Journal of Cellular Biochemistry 72:356–367 (1999)

180-kD Bullous Pemphigoid Antigen/Type XVII Collagen: Tissue-Specific Expression and Molecular Interactions With Keratin 18

Sirpa Aho* and Jouni Uitto

Departments of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania 19107
Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract The 180-kD bullous pemphigoid antigen (BPAG2) is a hemidesmosomal transmembrane protein, also known as type XVII collagen. In this study, potential interactions of BPAG2 with other proteins expressed in epidermal keratinocytes were explored by yeast two-hybrid system using the amino-terminal intracellular domain of BPAG2 as a bait. Several independent interacting clones encoding keratin 18 (K18) were identified when the keratinocyte cDNA library, cloned into the yeast two-hybrid activation domain vector, was screened. The peptide sequence responsible for the interaction of BPAG2 was restricted to amino acids 15–25, and substitution of a valine residue in the middle of this sequence by a proline (V23P) by site-directed mutagenesis abolished the interaction. Further examination of the K18 sequences by restricted cDNA constructs in yeast two-hybrid system identified a carboxyl-terminal segment corresponding to helix 2B domain as critical for BPAG2 binding. The interaction of BPAG2/K18 was confirmed by an in vitro protein-protein interaction assay, which also confirmed that normal human keratinocytes express K18 in culture. The tissue specific expression of BPAG2 was first examined using a multi-tissue RNA blot. Human multiple tissue cDNA panels representing a variety of adult and fetal tissues as well as tumor cells were used as PCR-templates to study the expression patterns of both BPAG2 and K18. The results demonstrated significant level of expression of BPAG2, besides in epidermal keratinocytes, also in a variety of tissues with predominant epithelial component, such as mammary, salivary and thyroid glands, colon, prostate, testis, placenta, and adult and fetal thymus, as well as in colon, pancreatic and prostatic adenocarcinoma cell lines, and an ovarian carcinoma. As expected, K18 transcript is present in liver, pancreas, colon, placenta, and in fetal kidney. Collectively, the results suggest that BPAG2 has a relatively broad tissue distribution including specialized and simple epithelia, and that within the tissues such as colon and placenta, BPAG2 may have direct interactions with K18, a keratin characteristically expressed in a simple epithelia. J. Cell. Biochem. 72:356–367, 1999.

Key words: BP180; cytokeratin 18; tissue specific expression; two-hybrid; protein-protein interactions

Stable association of epithelial layers to the underlying mesenchyme is mediated by a number of attachment complexes forming an adhesion zone at the epithelial-mesenchymal interface. The prototype of such adhesion zones is the dermal-epidermal interface which is shown by ultrastructural means to consist of a network of interconnecting attachment structures [Uitto and Pulkkinen, 1996; Burgeson and Christiano, 1997]. Among them, the dermal-epidermal junction contains hemidesmosomes which extend from the intracellular milieu of basal keratinocytes to the underlying extracellular matrix [Borradori and Sonnenberg, 1996]. The intracellular portion of hemidesmosomes attaches to the intermediate filament network which consists in basal keratinocytes of keratins 5 and 14 [Fuchs, 1995]. The extracellular portion of hemidesmosomes interacts with thread-like structures, known as anchoring filaments, which traverse the lamina lucida of the dermal-epidermal basement membrane zone and extend to the upper part of the lamina densa [Tidman and Eady, 1985]. At the lower portion of the dermal-epidermal basement mem-

Contract grant sponsor: United States Public Health Service, National Institutes of Health; Contract grant number: P01-AR38923.

*Correspondence to: Sirpa Aho, Ph.D., Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 S. 10th Street, Suite 450 BLSB, Philadelphia, PA 19107. E-mail: sirpaaho@hotmail.com

Received 27 July 1998; Accepted 21 August 1998

brane zone, morphologically recognizable structures, known as anchoring fibrils, extend from the lamina densa to the underlying dermis [Burgeson, 1993]. Thus, the dermal-epidermal basement membrane zone is a continuum of attachment molecules which are critical for stable association of epidermis to the underlying dermis.

As indicated above, the prototype of attachment complexes necessary for stable association of the epidermis to the underlying dermis is the hemidesmosome, which consists of both intracellular and extracellular macromolecular components. There are two forms of hemidesmosomes: Type I consists of a full complement of hemidesmosomal proteins with characteristic ultrastructural appearance, while type II represent a morphologically less well-developed entity and is devoid of some protein components [Jones et al., 1994; Uematsu et al., 1994]. At least four well-characterized proteins are now known to be integral components of type I hemidesmosomes, and these proteins contain subunit polypeptides representing five different gene products [Green and Jones, 1996]. A critical component of hemidesmosomes is the 180-kD bullous pemphigoid antigen, a collagenous protein also known as type XVII collagen [Li et al., 1993]. The essential role of this molecule in providing stability to the dermal-epidermal basement membrane zone is attested by the fact that mutations in the corresponding gene, BPAG2/COL17A1, are associated with a heritable blistering disease, known as generalized atrophic benign epidermolysis bullosa [Pulkkinen and Uitto, 1998].

The 180-kDa bullous pemphigoid antigen was originally identified as an autoantigen in a blistering skin disease, bullous pemphigoid [Diaz et al., 1990]. Subsequent cloning of BPAG2 cDNA and genomic sequences revealed that it is a collagenous protein, and specifically, the carboxyl-terminal domain of BPAG2/type XVII collagen consists of 15 separate collagenous segments with characteristic Gly-X-Y primary sequence [Giudice et al., 1992]. Further cell and molecular biological analyses indicated that BPAG2 has a transmembrane domain and that the amino-terminal segment is intracellular [Li et al., 1993; Hopkinson et al., 1992; Masunaga et al., 1997]. Thus, BPAG2 is a collagenous protein in type II topography.

BPAG2 has been shown to be expressed in stratified squamous epithelia, as exemplified by human skin [Nishizawa et al., 1993]. In addition, the epithelial basement membranes of cornea [Gordon et al., 1997], ocular conjunctiva, buccal mucosa, upper esophagus, placenta, umbilical cord, and transitional epithelium of the bladder have been shown to express BPAG2, when examined by immunofluorescence staining with antibodies recognizing BPAG2 epitopes [Fairley et al., 1995]. However, the expression of the corresponding gene at the mRNA level has not been examined in detail.

In this study, we have explored potential interactions of the intracellular domain of BPAG2 by screening a keratinocyte cDNA library in yeast two-hybrid system, which revealed putative interactions with K18. Furthermore, we have examined the tissue-specific expression of human BPAG2 and K18 by multi-tissue RNA blot and multi-tissue cDNA panel analyses of adult and fetal human tissues as well as of tumor cells.

