Plakin interactions in the hemidesmosome

Koster, J.J.B.

Citation for published version (APA):
Chapter 3

Analysis of the interactions between BP180, BP230, plectin and the integrin α6β4 important for hemidesmosome assembly

Chapter 3

Analysis of the interactions between BP180, BP230, plectin and the integrin α6β4 important for hemidesmosome assembly

Jan Koster, Dirk Geerts, Bertrand Favre, Luca Borradori and Arnoud Sonnenberg

Hemidesmosomes (HDs) are multi-protein complexes that promote stable adhesion of epithelial cells to the underlying extracellular matrix. We assessed the interactions between different hemidesmosomal components with each other, mapped the binding sites and studied the importance of these interactions for HD assembly in yeast two-hybrid and cell-transfection assays. The results show that: (1) bullous pemphigoid antigen (BP) 180 binds not only to BP230, but also to plectin. The interactions between these proteins are facilitated by the Y subdomain in the N-terminal plakin domain of BP230 and plectin, and residues 145-230 of the cytoplasmic domain of BP180; (2) different, but overlapping, sequences on BP180 mediate binding to β4, which, in turn associates with BP180 via its third fibronectin type III repeat; (3) sequences in the N-terminal extremity of BP230 mediate its binding to β4, which requires the C-terminal end of the connecting segment up to the fourth FNIII repeat of the β4 subunit. (4) Finally, cell-transfection studies showed that the localization of BP230 into hemidesmosome-like structures depends on its Z-Y subdomains as well as on the availability of BP180. By having further uncovered interactions between various hemidesmosomal components, mapped the involved binding sites and dissected a hierarchy of interactions relevant for their topogenic fate, our findings give novel insights into the molecular organization of hemidesmosomes.

Introduction

Hemidesmosomes (HDs) are multi-protein complexes that promote epithelial-stromal cohesion in stratified and complex epithelia, and connect the intermediate filament system of basal epithelial cells to proteins of the extracellular matrix. These complexes, which ultrastructurally appear as tripartite structures along the plasma membrane of basal cells, are composed of at least five different proteins: the laminin-5 receptor α6β4, the bullous pemphigoid antigens 180 (BP180, BPAG2 or type XVII collagen) and 230 (BP230 or BPAG1-e), CD151 and plectin (Jones et al., 1998; Borradori and Sonnenberg, 1999; Sterk et al., 2000). In certain tissues, such as intestinal epithelia, and cultured epithelial cells, a second type of HD has been identified, which is composed of α6β4 and plectin (Uematsu et al., 1994; Orian-Rousseau et al., 1996). These type II HDs, in contrast to the classical or type I HDs, do not exhibit the typical tripartite structure. BP230 and plectin are cytoplasmic proteins that belong to the plakin protein family, which also includes desmoplakin, envoplakin and periplakin. These proteins are crucially involved in the organization of the cytoskeleton (Ruhrberg and Watt, 1997; Leung et al., 2001). They are composed of domains that have considerable sequence homology. Their N-terminus consists of a plakin domain containing a number of subdomains of high α-helical content, designated NN, Z, Y, X, W and V, whereas the central coiled-coil rod domain is composed of heptad repeats thought to be involved in the dimerization of the plakin (Green et al., 1992). Finally, their C-terminal end exhibits one or more homologous repeat sequences designated A, B or C. In plectin as well as in neuronal and muscular isoforms of BP230 (BPAG1-a and BPAG1-b, respectively), a calponin-type actin-binding domain (ABD) precedes the plakin domain (Brown et al., 1995; McLean et al., 1996; Leung et al., 2001). The C-terminal end of plakins has intermediate filament binding properties (Meng et al., 1997; Wiche et al., 1993; Yang et al., 1996; Leung et al., 1999), whereas the N-terminal end harbors specific sequences that target the proteins to distinct membrane sites, such as HDs or desmosomes, cell-cell adhesion complexes in a variety of epithelia (Kowalczyk et al., 1997; Rezniczek et
al., 1998; Geerts et al., 1999; Hopkinson and Jones, 2000).

The $\alpha 6\beta 4$ integrin plays a central role in the assembly of HDs. Loss of $\alpha 6\beta 4$ due to mutations in the genes for either the $\alpha 6$ or $\beta 4$ subunit causes a distinct form of pyloric atresia associated with junctional epidermolysis bullosa (PA-JEB), and is characterized by fragility and extensive blistering of the skin. In affected patients HDs are rudimentary or completely absent (Vidal et al., 1995; Niessen et al., 1996; Ruzzi et al., 1997). A similar phenotype is observed in $\alpha 6$ or $\beta 4$ null mutant mice (van der Neut et al., 1996; Georges-Labouesse et al., 1996; Dowling et al., 1996). The large cytoplasmic domain of the integrin $\beta 4$ subunit is essential for the formation of HDs (Nievers et al., 1998; Murgia et al., 1998). It is over 1000 amino acids long and harbors two pairs of fibronectin type III (FNIII) repeats, separated by a connecting segment (CS) (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). The second FNIII repeat and the first 35 amino acid residues of the CS of the $\beta 4$ integrin are required for the recruitment of plectin into HDs (Nievers et al., 1997a; Nievers et al., 2000; Niessen et al., 1997b). The CS and the third FNIII repeat have been implicated in the binding to BP180 (Borradori et al., 1997; Aho and Uitto, 1998; Schaapveld et al., 1998). Furthermore, the cytoplasmic domain of $\beta 4$ has been reported to interact with BP230 (Hopkinson and Jones, 2000).

BP180 is a type II transmembrane protein with a 466-amino acid cytoplasmic domain and a large collagenous extracellular domain (Giudice et al., 1992). There is evidence that, like $\alpha 6\beta 4$, BP180 binds laminin-5 (Reddy et al., 1998). In cultured epithelial cell lines, the localization of BP180 in HDs is dependent on its interaction with the cytoplasmic domain of $\beta 4$ (Borradori et al., 1997; Schaapveld et al., 1998). Furthermore, the extracellular domain of BP180 is also able to interact with the $\alpha 6$ integrin subunit (Hopkinson et al., 1995; Hopkinson et al., 1998). Finally, BP180 is involved in the recruitment of BP230 into HDs (Borradori et al., 1998; Hopkinson and Jones, 2000).

