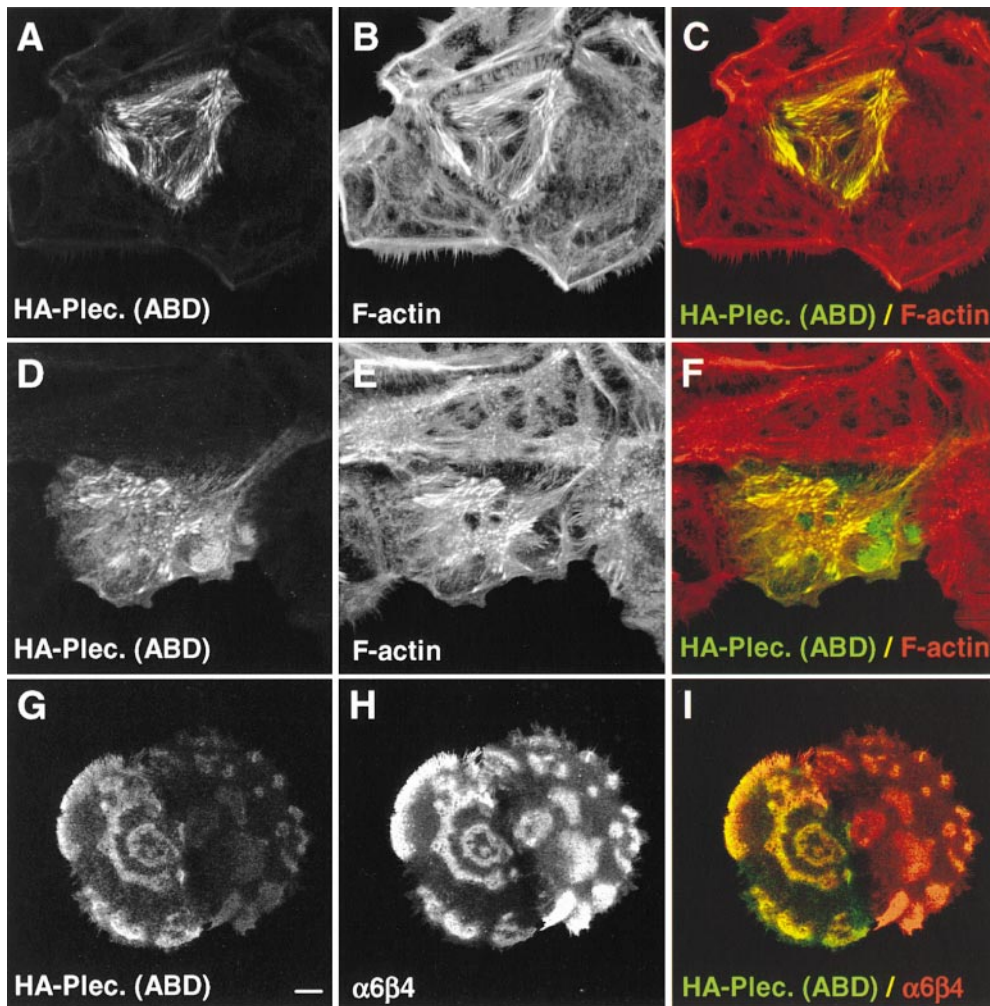
fourth FNIII repeat, alone or when present as a pair. Therefore, the interaction of the NH<sub>2</sub> terminus of plectin with  $\beta 4$  is specific for the first pair of FNIII repeats and 27 amino acids of the CS (amino acids 1115–1355).

#### **The R1281W $\beta 4$ Mutation Abrogates Binding to Plectin without Interfering with Other Protein Interactions of $\beta 4$**

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  $\beta 4$  (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  $\beta 4$  cDNA to study its effect on the binding of  $\beta 4$  to plectin. This was first tested in a yeast two-hybrid assay (Fig. 5 B). The results show that  $\beta 4$ -plectin binding was completely abrogated by this mutation, confirming that the second FNIII repeat of  $\beta 4$  is essential for binding to plectin. We then tested whether the intramolecular interaction within the wild-type  $\beta 4$  cytoplasmic domain, as described previously (Schaapveld et al., 1998), was affected in the  $\beta 4^{\text{R1281W}}$  mutant. Wild-type ( $\beta 4^{1115-1457}$ ) and R1281W ( $\beta 4^{1115-1457^*}$ ) constructs were expressed in yeast as GAL4 AD fusions together with a GAL4 BD fusion of  $\beta 4^{1457-1752}$ . Both wild-type ( $\beta 4^{1115-1457}$ ) and mutant ( $\beta 4^{1115-1457^*}$ ) bound strongly to the  $\beta 4^{1457-1752}$  fusion protein, indicating that intramolecular binding could still occur in the  $\beta 4^{\text{R1281W}}$  mutant protein.

Next, we tested the effect of the R1281W mutation on the function of  $\beta 4$  in hemidesmosome formation in im-





**Figure 3.** Subcellular distribution of HA-tagged plectin-ABD fragment in transfected MD-EBS keratinocytes. 48 h after transfection, cells were fixed, double-immunolabeled using mAb 12C05 against the HA epitope (green; A, D, and G) and polyclonal antiserum against  $\beta 4$  (red; H), and processed for immunofluorescence. TRITC-labeled phalloidin was used to stain F-actin (red; B and E). Composite images (C, F, and I) show the superimposition of the green and red signals, with areas of overlap appearing yellow. Cells were visualized by confocal microscopy. The plectin protein fragment is colocalized with actin stress fibers, or with  $\alpha 6\beta 4$ -containing hemidesmosome-like basal clusters. Sections were focused at the cell-substrate interface. Bar, 10  $\mu\text{m}$ .

mortalized keratinocytes isolated from a PA-JEB patient who completely lacked  $\beta 4$  (Schaapveld et al., 1998). PA-JEB keratinocytes were transiently transfected with full-length wild-type  $\beta 4$  or  $\beta 4^{\text{R1281W}}$  cDNA and analyzed by confocal immunofluorescence microscopy (Fig. 6). In transfected PA-JEB cells, both the wild-type  $\beta 4$  and the  $\beta 4^{\text{R1281W}}$  protein were found at the basal side of the cell in hemidesmosome-like clusters. Whereas wild-type  $\beta 4$  was colocalized with plectin, BP180, and BP230 in hemidesmosome-like structures (Fig. 6, A, B, and C, respectively), the  $\beta 4^{\text{R1281W}}$  protein was only colocalized with BP180 and BP230 (Fig. 6, D–F).

