See related Commentary on page vi

# Molecular Consequences of Deletion of the Cytoplasmic Domain of Bullous Pemphigoid 180 in a Patient with Predominant Features of Epidermolysis Bullosa Simplex

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**Bullous pemphigoid antigen 2 (BP180; COL17A1) collagen gene mutations typically result in nonlethal junctional epidermolysis bullosa. We have identified a patient, who had phenotypic features of mainly epidermolysis bullosa simplex and evidence for both intraepidermal and junctional blister formation. Mutation analysis disclosed compound heterozygous mutations in the COL17A1 gene, leading to deletion of Ile-18 to Asn-407 from the intracellular domain of BP180, BP180$^{\Delta 18-407}$. To gain insight into the mechanisms underlying the phenotype, we have investigated the functional consequences of this truncation in BP180. The results demonstrate that: (1) in cultured keratinocytes of the patient, the assembly of hemidesmosomes, and their linkage with intermediate filaments are impaired; (2) BP180$^{\Delta 18-407}$ is not capable of binding to the hemidesmosomal components BP230, plectin, and the β4 subunit of the α6β4 integrin in yeast two-hybrid assays; (3) BP180$^{\Delta 18-407}$ is recruited into hemidesmosome-like structures in both normal and BP180-deficient transfected keratinocytes when ectopically expressed, suggesting that the extracellular domain of BP180$^{\Delta 18-407}$ determines its topogenic fate; and, finally (4) the proteolytic shedding of the extracellular domain of BP180$^{\Delta 18-407}$ is not impaired in transfected COS-7 cells. Collectively, the data demonstrate that the truncation of the intracellular domain of BP180 impairs the organization of hemidesmosomes, affecting both the mechanical stability of basal keratinocytes and dermoepidermal cohesion.**

Key words: BP180/collagen XVII/epidermolysis bullosa/hemidesmosome/mutations.
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Epidermolysis bullosa (EB) comprises a large group of mechanobullous diseases characterized by fragility of the skin and mucosae (reviewed in Eady, 2001; Uitto and Pulkkinen, 2001). These disorders are caused by pathogenic mutations in genes encoding constituents of the hemidesmosomes and related structures, that critically promote strong stromal–epithelial cohesion (reviewed in Borradori and Sonnenberg, 1999). Three major variants of EB have been defined based on the level of tissue separation along the cutaneous basement membrane. First, EB simplex shows tissue separation within the cytoplasm of basal keratinocytes. It is typically due to mutations in genes encoding either keratin 5, keratin 14, or plectin. Secondly, in junctional EB, separation occurs within the basement membrane at the level of the lamina lucida; this group of EB results from mutations in either of at least six different genes coding for the two transmembrane hemidesmosomal components bullous pemphigoid (BP) 180 (also called collagen XVII or BPAG2) and the α6β4 integrin or laminin-5. Thirdly, in dystrophic EB, a disease caused by type VII collagen gene mutations, blistering takes places below the basement membrane within the upper papillary dermis (reviewed in Pulkkinen and Uitto, 1998; Bruckner-Tuderman, 1999; Irvine and McLean, 1999; Fine *et al*, 2000).

BP180, originally identified as the autoantigen of BP (Labib *et al*, 1986; Diaz *et al*, 1990; Giudice *et al*, 1992; Hopkinson *et al*, 1992), is a type II transmembrane protein with a large collagenous extracellular domain (ECD) (Giudice *et al*, 1992; Schäcke *et al*, 1998; Nykvist *et al*, 2001). Pathogenic mutations in the BP180 (*COL17A1*) gene are usually, but not always associated with nonlethal junctional EB variants. Most of the implicated mutations are premature termination codons resulting in non-sense-mediated mRNA decay with no or poor BP180 expression at the protein level (McGrath *et al*, 1995; Chavanas *et al*, 1997; Darling *et al*, 1997).

Recently, we described an unusual patient with phenotypic features of mainly EB simplex. Genetic analysis revealed compound heterozygous mutations in the BP180 gene leading to a large in frame deletion from residue 18 to 407 from the cytoplasmatic domain of BP180 (Fig 1). Blister formation occurred either at the level of lamina lucida or

Abbreviations: AD, transcription-activation domain; BD, DNA-binding domain; BP230, bullous pemphigoid antigen 1; BP180, bullous pemphigoid antigen 2; EB, epidermolysis bullosa; ECD, extracellular domain; IF, intermediate filaments; nt, nucleotides