MATERIALS AND METHODS

Yeast Strains and Vectors

The Matchmaker Two-Hybrid System (Clontech Laboratories, Inc., Palo Alto, CA) was used in this study. The two-hybrid vectors pGBT9 and pGAD424 were originally a generous gift from Dr. S. Fields (SUNY, Albany, NY). As a bait vector, pGB-*MEL1* containing the *MEL1* gene encoding α-galactosidase enzyme as an independent reporter was used [Aho et al., 1997]. Human keratinocyte Matchmaker cDNA library was purchased from Clontech, amplified according to the manufacturer's instructions, and the two-hybrid screening was done following the manual of Matchmaker Two-Hybrid System (Clontech). As a host yeast strain, competent *Saccharomyces cerevisiae* YRG-2 cells (Stratagene, La Jolla, CA) were used. Yeast transformation was carried out according to the manufacturer's instructions. The quantitation of α-galactosidase activity was done according to Lazo et al. [1977].

Construction of the Fusion Vectors

The BPAG2 intracellular domain fragments, devoid of the transmembrane domain, were produced by PCR amplification with specific primers made according to the published cDNA sequence [Giudice et al., 1992] using the foreskin keratinocyte cDNA library as template. For the original bait construct (p376), forward primer 5'-T*GG ATC C*TT GAA GTC ACT GAG AGA

ATT GT-3' and reverse primer 5'-GTA GCT GTG GAT ATC TGC AGT-'3 were used (the *Bam*HI site incorporated into the primer is italicized). After subcloning into the T-tailed Bluescript KS, the *Bam*HI-*Sal*I restriction fragment was isolated and ligated into the multiple cloning site of pGB-*MEL1* [Aho et al., 1997] previously digested with *Bam*HI-*Sal*I. For additional bait constructs, the same forward primer shown above and one of the following reverse primers: 5'-GGG CAG GGT GGA GGA CCA-3' (p409), or 5'-AGT GGA GGC AGG TGA GTG-3' (p430), were used. For p434 a forward primer 5'-ATG *GAA TTC* AGA CTT ACA TCC TTA CCA CC-3' and the reverse primer, 5'-TCC GAG GGT ACT CCG GAG-3' were used (the *Eco*RI site used for in-frame ligation is italicized).

The two-hybrid K18 cDNA clone was used as a PCR template to prepare shorter fragments. The primer sequences were based on the DNA sequence of the two-hybrid cDNA clone and checked against the published sequences [Oshima et al., 1986]. The 5' primers were equipped with an *Eco*RI restriction site designed to restore the correct reading frame after ligation and the reverse primers were equipped with the *Sal*I recognition site, both shown in italics. For the 5'-deletions, one of the forward primers, 5'-*GAA TTC* GCC AAG ATC ATG GCA GAC A-3' (p465); 5'-*GAA TTC* TCT CAG CAG ATT GAG GAG AG-3' (p466); 5'-*GAA TTC* GCC CTA CAG ATG GAG CAG-3' (p467); or 5'-*GAA TTC* CTT GGT GAT GCC TTG GAC A-3' (p468), and a reverse primer 5'-A*GT CGA C*TG GCT TAA TGC CTC AGA AC-3' were used. The PCR-products were ligated and subcloned into T-tailed [Holton and Graham, 1991] Bluescript, cut with *Eco*RI-*Sal*I and ligated into *Eco*RI-*Sal*I-digested GAL4 activation domain vector pGAD424. The plasmids for yeast transformation were isolated using Wizard Miniprep kit from Promega (Madison, WI) and sequenced to confirm the in-frame fusion.

GST-Fusion Protein Interaction Assay

The PCR product of BPAG2 corresponding to aa 1–89 [Giudice et al., 1992] was cloned into plasmid pGEX-4T-1 (Pharmacia, Gaithersburg, MD). Glutathione S-transferase (GST) or GST-fusion proteins were prepared from 250 ml cultures of *E. coli* XL1-Blue cells (Stratagene) and purified through binding to Glutathione Sepharose 4B according to the manufacturer's (Pharmacia) instructions.

Cells from a confluent culture of primary human foreskin keratinocytes in a 100-mm dish were collected. Cell lysate was prepared and the culture medium and the cell lysate were incubated with the BPAG2-GST/GT Sepharose 4B according to the Cellular Labeling and Immunoprecipitation Kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After careful washes, the pelleted GT-Sepharose 4B beads were boiled with sample buffer, proteins resolved by SDS-PAGE, and detected after Western blotting with the Monoclonal Anti-Cytokeratin Peptide 18 antibody (Sigma, St. Louis, MO) or Anti-GST Antibody (Pharmacia), and the secondary antibody conjugated to HRP was detected with Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products, Boston, MA).

Expression Studies

Human Multiple Tissue cDNA Panels (Human MTC Panels I and II, Human Fetal MTC Panel, and Human Tumor MTC Panel) were obtained from Clontech and used as templates for PCR analysis. Primers 5'-CAG AAG GCG GCA TGT ATG CT-3' and 5'-ACG GCT TGA CAG CAA TAC TTC-3' produced a 440 bp fragment from BPAG2 cDNA. PCR conditions were: 2 min at 94°C, followed by 38 cycles of 94°C 20 sec, 58°C 30 sec, and 72°C 1 min. K18 cDNA was detected using the same primers as for the construct p466 (see above). The PCR conditions for K18 were the same as for BPAG2. Due to the low level of expression of K18 in the tissues, 1/5 (5 µl) of the first PCR reaction product, mixed with 40 µl of PCR master mix, was used as a template and the PCR was continued for another 38 cycles. G3PDH-primers, provided by Clontech with each MTC panel, were used as a control, and the PCR was performed for 30 sec at 94°C, followed by 26 cycles of 94°C 20 sec and 68°C 2 min.

Human foreskin keratinocyte cDNA library was used as a template to study the abundance of BPAG2, K10, K13, K14, K16, and K18 transcripts. A series of 10-fold dilutions were prepared, and 1 µg, 100 ng, 10 ng, 1 ng, and 100 pg of library DNA were used as templates for PCR, which was conducted for 2 min at 94°C, followed by 38 cycles of 94°C 20 sec, 58°C 30 sec, and 72°C 1 min. Primers 5'-GAG AGC CTG ACT GAA GAG C-3' and 5'-GCT CGA ATC TCT TGC AAC TG-3' produced a 449-bp K10 specific

fragment (Genbank accession # J04029), primers 5'-AAG GAA TTT AGC AAC CAG GTG-3' and 5'-TGA TGT CCG TAG GCC TTA A-3' produced a 636-bp K13 specific fragment (Genbank accession # X14640), primers 5'-CTG AGA GGC CAG GTG GGT-3' and 5'-CTC AGT TCT TGG TGC GAA GG-3' produced a 654-bp K14 specific fragment (Genbank accession # M28646) [I. McLean, personal communication], and primers 5'-CTG AGA GGT CAG ACC GGC-3' and 5'-CTA GGA GCT CTG GCC CTG-3' produced a 639-bp K16 specific fragment (Genbank accession # S79867). The PCR products were confirmed by DNA sequencing using each 5' primer as a sequencing primer.