The aim of our study was: (1) to further assess the potential of the $\alpha 6\beta 4$, BP180, BP230 and plectin to associate with each other; (2) to map the involved binding sites and, finally, (3) to define the importance of these interactions for the recruitment of these proteins into HD in combined yeast two-hybrid assays and cell-transfection studies. Our findings show that interactions between these hemidesmosomal components are more complex than previously anticipated, uncover the potential of BP180 to interact with plectin and reveal that the recruitment of these proteins into HDs is regulated by a hierarchy of interactions that each appear to have a different impact on HD assembly.

**Materials and Methods**

**Cell lines and antisera**

Immortalized $\beta 4$-deficient PA-JEB keratinocyte cell line has been described previously (Schaapveld et al., 1998). BP180-deficient keratinocytes, immortalized by transfection with human papilloma virus-18 E6 and E7 genes were kindly provided by P. Marinkovich (University of California San Francisco, San Francisco, CA). The PAJEB and GABEB (generalized atrophic benign epidermolysis bullosa) keratinocytes were grown in keratinocyte serum-free medium (SFM) (Gibco-BRL) supplemented with bovine pituitary extract, 5 ng/ml epidermal growth factor, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were grown at 37°C in a humidified, 5% CO2 atmosphere. Mouse monoclonal antibody (mAb) 233 against BP180 (Nishizawa et al., 1993) and mouse mAb 121 against plectin/HD1 (Hieda et al., 1992) were kind gifts from K. Owaribe (University of Nagoya, Nagoya, Japan). Rat mAb 439-98 recognizes an extracellular epitope on the integrin $\beta 4$ subunit and mouse mAb 450-11A directed against the cytoplasmic domain of $\beta 4$ were purchased from Pharmingen (San Diego, CA). The rabbit polyclonal antiserum against BP230 (Tanaka et al., 1990) was a kind gift from J. R. Stanley University of Pennsylvania, Philadelphia, PA). The human mAbs 5E and 10D against BP230 were generously provided by T. Hashimoto (Keio University, Tokyo, Japan) (Hashimoto et al., 1993). Rabbit polyclonal antibody against laminin-5 was kindly provided by P. Rouselle (Lyon, France). The rabbit polyclonal antibodies against the extracellular domain of $\beta 4$ (sc-9090) and against hemagglutinin (HA) epitope tag (sc-805) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were purchased from Rockland (Gilbertsville, PA) (FITC-conjugated goat anti-mouse immunoglobulin (Ig) G) and Molecular Probes (Eugene, OR) (Alexa-488 conjugated goat anti-human IgG and Texas-Red-conjugated goat anti-rat and anti-rabbit IgG).
**Fig. 1.** Immunofluorescence analysis of hemidesmosomal components in PA-JEB/\(\beta 4\) cells. PA-JEB/\(\beta 4\) cells were fixed and immunolabeled for laminin-5 (A) or the hemidesmosomal markers \(\beta 4\) (A,B,D,F), plectin (B,G,I), BP180 (C,E,F) or BP230 (C,H,I). Merged images (A,B,C,F,I) show laminin-5, plectin and BP180 in green, and \(\beta 4\) and BP230 in red; colocalized staining appears in yellow. Note that \(\beta 4\) and plectin (B), as well as BP230 and BP180 (C), are nearly completely co-localized, whereas \(\beta 4\) and BP180 (F) or plectin and BP230 (I) show only partial co-localization. Bar, 10 μm.

**DNA transfections**
PA-JEB or GABEB cells were grown to 40% confluence in 12-well tissue-culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ). Transient transfections were performed with 0.8 μg cDNA using Lipofectin, according to the manufacturer's instructions (Gibco-BRL). Transfection mixtures were replaced by SFM medium after 6 hours and incubated in this medium for 12 hours. Subsequently, the SFM medium was replaced by Nutrient Mixture Ham's F12/Dulbecco's MEM (1:3) for an additional 24 hours after which the cells were assayed for gene expression.

**Immunofluorescence microscopy**
PA-JEB and GABEB cells grown on glass coverslips were fixed with 1% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at room temperature. After rinsing in PBS and blocking with 2% BSA in PBS for 30 minutes at room temperature, the cells were incubated with primary antibodies for 60 minutes at room temperature and then washed three times with PBS. Cells were subsequently incubated with Alexa-488, FITC- and Texas Red-conjugated...
secondary antibodies directed against mouse, rat, rabbit or human IgG for 45 minutes at room temperature. Coverslips were washed three times, mounted in Mowiol/DABCO and viewed under a Leica confocal scanning laser microscope.

Yeast two-hybrid interaction assay

The yeast strain Saccharomyces cerevisiae PJ69-4A (a kind gift from P. James, University of Wisconsin, Madison, WI), which contains the following genetic markers: trp1-901, leu2-3, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 and GAL2-ADE2 (James et al., 1996) was used as host for the two-hybrid assays. This strain contains two tightly regulated selectable Gal4-driven reporter genes, his and ade, allowing sensitive detection of protein-protein interactions between Gal4 fusion proteins. The Gal4 activation domain (AD)- and Gal4 binding domain (BD)-fusion plasmids were co-transformed into PJ69-4A, as described previously (Schaapveld et al., 1998), and equal aliquots of transformed cells were spread out on plates containing yeast synthetic complete medium lacking leu and trp (vector markers) or leu, trp, his and ade (vector and interaction markers). Plates were incubated at 30°C and growth of colonies was scored after 6 and 10 days.