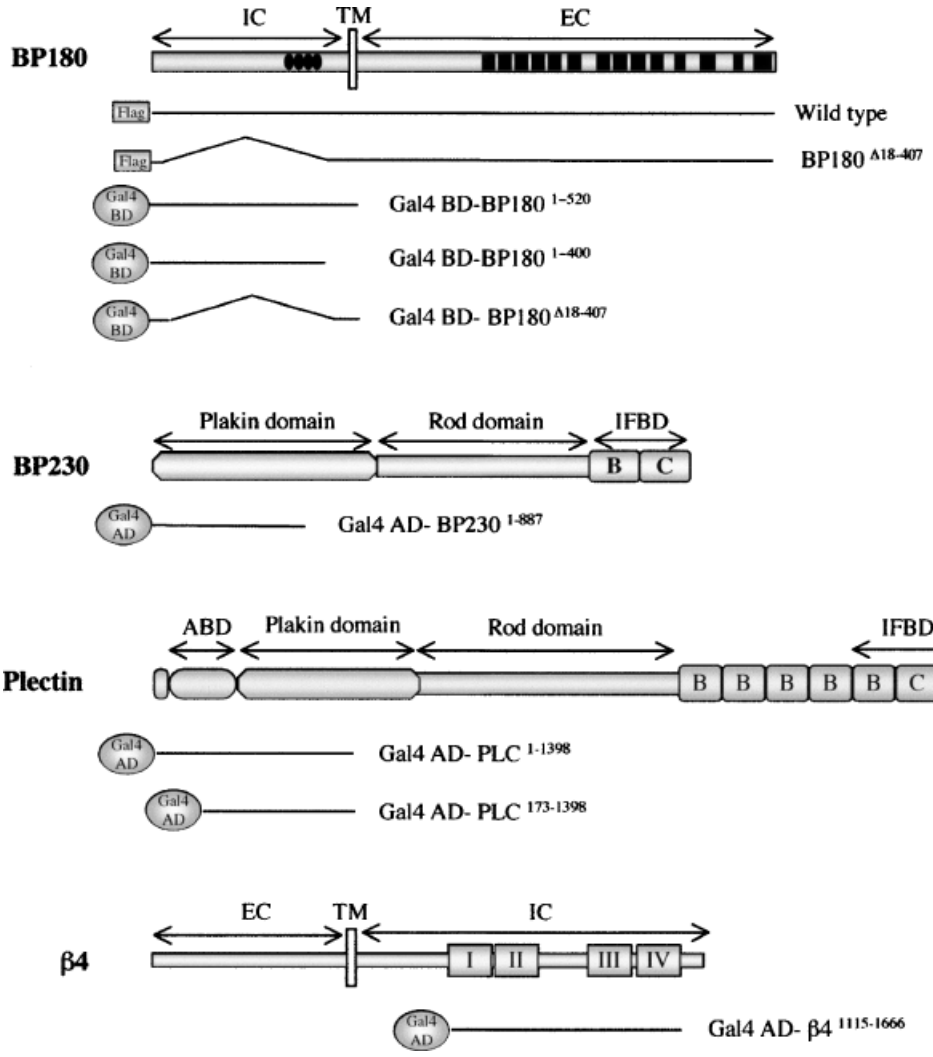


**Figure 1**
**Schematic diagram of BP180, BP230, plectin, the β4 integrin subunit, and the various deletion mutants utilized**. IC, intracellular domain; EC, Extracellular domain; TM, Transmembrane domain; IFBD, IF-binding domain; ABD, Actin-binding domain.

within the basal keratinocytes. Ultrastructurally, the attachment of keratin filaments to the hemidesmosomal plaque appeared to be impaired (Huber *et al*, 2002). As there is no evidence for a direct linkage of BP180 with epidermal keratins, it is likely that the severing of the keratin bundles is indirect. Recent studies found that BP180 binds to BP230 and plectin, two cytoplasmic hemidesmosomal components of the plakin family of cytolinkers (Aho and Uitto, 1999; Koster *et al*, 2003). The C-terminal tail domain of these two proteins is able to bind to intermediate filament (IF) proteins, whereas their N-terminal head domain is associated with the cytoplasmic domain of the β4 subunit of the α6β4 integrin and to BP180 (Hopkinson and Jones, 2000; Favre *et al*, 2001; Fontao *et al*, 2003; Koster *et al*, 2003). Hence, BP230 and plectin act as linkers, connecting the IF network with BP180 and the α6β4 integrin at the basal cell side (Borradori and Sonnenberg, 1999).

To explain better the EB simplex phenotype in our patient, we assessed the functional consequences of the large deletion within $BP180^{\Delta 18–407}$ and carried out morphologic, cell biologic, and yeast assays. The results indicate that in the presence of $BP180^{\Delta 18–407}$ the molecular organization of hemidesmosome is severely altered. Although its potential to be recruited into hemidesmosome-like structures in keratinocytes and its shedding from the cell surface are unaffected, $BP180^{\Delta 18–407}$ has lost the ability to bind to BP230, plectin, and the β4 integrin subunit.

## Results

**Electron microscopic analysis of cultured primary keratinocytes** To assess the impact of the large deletion from the cytoplasmic domain of BP180 on the formation of hemidesmosomes, electron microscopy studies were performed on cultured primary keratinocytes derived from our EB patient expressing $BP180^{\Delta 18–407}$ ($BP180^{\Delta 18–407}$ keratinocytes) and from a healthy volunteer (NHK), respectively. $BP180^{\Delta 18–407}$ keratinocytes exhibited only rare membrane-associated electron-dense structures at sites of cell-substrate contact (Fig 2*A*). Their appearance was rudimentary, as there was no obvious inner and outer cytoplasmic plate, and they did not associate with keratin filament bundles. By contrast, NHK displayed normal looking hemidesmosome with an outer and inner plate that was frequently linked with keratin filament bundles (Fig 2*B*).

**Distribution of hemidesmosomal components in cultured primary keratinocytes** We next examined by confocal immunofluorescence microscopy the expression and

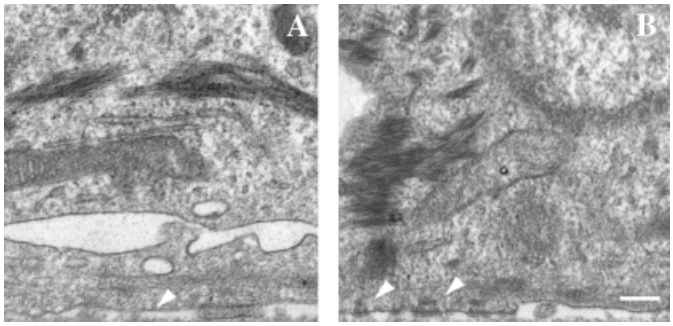


**Figure 2**
**Electron microscopic examination of the basal regions of cultured BP180$^{\Delta 18–407}$ keratinocytes and normal human keratinocytes.** A few rudimentary membrane-associated electron-dense plaques at sites of cell-substrate contact (*arrowheads*) are observed in cultured BP180$^{\Delta 18–407}$ keratinocytes. Keratin filament bundles (CK) are not significantly connected to these junctional complexes (*A*). In normal human keratinocytes (*B*) the morphology of hemidesmosomes (*arrowheads*) appear normal with a sub-basal dense plate and an inner plate connected to keratin filaments. Cells were grown on coverslips coated with conditioned medium from 804G cells enriched in laminin-5 as described in the *Materials and Methods*. *Scale bar* = 0.2 μm.