A Human RNA Master Blot was purchased from Clontech and hybridized according to the manufacturer's instructions. A 1273-bp fragment of the intracellular domain of BPAG2 was prepared by PCR using primers 5'-GAA GTC ACT GAG AGA ATT GTC-3' and 5'-GTA GCT GTG GAT ATC TGC AGT-3', and was labeled with [α-^{32}P]-dCTP (Amersham, Arlington Heights, IL) using a random priming kit (Boehringer).

RESULTS

Molecular Interactions of BPAG2 With Keratin 18

To identify molecules potentially interacting with BPAG2, a 1273 bp cDNA corresponding to the intracellular domain of BPAG2 (amino acids 13–437) was cloned into the yeast-two-hybrid GAL4 binding domain vector to develop the construct p376 (see Fig. 1). This construct

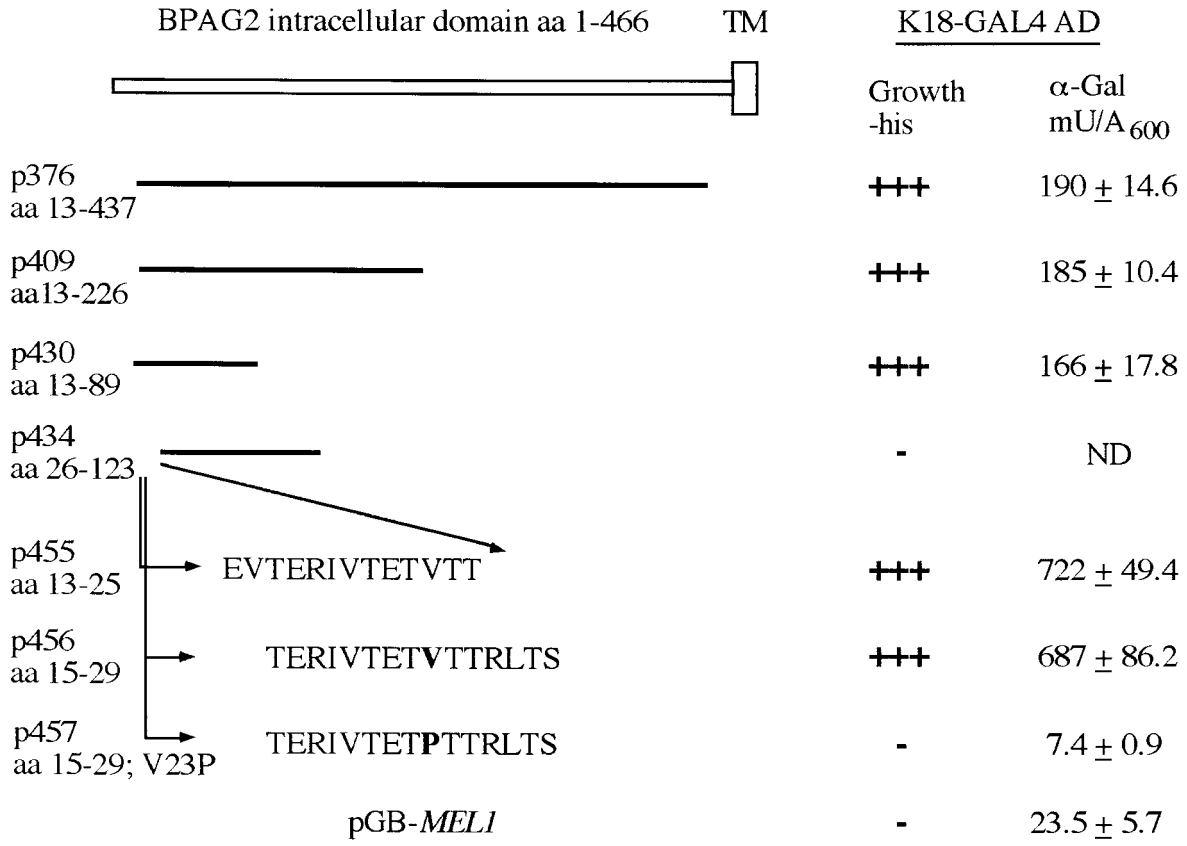

Fig. 1. Interactions of the BPAG2 intracellular domain and keratin 18, as determined in yeast two-hybrid system. PCR amplified cDNA fragments corresponding to the intracellular domain of BPAG2 (amino acids 1–466) were cloned into the binding domain vector of yeast two-hybrid system to generate constructs indicated on the left (p376-p434). In addition, three constructs (p455, p456, and p457) consisting of double stranded oligomers with *EcoR*I and *Sal*I overhang ends (see Fig. 2) were also inserted into the binding domain vector. In case of the vector p457, a valine residue was mutated to proline by substitution of two nucleotides. These constructs contained yeast *MEL1* reporter gene which encodes α-galactosidase. The interactions of these constructs were examined with a GAL4 activation domain vector containing full-length keratin 18 cDNA. The interactions were screened by growth in His$^-$ medium and were quantitated by assay of α-galactosidase activity as shown on the right.

was used as a bait to screen a human keratinocyte cDNA library cloned into the GAL4 activation domain vector. The screening of ~8 × 10^5 library colonies resulted in identification of approximately 70 colonies capable of growing in His^- medium, suggestive of protein-protein interactions. The GAL4 activation domain plasmids were isolated and retransformed into yeast with the original bait plasmid. The plasmids which consistently showed interaction, were subjected to DNA sequencing. Among the positive clones, a total of 10 encoded K18 polypeptide. Eight of the clones were full-length (amino acids 1–429), while two of them corresponded to partial carboxy-terminal sequences (amino acids 142–429 and 205–429, respectively; Fig. 3). Thus, the intracellular domain of BPAG2 was capable of binding to K18.

To identify the region of BPAG2 intracellular domain responsible for K18 binding, a number of 3' deletion constructs truncating the BPAG2 intracellular domain polypeptide at different distances from its carboxyl-terminal end were tested as a bait, representatives of which are shown in Figure 1. They all showed activity comparable to the original bait. However, a 5'-end deletion construct (construct p434) was able to pinpoint a discrete 13-amino acid segment (amino acids 13–25), in the amino-terminal portion of the polypeptide as the site responsible for K18 binding (Figs. 1 and 2). Synthetic oligonucleotides were used to generate a bait construct consisting of amino acids 13–25 and 15–29 (Fig. 2). These both showed more than 3 times higher activity than the original bait construct (Fig. 1). Thus, the critical binding region consists of 11 amino acids, numbers 15–25, at the amino-terminal region of BPAG2. The third oligonucleotide-generated bait construct replacing valine 23 by a proline (V23P), a substitution which is predicted to disrupt the β-sheet conformation of the amino-terminal end of the BPAG2 polypeptide, entirely abolished the binding activity (Figs. 1 and 2). Thus, the binding of K18 with the amino-terminal segment of BPAG2 appears to be sequence-specific and conformation dependent.