Fig. 2. Immunofluorescence analysis of hemidesmosomal components in GABEB cells. GABEB cells were fixed and immunolabeled for plectin (A), BP180 (B), BP230 (C) and β4 (A-C). In D-I, GABEB cells were transfected with cDNA encoding wild-type BP180 tagged at the N-terminus with a FLAGepitope (yellow square in diagrams at the bottom of D and H; diagrams represent cDNA encoding wild-type BP180) stained for BP180 (D,F,H,I), β4 (E,F) or BP230 (G,I). Merged images (A-C,F) show plectin, BP180 and BP230 in green and β4 in red. In the merged image I, BP180 is in green and BP230 is in red. Note that on reconstitution of BP180, BP230 is recruited into HD-like structures, whereas it is not recruited when BP180 expression is absent. Bars, 10 µm.
The plating efficiencies on -leu,-trp,-his,-ade (SC-LTHA) plates, as compared with the plating efficiency on -leu,-trp (SC-LT), was used as a measure for the strength of the two-hybrid protein interaction. Autonomous activation of the reporter genes was suppressed by the addition of 2 mM 3-amino-1,2,4-triazole (a His antagonist) (A8506; Sigma Chemical Co.). Expression of the Gal4-fusion proteins was analyzed by immunoblotting with antibodies against the Gal-activation or -DNA binding domain (sc-1663 and sc-510, respectively; Santa Cruz). Constructs for the yeast two-hybrid studies were generated using standard cloning techniques. All nucleotide and amino acid positions are numbered with the ATG initiation codon at position one. The cDNA sequences used for alignment and designation of primers are available from GenBank under accession number m77830 (desmoplakin); mn_000494 (BP180); m69225 (BP230); u53204 (plectin) and x53587 (β4). Plasmid inserts were generated by restriction digestion or PCR using the proofreading Pwo DNA polymerase (Boehringer Mannheim) and gene-specific sense and antisense primers containing restriction site tags. Numbers in superscript correspond to the amino acid residues of subclones encoded within the Gal4 (AD) or (BD) fusion proteins. Vectors were used the yeast Gal4 AD or BD expression vectors pACT2 or pAS2.1 (Clontech).

**β-Galactosidase assay**

For the quantitative analysis of β-galactosidase activity, five yeast colonies were combined and grown to an OD660 of approximately 1.0 in selective medium lacking Leu and Trp. β-Galactosidase activity was determined at 37°C using the Pierce yeast β-galactosidase assay kit (75768) with O-nitrophenyl β-D-galactopyranoside as substrate. The A405 was measured in an ELISA-reader and the time at which the reaction reached a value of 0.2 was taken to calculate the β- galactosidase activity using the equation:

![Fig. 3. Yeast two-hybrid analysis of interactions between BP180 and BP230 and BP180 and β4. (Top) Schematic representation of BP180. DSR, direct sequence repeat; TM, transmembrane region. (Bottom) Cotransformation of yeast host strain PJ69-4A with BP230β1-555 or β4115-1666 cDNA constructs fused to the Gal4 (AD) domain (in pACT2) and cDNA constructs encoding various fragments of BP180 fused to Gal4 (BD) domain (in pAS2.1) as indicated. Transformation mixtures were spread on SC-LT and SC-LTHA plates and grown at 30°C. Plating efficiency on selective SC-LTHA plates is expressed as a percentage of the plating efficiency on nonselective SC-LT plates from the same transformation. Plates were scored after 6 and 10 days. All efficiencies listed represent an average of multiple independent transformations. ++, plating efficiency on SC-LTHA is ≥80% of the plating on SC-LT, colonies are fully developed on day 5; +, 40-80% of the plating on SC-LT, small and large colonies on day 5; +, ≥50% of the plating on SC-LT at 10 days of growth; –, no colonies on selective plates after 10 days of growth. Note that the interaction between BP180 and BP230 requires a fragment of BP180 containing amino acids 145-230, whereas for the interaction of BP180 with β4115-1666 other sequences are required. Identical results were obtained when, instead of BP230β1-555, BP230β1-1156 was used for assessment of the interactions with the different BP180 mutants.
Fig. 4. Effects of expression of BP180 mutants on the localization of BP230 in GABEB keratinocytes. GABEB keratinocytes were transfected with cDNA encoding BP180<sup>145-230</sup> (A,B) or BP180<sup>1-36</sup> (C,D) and immunolabeled for BP180 (A-D), β4 (A,C) or BP230 (B,D). In the merged images (A,C), BP180 is in green and β4 in red, whereas in B and D, BP180 is in red and BP230 is in green. Staining for co-localization is in yellow. Diagrams at the bottom of panels A-D represent the various BP180 mutants. Note that deletion of amino acids 145-230 from BP180 does not abrogate the ability of BP180 to be recruited into HDs, whereas the recruitment of BP230 is impaired. Deletion of the N-terminal 36 residues of BP180 has no effect on the recruitment of either BP180 or BP230 into HDs. Bar, 10 μm.

Fig. 5. Interaction between BP230 and BP180 involves the conserved Y subdomain of plakins and mapping of the interaction site on BP230 for β4. The PJ69-4A yeast strain was co-transformed with a pAS2.1- or pPACT2-derived vector encoding BP180<sup>1-401</sup> or β4<sup>1115-1666</sup> and the corresponding complementary vectors encoding various fragments of BP230, desmoplakin or plectin as indicated. Two-hybrid interactions were analysed by growth on selective SCLTHA plates. (A) Top, schematic representation of BP230. The subdomains NN, Z, Y, X, W and V are those described by Green et al. (Green et al., 1992). These subdomains as well as the homologous repeat sequences designated B and C are indicated by boxes, shaded from white to gray. The box representing the rod domain has been shaded more darkly. Bottom, the interaction of BP180<sup>1-451</sup> with the various fragments of BP230 could be shown independently of whether the cDNA constructs were cloned into the pAS2.1 or the pACT2 vector. Because of an autonomous transactivation of pAS2.1-β4<sup>1115-1666</sup>, the BP230 binding activity of β4<sup>1115-1666</sup> could only be determined with the combination of pACT2-β4<sup>1115-1666</sup> and pAS2.1-BP230. (B) Interactions of BP180<sup>1-401</sup> with the Z-Y and Y domains of BP230, plectin and desmoplakin (DP) were only revealed when the cDNAs encoding the various domains of BP230, plectin and desmoplakin were inserted into pACT2 and the cDNA for BP180<sup>1-401</sup> was inserted into pAS2.1. (C) The β-galactosidase activity of the yeast transformants expressing BP180<sup>1-441</sup> and the indicated BP230, plectin and desmoplakin constructs was quantified in a liquid culture assay using O-nitrophenyl β-D-galactopyranoside as substrate. The negative interaction controls are pAS2.1-BP180<sup>1-401</sup>/pACT2 (2.9±0.3 β-galactosidase units) and pAS2.1/pACT2 (2.4±0.2 β-galactosidase units) and the positive controls (not shown) are p53/pSV-40 large T (77.3±10.1 β-galactosidase units) and the complete Gal4 transcription factor in pC11 (266.8±16.3 β-galactosidase units). (D) Interactions between BP230 mutants and β4<sup>1115-1666</sup> were assayed with the BP230 mutants fused to the Gal4 (BD) in pAS2.1 and β4<sup>1115-1666</sup> fused to the Gal4 (AD) in pACT2. For further details, see Fig. 3. In panel D, the amino acids GGG, GSG and G correspond to the linker sequences placed in between the Gal4 (AD)- and the BP230-specific sequences. In B and D note that the interaction between BP230 and the cytodomain of BP180 only requires the Y domain of BP230, whereas for the interaction with β4 the 56-most N-terminal residues of BP230 are involved. Panel E shows an alignment of the Z-Y regions in the different plakin proteins.
1,000-A405/(cell volume (ml) x time of reaction (min) x OD660). Samples that did not reach this value within 4 hours were left overnight and measured the next morning. The final values are the results from three independent determinations.