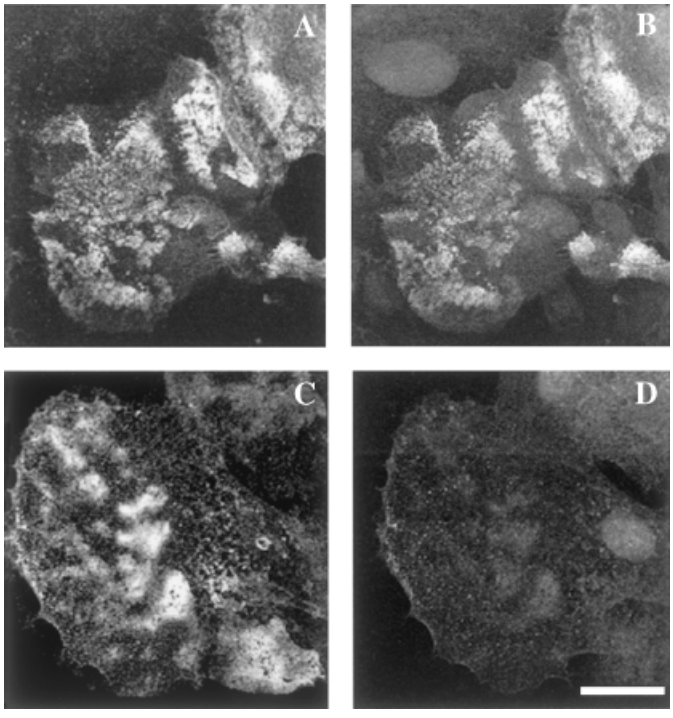


**Figure 3**
**Immunolocalization of BP180 and BP230 in cultured BP180$^{\Delta 18–407}$ keratinocytes by confocal laser microscopy.** Normal human keratinocytes (*A,B*) and BP180$^{\Delta 18–407}$ keratinocytes (*C,D*) cultured on glass coverslips were double stained with the MoAb 1D1 directed against BP180 (*A,C*) and the human MoAb 5E recognizing BP230 (*B,D*). Confocal optical sections made at the basal side of the cells shows a clear colocalization of BP180 and BP230 in large patches typical for hemidesmosome-like structures in normal human keratinocytes. In BP180$^{\Delta 18–407}$ keratinocytes, BP180 is clustered at the basal aspect of the cell, whereas BP230 remains diffusely in the cytoplasm. *Scale bar* = 15 μm.

localization of hemidesmosomal components in primary keratinocytes. Staining using the MoAb 1D1 demonstrated that in BP180$^{\Delta 18–407}$ keratinocytes BP180$^{\Delta 18–407}$ localized in discrete clusters and patches, the staining pattern of which

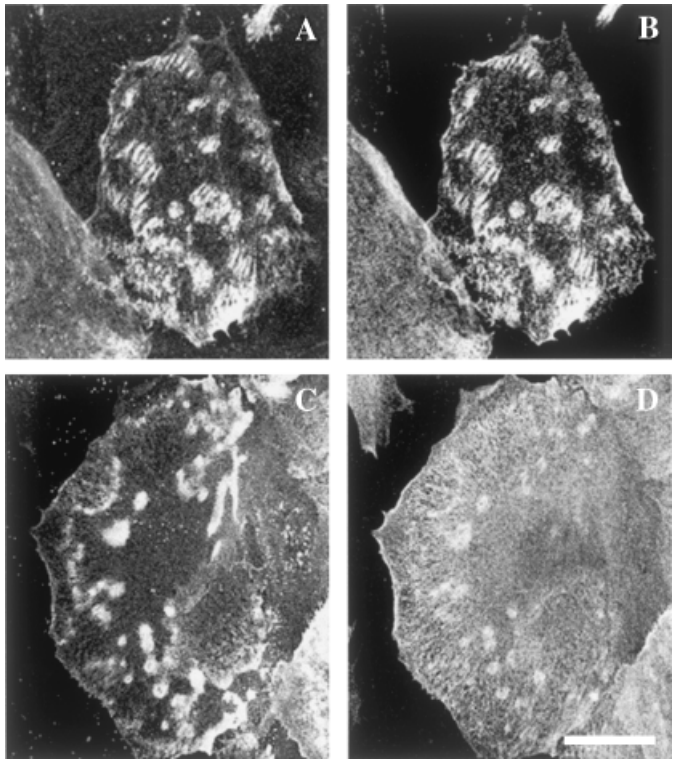


**Figure 4**
**Immunolocalization of the β4 integrin subunit and plectin in cultured BP180$^{\Delta 18–407}$ keratinocytes by confocal laser microscopy.** Normal human keratinocytes (*A,B*) and BP180$^{\Delta 18–407}$ keratinocytes (*C,D*) grown on glass coverslip were double stained with a guinea pig anti-plectin antibody (*B,D*) and the MoAb 3E1 recognizing the β4 integrin subunit (*A,C*). Plectin is colocalized with β4 in large patches typical of hemidesmosome-like structures in normal human keratinocytes. In BP180$^{\Delta 18–407}$ keratinocytes, clusters of plectin are found in only about 10% of cells and show reduced colocalization with β4. A predominant diffuse cytoplasmic staining is observed for plectin. *Scale bar* = 15 μm.

was typical for hemidesmosome-like structures (Schaapveld *et al*, 1998) (Fig 3*C*). As expected, no staining was detected with the MoAb 1A8c directed against the cytoplasmic domain of BP180 (not shown). Furthermore, in BP180$^{\Delta 18–407}$ keratinocytes, BP230 was not co-distributed with either β4 (not shown) or BP180 in hemidesmosome-like structures but remained diffusely distributed over the cytoplasm of these cells (Fig 3*D*). Plectin was no longer exclusively concentrated in hemidesmosome-like structures together with the α6β4 integrin but was also found diffusely distributed in the cytoplasm (Fig 4*D*). In contrast, in NHK BP180, BP230, plectin, and α6β4 integrin were normally concentrated at the basal sites of the cells in a pattern typical for hemidesmosome-like structures (Figs 3 and 4).

**BP180$^{\Delta 18–407}$ is recruited into hemidesmosome-like structures upon ectopic expression in normal and BP180-deficient keratinocytes** As sequences within the cytoplasmic domain of BP180 regulate its subcellular localization (Borradori *et al*, 1997), it was unexpected to find BP180$^{\Delta 18–407}$ concentrated in hemidesmosome-like structures. Therefore, we tested the topogenic fate of BP180$^{\Delta 18–407}$ upon its expression in 804G cells and a

previously described PAJEB/β4 keratinocyte cell line (Sterk *et al*, 2000). Both wild-type and BP180$^{\Delta 18–407}$ recombinant proteins were found co-distributed with the α6β4 integrin in hemidesmosome-like structures when ectopically expressed in these cell lines (not shown). To assess whether the subcellular distribution of BP180$^{\Delta 18–407}$ depends on its ability to form heterotrimers with endogenous BP180, we carried out transfection studies using an immortalized BP180-deficient cell line (Koster *et al*, 2003). In few of the transfected cells, BP180$^{\Delta 18–407}$ was found colocalized with α6β4 in hemidesmosome-like structures (Fig 5). Overall, these findings indicate that the topogenic fate of BP180$^{\Delta 18–407}$ most likely depends on its interaction with an extracellular matrix protein present on the glass coverslips and/or the ability of its ECD to bind to the α6 subunit (Hopkinson *et al*, 1998).