To identify the reciprocal region(s) of K18 responsible for the interactions with BPAG2, a number of 5' deletion clones of K18 cDNA were inserted into the GAL4 activation domain vector of the yeast two-hybrid system and were tested for their ability to interact with the BPAG2 clone p376 as a bait (Fig. 3). The results indicated that significant binding activity was noted with the full-length cDNA (amino acids 1–429), as well as with those shortened in the 5' end down to amino acid residue 272 (construct p466; Fig. 3). Further deletion to amino acid 332 (construct p467) resulted in partial reduction of activity, as quantitated by the α-galactosidase assay, while further deletion to amino acid 392 (construct p468) completely abolished the activity. Thus, intact helix segment 2B appears to be necessary for K18 binding to BPAG2 (see Fig. 3).

p455 aa 13-25

```
AA TTC GAA GTC ACT GAG AGA ATT GTC ACT GAA ACA GTA ACC ACA G
     G CTT CAG TGA CTC TCT TAA CAG TGA CTT TGT CAT TGG TGT CAG CT
        E   V   T   E   R   I   V   T   E   T   V   T   T
```

p456 aa 15-29

```
        AA TTC ACT GAG AGA ATT GTC ACT GAA ACA GTA ACC ACA AGA CTT AGA TCC G
             G TGA CTC TCT TAA CAG TGA CTT TGT CAT TGG TGT TCT GAA TGT AGG CAG CT
                T   E   R   I   V   T   E   T   V   T   T   R   L   T   S
```

p457 aa 15-29; V23P

```
        AA TTC ACT GAG AGA ATT GTC ACT GAA ACA CCA ACC ACA AGA CTT AGA TCC G
             G TGA CTC TCT TAA CAG TGA CTT TGT GGT TGG TGT TCT GAA TGT AGG CAG CT
                T   E   R   I   V   T   E   T   P   T   T   R   L   T   S
```

Fig. 2. Nucleotide and translated amino acid sequences of oligomers used to make bait constructs p455, p456, and p457 (see Fig. 1). The oligomers consisted of two complementary strands and generated *EcoR*I and *Sal*I overhang ends after annealing, which facilitated the cloning into the binding domain vector pGB-*MEL1*. Note the 2 bp substitution in the construct p457 which replaces valine 23 by a proline (V23P).

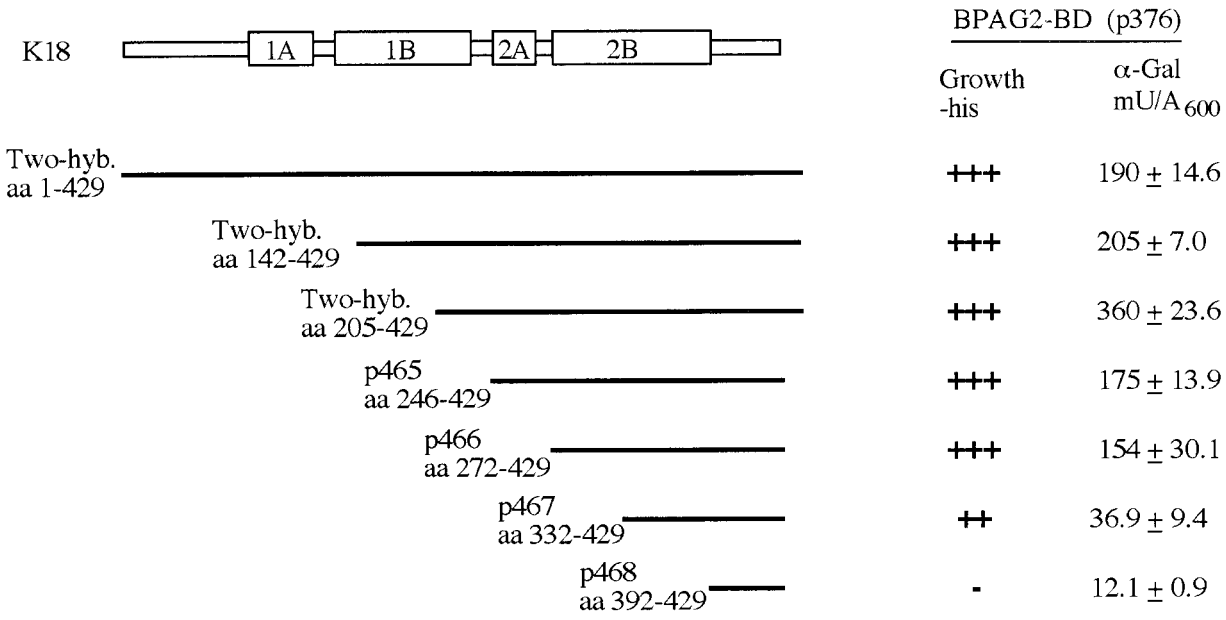

Fig. 3. Identification of K18 domains binding to BPAG2 in yeast two-hybrid system. cDNAs corresponding to full-length (amino acids 1–429) K18, the domain organization of which is schematically shown on the top, or partial sequences devoid of the amino-terminal amino acids, were cloned into pGAD424 vector. The three top cDNAs were isolated from keratinocyte cDNA library in the yeast two-hybrid system through binding to the BPAG2 bait (see Fig. 1). The four other clones (p465–468) were generated by PCR, and contained a shortened 3'-UTR. The binding with BPAG2 binding domain vector (p376; see Fig. 1) was determined by growth on His^- medium and quantitated by α-galactosidase activity assay.

In Vitro Protein-Protein Interaction Assay Confirms the BPAG2/K18 Interaction

To verify the interaction between BPAG2 and K18 polypeptides, the amino-terminal domain, aa 1–89 of BPAG2, was expressed as a GST-fusion protein in *E. coli*. The fusion protein/GT-Sepharose 4B complex was incubated with a keratinocyte cell lysate, and after careful washes the proteins were separated on SDS-page and identified with a monoclonal antibody specific for K18 polypeptide. Indeed, BPAG2 pulled down the K18 polypeptide, and detectable quantities of K18 polypeptide were present in the lysate of normal human keratinocytes (Fig. 4).