Results

Different requirements for the recruitment of BP180 and BP230 into HD-like structures

To specify the molecular interactions important for the formation of HDs, we first analyzed, by immunofluorescence microscopy, the distribution of various hemidesmosomal components in keratinocyte cell lines lacking either β4 or BP180. In β4-deficient PA-JEB cells, the transient expression of the β4 subunit was shown to restore the cells’ ability to form HD-like structures (Schaapveld et al., 1998). In extension to these studies, we found that, in many cells of a PA-JEB keratinocyte cell line that stably express β4 (PA-JEB/β4 cells), the subcellular localization of laminin-5 and plectin was largely identical to that of α6β4 (Fig. 1A,B). Furthermore, the distribution of BP180 and BP230 was the same, i.e. in structures appearing as dots and patches, which are typical for HD-like structures or stable anchoring contacts (Fig. 1C). By contrast, co-localization of either β4 with BP180 or of plectin with BP230 was mostly only partial (Fig. 1D-I). These results indicate that certain, but not all, HD-like structures contain, in addition to α6β4 and plectin, BP180 and BP230 as shown by immunofluorescence microscopy studies, i.e. they represent both type I and type II HDs (Uematsu et al., 1994).

When BP180-deficient keratinocytes obtained from a patient with GABEB were analyzed by immunofluorescence microscopy, the distribution of β4 appeared to be normal; it was concentrated in patches at sites of cell-substrate contacts (Fig. 2A-C). However, although in nearly all PA-JEB/β4 cells the staining of β4 and plectin largely overlapped, many of the BP180-deficient GABEB cells there were several patches containing β4 but not plectin (compare Fig. 2A with Fig. 1B). Furthermore, consistent with previous observations Borradori et al., 1998), BP230 was not present in these HD-like structures (Fig. 2C). Nevertheless, on transient transfection with cDNA for BP180, we found that BP230 was recruited into HD-like structures together with BP180 (Fig. 2D-I). Together, these findings obtained in PA-JEB and GABEB keratinocytes indicate that the recruitment of BP230 into HD-like structures is regulated distinct – either direct or indirect – interactions, involving mainly β4 and BP180. Furthermore, for the localization of plectin HD-like structures to be efficient, it seems that BP180 is also required.

The cytoplasmic domain of BP180 contains two distinct binding sites for β4

Previous studies have shown that the recruitment of BP180 into HDs depends on a direct association of BP180 with β4 (Borradori et al., 1997). To identify the β4 binding region on the cytoplasmic domain of BP180, several deletion mutants were generated and expressed in yeast cells together with a β4 construct containing all four FNIII repeats of the β4 cytoplasmic domain, 145-1145-1666 (Fig. 3). Interactions were detected by growth of yeast cells on plates lacking histidine and adenine (see Materials and Methods). Although constructs BP1801-230 and BP180145-401 bound to 145-1145-1666, there was no interaction with either BP1801-147 or BP180145-230. Notably, the latter contains the stretch of amino acids 145-230 shared by the above two BP180 constructs, which had binding activity. Because proper expression of the BP180145-230 mutant was ascertained by immunoblotting of yeast cell lysates, the lack of binding could not be due to defective protein expression (data not shown). Furthermore, deletion of the stretch of amino acids 145-230 from the cytoplasmic domain of BP180, BP180141-401,145-230 had no impact on its interaction with 145-1145-1666. Together, these results strongly suggest that there are at least two distinct binding sites for β4 on BP180: one located in the first 230-amino-acid stretch of BP180 and a second in a more C-terminally located region encompassing amino acids 231-401.

A stretch of 85 amino acids in the cytoplasmic domain of BP180 is crucial for its binding to BP230

BP180 has recently been shown to associate with BP230 via a region of 280 amino acids (residues 180-460) spanning half the cytoplasmic domain (Hopkinson and Jones, 2000). To define the region on BP180 that binds to BP230, we carried out additional yeast twohybrid assays. As shown
Fig. 6. Expression of BP230 mutants in PA-JEB/β4 keratinocytes. PA-JEB/β4 cells were transfected with cDNAs encoding BP230-887 (A,B), BP230\(^{1-2161}\) (C,E) or BP230\(^{1-2161, AZY}\) (D,F), tagged at the N-terminus with an HA epitope (red circle). Cells were fixed and immunolabeled for BP180 (A-D), the HA-tagged BP230 mutants (A-F) and total BP230 (endogenous and transfected BP230) (E,F). In the merged images (A-D), BP180 is in green and the HA-tagged mutants are in red, whereas in (E,F), the HA-tagged BP230 mutant is in green and total BP230 is in red. Co-localized staining appeared in yellow. Diagrams at the bottom of panels A-F represent the various BP230 mutants. Note that the cell depicted in B represents a rare event, whereas the majority of the cells resemble those depicted in A. Also note that removal of the Z-Y domains results in the loss of BP230 recruitment into HD-like structures. Bars, 10 μm.
in Fig. 3, the BP180 mutants that contain the region of 85 amino acids 145-230 bound to BP230\(^{1-155}\) and BP230\(^{1-1156}\), whereas those lacking it did not. Furthermore, the BP180\(^{145-230}\) mutant that only contains this stretch of amino acids was also able to bind, albeit less efficiently. Thus, although additional sequences might further strengthen their interaction, this 85-amino-acid stretch is necessary and sufficient for the binding of BP180 to BP230. Finally, two mutants carrying a deletion of the first 37 N-terminal amino acids (BP180\(^{38-422}\) and BP180\(^{38-422,\Delta 229-324}\)), a region previously identified as being crucial for recruiting BP230 into HDs in transfection studies (Hopkinson et al., 1995; Borradori et al., 1998), were both able to interact with BP230. These results suggest that BP180 interacts with BP230 through a region of 85 amino acids (BP180\(^{145-230}\)) and that the first 37 N-terminal amino acids are dispensable for binding in yeast.