**Deletion of residues 18 to 407 in BP180 impaired its association with the β4 integrin subunit, BP230 and plectin** The cytoplasmic domain of BP180 contains binding sites for BP230, plectin, and the β4 subunit (Borradori *et al*, 1997; Aho and Uitto, 1998; Schaapveld *et al*, 1998; Hopkinson and Jones, 2000; Koster *et al*, 2003). Therefore, we tested whether deletion of residues 18 to 407 from the cytoplasmic domain of BP180 impairs its ability to interact with β4, plectin, and BP230 by using yeast two-hybrid assays. For this purpose, PJ69 yeast was cotransformed with a pAS2-1 plasmid construct encoding residues 1 to 400 of BP180 as GAL4-BD fusion protein, GAL4-BD-BP180$^{1–400}$ together with a pACT2 plasmid construct encoding deletion mutants of the β4 subunit, plectin, or BP230, all fused to the GAL4-AD (Figs 1 and 6). Cotransformed yeasts were selected and tested for protein–protein interactions using the β-galactosidase assays. The results demonstrate that GAL4-BD-BP180$^{1–400}$ encoding residues 1 to 400 of the cytoplasmic domain of BP180 strongly bound to the GAL-AD-β4$^{1115–1666}$ construct encoding a region of the β4 cytoplasmic domain encompassing both pairs of type III fibronectin repeats. The same GAL4-BD-BP180$^{1–400}$ also strongly interacted with GAL4-AD-BP230$^{1–887}$, GAL4-AD-PLC$^{1–1398}$, and GAL4-AD-PLC$^{173–1398}$, encoding the N-terminal parts of BP230 and plectin, respectively. In BP230, residues 1 to 887 encompassing repeat NN to W of its plakin domain were sufficient to mediate strong binding to BP180. Furthermore, both the constructs GAL4-AD-PLC$^{1–1398}$ and GAL4-AD-PLC$^{173–1398}$, lacking half the actin-binding domain, bound to BP180 to a similar extent. In contrast, GAL4-BD-BP180$^{\Delta 18–407}$ containing the cytoplasmic deletion showed no binding activity towards GAL4-AD constructs of plectin, BP230, and β4 (Fig 6). We could not test a construct encoding the cytoplasmic domain of BP180, the transmembrane and part of the ECD, GAL4-BD-BP180$^{1–520}$, as transformed yeast had a tendency for transactivation. Finally, we tested the ability of BP180 to bind to K5/K14 keratins in yeast three-hybrid assays. We found that a large portion of the cytoplasmic domain of BP180, GAL4-BD-BP180$^{1–400}$, bound to monomeric K5 and K14 and the K5/K14 heterodimer (not shown).

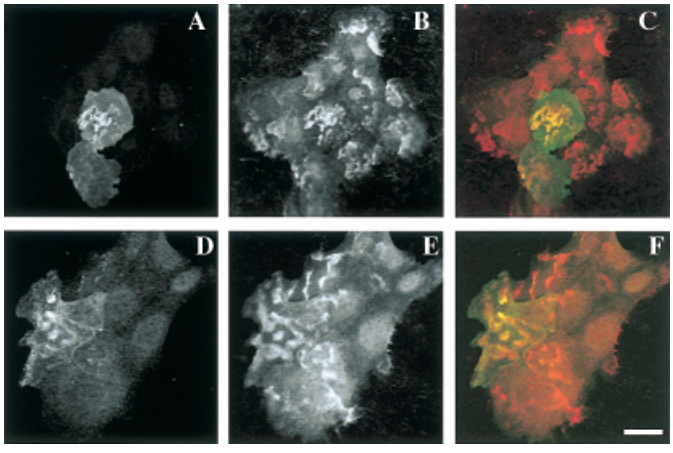


**Figure 5**
**BP180$^{\Delta 18–407}$ becomes localized into hemidesmosome-like structures upon its expression in BP180-deficient keratinocytes.** BP180-deficient keratinocytes were transiently transfected with cDNA encoding wild-type BP180 (*A–C*) and BP180$^{\Delta 18–407}$ (*D–F*), that were FLAG-tagged at their N-terminus. Forty-eight hours after transfection cells were double stained with an anti-α6 integrin subunit antibody (*B,E*) and the anti-FLAG antibody (*A,D*) and viewed under confocal laser microscope. Both BP180 and BP180$^{\Delta 18–407}$ show a colocalization with the α6 integrin subunit in hemidesmosome-like structures. Overlay of red and green channel is shown in *C* and *F*. *Scale bar* = 25 μm.

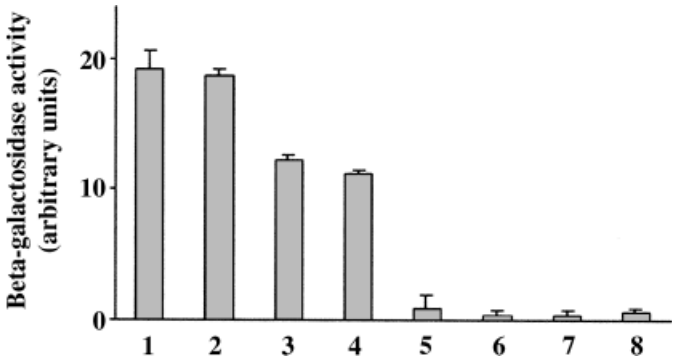


**Figure 6**
**Assessment of the binding ability of BP180 recombinant proteins fused to GAL4-BD with distinct regions of BP230, of plectin, and of the cytoplasmic domain of β4 fused to GAL4-AD in yeast two-hybrid assays.** The PJ69-4A yeast strain was cotransformed with pAS2-1 plasmid encoding either residues 1 to 400 (bars 1–4) or residues 1 to 520 with the Δ18-407 deletion (bars 5–8) of BP180 as GAL4-BD fusion proteins, and pACT2 plasmids encoding residues 1 to 887 of BP230 (bars 1 and 5), residues 1115 to 1666 of the β4 integrin subunit (bars 2 and 6), residues 1 to 1398 of plectin (bars 3 and 7) or residues 173 to 1398 of plectin (bars 4 and 8) as GAL4-AD fusion proteins. The β-galactosidase activity of yeast transformants was quantified in liquid assays using *O*-nitrophenyl β-D-galactopyranoside as a substrate as described in *Materials and Methods*. In these assays the β-galactosidase activity is proportional to the strength of the interaction.