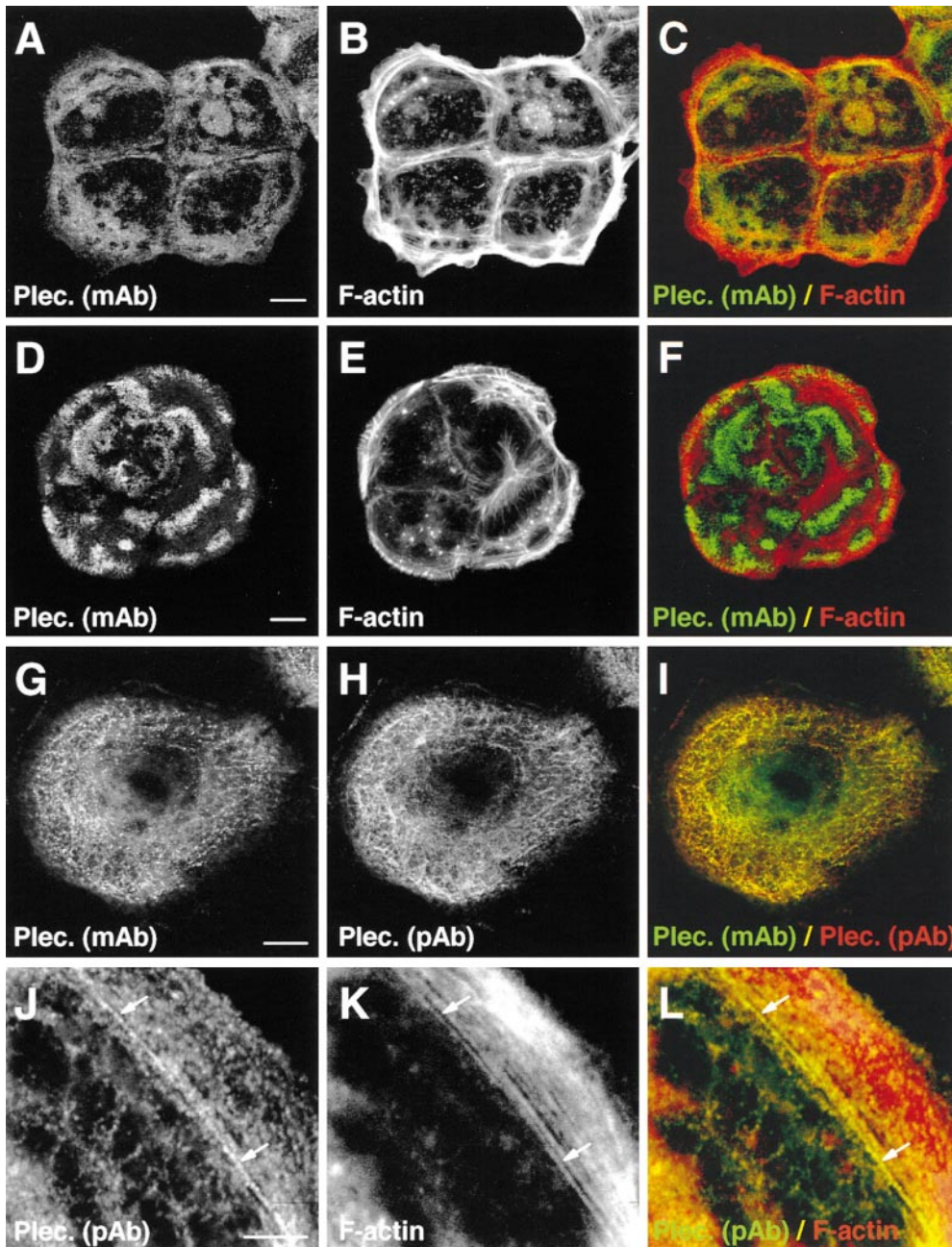
Thus, the data show that plectin can directly bind  $\beta 4$ . The recruitment of BP180 into hemidesmosomes does not seem to require plectin; BP180 (and BP230) and plectin can be independently recruited by  $\beta 4$ .

#### **Direct Binding of both $\beta 4$ 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  $\beta 4$  can bind to the plectin-ABD. Binding of  $\beta 4$  to an  $\text{NH}_2$ -terminal part of plectin (residues 1–1128) has been shown previously by Reznicek et al. (1998) using in vitro binding assays, but this interaction ap-

peared 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  $\beta 4$ . To examine the binding of  $\beta 4$  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  $\text{NH}_2$  terminus (residues 1–1155) was generated. Each of these was expressed in yeast as a GAL4 BD fusion, together with  $\beta 4^{\text{1115–1457}}$  as a GAL4 AD fusion (Fig. 7 A). Binding of  $\beta 4^{\text{1115–1457}}$  to the plectin  $\text{NH}_2$  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  $\beta 4$ -binding site and for comparing it with that of actin, plectin-ABD subclones were constructed and tested in a yeast two-hybrid assay with  $\beta 4^{\text{1115–1457}}$  or with full-length actin. As shown in Fig. 7 B, the plectin-ABD binds not only  $\beta 4$ , but also actin. The sequences encoded by exons



**Figure 4.** Distribution of plectin compared with F-actin in PA-JEB cells. PA-JEB keratinocytes and PA-JEB cells stably expressing the integrin  $\alpha 6\beta 4$  were fixed, double-immunolabeled using mAb 121 against plectin (green; A, D, and G) or an affinity-purified polyclonal antiserum against the plectin-ABD (green; H and J) and F-actin (red; B, E, and K), and processed for immunofluorescence. Composite images (C, F, I, and L) were generated by superimposition of the green and red signals, with areas of overlap appearing yellow. Cells were visualized by confocal microscopy. In PA-JEB cells (A–C), the staining patterns of plectin and F-actin partly overlapped, whereas in the  $\alpha 6\beta 4$ -positive cells (D–F) plectin was exclusively localized in hemidesmosome-like basal clusters that did not stain for F-actin. These sections (A–F) were taken at the optical plane corresponding to the cell–substrate interface. In another plane of focus (G–I), it is shown that the two anti-plectin antibodies produce a similar pattern of staining of the cytoskeleton in PA-JEB keratinocytes. (J–L) Higher magnification shows plectin associated with F-actin filaments in PA-JEB keratinocytes (arrows). Bars: 10  $\mu\text{m}$  (A, D, and G); and 5  $\mu\text{m}$  (J).