Cultured human keratinocytes express a variety of keratins, but the relative abundance of the corresponding mRNAs, particularly those encoding keratin 18, is not clear. To examine the relative abundance of various keratin mRNAs, serial 10-fold dilutions of the human foreskin keratinocyte two-hybrid cDNA library were used as template for PCR with primers specific for various keratins. A number of dilutions established that keratins 14 and 16 were the most abundant keratin cDNAs in this library, followed by keratins 16, 10, and 13

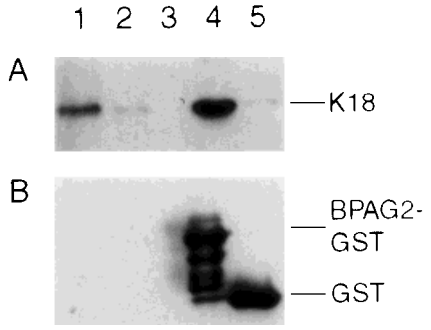

Fig. 4. The in vitro protein-protein interaction assay confirming that the BPAG2-GST-fusion protein interacts with K18. **Lanes 1,2:** Keratinocyte extract, 20 μg and 2 μg of protein, respectively; **lane 3:** blank lane; **lane 4:** keratinocyte cell extract (100 μg of protein) incubated with BPAG2-GST-Sepharose 4B; **lane 5:** keratinocyte cell extract (100 μg of protein) incubated with GST-Sepharose 4B. **A:** Western blot was developed with an anti-K18 antibody; **B:** Western blot was developed with an anti-GST antibody.

(Fig. 5). Keratin 18 cDNA was present in a relatively low abundance, the apparent concentration being at least four orders of magnitude lower than that of keratin 14 (Fig. 5). The fact that the original yeast two-hybrid screen of this cDNA library with the BPAG2 cDNA construct as a bait did not result in isolation of any other

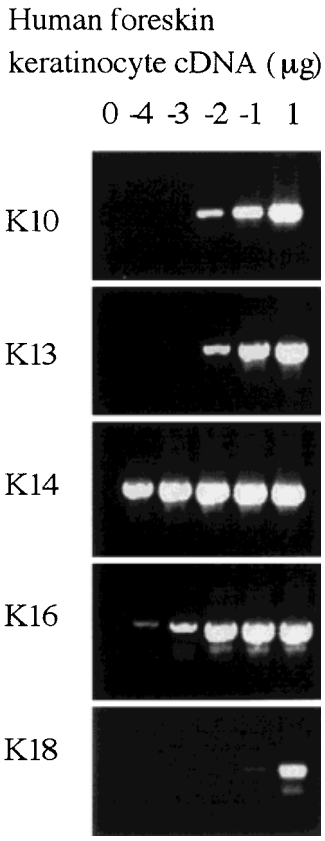

Fig. 5. Relative abundance of various type I keratin cDNAs in human foreskin keratinocyte library. A series of 10-fold dilutions of the library DNA (**1**, **-1**, **-2**, **-3**, and **-4**, representing 1 μg, 100 ng, 10 ng, 1 ng, and 100 pg of template, respectively) as indicated on the top of the panel, was used as a template for PCR amplification. The control lane (**0; left lane**), was devoid of DNA template. The primers and the conditions for PCR amplification are indicated in the Materials and Methods section.

keratin cDNAs, besides keratin 18, appears to attest to the specificity of the BPAG2/K18 interaction.

Tissue-Specific Expression of BPAG2

BPAG2 is known as a hemidesmosomal transmembrane molecule expressed by the basal epidermal keratinocytes. To examine a possible wider tissue distribution, a multiple tissue RNA blot representing a variety of adult and fetal tissues was used to screen the expression of BPAG2 at the mRNA level. The results, shown in Figure 6, indicated strong expression in tissues with a prominent epithelial component, including placenta, trachea, and colon, as well as in mammary, salivary and thyroid glands, and prostate tissues. Relatively high level of expression was also noted in both adult and fetal thymus (Fig. 6). Low, but detectable, levels were noted in bladder, uterus, testis, and small intestine, while a variety of other tissues were apparently negative for BPAG2 expression (Fig. 6).

To examine the BPAG2 expression in further detail, we utilized multi-tissue cDNA panels as a template for PCR amplification with BPAG2 3'-end specific primers. The advantages of this technique over the multiple tissue RNA blot include significantly enhanced sensitivity and specificity, but for maximal representation of the transcripts, the primers need to target the 3'-end of the mRNA. The results, shown in Figure 7, revealed, in general, a similar expression pattern as noted with RNA hybridization, but the fetal skeletal muscle and the fetal spleen were also weakly positive, attesting to the sensitivity of this technique. Thus, in addition of the well characterized expression in epidermal basal keratinocytes, BPAG2 is expressed in a variety of normal tissues with an epithelial component.

Previous studies have suggested that the expression of BPAG2 is aberrant in cancerous and precancerous tissues, as examined by immunohistochemical means [Yamada et al., 1996]. To examine BPAG2 expression in such tissues at mRNA level, a human tumor multiple tissue cDNA panel was tested. The results revealed clearly detectable level of expression in adenocarcinomas of colon, pancreas and prostate, (lanes 2–5 in Fig. 8), which is in agreement with the expression of the BPAG2 transcript in the corresponding normal tissues. The expression was essentially undetectable in two lung carcinomas and in one breast carcinoma (lanes 6–8, Fig. 8).

Keratin 18 Expression in Various Tissues

Keratin 8/18 is known as a component of simple epithelia. To further examine the tissue-specific expression of keratin 18, PCR amplification of multiple-tissue cDNA panels, which were originally used to examine the BPAG2 expression (see above), was performed. The results indicated a detectable level of expression in a variety of both adult and fetal tissues (Fig. 7). Among the tissues that were originally shown to express BPAG2, keratin 18 expression was also noted in placenta, colon, prostate, and the lower intestine (Fig. 7). These findings raise the possibility that BPAG2 in intestinal tissues, particularly in colon, may serve as a link to