**BP180$^{\Delta 18–407}$ is normally post-translationally processed** To re-evaluate in detail the impact of the truncation on shedding of the ECD of BP180 from the cell surface (Schäcke *et al*, 1998; Franzke *et al*, 2002), we utilized transiently transfected COS-7 cells encoding wild-type BP180 or BP180$^{\Delta 18–407}$. As depicted in Fig 7, Western blot analysis of extracts from COS-7 cells transfected with cDNA for wild-type BP180 identifies two proteins with an electrophoretic mobility of approximately 180 and 120 kDa, respectively. Whereas the former corresponds to the unprocessed form of BP180, the latter represents the shed

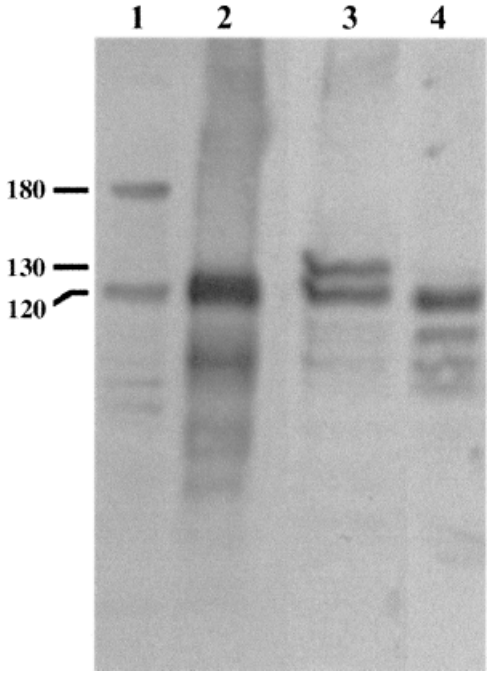


**Figure 7**
**The shedding of the ECD of BP180$^{\Delta 18–407}$ is unaffected in transfected COS-7 cells.** COS-7 cells were transfected with cDNA encoding wild-type BP180 (*lanes 1 and 2*) and BP180$^{\Delta 18–407}$ (*lanes 3 and 4*). Cell extracts (*lanes 1 and 3*) and cell culture media (*lanes 2 and 4*) were immunoblotted with a rabbit anti-serum directed against the NC16A domain of BP180. The shed ectodomain of 120 kDa could be detected in media (*lanes 2 and 4*) and cell extracts (*lanes 1 and 3*) obtained from transfected COS-7 cells expressing either wild-type BP180 or BP180$^{\Delta 18–407}$. Furthermore, proteins of 180 kDa (*lane 1*) and 130 kDa (*lane 3*) corresponding to unprocessed full-length BP180 and BP180$^{\Delta 18–407}$, respectively, were detected in extracts from transfected cells.

ECD (Schäcke *et al*, 1998). This 120 kDa fragment was also identified in the culture medium of COS-7 cells. When extracts of COS-7 cells transfected with cDNA encoding BP180$^{\Delta 18–407}$ were assayed, reactivity with a 130 kDa polypeptide corresponding to unprocessed BP180$^{\Delta 18–407}$ and a 120 kDa polypeptide were identified. The latter was also detected in the culture medium. Hence, the deletion from the cytoplasmic domain of BP180 has no deleterious effect on the shedding of its ECD.

## Discussion

We investigated the impact of the large deletion from the cytoplasmic domain of BP180$^{\Delta 18–407}$ on the formation and organization of hemidesmosome. Our results demonstrate that: (1) in the presence of BP180$^{\Delta 18–407}$ the assembly of hemidesmosomes and their linkage with the IF cytoskeleton are severely impaired; (2) BP180$^{\Delta 18–407}$ is recruited into hemidesmosome-like structures when ectopically expressed in both normal and BP180-deficient keratinocytes, although it has lost the ability to bind to the hemidesmosomal components BP230, plectin, and the β4 subunit of the α6β4 integrin; and finally (3) the shedding of the ECD of BP180$^{\Delta 18–407}$ is unaffected by the deletion.

The ultrastructural studies provide evidence that primary cultured BP180$^{\Delta 18–407}$ keratinocytes exhibit few and rudimentary hemidesmosomes, which appear to be poorly connected with keratin IF bundles. These observations indicate, in analogy to what is observed *in vivo* in the skin of the EB patient (Huber *et al*, 2002), that BP180 is necessary *in vitro* for the formation of hemidesmosomes and for their linkage with the keratin IF network. This idea is further substantiated by our immunofluorescence microscopy studies, which demonstrated that the molecular organization of hemidesmosome-like structures in BP180$^{\Delta 18–407}$ keratinocytes is disturbed *in vitro*. These observations are in line with results of previous studies utilizing immortalized keratinocyte cell lines, which completely lacked BP180 expression (Borradori *et al*, 1998; Koster *et al*, 2003).

Immunofluorescence microscopy analyses of cultured BP180$^{\Delta 18–407}$ keratinocytes (this study) and of the patient's skin (Huber *et al*, 2002) disclosed that the distribution pattern of BP180$^{\Delta 18–407}$ was not significantly impaired. Therefore, the behavior of BP180$^{\Delta 18–407}$ was further evaluated in transfection studies. The findings confirm that BP180$^{\Delta 18–407}$ has the potential to become recruited into hemidesmosome-like structures upon ectopic expression. These results are at apparent variance with previous reports indicating that the cytoplasmic domain of BP180 is required for its localization in hemidesmosomes (Borradori *et al*, 1997, 1998). A plausible explanation for these discrepancies is that, in contrast to these latter studies, in our case BP180$^{\Delta 18–407}$ has an intact ECD, which may bind to an as yet undefined extracellular ligand deposited on the coverslip, or, alternatively, to the ECD of the α6 subunit of the α6β4 integrin, as previously reported (Hopkinson *et al*, 1998). Finally, BP180$^{\Delta 18–407}$ potentially form heterotrimers with endogenous wild-type BP180. Nevertheless, our results, utilizing BP180-deficient keratinocytes, demonstrate that BP180$^{\Delta 18–407}$ can be recruited into hemidesmosome-like structures even in the absence of endogenous BP180. These and previous observations (Borradori *et al*, 1997, 1998) indicate that the localization of BP180 to the basal side of keratinocytes occurs by two independent mechanisms involving interactions with either cytoplasmic components of hemidesmosomes or, alternatively, with extracellular matrix proteins and/or the ECD of the α6 subunit.