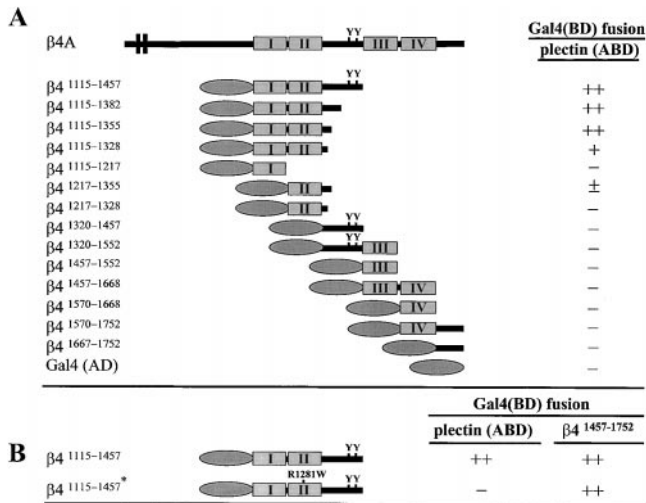
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1c and 9 are not involved in binding to  $\beta 4$ . Deletion of  $\text{NH}_2$ -terminal sequences within the ABD, even of only the first four residues, disrupted binding to  $\beta 4$  completely, suggesting that the first part of the ABD is essential. 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<sup>65–339</sup> fusion protein, since data in the literature do not reveal that amino acids  $\text{NH}_2$ -terminal of the ABD are required for binding to actin in other actin-binding proteins (Fabrizio 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-





**Figure 5.** Mapping of the binding site between the NH<sub>2</sub>-terminal part of plectin and the cytoplasmic domain of  $\beta 4$  and of the internal folding of the  $\beta 4$  cytoplasmic domain by yeast two-hybrid analysis. (A) Cotransformation of yeast host strain PJ69-4A with pAS2-plectin<sup>1-339</sup> and one each of the listed pACT2- $\beta 4$  constructs or empty pACT2 vector to determine the plectin- $\beta 4$  interaction site. (B) Cotransformation with pAS2-plectin<sup>1-339</sup> or pAS2- $\beta 4^{1457-1752}$ , and pACT2- $\beta 4^{1115-1457}$  (wild-type) or - $\beta 4^{1115-1457}^*$  (R1281W) to characterize plectin- $\beta 4$  binding and the internal folding of the  $\beta 4$  cytoplasmic domain. Transformation mixtures were spread on SC-LT and SC-LTHA plates and grown for 12 d at 30°C. Plating efficiency on selective SC-LTHA plates is expressed as a percentage of plating efficiency on nonselective SC-LT plates of the same transformation. ++, >50%; +, >50% (slow-growing colonies); ±, 5–25%; –, 0%. Plates were scored after 6 and 12 d of growth. Slow-growing colonies could only be scored after 12 d of growth. Plating efficiencies of <25% always represented slow-growing colonies. All efficiencies listed represent an average of multiple independent transformations on at least three separate occasions.

action with plectin<sup>1-339</sup>,  $\alpha$  skeletal muscle actin,  $\beta$  cytoplasmic actin, and  $\gamma$  cytoplasmic actin, and all effectively bind plectin (data not shown).

In conclusion, the binding sites for  $\beta 4$  and actin both reside in the plectin-ABD. Both sites start at the beginning of the ABD; actin binding only requires ABS1 and ABS2; the binding of  $\beta 4$  also requires ABS3 and more COOH-terminal sequences.  $\beta 4$  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  $\beta 4$  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 $\beta 4$ and F-Actin to the Plectin-ABD

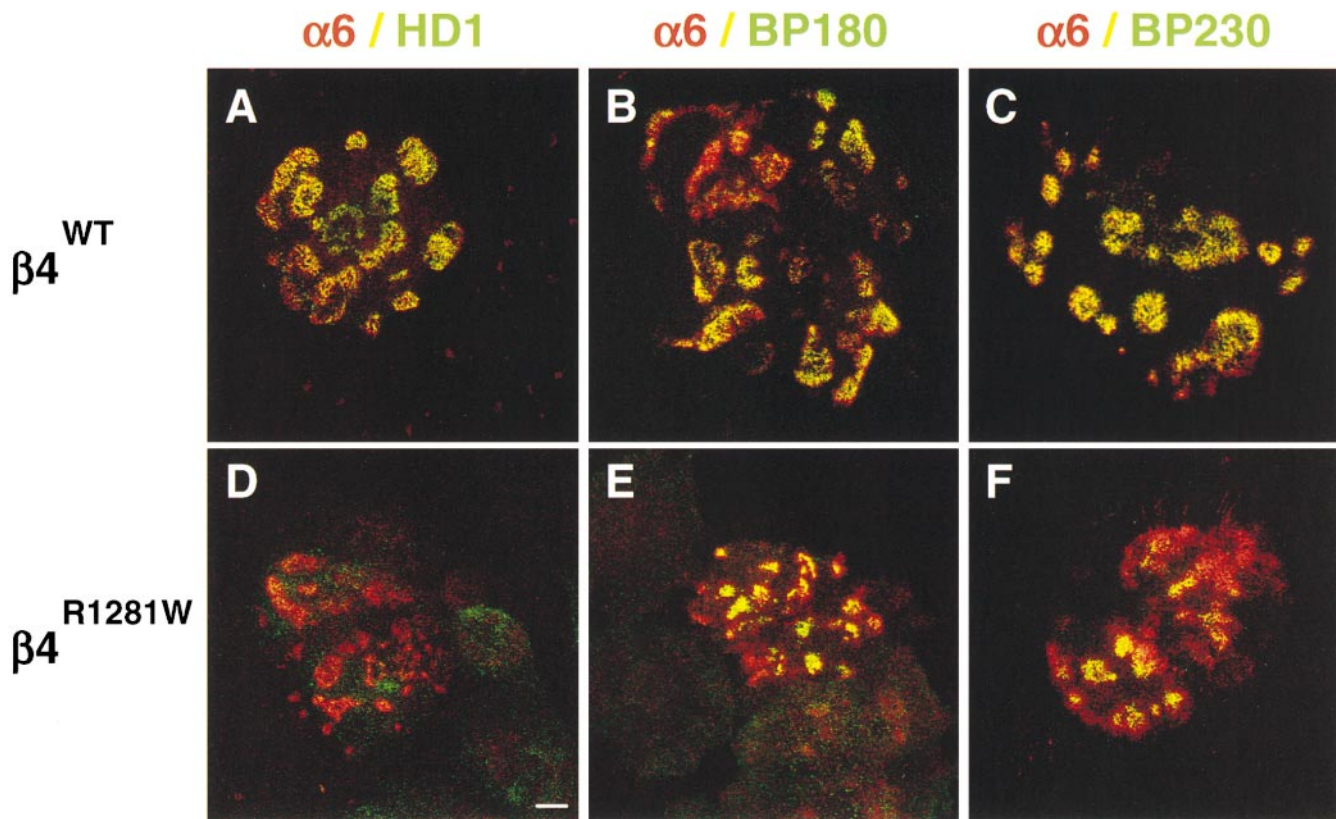
To confirm the yeast two-hybrid results described above, an in vitro binding assay was performed. GST- $\beta 4$  fusion proteins or F-actin were spotted on nitrocellulose using a dot blot system and the immobilized proteins were overlaid with in vitro translated, radio-labeled plectin-ABD protein (Fig. 8, A and B). Two different buffers were used in the overlay assay: APB, a high-salt buffer which maintains F-actin in a polymerized state, and AGB, a low-salt