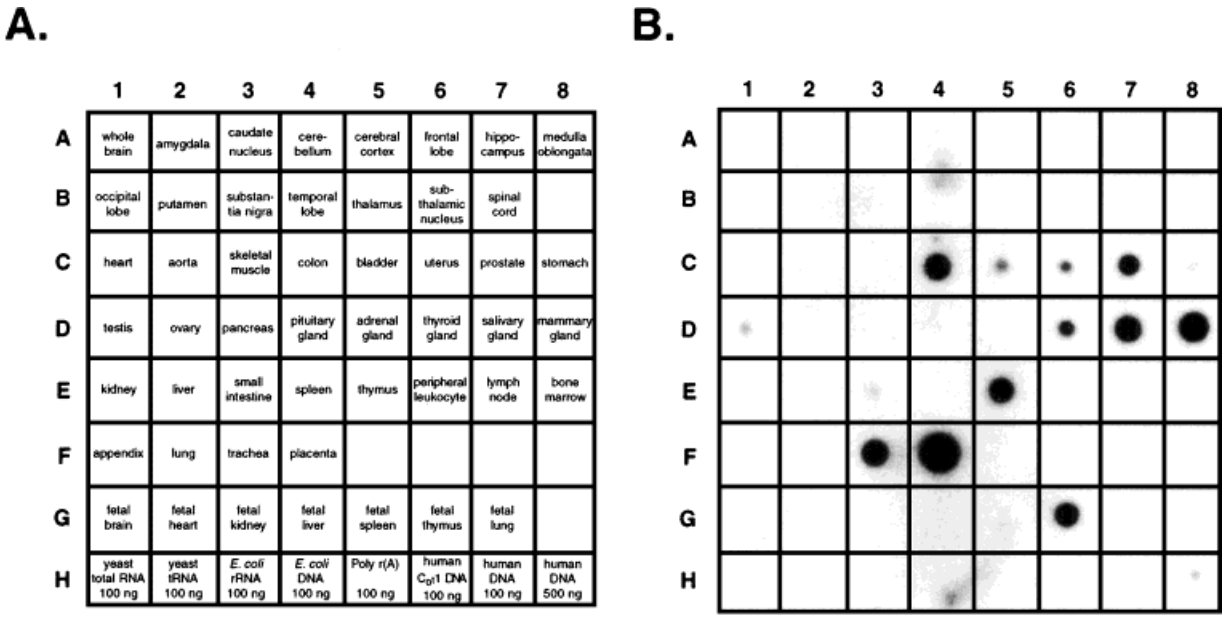

Fig. 6. Tissue-specific expression of the 180-kD bullous pemphigoid antigen/type XVII collagen at the mRNA level. A Master RNA Blot (Clontech) was hybridized with a 1273 bp cDNA corresponding to the intracellular domain of BPAG2. The tissue origins of RNA are indicated in **A**, while autoradiograph of the filter is shown in **B**.

keratin 18, a keratin primarily expressed in simple epithelia.

To further explore keratin 18 expression at the mRNA level, the human tumor multiple tissue cDNA panel, examined for BPAG2 expression (see above), was also tested for K18 expression. Prominent expression was noted in a lung carcinoma (lane 7, Fig. 8), while the remaining tumor tissues showed lower levels of expression (Fig. 8). However, when compared to the normal tissues, the expression of K18 was considerably higher in all tumor tissues, and strong bands were detected after an equal number of PCR-cycles was applied (data not shown), which is in agreement with earlier immunohistochemical studies [Debus et al., 1984].

DISCUSSION

Recent studies have explored the molecular complexity of the cutaneous basement membrane zone with particular emphasis on hemidesmosomes and the specific interactions of these components [Borradori et al., 1998]. It has been demonstrated that the intracellular domain of BPAG2 is able to bind the β4 integrin polypeptide [Aho and Uitto, 1998; Borradori et al., 1998] which in turn binds plectin [Reznicczek et al., 1998; Sanchez-Aparicio et al., 1997], a versatile multi-functional binding protein, which clearly has binding domains for intermediate filament keratins [Nikolic et al., 1996; Svitkina et al., 1996; Wiche et al., 1993]. Thus, in stratifying squamous epithelia, the link between BPAG2 and the intermediate filaments may not be direct but could be mediated by other hemidesmosomal proteins. In contrast, in simple epithelia, this interaction may be direct, as suggested by our studies.

Based on the computer analysis, the amino-terminal end of BPAG2 forms a β-sheet, a structure predicted to be responsible for protein-protein interactions [Aho and Uitto, 1998]. The sequence seems to be unique for BPAG2, with no significant homology to any known proteins or peptide motifs. A single point mutation, which is predicted to abolish the β-sheet conformation, completely abolished the interaction with K18, attesting to the direct interaction between the two proteins.

The deletion analysis showed that the intact helix 2B of K18 is necessary for the interaction with BPAG2. The deletion construct encoding the carboxy-terminal half of the helix 2B showed a weak interaction but the tail domain alone did not interact with BPAG2. The conserved region at the carboxy-terminus of helix 2B is required for the filament stabilization [Wilson et al., 1992] and point mutations changing such