The results in yeast demonstrate that BP180$^{\Delta 18–407}$ has lost the capacity to associate with the N-terminal portions of both plectin and BP230 as well as with the β4 cytoplasmic domain of the α6β4 integrin, a transmembrane protein playing a key role in the formation of hemidesmosomes and their regulation (reviewed in Borradori and Sonnenberg, 1999). These findings confirm a recent study showing that the cytoplasmic tail of BP180 is crucial for its association with BP230 and plectin via their Y domain (Koster *et al*, 2003). Specifically, the stretch of residues 145 to 230 in the cytoplasmic domain of BP180 was critical for binding to BP230. It should be noted that, in contrast to BP230, plectin was found, although to a reduced extent, in hemidesmosome-like structures of cultured BP180$^{\Delta 18–407}$ keratinocytes. Whereas the presence of both BP180 and β4 is critical for the localization of BP230 into hemidesmosomes (this study; Schaapveld *et al*, 1998; Koster *et al*, 2003), plectin can be recruited into hemidesmosomes even in the

absence of BP180, by means of a direct association with the β4 subunit (Niessen *et al*, 1997; Schaapveld *et al*, 1998; Koster *et al*, 2003). Finally, although we found that the cytoplasmic domain of BP180 interacts with monomeric K5 and K14 and the K5/K14 heterodimers in yeast, these results may not be biologically relevant, as: (1) in transfection studies, BP180 mutant proteins encompassing the intracellular domain do not become colocalized with the keratin network (Borradori *et al*, 1997, 1998; Koster *et al*, 2003); (2), in recent yeast assays the binding ability of certain BP230 and desmoplakin mutant proteins with monomeric keratins, such as K5, K14, and K18, could not be confirmed by biochemical and cell biologic approaches, and appeared to be an artifact related to the truncation procedures (Fontao *et al*, 2003). Collectively, these data demonstrate that the deletion in the cytoplasmic domain of $BP180^{\Delta 18–407}$ has two major consequences explaining the phenotype of the patient: (1) it disturbs the molecular composition and stability of hemidesmosomes, resulting in defective dermo-epidermal cohesion, and (2) it affects the tethering of the keratin IF network to the basal cell membrane, making the basal keratinocytes fragile with cell rupturing and development of an EB simplex phenotype. In this regard, gene-targeted elimination of BP230 and plectin in mice has demonstrated the critical role of these proteins for the linkage of the keratin IF network to hemidesmosomes (Guo *et al*, 1995; Andrä *et al*, 1997; Steinbock and Wiche, 1999). Furthermore, in EB simplex patients carrying pathogenic mutations in the plectin-gene, the connection of the IF network with hemidesmosomes is also affected (Smith *et al*, 1996).

Finally, we assessed whether the truncation in $BP180^{\Delta 18–407}$ alters its proteolytic processing and the shedding of its ECD. Shedding has been shown to depend on a disintegrin and metalloprotease domain (ADAM) proteases and is thought to affect keratinocyte adhesion, differentiation, and migration (Franzke *et al*, 2002). Here we show that the 120 kDa ECD of $BP180^{\Delta 18–407}$, similarly to what was observed in keratinocytes (Huber *et al*, 2002), is properly shedded in transfected COS-7 cells. Based on these observations, it is likely that in our patient dermo-epidermal fragility and blistering result from disturbed assembly of hemidesmosomes rather than from the altered incorporation of BP180 and its shed ECD into the cutaneous basement membrane.

In conclusion, our findings demonstrate that the truncation of the cytoplasmic domain in $BP180^{\Delta 18–407}$ has a profound impact on the assembly of hemidesmosomes, impairing both the mechanical stability of basal keratinocytes and dermo-epidermal cohesion. This study indicates that without the cytoplasmic domain of BP180, BP230, plectin, and α6β4 are ineffective at connecting the IF network to hemidesmosomes and, thus, to extracellular matrix proteins.

## Materials and Methods

**EB patient** The $BP180^{\Delta 18–407}$ keratinocytes were derived from a previously described EB patient as reported (Huber *et al*, 2002). Mutational analysis disclosed one mutation (R1226X) leading to a premature termination codon accompanied by a non-sense mediated mRNA decay, whereas the other resulted in skipping of exons 3 to 15 in COL17A1 gene and in synthesis of a truncated 130 kDa BP180 protein.

**cDNA constructs** cDNA constructs encoding $BP180^{1–520}$ and $BP180^{\Delta 18–407}$ were generated using primers that added appropriate stop codons along with restriction sites allowing subcloning in a polymerase chain reaction with proof-reading *Pfu* polymerase (Promega, Madison, WI). cDNA fragments covering different parts of plectin were obtained by reverse transcription–polymerase chain reaction using a human keratinocyte cDNA library, as template, with a superscript one-step reverse transcription–polymerase chain reaction system with platinum *Taq* according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Correctness of all constructs was verified by DNA sequence analysis. Plasmids containing cDNA encoding full-length FLAG-tagged BP180 and $BP180^{1–400}$, $BP230^{1–887}$, and $\beta4^{1115–1667}$ have been previously described (Borradori *et al*, 1997; Koster *et al*, 2003) (Fig 1).

**Cell culture and transfection** The immortalized PA-JEB/β4 (Sterk *et al*, 2000) and the BP180-deficient (Koster *et al*, 2003) keratinocyte cell lines, primary normal human keratinocytes (NHK) and $BP180^{\Delta 18–407}$ keratinocytes were cultured in Keratinocyte-SFM medium (Invitrogen). NHK and $BP180^{\Delta 18–407}$ keratinocytes were isolated as described (Limat and Noser, 1986; Huber *et al*, 2002). Keratinocytes differentiation was induced by incubation for 48 h in HAMF12/Dulbecco minimal Eagle's medium (1:3) (Invitrogen) (Schaapveld *et al*, 1998). 804G and COS-7 cells were grown in Dulbecco minimal Eagle's medium (Invitrogen) supplemented with 10% (v/v) bovine fetal bovine serum, 100 U per mL penicillin, and 100 U per mL streptomycin.

For immunofluorescence staining, cells were plated on glass coverslip coated with laminin-5 enriched medium produced by 804G cells (Hormia *et al*, 1995). Cells were grown up to 50 to 60% confluency, then made to differentiate (see above) for 48 h before processing. For transfection, cells were plated at 40 to 60% confluency on glass coverslips in six-well tissue culture plates. Keratinocytes were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's procedure. COS-7 cells were transfected using the DEAE-dextran method. Two days after, transfected cells were processed for confocal microscopy (Favre *et al*, 2001) and viewed under a Zeiss LSM410 confocal inverted laser scanning microscope.