buffer in which F-actin can depolymerize to monomeric G-actin. The plectin-ABD strongly bound F-actin, GST- $\beta 4^{1115-1382}$ , but not GST- $\beta 4^{1115-1382}^*$  (R1281W) or GST- $\beta 4^{1457-1752}$ , which is in accordance with the yeast two-hybrid assay results (Figs. 5 and 7). No significant binding was found to GST- $\beta 4^{1115-1328}$ , although this mutant protein interacted weakly with the plectin-ABD 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  $\beta 4^{1115-1328}$  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- $\beta 4^{1115-1382}$  fusion protein was stronger in the presence of low salt (AGB). Thus, binding is probably ionic in nature, explaining why charged residues, i.e., R1281, are important for  $\beta 4$ -plectin interactions. In contrast, binding of the plectin-ABD to actin was significantly weaker in the presence of low salt.

To obtain evidence that  $\beta 4$  and F-actin indeed compete for binding to the plectin-ABD, we performed an in vitro competition assay. F-actin, polymerized in the presence of MBP-plectin-ABD 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- $\beta 4$  fusion protein, or in the presence of GST- $\beta 4^{1115-1328}$  or GST- $\beta 4^{1115-1382}^*$ , all MBP-plectin protein was found in the pellet together with actin. However, in the presence of GST- $\beta 4^{1115-1382}$ , about half of the MBP-plectin protein appeared in the supernatant due to binding to the soluble GST- $\beta 4$  fusion protein. The results show that  $\beta 4$  can efficiently compete with F-actin for binding to the plectin-ABD.

### The Plectin COOH Terminus Interacts with Intermediate Filament Proteins, but Not with $\beta 4$

The results presented so far provide evidence for a role of the NH<sub>2</sub> terminus of plectin in the binding of both F-actin and  $\beta 4$ . 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 GFAP. 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  $\beta 4^{1115-1457}$ ,  $\beta 4^{1320-1668}$ , or  $\beta 4^{1457-1752}$ , or of different intermediate filament proteins (Fig. 9). No interaction was found between the plectin fragments and any of the  $\beta 4$  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.



**Figure 6.** Localization of hemidesmosomal components in PA-JEB keratinocytes transiently transfected with cDNAs encoding full-length wild-type  $\beta 4$  or  $\beta 4^{R1281W}$ . Cells were fixed, double-immunolabeled using polyclonal antiserum against  $\alpha 6$  (red; A–F) and mAb 121 against plectin/HD1 (green; A and D), mAb 233 against BP180 (green; B and E), or polyclonal antiserum against BP230 (green; C and F), and processed for immunofluorescence. Cells were visualized by confocal microscopy. Expression of  $\beta 4$  results in the formation of hemidesmosome-like 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  $\mu\text{m}$ .

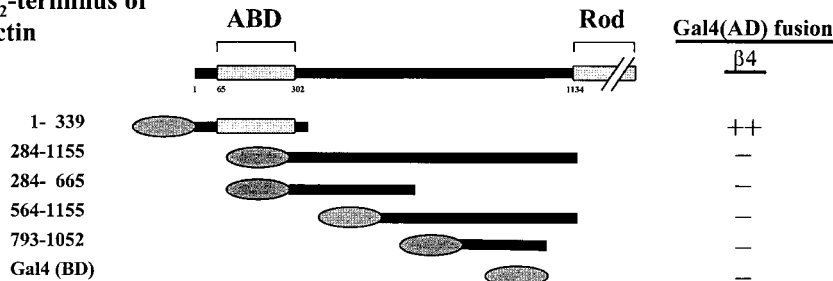
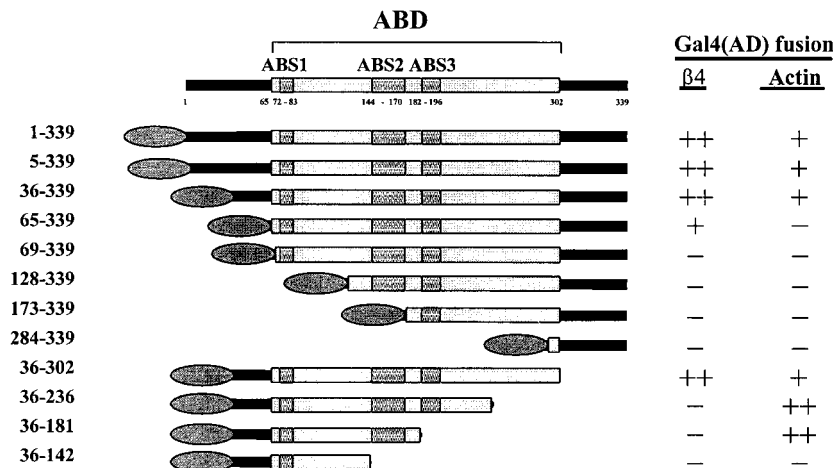
Binding was observed with keratins 14 and 18, but not with keratins 5 or 8, and with both vimentin and GFAP.

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 coex-

pressed 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 (Fig. 10, D–F). Although the expression of plectin-IFBD had no apparent effect on the keratin intermediate filament network, in many cells it induced a collapse of the vimentin network into dense clusters around the nucleus (Fig. 10, G–I). Colocalization of these two proteins in filamentous structures was only observed in a few cells (data not shown). The dramatic effect of the plectin-IFBD on the integrity of the vimentin intermediate filament network suggests that it interferes with another protein with properties similar to plectin that mediates the anchorage of these filaments at distal or peripheral sites in the cell. It may also indicate that plectin-IFBD binds more strongly to vimentin than to keratins. The  $\alpha 6\beta 4$  integrin was always present in basally located hemidesmosome-like clusters, and showed no obvious colocalization with plectin-IFBD (Fig. 10, A–C). These results suggest that in a normal situation, when the intact plectin protein is present, it helps to provide a scaffold for the vimentin cytoskeleton.

**A****NH<sub>2</sub>-terminus of Plectin****B**

**Figure 7.** Mapping of the interaction site for β4 and for actin on the NH<sub>2</sub> terminus of plectin by yeast two-hybrid analysis. (A) Cotransformation of yeast host strain PJ69-4A with one each of the pAS2-plectin subclones listed together with pACT2-β4<sup>1115-1457</sup> to determine the interaction site for β4 on plectin (A) and with one each of the pAS2-plectin ABD subclones listed together with pACT2-β4<sup>1115-1457</sup> or actin for a comparison of the β4- and actin-binding sites on plectin (B). The results shown for plectin<sup>36-302</sup>, plectin<sup>36-236</sup>, plectin<sup>36-181</sup>, and plectin<sup>36-142</sup> are identical for those obtained with two sets of four similar subclones starting at residues 1 or 5, respectively. The results shown are for β cytoplasmic actin. No binding was found between pACT2-β4<sup>1115-1457</sup> and pAS2-actin (data not shown). Details are as for Fig. 5.