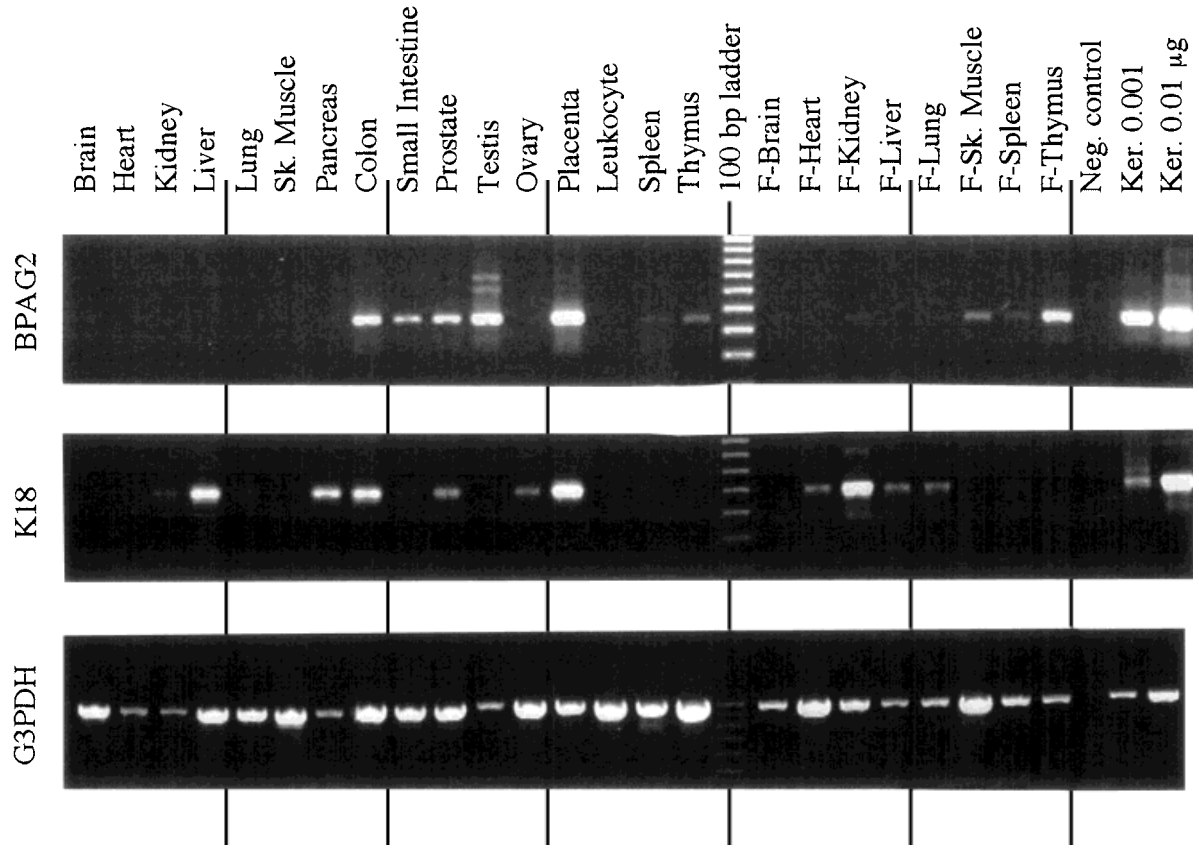

Fig. 7. Tissue-specific expression of the BPAG2 and K18 genes, as determined by multi-tissue cDNA panels. The panels consist of cDNAs derived from polyA$^+$ RNA isolated from tissues indicated on the top of the panel, representing 16 adult tissues (left panel), eight fetal tissues (middle panel), and epidermal keratinocytes (two right lanes). Note the negative control lane (third from the right) and the one representing 100 bp size marker ladder (middle). The panel depicted on the top represents amplification with BPAG2 specific primers, as described in the Methods, while the center panel represents expression of K18. G3PDH, a ubiquitously expressed housekeeping gene, was amplified as a control (lower panel).

residues had profound effect upon filament assembly [Hatzfeld and Weber, 1991].

The activation domain library used in yeast two-hybrid system was derived from human foreskin keratinocytes, which are known to express a variety of other keratins, including keratins 5 and 14 in basal layers and keratins 1 and 10 in suprabasal layers [Corden and McLean, 1996]. The observation that only keratin 18 cDNAs were isolated in the yeast two-hybrid system, while no evidence for BPAG2 interactions with other keratins was noted, suggests specificity of the binding. To examine the relative abundance of the expression of different keratins in cDNA library cloned into the activation domain of the yeast two-hybrid system, PCR amplification of the keratinocyte cDNA library with primers specific for a variety of type I (acidic) keratins was performed. The results indicated that the level of keratin 18 expression was two to four orders of magnitude lower than that of other keratins tested. Thus, the lack of binding of other keratins, besides keratin 18, to the BPAG2 bait can not be explained by lack of expression of these other keratins in keratinocytes. Thus, it is highly probable that the other keratin polypeptides present in the keratin cDNA library do not have direct interactions with the 180-kD bullous pemphigoid antigen polypeptide.

During intermediate filament assembly, keratin molecules initially form obligate heterodimers, and the counterpart of keratin 18 is keratin 8 [Jackson et al., 1980; Meng et al., 1997]. Nevertheless, in the yeast two-hybrid system, the interactions must occur between discrete individual polypeptides, since the interacting clones in the activation domain were retransformed, and the interactions were confirmed using purified plasmids.

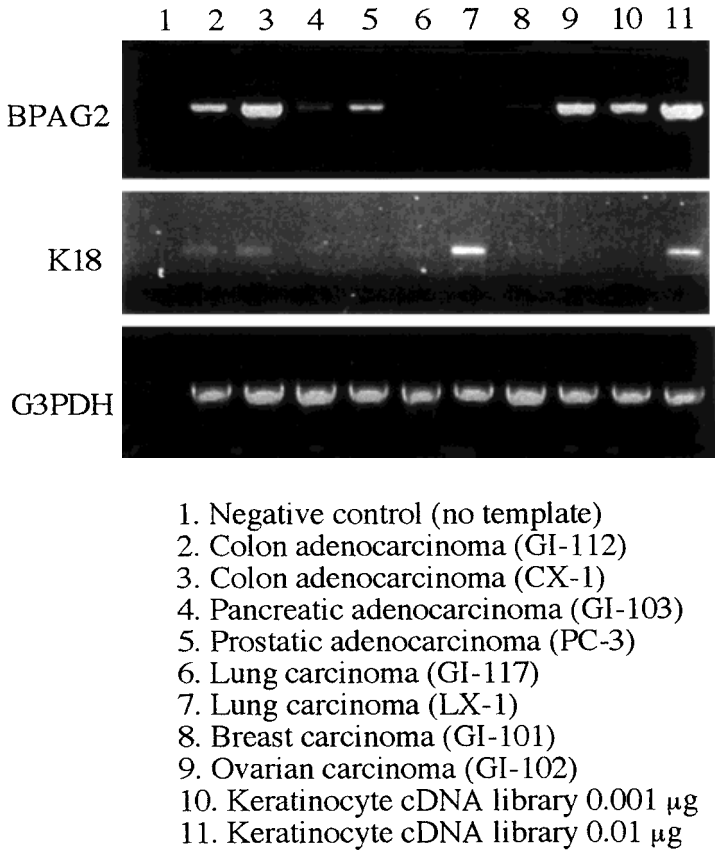

Fig. 8. Expression of BPAG2 and K18 in human tumor multiple tissue cDNA panel. The amplification of BPAG2 and K18 sequences was performed with specific primers (see Materials and Methods, and Fig. 7) with 38 cycles of PCR with each primer pair.

Previous studies have clearly demonstrated that BPAG2 is the major component of hemidesmosomes at the dermal-epidermal basement membrane zone, while immunohistochemical analyses have suggested more widespread distribution [Nishizawa et al., 1993; Fairley et al., 1995]. Utilizing multiple-tissue mRNA blots and multi-tissue cDNA panels, we demonstrated that BPAG2 is expressed in a variety of tissues with prominent epithelial component. Some of these tissues have ultrastructurally demonstrable type I hemidesmosomes, i.e., those presumably composed of the full complement of hemidesmosomal proteins, including the 230-kD and the 180-kD bullous pemphigoid antigens, plectin, and the $\alpha 6\beta 4$ integrin. In other tissues, such as those with simple epithelia and simple epithelium derived adenocarcinomas, BPAG2 expression was clearly demonstrated at the mRNA level, yet these tissues do not contain type I hemidesmosomes [Jones et al., 1994]. On the other hand, it has been suggested that some of these tissues contain so-called type II hemidesmosomes, presumed to be incomplete forms of hemidesmosomes and suggested to lack bullous pemphigoid antigens [Jones et al., 1994; Uematsu et al., 1994]. However, our results demonstrating the expression of BPAG2 at the mRNA level using two independent sets of starting material, suggest the presence of the corresponding protein component in these tissues as well, although the assembly of morphologically complete type I hemidesmosomes is not evident by ultrastructural examination [Hieda et al., 1992].

Keratin 18 has been previously suggested to be expressed primarily in simple epithelia [Leube et al., 1986; Oshima et al., 1986], and utilization of multiple-tissue cDNA panels in our study confirmed this general distribution. Other reports have shown that the genes encoding simple epithelial cytokeratins can also be expressed in stratified epithelia [Bosch et al., 1988]. Direct comparison of tissue specific expression between BPAG2 and keratin 18 transcripts revealed particularly strong expression

of both of these genes in colon, in addition to prostate and placenta. These observations suggest that BPAG2 and keratin 18 may indeed participate in functional interactions in simple epithelia.