**Deletion of a stretch of 85 amino acids from the cytoplasmic domain of BP180**

**Integrin \(\beta 4\)**

*Fig. 7.* Yeast two-hybrid analysis of interactions between \(\beta 4\) and BP230 and \(\beta 4\) and BP180. (Top) Schematic representation of the cytoplasmic domain of \(\beta 4\). FNII repeats are represented by boxes in which the number of the repeats is shown. TM, transmembrane region. (Bottom) Cotransformation of yeast host strain PJ69-4A with \(\beta 4\) mutants in the pACT2 (AD) vector and BP230\(^{1-155}\) or BP180\(^{1-401}\) in the pAS2.1 (BD) vector. For further details see Fig. 3. Note that BP230 requires the third and fourth FNIII repeats, as well as the last 20 amino acids of the CS of \(\beta 4\), to allow an interaction, whereas BP180 only requires the third FNIII repeat.

**Reduces the recruitment of BP230 in HDs in transfected cells**

Next, we analyzed in transfection studies the importance of the stretch of 85 amino acids of BP180, which contains a binding site for BP230, for the recruitment of the latter into HDs. When introduced in BP180-deficient keratinocytes, BP180\(^{145-230}\) is correctly recruited into HDs and, like wild-type BP180, is colocalized with \(\beta 4\) (Fig. 4A). The same was observed in cells expressing BP180\(^{\Delta 1-36}\) (Fig. 4C). However, at variance with cells expressing either wild-type BP180 (Fig. 2I) or the BP180\(^{\Delta 1-36}\) mutant (Fig. 4D), in cells expressing BP180\(^{145-230}\), BP230 was found only rarely in HD-like structures together with the BP180 mutant (Fig. 4B). Thus, consistent with the results in yeast two-hybrid interaction assays, in which a region encompassing amino acids 145-230 of BP180 is required and sufficient for its interaction with BP230, the deletion of this sequence from BP180 dramatically reduced recruitment of BP230 into HDs. Deletion of the 36 most N-terminal amino acid residues of BP180 reduces the recruitment of BP230 in HDs in transfected cells.
had no effect on the recruitment of either BP230 or β4 into HD-like structures.

**BP230 and plectin interact with BP180 via their Y domain**

We next investigated which region of BP230 is involved in binding to BP180. For this purpose, a series of mutant forms of BP230 were generated and tested together with BP180 in yeast two-hybrid assays. The results show that BP230 interacted with BP180 by the Z-Y subdomains (Fig. 5A,B) and, although the Y domain alone was able to bind to BP180, albeit slightly less efficiently (Fig. 5B), no binding of the Z domain alone could be detected. In turn, BP180 and BP230, which interacted weakly with BP230 (Fig. 3), did not bind to the isolated Z-Y or Y subdomains of BP230 (data not shown), possibly because interaction of this short stretch of BP180 with BP230 requires additional sequences flanking the Z-Y subdomains of BP230. Indeed, when two larger fragments of BP230 containing the Z-Y subdomains (BP230 and BP230) were tested for binding to BP180 using a β-galactosidase assay, they did bind more strongly than the isolated Z-Y or Y subdomains (Fig. 5C).

The observations that in BP180-deficient keratinocytes the localization of plectin in HD-like structures appeared to be somewhat impaired and that the Y domain of plectin is homologous to that of BP230 (Fig. 5E) prompted us to investigate whether plectin can directly bind to BP180. We therefore generated two constructs encoding the Z-Y or Y domains of plectin and tested their binding ability in yeast. As shown in Fig. 5B, both constructs interacted with BP180, although less strongly than the corresponding constructs of BP230 as confirmed by a quantitative β-galactosidase assay (Fig. 5C). Hence, BP180 is capable of binding to the Y domain of not only BP230 but also of plectin. Surprisingly, we found that the Z-Y and Y domains of desmoplakin, which is a component of desmosomes, also interacted with BP180. As in the case of BP230, the isolated Z-Y and Y subdomains of plectin or desmoplakin did not interact with BP180 (data not shown).

**The Z-Y domains of BP230 contain sequences important for its recruitment into HDs in transfected cells**

To assess whether an interaction of BP230 with BP180 is required for the localization of BP230 in HDs, we generated various HA-tagged BP230 mutants in which the Z-Y domains had been deleted. Because human keratinocytes lacking BP230 expression have not yet been identified, we expressed these mutants in PA-JEB/β4 cells, whereas immunofluorescence microscopy analyses were carried out with polyclonal antibodies against the HA-epitope tag to distinguish the localization of the ectopically expressed BP230 mutant proteins from that of endogenous BP230. Although, in most cells, a BP230 construct containing the first 1-887 N-terminal amino acids (BP230) was found diffusely distributed over the cytoplasm (Fig. 6A), in very few of the transfected cells it was concentrated in HD-like structures, together with BP180 (Fig. 6B). By contrast, a mutant form of BP230, BP230 (1-2161), which encompasses the rod domain, was found not only in HD-like structures in a larger proportion of the cells, but also in aggregates in the cytoplasm, particularly in transfected cells that strongly expressed the cDNA (Fig. 6C). Other than BP230 (1-2161), BP230 (1-2161,ΔY) from which the Y domains had been deleted was not colocalized with BP180 in HD-like structures, but was found in small aggregates (Fig. 6D).