### **The Integrin α6β4 Organizes the Vimentin Intermediate Filament Network through Plectin upon Expression in PA-JEB Keratinocytes**

To study a possible indirect role for the integrin α6β4 (via plectin) in the organization of the vimentin intermediate filament network, immortalized β4-deficient PA-JEB keratinocytes were transiently transfected with cDNAs encoding wild-type or mutant β4 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 β4 it was also detected in dense basally located clusters colocalized with α6β4 (Fig. 11, A–C). These results show that expression of β4 leads to a reorganization of the vimentin intermediate filament network. This most likely occurs by binding of β4 to the ABD in the plectin NH<sub>2</sub> terminus, accompanied by binding of vimentin to the IFBD in its COOH terminus. The effect of β4 on the vimentin network would then be indirect and dependent on the binding of β4 to plectin.

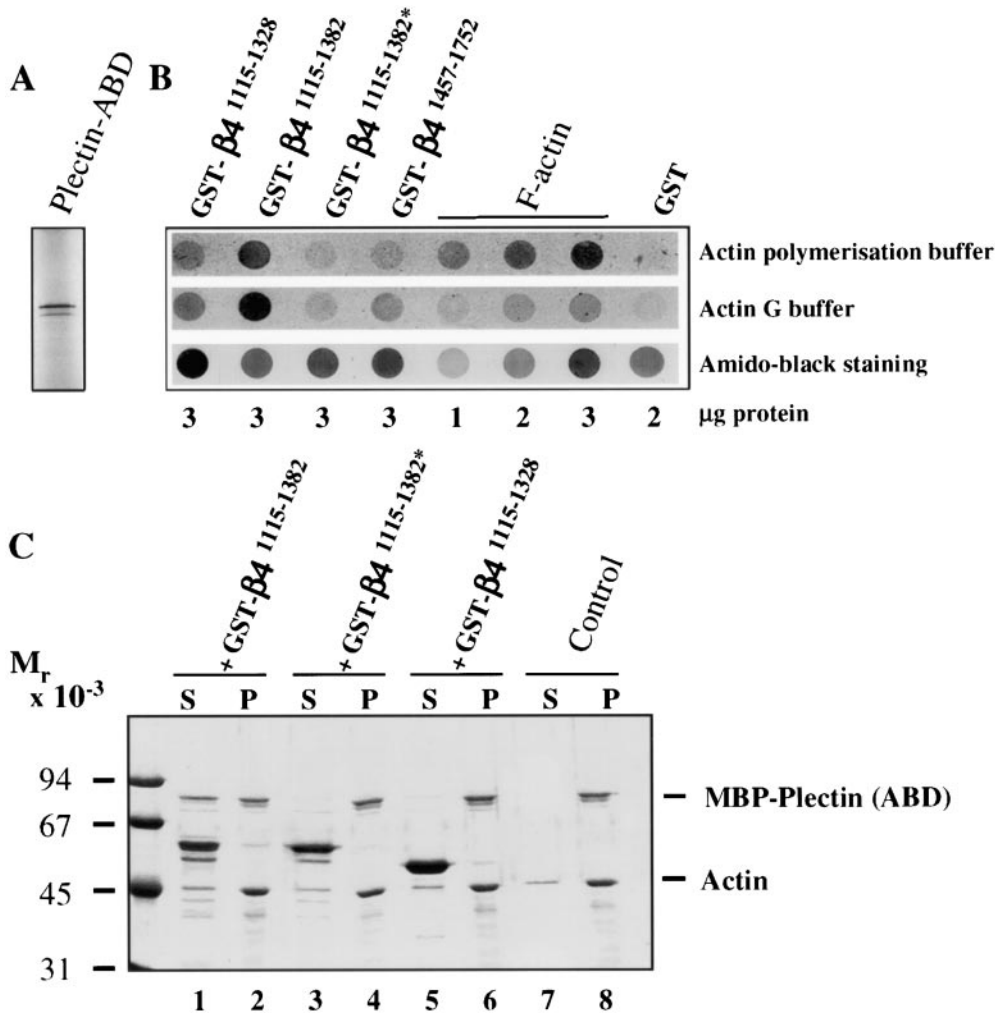
Further evidence for this was obtained by transfection of one mutant β4 protein, β4<sup>1355</sup>, that can efficiently bind plectin, and one β4 mutant, β4<sup>1328</sup>, that binds plectin in yeast two-hybrid assays, but much less efficiently. In cells transfected with β4<sup>1355</sup> cDNA, the β4 protein fragment was present in basal hemidesmosome-like clusters colocalized with vimentin (Fig. 11, D–F). The β4<sup>1328</sup> mutant was also found in basal clusters, but it only occasionally colocalized with vimentin (Fig. 11, G–I).

These results show that β4 is capable of organizing the vimentin intermediate filament cytoskeleton. It does so indirectly, via plectin, since only β4 mutants that contained the plectin binding site (like β4<sup>1355</sup>) were capable of organizing vimentin filaments into hemidesmosome-like basal clusters. Since this β4 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 α6β4, 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 α6β4 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<sup>®</sup> analysis of the MD-EBS cells gave expression levels of the α6 and β4 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 β4 at the basal side of the epidermal cells was reduced, indicating an important role for plectin in maintaining the



no GST fusion protein (lanes 7 and 8). The F-actin complexes were then isolated by centrifugation, after which supernatant (S; odd-numbered lanes) and pellet (P; even-numbered lanes) were isolated and analyzed using SDS-PAGE. Proteins were visualized using Coomassie brilliant blue staining.

integrity of hemidesmosomes (Andrä et al., 1997). Evidently, normal basal localization of  $\alpha 6\beta 4$  into hemidesmosome-like clusters can occur in MD-EBS keratinocytes, but plectin might have a role in retaining  $\alpha 6\beta 4$  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 $\beta 4$ 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 (Andrä 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  $\alpha 6\beta 4$ . Direct binding of  $\beta 4$  to the plectin-ABD was demonstrated using yeast two-hybrid and dot blot overlay assays. In addition, we showed that  $\beta 4$  can effectively compete plectin-ABD out of an F-actin-plectin complex in an in vitro actin cosedimentation assay. This suggests that competition between  $\beta 4$  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  $\beta 4$ -containing hemidesmosomes at the basal side of keratinocytes when  $\beta 4$  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 (PIP<sub>2</sub>), for example (Andrä et al., 1998).