**Antibodies** The following immuno-reagents were used: rabbit anti-α6 integrin subunit anti-serum (Delwel *et al*, 1993); mouse monoclonal antibody (MoAb) anti-Flag M2 (Sigma); mouse MoAb 3E1 anti-β4 integrin subunit (Hessle *et al*, 1984); mouse MoAb 1A8c and 1D1, that recognize the cytoplasmic domain and the ECD of BP180, respectively (Nishizawa *et al*, 1993), rabbit antiserum directed against the NC16A domain of BP180 (Schumann *et al*, 2000); mouse MoAb 121 anti-plectin (Hieda *et al*, 1992); a guinea pig anti-plectin antibody (Progen, Heidelburg, Germany), the human MoAb 5E anti-BP230 (Hashimoto *et al*, 1993), Alexa-488 conjugated-goat anti-rabbit IgG (Molecular Probes), Alexa-568 conjugated-goat anti-mouse and anti-guinea pig IgG (Molecular Probes, Leiden, The Netherlands); Alexa-568 conjugated-goat anti-human IgG (Molecular Probes); the mouse MoAb and rabbit anti-serum anti-Gal4-AD or Gal4-BD (Santa Cruz, Santa Cruz, CA), peroxidase-coupled anti-rabbit IgG or anti-mouse IgG (Bio-Rad, Hercules, CA).

**Electron microscopy** $BP180^{\Delta 18–407}$ keratinocytes and NHK were plated on themanox coverslips coated with conditioned medium from 804G cells enriched in laminin-5 to ensure optimal culture conditions (Hormia *et al*, 1995) and grown to confluency in Keratinocyte-SFM medium. Cell monolayers were made to differentiate for 72 h, then fixed for 2 h in 2% glutaraldehyde (v/v) in phosphate-buffered saline at reverse transcription and processed for electron microscopy. Ultrathin sections were examined with CM10 Philips electron microscope at 80 kV.

**Ectodomain shedding of BP180** For the shedding experiments, COS-7 cells were transiently transfected with a cDNA construct encoding wild type or $BP180^{\Delta 18–407}$ as described earlier (Borodori *et al*, 1997). Medium from the COS-7 cells was collected 48 h after transfection, cooled, centrifuged to remove cellular debris, and supplemented with 1 mM Pefablock (Merck, Glattburg, Switzerland) and 1 mM *N*-ethylamide (Sigma, St. Louis MO). The proteins from 1 mL of medium or 100 μL of cell extract were concentrated by precipitation with 70% ethanol and separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 6–22% polyacrylamide gradient gel.

**Western blot analysis** Protein extracts were prepared in sodium dodecyl sulfate sample buffer, heated for 5 min at 95°C, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western blotting as described (Favre *et al*, 2001).

**Yeast two- and three-hybrid analyses** The *Saccharomyces cerevisiae* strain PJ69-4A, which contains the genetic markers trp1-901, leu2-3112 ura3-52, his3-200, gal4Δ, gal80 Δ, LYS2::-GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ (James *et al*, 1996) was used for all assays. It contains three tightly regulated reporter genes, HIS3, ADE2, and Lac-Z each driven by a different promoter under the control of GAL4 transcriptional factor. Cells were cotransformed with two defined constructs in pAS2-1, pACT2 (Clontech, Palo Alto, CA), according to Clontech's procedure. Double transformants were selected on agar synthetic complete (SC) medium lacking leucine and tryptophan (SC-LW). For each transformation, eight colonies were arrayed in 96 well microtiter plates and then transferred on to agar SC-LW (positive control), SC-LW without adenine, and SC-LW without histidine and supplemented with 2 mM 3-amino 1,2,3-triazole. After a 5 d incubation at 30°C, three independent colonies were tested for protein–protein interactions using the semi quantitative β-galactosidase assay as described (James *et al*, 1996). Yeast three-hybrid assays were carried out as reported in detail elsewhere (Fontao *et al*, 2003). *Trans*-activation controls were performed for each construct with the opposite vector without insert.

To ascertain that the transformed yeast expressed the various recombinant proteins, cell extracts were prepared by the trichloroacetic acid method (Clontech Protocol Handbook) and subjected to immunoblotting with antibodies directed to the GAL4-AD or GAL4-BD. Protein bands of the expected size were observed (not shown).

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## References

Aho S, Uitto J: Direct interaction between the intracellular domains of bullous pemphigoid antigen 2 (BP180) and beta 4 integrin, hemidesmosomal components of basal keratinocytes. Biochem Biophys Res Commun 243:694–699, 1998

Aho S, Uitto J: 180-kD bullous pemphigoid antigen/type XVII collagen. Tissue-specific expression and molecular interactions with keratin 18. J Cell Biochem 72:356–367, 1999

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

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

Borradori L, Koch PJ, Niessen CM, Erkeland S, van Leusden MR, Sonnenberg A: The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. J Cell Biol 136:1333–1347, 1997

Borradori L, Chavanas S, Schaapveld RQ, Gagnoux-Palacios L, Calafat J, Meneguzzi G, Sonnenberg A: Role of the bullous pemphigoid antigen 180 (BP180) in the assembly of hemidesmosomes and cell adhesion—re-expression of BP180 in generalized atrophic benign epidermolysis bullosa keratinocytes. Exp Cell Res 239:463–476, 1998

Bruckner-Tuderman L: Hereditary skin diseases of anchoring fibrils. J Dermatol Sci 20:122–133, 1999

Chavanas S, Gache Y, Tadini G, Pulkkinen L, Uitto J, Ortonne JP, Meneguzzi G: A homozygous in-frame deletion in the collagenous domain of bullous pemphigoid antigen BP180 (type XVII collagen) causes generalized atrophic benign epidermolysis bullosa. J Invest Dermatol 109:74–78, 1997

Darling TN, Bauer JW, Hintner H, Yancey KB: Generalized atrophic benign epidermolysis bullosa. Adv Dermatol 13:87–119, 1997

Delwel GO, Hogervorst F, Kuikman I, Paulsson M, Timpl R, Sonnenberg A: Expression and function of the cytoplasmic variants of the integrin alpha 6 subunit in transfected K562 cells. Activation-dependent adhesion and interaction with isoforms of laminin. J Biol Chem 268:25865–25875, 1993