ACKNOWLEDGMENTS

We thank Hua Xie and Kyle Rothenberger for excellent technical assistance and Carol Kelly and Carol Biscieglia for secreterial help.

REFERENCES

Aho S, Arffman A, Pummi T, Uitto J. 1997. A novel reporter gene *MEL1* for the yeast two-hybrid system. Anal Biochem 253:270–272.

Aho S, Uitto J. 1998. Direct interaction between the intracellular domains of bullous pemphigoid antigen 2 (BP180) and β4 integrin, hemidesmosomal component of basal keratinocytes. Biochem Biophys Res Com 243:694–699.

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

Borradori L, Sonnenberg A. 1996. Hemidesmosomes: Roles in adhesion, signaling and human diseases. Curr Opin Cell Biol 8:647–656.

Bosch FX, Leube RE, Achtstätter T, Moll R, Franke WW. 1988. Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. J Cell Biol 106:1635–1648.

Burgeson RE. 1993. Type VII collagen, anchoring fibrils, and epidermolysis bullosa. J Invest Dermatol 101:252–255.

Burgeson RE, Christiano AM. 1997. The dermal-epidermal junction. Curr Opin Cell Biol 9:651–658.

Corden LD, McLean WHI. 1996. Human keratin diseases: Hereditary fragility of specific epithelial tissues. Exp Dermatol 5:297–307.

Debus E, Moll R, Franke WW, Weber K, Osborn M. 1984. Immunohistochemical distinction of human carcinomas by cytokeratin typing with monoclonal antibodies. Am J Pathol 114:121–130.

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

Fairley JA, Heintz PW, Neuburg M, Diaz LA, Giudice GJ. 1995. Expression pattern of the bullous pemphigoid-180 antigen in normal and neoplastic epithelia. Br J Dermatol 133:385–391.

Fuchs E. 1995. Keratins and the skin. Annu Rev Cell Dev Biol 11:123–153.

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

Gordon MK, Fitch JM, Foley JW, Gerecke DR, Linsenmayer C, Birk DE, Linsenmayer TF. 1997. Type XVII collagen (BP180) in the developing avian cornea. Invest Ophthalmol Vis Sci 38:153–166.

Green KJ, Jones JCR. 1996. Desmosomes and hemidesmosomes: Structure and function of molecular components. FASEB J 10:871–881.

Hatzfeld M, Weber K. 1991. Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain. J Cell Biol 99:351–362.

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

Holton TA, Graham MW. 1991. A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucl Acids Res 19:1156.

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

Jackson BW, Grund C, Schmid E, Burke K, Franke W, Illmensee K. 1980. Formation of cytoskeletal elements during mouse embryogenesis: Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. Differentiation 17:161–179.

Jones JCR, Asmuth J, Baker SE, Langhofer M, Roth SI, Hopkinson SB. 1994. Hemidesmosomes: Extracellular matrix/intermediate filament connectors. Exp Cell Res 213:1–11.

Lazo PS, Ochoa AG, Gascon S. 1977. α-Galactosidase from *Saccharomyces carlsbergensis*. Eur J Biochem 77:375–382.

Leube RF, Bosch FX, Romano V, Zimbelmann R, Höfler H, Franke WW. 1986. Cytokeratin expression in simple epithelia: III. Detection of mRNAs encoding human cytokeratins nos. 8 and 18 in normal and tumor cells by hybridization with cDNA sequences in vitro and in situ. Differentiation 33:69–85.

Li K, Tamai K, Tan EML, Uitto J. 1993. Cloning of type XVII collagen. Complementary and genomic DNA sequences of mouse 180-kilodalton bullous pemphigoid antigen (BPAG2) predict an interrupted collagenous domain, a transmembrane segment, and unusual features in the 5'-end of the gene and the 3'-untranslated region of the mRNA. J Biol Chem 268:8825–8834.

Masunaga T, Shimizu H, Yee C, Borradori L, Lazarova Z, Nishikawa T, Yancey KB. 1997. The extracellular domain of BPAG2 localizes to anchoring filaments and its carboxyl terminus extends to the lamina densa of normal human epidermal basement membrane. J Invest Dermatol 109:200–206.

Meng J-J, Bornslaeger EA, Green KJ, Steinert PM, Ip W. 1997. Two-hybrid analysis reveals fundamental differences in direct interactions between desmoplakin and cell type-specific intermediate filaments. J Biol Chem 272:21495–21503.

Nikolic B, Nulty EM, Mir B, Wiche G. 1996. Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions. J Cell Biol 134:1455–1467.

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

Oshima RG, Millan JL, Cecena G. 1986. Comparison of mouse and human keratin 18: A component of intermediate filaments expressed prior to implantation. Differentiation 33:61–68.

Pulkkinen L, Uitto J. 1998. Hemidesmosomal variants of epidermolysis bullosa. Mutations in the α6β4 integrins and the 180-kD bullous pemphigoid antigen/type XVII collagen genes. Exp Dermatol 7:46–64.

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

Sanchez-Aparicio P, Martinez de Velasco AM, Niessen CM, Borradori L, Kuikman I, Hulsman EHM, Fässler R, Owaribe K, Sonnenberg A. 1997. The subcellular distribution of the high molecular mass protein, HD1, is determined by the cytoplasmic domain of the integrin β4 subunit. J Cell Sci 110:169–178.

Svitkina TM, Veskhovsky AB, Borisy GG. 1996. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J Cell Biol 135:991–1007.

Tidman MJ, Eady RAJ. 1985. Evaluation of anchoring fibrils and other components of the dermal-epidermal junction in dystrophic epidermolysis bullosa by a quantitative ultrastructural technique. J Invest Dermatol 84: 374–377.

Uematsu J, Nishizawa Y, Sonnenberg A, Owaribe K. 1994. Demonstration of Type II hemidesmosomes in mammary gland epithelial cell line, BMGE-H. J Biochem 115:469–476.

Uitto J, Pulkkinen L. 1996. Molecular complexity of the cutaneous basement membrane zone. Mol Biol Report 23:35–46.

Wiche G, Gromov D, Donovan A, Castanon MJ, Fuchs E. 1993. Expression of plectin mutant cDNA in cultured cells indicates a role of COOH-terminal domain in intermediate filament association. J Cell Biol 121:607–619.

Wilson AK, Coulombe PA, Fuchs E. 1992. The roles of K5 and K14 head, tail, and R/KLLEGED domains in keratin filament assembly in vitro. J Cell Biol 119:401–414.

Yamada T, Endo R, Tsukagoshi K, Fujita S, Honda K, Kinoshita M, Hasebe T, Hirohashi S. 1996. Aberrant expression of a hemidesmosomal protein, bullous pemphigoid antigen 2, in human squamous cell carcinoma. Lab Invest 75:589–600.