Since hemidesmosomes are generally seen as stable ad-

Figure 8. Dot blot overlay and actin sedimentation assay of recombinant purified  $\beta 4$  protein fragments with radiolabeled plectin-ABD fragment. (A) SDS-PAGE of 4 ng in vitro translated [<sup>35</sup>S]-methionine/cysteine-labeled HA-tagged plectin-ABD protein (40 kD). Autoradiogram. (B) Recombinant purified GST fusion proteins encoding  $\beta 4^{1115-1328}$ ,  $\beta 4^{1115-1382}$  (wild-type sequence),  $\beta 4^{1115-1382*}$  (R1281W),  $\beta 4^{1457-1752}$  (3  $\mu$ g each), or GST alone (2  $\mu$ g), and purified F-actin (1, 2, or 3  $\mu$ g) were immobilized on nitrocellulose by dot blotting and incubated with 100 ng [<sup>35</sup>S]-methionine/cysteine-labeled plectin-ABD in 3 ml APB or AGB (upper and middle lanes, respectively). Protein loading was verified by amido-black staining (bottom lanes). The GST protein alone does not bind to plectin-ABD. (C) Actin filaments polymerized in the presence of and complexed with MBP-plectin ABD fusion protein were incubated with a 10-fold molar excess of GST fusion proteins encoding  $\beta 4^{1115-1382}$  (lanes 1 and 2),  $\beta 4^{1115-1382*}$  (lanes 3 and 4),  $\beta 4^{1115-1328}$  (lanes 5 and 6), or



	Gal4(BD) fusion plectin	
	R5-R6	R6
Keratin 5	-	-
Keratin 14	++	-
Keratin 8	-	-
Keratin 18	++	-
Vimentin	++	-
GFAP	++	-
$\beta 4$ <sup>1115-1457</sup> (I-II-CS)	-	-
$\beta 4$ <sup>1320-1668</sup> (CS-III-IV)	-	-
$\beta 4$ <sup>1457-1752</sup> (III-IV-CT)	-	-
Gal4 (AD)	-	-

**Figure 9.** Mapping of the interaction site between the COOH terminus of plectin and the cytoplasmic domain of  $\beta 4$  and of full-length intermediate filament proteins by yeast two-hybrid analysis. Yeast host strain PJ69-4A was cotransformed with pAS2-plectin and one each of the listed pACT2-IF or pACT2- $\beta 4$  subclone constructs, or with empty pACT2 vector to determine the interaction sites between plectin and  $\beta 4$  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.

hesion complexes that are less compatible with cell motility than the actin structures, it is clear that plectin recruitment into hemidesmosomes might represent a pivotal factor in adhesion and motility properties of epithelial cells.

### ***$\beta 4$ Contains a Single Binding Site for Plectin***

In a recent study, two distinct plectin-binding sites were identified on the  $\beta 4$  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). NH<sub>2</sub>-terminal fragments of epithelial rat plectin (both with and without the ABD) were found to interact with both binding sites on  $\beta 4$  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  $\beta 4$  and plectin protein fragments were shown to be colocalized mainly in dense perinuclear structures in the cytoplasm (Reznicek et al., 1998).

Previously, we have identified a region on the  $\beta 4$  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 NH<sub>2</sub>-terminal plectin-binding site on the  $\beta 4$  protein, and mapped it to residues 1217–1355. No evidence was found for the more COOH-terminal binding site reported by Reznicek et al. (1998). Full-length  $\beta 4$

containing the amino acid mutation R1281W, in marked contrast to wild-type  $\beta 4$  (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 NH<sub>2</sub>-terminal plectin-binding site on  $\beta 4$  is required for the proper localization of plectin into hemidesmosomes.

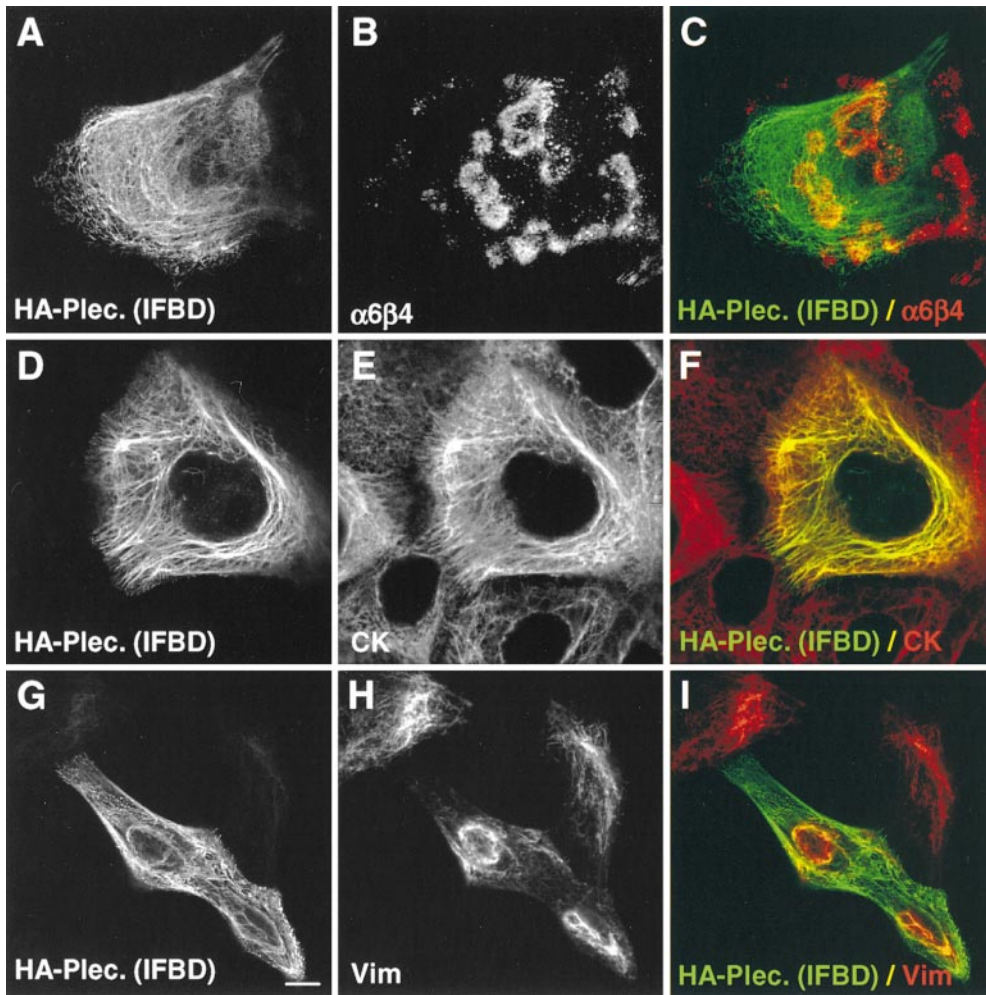
Another difference with the results obtained by Reznicek et al. (1998) is that we located the binding site for  $\beta 4$  in the plectin-ABD and were not able to demonstrate interaction of  $\beta 4$  with NH<sub>2</sub>-terminal fragments of plectin from which the ABD is absent. Nevertheless, it remains possible that sequences downstream of the ABD, although not able to mediate binding by themselves, could enhance the binding of the ABD to  $\beta 4$ . Such a scenario of cooperative binding may proffer 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 Reznicek et al. (1998) used denatured proteins for the in vitro overlay experiments, whereas the  $\beta 4$  cytoplasmic domain in vivo is most probably intricately folded (Reznicek et al., 1998; Schaapveld et al., 1998). Our concern with the cell transfection experiments presented by these authors is that overexpression of the  $\beta 4$  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  $\beta 4$  clones used for cell transfections contain the complete extracellular and transmembrane regions, and therefore the expression of  $\beta 4$  at the basal cell surface (the physiological location for  $\alpha 6\beta 4$ ) is limited by the amount of endogenous integrin  $\alpha 6$  subunit.