Because both BP230 (1-2161) and BP230 (1-2161,ΔY) contain the central rod domain of BP230 and thus have the potential to form dimers with endogenous BP230, transfected cells were also stained with an anti-BP230 antiserum raised against a C-terminal fragment of BP230 (amino acids 1722-2203). Because a large portion of this fragment is part of the two BP230 mutants, the anti-BP230 antiserum will stain not only endogenous BP230, but also the two BP230 mutants. Consistent with the localization of transfected BP230 (1-2161) in HDs, the staining pattern with the anti-BP230 and anti-HA antibodies overlapped in transfected cells and was comparable to that seen with anti-BP230 in untransfected cells, i.e. in HDs (Fig. 6E). By contrast, BP230 (1-2161,ΔY) was again found in small aggregates and it was not obviously co-localized with endogenous BP230 in HDs (Fig. 6F). Thus, even if dimerization of BP230 (1-2161,ΔY) mutants with endogenous BP230 occurs, this did not result in the recruitment of the mutated molecule into HDs.
Collectively, these results suggest that: (1) the Z-Y domain contains sequences important for the localization of BP230 into HDs, most likely via binding to BP180, and (2) the presence of the rod domain of BP230 thought to be implicated in the formation of dimers (Ruhberg and Watt, 1997; Leung et al., 2001) increases the correct targeting of the protein into HDs.

**BP230 interacts with the third and fourth FNIII repeat of β4**

In BP180-deficient keratinocytes, the recruitment of BP230 into HD-like structures is crucially dependent on the re-expression of BP180 (Borradori et al., 1998). However, in PA-JEB keratinocytes, i.e. in the absence of α6β4, no HDs are formed, despite the fact that these cells express BP180 and BP230. We therefore wondered whether the β4 subunit might directly bind to BP230 and thus also affects its localization. We tested the binding activity of several β4 constructs, in which one or more FNIII repeats were deleted or in which the CS was...
progressively shortened, with a BP230 construct containing the first 1156 amino acids of the N-terminal in yeast two-hybrid assays. As depicted in Fig. 7, removal of part of the fourth FNIII repeat resulted in loss of interaction. The deletion of the first pair of FNIII repeats from the cytoplasmic domain of β4 had no effect on binding, but the removal of also the CS abolished the interaction. Specifically, an interaction between BP230 and β4 still occurred with progressive deletions up to amino acid 1436, but not beyond. These data show that the extreme C-terminal portion of the CS in combination with the third and the fourth FNIII repeats of β4 are required for its binding to BP230.

Finally, as a control, the various β4 constructs were also tested against the cytoplasmic domain of BP180, BP180(1-401). In extension to previous studies (Geert et al., 1999), we found that construct β4(1457-1552) encompassing the third FNIII contains the minimal sequences necessary for the interaction with BP180.

The β4 subunit interacts with N-terminal sequences specific for the epidermal isoform of BP230

We next investigated which sequences within BP230 are involved in its binding to β4 in yeast. Various BP230 constructs were tested with a β4 construct containing the first FNIII repeat up to the fourth repeat, β4(1115-1666) (Fig. 5A). The results indicate that the first stretch of 92 amino acids at the N-terminus of BP230 is sufficient for its interaction with β4.

To ascertain that a binding site for β4 was not accidentally introduced by fusing the Gal4 domain to the N-terminal extremity of BP230, we generated additional mutants as depicted in Fig. 5D. All these constructs were able to interact with β4, providing evidence that the interaction was indeed dependent on BP230-specific sequences and not on the Gal4 moiety. Furthermore, the results show that the first three amino acids of BP230 are dispensable for this interaction.

Except for the first 56 amino acids, the N-terminal region of BP230 is almost identical to that of two isoforms, dystolin-1 and -2, encoded by the BPAG1 gene that also encodes BP230. These two isoforms, previously thought to be specifically expressed in the nervous system (Yang et al., 1996), differ from the BP230 isoform by the presence of an ABD at their extreme N-terminus that is absent from BP230. To determine whether or not the interaction between BP230 and β4 is mediated by the stretch of amino acids 56-92 common to BP230 and the two dystolin isoforms, we generated constructs comprising amino acids 1-92, 1-56 or 56-190 of BP230. When these constructs were assayed for an interaction with β4(1115-1666), it was found that amino acids 1-56 of BP230 interacted, although less efficiently than amino acids 1-92, with β4, whereas amino acids 56-190 did not (Fig. 5D). These data indicate that the interaction of BP230 with β4 is mediated by sequences specific for the epidermal isoform, although obviously the common part also influences binding.

BP230 cannot replace plectin in supporting the formation of HDs

The finding that BP230 can interact with both β4 and BP180 prompted us to investigate whether BP230 can replace plectin in supporting the formation of HDs. For this, we made use of a mutant β4 subunit (β4R1281W) that is unable to interact with plectin, but can bind to BP180 and BP230 (Geert et al., 1999; Koster et al., 2001). Stable expression of β4R1281W in PA-JEB cells by using retroviral transduction revealed that this mutant can support the formation of HD-like structures containing BP180 and BP230, consistent with previous observations using transient transfection protocols (Geert et al., 1999). However, these HD-like structures appear to be less conspicuous and dense than those formed in the presence of wild-type β4 (compare Fig. 1 and Fig. 8). Furthermore, in the vast majority of cells, the β4 mutant was not co-localized with BP180 or BP230. From these results, we conclude that: (1) when plectin is not bound to β4, β4 binding to BP180 is strongly reduced, and (2) BP230 cannot replace plectin in supporting proper localization of BP180 into HDs.

Discussion

In this study we have further characterized the interactions between different hemidesmosomal components involved in the assembly of HDs. In extension to recent studies, we show that the β4 cytoplasmic domain interacts with both BP180 and BP230 (Schaapveld et al., 1998;
Fig. 9. Model for the assembly of HDs in cultured keratinocytes. The association of the α6β4 integrin with plectin via the β4 subunit and their clustering is crucial for the formation of type II HDs. When BP180 is present, it will be incorporated into this complex through interactions with both β4 and plectin. This is followed by the recruitment of additional plectin and BP180 molecules, resulting in a further increase of the size and the stability of the ternary complexes. In a final step, BP230 becomes incorporated into these complexes, and the HD-like structures containing α6β4, BP180 and plectin are turned into type I HDs. Because both plectin and BP230 interact with the same site on BP180, it is likely that they may compete for this binding site.