Diaz LA, Ratrie H 3rd, Saunders WS, Futamura S, Squiquera HL, Anhalt GJ, Giudice GJ: Isolation of a human epidermal cDNA corresponding to the 180-kD autoantigen recognized by bullous pemphigoid and herpes gestationis sera. Immunolocalization of this protein to the hemidesmosomes. J Clin Invest 86:1088–1094, 1990

Eady RA: Epidermolysis bullosa: Scientific advances and therapeutic challenges. J Dermatol 28:638–640, 2001

Favre B, Fontao L, Koster J, *et al*: The hemidesmosomal protein bullous pemphigoid antigen 1 and the integrin beta 4 subunit bind to ERBIN. Molecular cloning of multiple alternative splice variants of ERBIN and analysis of their tissue expression. J Biol Chem 276:32427–32436, 2001

Fine JD, Eady RAJ, Bauer EA, *et al*: Revised classification system for inherited epidermolysis bullosa: Report of the Second International Consensus Meeting on Diagnosis and Classification of Epidermolysis Bullosa. J Am Acad Dermatol 42:1051–1066, 2000

Fontao L, Favre B, Riou S, *et al*: Interaction of the bullous pemphigoid antigen 1 (BP230) and desmoplakin with intermediate filaments is mediated by distinct sequences within their COOH terminus. Mol Biol Cell 14:1978–1992, 2003

Franzke CW, Tasanen K, Schäcke H, *et al*: Transmembrane collagen XVII, an epithelial adhesion protein, is shed from the cell surface by ADAMs. EMBO J 21:5026–5035, 2002

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

Guo L, Degenstein L, Dowling J, Yu QC, Wollmann R, Perman B, Fuchs E: Gene targeting of BPAG1: Abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. Cell 81:233–243, 1995

Hashimoto T, Amagai M, Ebihara T, *et al*: Further analyses of epitopes for human monoclonal anti-basement membrane zone antibodies produced by stable human hybridoma cell lines constructed with Epstein-Barr virus transformants. J Invest Dermatol 100:310–315, 1993

Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E: Basement membrane diversity detected by monoclonal antibodies. Differentiation 26:49–54, 1984

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

Hopkinson SB, Jones JC: The N terminus of the transmembrane protein BP180 interacts with the N-terminal domain of BP230, thereby mediating keratin cytoskeleton anchorage to the cell surface at the site of the hemidesmosomes. Mol Biol Cell 11:277–286, 2000

Hopkinson SB, Riddelle KS, Jones JC: Cytoplasmic domain of the 180-kD bullous pemphigoid antigen, a hemidesmosomal component: Molecular and cell biologic characterization. J Invest Dermatol 99:264–270, 1992

Hopkinson SB, Findlay K, deHart GW, Jones JC: Interaction of BP180 (type XVII collagen) and alpha6 integrin is necessary for stabilization of hemidesmosomes structure. J Invest Dermatol 111:1015–1022, 1998

Hormia M, Falk-Marzillier J, Plopper G, Tamura RN, Jones JC, Quaranta V: Rapid spreading and mature hemidesmosomes formation in HaCaT keratinocytes induced by incubation with soluble laminin-5r. J Invest Dermatol 105:557–561, 1995

Huber M, Floeth M, Borradori L, *et al*: Deletion of the cytoplasmatic domain of BP180/collagen XVII causes a phenotype with predominant features of epidermolysis bullosa simplex. J Invest Dermatol 118:185–192, 2002

Irvine AD, McLean WHI: Human keratin diseases. The increasing spectrum of disease and. subtlety of the phenotype ± genotype correlation. Br J Dermatol 140:815–828, 1999

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

Koster J, Geerts D, Favre B, Borradori L, Sonnenberg A: Analysis of the interactions between BP180, BP230, plectin and the integrin alpha6beta4 important for hemidesmosomes assembly. J Cell Sci 116:387–399, 2003

Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA: Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J Immunol 136:1231–1235, 1986

Limat A, Noser FK: Serial cultivation of single keratinocytes from the outer root sheath of human scalp hair follicles. J Invest Dermatol 87:485–488, 1986

McGrath JA, Gatalica B, Christiano AM, *et al*: Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa. Nat Genet 11:83–86, 1995

Niessen CM, Hulsman EH, Rots ES, Sanchez-Aparicio P, Sonnenberg A: Integrin alpha 6 beta 4 forms a complex with the cytoskeletal protein HD1 and induces its redistribution in transfected COS-7 cells. Mol Biol Cell 8:555–566, 1997

Nishizawa Y, Uematsu J, Owaribe K: HD4, a 180 kDa bullous pemphigoid antigen, is a major transmembrane glycoprotein of the hemidesmosomes. J Biochem (Tokyo) 113:493–501, 1993

Nykvist P, Tasanen K, Viitasalo T, *et al*: The cell adhesion domain of type XVII collagen promotes integrin-mediated cell spreading by a novel mechanism. J Biol Chem 276:38673–33869, 2001

Pulkkinen L, Uitto J: Hemidesmosomal variants of epidermolysis bullosa. Mutations in the $\alpha 6\beta 4$ integrin, and the 180-kD bullous pemphigoid antigen/type XVII collagen genes. Exp Dermatol 7:46–64, 1998

Schaapveld RQ, Borradori L, Geerts D, *et al*: Hemidesmosome formation is initiated by the beta4 integrin subunit, requires complex formation of beta4 and HD1/plectin, and involves a direct interaction between beta4 and the bullous pemphigoid antigen 180. J Cell Biol 142:271–284, 1998

Schäcke H, Schumann H, Hammami-Hauasli N, Raghunath M, Bruckner-Tuderman L: Two forms of collagen XVII in keratinocytes. A full-length transmembrane protein and a soluble ectodomain. J Biol Chem 273:25937–25943, 1998

Schumann H, Baetge J, Tasanen K, Wojnarowska F, Schäcke H, Zillikens D, Bruckner-Tuderman L: The shed ectodomain of collagen XVII/BP180 is targeted by autoantibodies in different blistering skin diseases. Am J Pathol 156:685–695, 2000

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

Steinbock FA, Wiche G: Plectin: A cytolinker by design. Biol Chem 380:151–158, 1999

Sterk LM, Geuijen CA, Oomen LC, Calafat J, Janssen H, Sonnenberg A: The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. J Cell Biol 149:969–982, 2000

Uitto J, Pulkkinen L: Molecular genetics of heritable blistering disorders. Arch Dermatol 137:1458–1961, 2001