### ***Plectin and BP180 Can Bind $\beta 4$ 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  $\beta 4$ -deficient PA-JEB keratinocytes, in which mutant  $\beta 4$  proteins were transiently expressed. Two mutant  $\beta 4$  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  $\alpha 6\beta 4$  in basal hemidesmosome-like clusters in the absence of plectin. Furthermore, we have found that when expressed in PA-JEB keratinocytes, the  $\beta 4$ <sup>R1281W</sup> 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).



**Figure 10.** Subcellular distribution of HA-tagged plectin-IFBD fragment in MD-EBS keratinocytes after transient transfection. Cells were transiently transfected with an HA-tagged plectin R5-R6 cDNA construct. Cells were fixed, double-immunolabeled using polyclonal antiserum HA-11 against the HA tag (green; A, D, and G) and a mixture of mAbs 58XB4 and 4.3E1 against  $\beta 4$  (red; B), a mixture of mAbs SPK-14 and KL1 against keratins (red; E), or mAb V9 against vimentin (red; H), and processed for immunofluorescence. Composite images (C, F, and I) were generated by superimposition of the green and red signals, with areas of overlap appearing yellow. Cells were visualized by confocal microscopy. The plectin-IFBD protein fragment is exclusively found in cytoplasmic filament structures (A, D, and G).  $\alpha 6\beta 4$  is present in basally located hemidesmosome-like clusters (B), together with the plectin-IFBD (C). Keratins are observed in a dense cytoplasmic filament network (E), which is decorated by the plectin-IFBD (F). Expression of the IFBD-fragment of plectin causes a collapse of the vimentin filament network (H and I). Bar, 10  $\mu\text{m}$ .

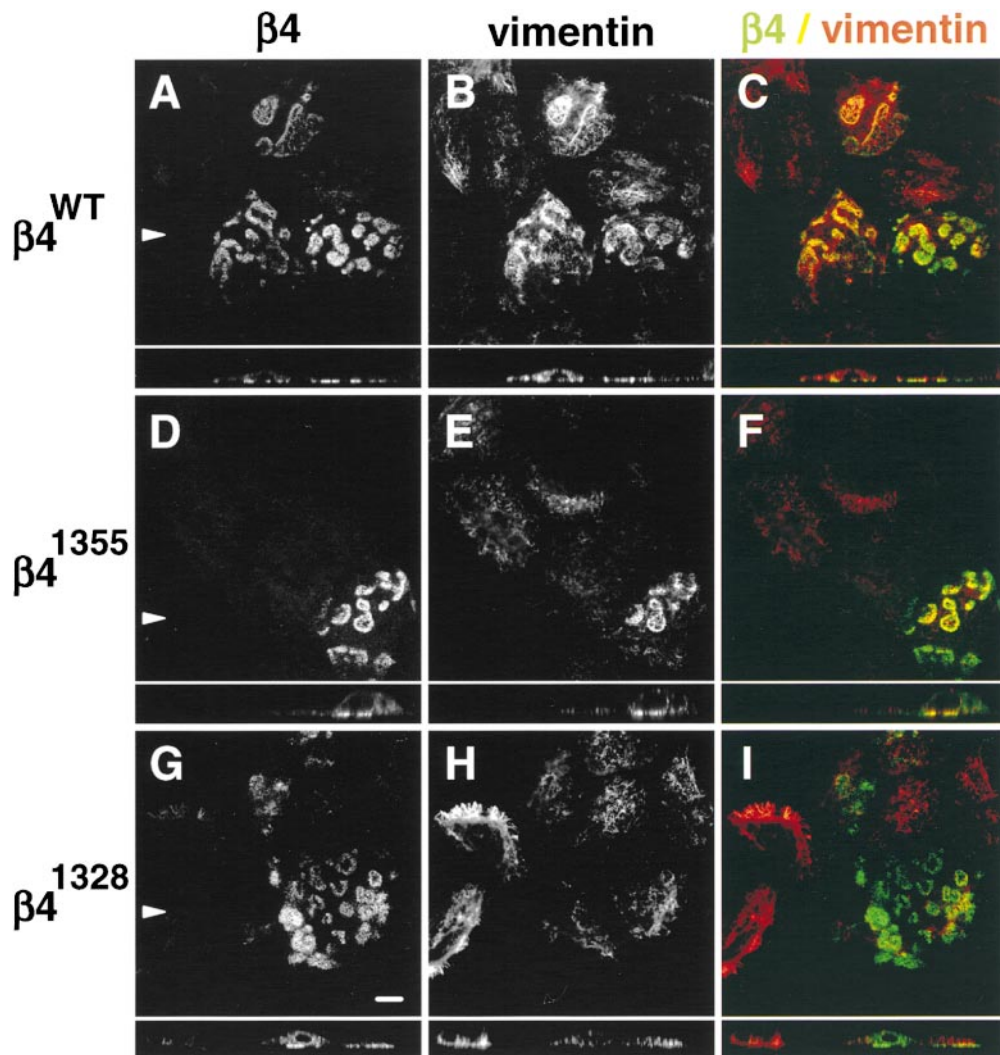
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We reasoned that the two  $\beta 4$  mutant proteins used in our previous study,  $\beta 4^{\Delta 1219-1319}$ , in which the complete second FNIII repeat had been deleted, and  $\beta 4^{\Delta 17}$ , in which amino acids 1249–1265 of the second FNIII repeat are absent (Vidal et al., 1995), were incorrectly folded in such a way that the binding site for BP180 on  $\beta 4$  was rendered dysfunctional. There is evidence for an intramolecular folding in vitro of the  $\beta 4$  cytoplasmic domain (Reznicek et al., 1998; Schaapveld et al., 1998), in which the COOH-terminal part of the  $\beta 4$  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  $\beta 4^{\text{R1281W}}$  mutant, this intramolecular association is still intact. We conclude that binding of  $\beta 4$  to plectin and BP180 most probably occurs independently.