Hopkinson and Jones, 2000) via sequences located in its C-terminal half, encompassing the third FNIII repeat (for BP180) and the C-terminal sequences of the CS up to the fourth FNIII repeat (for BP230). These sites are different from those implicated in the binding of plectin and its localization into HDs, which are located in the first pair of FNIII repeats and the N-terminal portion of the CS (Geerts et al., 1999; Koster et al., 2001). Furthermore, we show that the binding sites on BP180 for plectin and BP230 are different from those involved in the binding to β4, and encompass the same stretch of 85 amino acids in the N-terminal half of the cytoplasmic domain of BP180.

The different interactions between the various hemidesmosomal components are depicted in Fig. 9, along with a model that shows how these interactions are likely to contribute to the formation of stable type I HDs.

**Interaction of BP180 with plectin contributes to their localization in HDs**

The localization of BP180 in HDs containing α6β4 and plectin has previously been shown to depend on an interaction of BP180 with the cytoplasmic domain of β4 (Borradori et al., 1997; Schaapveld et al., 1998). Interactions between the α6 subunit and the extracellular NC16a domain of BP180 are probably also implicated (Hopkinson et al., 1998). Here, we identified a third interaction partner of BP180, i.e. plectin.

The interaction between BP180 and plectin is not sufficiently strong to induce the formation of HDs in the absence α6β4, because in β4-deficient PA-JEB cells, which contain BP180 and plectin, no HDs are formed. However, it may strengthen the interaction between α6β4, BP180 and plectin in a three-molecular complex, thereby ensuring their proper incorporation in HDs. Consistent with this idea, we found less plectin in the β4-positive adhesion structures of the BP180-deficient GAEB keratinocytes than in those of PAEB/β4 keratinocytes, at least as assessed by immunofluorescence. Moreover, studies with PA-JEB keratinocytes stably expressing a mutant β4 subunit (β4<sup>R1281W</sup>) that is unable to interact with plectin (Geerts et al., 1999; Koster et al., 2001) revealed that the incorporation of BP180 into cell-substrate
structures is severely compromised. Only few cells formed HD-like adhesion structures containing α6β4, BP180 and BP230. Thus, although α6β4 can bind to BP180, proper incorporation of this protein into HDs only occurs when plectin is also available.

The results with the PA-JEB/β4R1281W cells also suggest that the role of plectin in supporting efficient localization of BP180 in HDs cannot be replaced by BP230. This is surprising considering the fact that BP230 can interact with both β4 and BP180. Both BP230 and plectin probably bind via sequences contained in their Y subdomain to the same region (amino acids 145-230) on BP180 (see below). Thus, it is possible that, when plectin is not bound to α6β4, it competes with BP230 for binding to BP180. However, given the fact that the binding activity of BP180 for BP230 is greater than that for plectin, this is not very likely. An alternative possibility, which we favor, is that binding of plectin to β4 is required for activating the β4 cytoplasmic domain, by unfolding it and rendering it accessible for interaction with BP230 (see also Fig. 9). Evidence for such an intramolecular folding of the β4 cytoplasmic domain has been presented in both biochemical and yeast two-hybrid assays (Reznicek et al., 1998; Geerts et al., 1999). Regardless of what the mechanism might be, it is clear that plectin and BP230 are not exchangeable in their ability to support the formation of proper HDs.

In view of our finding that binding to BP180 is mediated by the Y domain of plectin, it is interesting to note that Pulkkinen et al. (Pulkkinen et al., 1996) have described a deletion of three amino acids, QEA, in this region in a patient with epidermolysis bullosa simplex associated with muscular dystrophy (EBS-MD). A pathological consequence of this deletion, therefore, could be that plectin is unable to bind to BP180, and that the ternary complex of α6β4, BP180 and plectin, which serves as a platform for the incorporation of BP230 into HDs (see below), cannot be (properly) formed, leading to deficient assembly of HDs. Future experiments, however, should reveal whether indeed the deletion of these three amino acids in the Y domain prevents the binding of plectin to BP180.

In previous studies (Hopkinson et al., 1995; Borradori et al., 1997), it was found that the 36 most N-terminal amino acids of the cytoplasmic domain of BP180 were implicated in the recruitment of BP180 into HDs. However, in this study, no evidence was found for the involvement of this region in the localization of BP180 in HDs. An important difference between our study and the study by Borradori et al. (Borradori et al., 1997) is that the latter used a chimeric protein, which consisted of the membrane targeting sequence of K-ras fused to the cytoplasmic region of BP180, whereas in the present study full-length BP180 has been used. Full-length BP180 forms trimers (Hirako et al., 1996) and, therefore, it is predicted that it interacts more strongly with β4 than the chimeric monomeric molecule lacking the collagenous extracellular domain. In that case, binding of full-length BP180 to β4 may have become less dependent on additional sites of interaction that reside on the cytoplasmic domain of BP180. However, it remains unexplained why we have not been able to detect an interaction between β4 and a BP180 mutant containing the first 36 amino acids (BP1801-147) in yeast two-hybrid interaction assays. Perhaps the fusion of the N-terminus of BP180 to the Gal4 (BD) has destroyed the β4-binding site in the N-terminus of BP180. Although the importance of the first 36 amino acids of BP180 for the binding to β4 could not be confirmed in the yeast two-hybrid system, the finding that both BP1801-131 and BP180145-401, but not the amino acids 145-230 that are shared by these two constructs, interact with β4 suggests that there are two β4 binding sites on BP180. One of them is located in the first 230 N-terminal amino acids of the BP180 cytoplasmic domain and the other is located C-terminally to this region. Although the β4-binding site in the first 230 N-terminal amino interactions of BP180 may overlap with that for BP230 (see below), the two sites are clearly not identical. Binding of β4 still occurs when the region of amino acids 145-230, which is essential and sufficient for the interaction with BP230, has been deleted from the cytoplasmic domain of BP180 (BP1801-401,145-230). In line with the results obtained in yeast, a full-length BP180 molecule carrying this deletion became co-aligned with β4 in HD-like structures.