#### ***An Arginine to Tryptophan Substitution in $\beta 4$ Results in a JEB Rather than an MD-EBS Phenotype***

From our data it is expected that a patient who is homozy-

gous for the R to W mutation at residue 1281 in  $\beta 4$ , would have a phenotype similar to that of plectin-deficient patients, i.e., EBS. However, a  $\beta 4$  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  $\alpha 6\beta 4$  at the cell surface, thereby compromising the adhesion function of the cell. A role for plectin in facilitating or maintaining high surface levels of  $\alpha 6\beta 4$  is suggested, because as mentioned previously, in the two MD-EBS cell lines used in this study a reduction in the levels of  $\alpha 6\beta 4$  has been observed. Indeed, the expression of  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$ , 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  $\beta 4^{\Delta 17}$  (Schaapveld et al., 1998) and  $\beta 4^{\text{R1281W}}$  mutants (this study), which are both mutations identified in patients (Vidal et al., 1995; Pulkkinen et al., 1998), show that differ-



**Figure 11.** Subcellular distribution of wild-type and mutant  $\beta 4$  and study of the effect of  $\beta 4$  expression on the vimentin intermediate filament network in transfected PA-JEB keratinocytes. PA-JEB cells were transfected with cDNA encoding full-length  $\beta 4$  (A–C), COOH-terminal deletion mutants  $\beta 4^{1355}$  (D–F), and  $\beta 4^{1328}$  (G–I). Cells were fixed, double-immunolabeled, and processed for confocal immunofluorescence microscopy using polyclonal antiserum against the  $\alpha 6$  subunit (green; A, D, and G), or mAb V9 against vimentin (red; B, E, and H). Composite images (C, F, and I) were generated by superimposition of the green and red signals, with areas of overlap appearing yellow. Expression of wild-type full-length  $\beta 4$  results in the formation of hemidesmosome-like structures, in which  $\alpha 6\beta 4$  is concentrated at sites of cell-substrate contact, as is shown by staining for  $\alpha 6$ , and codistributes with the vimentin network, most probably dependent upon its binding to plectin (A–C). The  $\beta 4^{1355}$  mutant protein, which efficiently binds to plectin, is found colocalized with the vimentin network

(D–F). The  $\beta 4^{1328}$  mutant protein, which can only bind plectin with low efficiency in vitro, was only very occasionally found to be colocalized with vimentin (G–I). Bar, 10  $\mu\text{m}$ .

ences in the severity of the disease in different PA-JEB patients can also result from an impaired function of  $\beta 4$  to recruit BP180 and BP230 into hemidesmosomes. Both mutants are unable to recruit plectin, but only the  $\beta 4^{\Delta 17}$  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 vi-

mentin intermediate filament network and its association with hemidesmosomes. The significance of anchoring vimentin to hemidesmosomes (seen in the PA-JEB cells after transfection with  $\beta 4$  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  $\alpha 6\beta 4$ -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  $\beta 4$  subunit of the  $\alpha 6\beta 4$  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-



cated at the end of domain R5), first identified by Wiche and co-workers (Foisner et al., 1988, 1991), since clones missing this region do not interact with this intermediate filament protein. In addition, plectin binds the type I (or acidic) keratins 14 and 18, and the type III intermediate filament protein GFAP. The type II (or basic) keratins 5 and 8 do not bind, which for the first time demonstrates specificity of the binding of plectin to keratins. In a previous study (Foisner et al., 1988) a mixture of keratins was used, and thus binding to particular keratins could not be distinguished. Since the crucial region in the plectin-IFBD (Nikolic et al., 1996) is strongly basic, it is suggested that binding to the acidic type I keratins is ionic in nature.

In vivo keratin filaments are exclusively composed of heterodimers, i.e., specific combinations of a type I and a type II monomer. Keratin 5 dimerizes with keratin 14 and keratin 8 with keratin 18. In the yeast two-hybrid assay, only a single keratin was available for interaction with the plectin-IFBD and since keratins cannot form stable homopolymers (Quinlan et al., 1994), plectin most probably is capable of binding monomeric keratin. The fact that plectin associates with intermediate filaments demonstrates that in the formation of keratin dimers the binding site for plectin is not blocked. The association of plectin with intermediate filament proteins can be influenced by specific plectin phosphorylation events. Together, these findings support the idea that plectin-intermediate filament association is a well-regulated event in the organization of the cytoskeleton (for review see Wiche, 1998).

In apparent contrast with the findings published by Reznicek et al. (1998), which described a binding site for  $\beta 4$  on a COOH-terminal fragment of plectin starting with the last part of domain R5 using in vitro binding assays, we were not able to detect binding of the plectin fragment containing these sequences to the  $\beta 4$  cytoplasmic domain (see previous section for a possible explanation); no interaction was found between GAL4-plectin and GAL4- $\beta 4$  fusion proteins in a yeast two-hybrid assay and similarly, plectin-IFBD protein was not found to be colocalized with  $\alpha 6\beta 4$  in hemidesmosome-like basal clusters upon expression in MD-EBS keratinocytes. Thus, the COOH terminus of plectin appears to be primarily involved in the binding of intermediate filament proteins, rather than of  $\beta 4$ .

### ***Plectin as a Linker Protein***

Plectin can bind at least three different actin isoforms with similar efficiencies (data not shown), in contrast to related actin-binding proteins like utrophin, which have different binding efficiencies for various actin isoforms (Winder et al., 1995). Actin isoforms are expressed at specific stages of development and only in certain tissues, and can have specific subcellular distributions within one cell (for review see Gunning et al., 1998). Thus, plectin can act as an actin linker protein in all these tissues and cells.

Like plectin, other plakins also show specificity in binding intermediate filament proteins: BP230 binds neurofilament proteins but not vimentin (Yang et al., 1996; Leung et al., 1999), and desmoplakin (a desmosomal plakin) has a much higher affinity for type II keratins than for type I keratins or vimentin (Stappenbeck et al., 1993; Kouklis et al., 1994; Meng et al., 1997). 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; Gache et al. 1996; Andr a et al., 1997).

The data presented in this study conclusively prove that the NH<sub>2</sub> terminus of plectin is involved in interactions with actin filaments or with  $\beta 4$  and its COOH terminus in binding to intermediate filament proteins. A similar organization of binding sites has been found for desmoplakin: its NH<sub>2</sub> 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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