Expression of BP180 is required for proper localization of BP230 in HDs

Chapter 3
The observation that in BP180-deficient keratinocytes, BP230 was not co-localized with α6β4 and plectin in HD-like structures but diffusely distributed over the cytoplasm indicates that β4 and plectin are not sufficient for the proper localization of BP230 into HDs. Efficient recruitment of BP230 only occurs when BP180 is also expressed in these cells (Schaapveld et al., 1998). However, it is likely that the additional binding site for BP230 that is provided on β4 also contributes to the recruitment of BP230 in HDs. Thus, a model emerges in which α6β4 interacts with plectin, after which they bind BP180 in a ternary complex. Ultimately, BP230 binds to two components in the complex, i.e. α6β4 and BP180, and stable type I HDs are then formed (Fig. 9).

Our results show that sequences in the C-terminal domain of β4 (the second pair of FNIII repeats) interact with residues 1-56 of BP230, which are contained within the N-terminal domain of BP230 (residues 1-980) that has been shown to be involved in the interaction with β4 (Hopkinson and Jones, 2000). In the same study, a second β4 binding site on BP230 has been identified in its C-terminal domain, residues 1812-2649. By contrast, we found that a fragment of BP230 encompassing residues 2077-2649 did not associate with β4, but bound to intermediate filaments in yeast and was able to decorate intermediate filaments in transfected cells (J. Koster, unpublished observations). It is possible that the binding site for β4 is located in the region of amino acids 1812-2077 of BP230 and that this region contributes to the recruitment of BP230 into HDs. In support of this, in transfection experiments with PA-JEB/β4 cells, BP2301-2649 was localized in only very few of the HDs, whereas a BP230 construct, which also includes the rod domain and the region 1812-2077 (BP2301-2161), was detected in a higher percentage of the HDs. The rod domain, which is an integral part of all members of the plakin family and is responsible for the dimerization of these molecules, may also contribute to the localization of BP230 in HDs by its ability to dimerize the binding sites in the N- and C-terminal domains.

**BP230 interacts with BP180 via its Y domain**

In a recent study (Hopkinson and Jones, 2000) it was shown that the first 170 amino acids, as well as amino acids 555-700 of an N-terminal 700 amino acid fragment of BP230, are required for its binding to BP1801517. Our finding that BP2301-1555 interacted with BP1801401 shows that the amino acids 555-700 of BP230 are not essential. Furthermore, no binding was detected when a fragment containing the first 172 amino acids of BP230 was used, whereas this fragment did bind to β4. Binding of BP180 to BP230 appeared to be mediated by the Y subdomain of BP230.

The importance of the Y subdomain for binding is further supported by the fact that in transient transfection experiments using PA-JEB/β4 cells, BP2301-2161, but not the construct from which the Z-Y domain had been deleted (BP23012161,3ZY) was recruited into HDs. However, the latter construct had a tendency, when expressed in transfected cells, to form aggregates in the cytoplasm, which might prevent its recruitment into HDs. The fact that Hopkinson and Jones (Hopkinson and Jones, 2000) found that sequences additional to the Z-Y subdomains of BP230 are required for interaction with BP1801517, in contrast to our results with BP1801461, may be explained by their use of a less-sensitive assay system. This is supported by the finding that BP1801-401 binds more strongly to BP2301555 and BP2301887 than to the isolated Z-Y and Y subdomains of BP230 and, furthermore, that BP1801401, but not BP180145-230, can bind to the Z-Y and Y subdomains of BP230. Thus, it seems that additional sequences stabilize the interaction of the Y subdomain with BP230. We found, unexpectedly, that the Y subdomain of desmoplakin was also able to interact with BP180. In fact, desmoplakin is not found in HDs (Green and Jones, 1996), but in desmosomes, in which BP180 is not present. It is possible that desmoplakin does not recruit BP180 into desmosomes (despite its ability to directly interact with BP180) because α6β4 is absent from these adhesion complexes. This assumption is indirectly supported by the observation that α6β4 is necessary for the efficient recruitment of BP230 and plectin into HDs, despite the ability of these two molecules to bind to BP180 (Borradori et al., 1997; Schaapveld et al., 1998).
Alternatively, the interaction between plakins and cell-surface receptors might be subject to regulation. Clearly, these results further stress the important role of α6β4 in the assembly of HDs.

Comparison of HD formation in vivo and in cultured keratinocytes in vitro

The crucial role of plectin in the formation of HDs as observed in cultured keratinocytes does not seem to be supported by findings in vivo. Indeed, it has been shown that HDs can be formed in plectin-deficient mice, although the HDs appear to be rudimentary and their number is reduced (Andrä et al., 1997). We think that this discrepancy is partly due to a different role of BP180 in vitro and in vivo. As we have shown in vitro, the clustering and polarization of BP180 at the cell basis is entirely dependent on the presence of α6β4 and plectin, probably because the specific ligand for BP180 is not produced by keratinocytes in culture. No polarized expression of BP180 was observed in β4-deficient PA-JEB keratinocytes (Schaapveld et al., 1998). By contrast, in vivo, where the ligand for BP180 is almost certainly present and deposited into the basement membrane (it may be produced by the mesenchymal cells), clustering of BP180 is probably less dependent on plectin and α6β4, and thus can also occur in the absence of plectin.

BP230 may then subsequently interact with the BP180-α6β4 complexes, resulting in the formation of rudimentary HDs. In support of this, we have found that when α6β4 is absent, as in patients with PA-JEB, some HDs can be observed, the formation of which may have been initiated by interaction of BP180 with an unidentified ligand in the epidermal basement membrane (Nissen et al., 1996).

In conclusion, we have characterized interactions between several molecules that are crucially involved in the assembly of HDs. We show that the different hemidesmosomal components can interact with multiple other components and that often interactions with more than one component are required for their efficient recruitment into HDs. Our findings that the cytoplasmic domain of β4 bears a scaffold of FNIII repeats responsible for interactions with the three major hemidesmosomal components plectin, BP180 and BP230, underscores its essential role in the formation of HDs.

Acknowledgements

We thank our colleagues for their generous gifts of antibodies and P. Marinkovich for providing the GABEB keratinocyte cell line. We are grateful to C.P.E. Engelfriet for comments on the manuscript. This work was supported by a grant from the Dystrophic Epidermolysis Bullosa Research Association (DEBRA Foundation, Crawthorne, UK) to A.S. Part of this work was supported by the Swiss National Foundation of Scientific Research (32-51083.97 and 32-56727.99) to L.B.

References


