

# Molecular Genetic Studies of a Human Epidermal Autoantigen (the 180-kD Bullous Pemphigoid Antigen/BP180): Identification of Functionally Important Sequences within the BP180 Molecule and Evidence for an Interaction between BP180 and $\alpha 6$ Integrin

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**Abstract.** The 180-kD bullous pemphigoid autoantigen (BP180) is a component of the hemidesmosome, a cell-matrix connector. This protein is oriented in a type II fashion in the membrane of the hemidesmosome and is a hybrid collagen (classified as type XVII). We have analyzed the fate of various mutant BP180 molecules transfected into several different cell types. A protein, D1, lacking the collagen-like extracellular domains of BP180 polarizes normally in 804G epithelial cells and colocalizes with other hemidesmosomal components in the plane of the basal cell surface. However, deletion of a stretch of 36 amino acids located at the NH<sub>2</sub> terminus of D1 induces an apical polarization of the protein (D1-36N) in the cell surface of 804G cells. Deletion of the 27-amino acid noncollagenous extracellular domain that is located immediately after the membrane spanning domain of BP180 results in a failure of D1-27C

protein to codistribute with other hemidesmosomal components despite its basal localization in transfected 804G cells. In FG cells, which lack their own BP180, transfected D1 protein localizes with the  $\alpha 6\beta 4$  integrin heterodimer. In HT1080 cells, which do not possess BP180 or  $\beta 4$  integrin, D1 protein localizes with  $\alpha 6\beta 1$  integrin while both the D1-27C and D1-36N proteins do not. Moreover, D1 protein coprecipitates with  $\alpha 6$  integrin from extracts of HT1080 transfectants. Taken together, these results suggest that the NH<sub>2</sub>-terminal domain of BP180 determines polarization of BP180 while the noncollagenous extracellular domain of BP180 stabilizes its interactions with other hemidesmosomal components, such as  $\alpha 6$  integrin. Perturbation of this latter domain by human bullous pemphigoid autoantibodies may explain the loss of epidermal cell-dermis attachment that characterizes the BP disease.

THE skin provides protection from physical, chemical, and biological trauma. Its outer surface is covered by a stratified layer of epithelial cells that must be firmly attached to the connective tissue to maintain skin integrity. This adherence is, in part, mediated by certain cell-matrix junctions called hemidesmosomes that integrate the cytoskeleton of the cell with the extracellular matrix (Staehelein, 1974; Jones et al., 1994). A number of adhesion molecules have now been identified in the hemidesmosome including a human autoantigen of 180 kD and the extracellular matrix receptor  $\alpha 6\beta 4$  integrin (Jones et al., 1994). Evidence for their roles in adhesion comes from studies where antibodies directed against their extracellular domains are capable of inducing a disruption of cell connective tissue adherence (Kurpakus et al., 1991; Liu et al., 1993).

The 180-kD hemidesmosomal autoantigen was first

identified using human autoantibodies from the serum of patients with a blistering skin disease called bullous pemphigoid (BP)<sup>1</sup> (Labib et al., 1986; Klatte et al., 1989). For this reason it has been called the 180-kD bullous pemphigoid antigen or BP180. Using BP autoantibodies, cDNAs that encode human BP180 have been isolated and its entire coding sequence has now been characterized (Giudice et al., 1991, 1992; Hopkinson et al., 1992). BP180 is transmembranous and is inserted into the membrane of the hemidesmosome in a type II orientation (Hopkinson et al., 1992). In other words, the collagen-like domain of BP180 is extracellular while its NH<sub>2</sub> terminus is located in the cytoplasmic plaque of the hemidesmosome (Hopkinson et al., 1992). The COOH terminus of BP180 contains a series of collagen-like repeats, leading some to classify BP180 as collagen type XVII (Giudice et al., 1991; Li et al., 1993).

With the exception of a hemidesmosomal component of 300 kD that binds keratin and may play a role in cytoskeleton anchorage to the hemidesmosomal plaque, we know

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1. *Abbreviation used in this paper:* BP, bullous pemphigoid.

little about protein-protein interactions of hemidesmosome elements (Jones et al., 1994; Skalli et al., 1994). Furthermore, because of the inherent insolubility of hemidesmosomes and their lack of abundance in tissues, few hemidesmosome elements have been purified (Klatte and Jones, 1994). Consequently, we have turned to molecular genetics for characterization of protein interactions within the hemidesmosome. In this study we have concentrated on analyses of BP180. Our investigations are possible because we have made use of a cell line (804G) that assembles hemidesmosomes in vitro as well as several other cell lines, selected because they fail to express BP180 while expressing one or more of the other components of the hemidesmosome (Riddelle et al., 1991). During the course of our experiments we have identified two functionally important domains in the BP180 molecule and we present evidence for an interaction between BP180 and the  $\alpha 6$  integrin subunit.

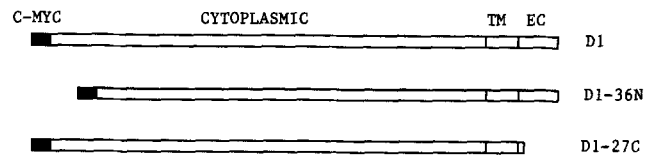
## Materials and Methods

### Cell Culture

804G and FG cells were maintained in culture as reported previously (Riddelle et al., 1991; Hopkinson and Jones, 1994). HT1080 cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD) and maintained in Minimum Essential Medium containing 10% fetal calf serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The hybridoma line 9E10, purchased from ATCC, was maintained in RPMI medium containing 10% fetal calf serum and 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin.

### Antibodies

A rabbit antiserum and monoclonal antibodies against the NH<sub>2</sub> terminus of BP180 were described in Hopkinson et al. (1992). 9E10 antibodies against c-myc were collected as supernatant from subconfluent dishes of the 9E10 hybridoma line. A  $\beta 4$  integrin monoclonal antibody (3E1) was purchased from GIBCO BRL (Gaithersburg, MD). The rat mAb 135-13C against  $\alpha 6$  integrin was provided by Dr. Stephen Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). GoH3 rat monoclonal antibody was



**Figure 1.** Diagram of mutant BP180 constructs. D1 consists of the entire cytoplasmic, membrane spanning (TM) and a portion of the noncollagenous extracellular (EC) domains of BP180 (residues 1–520) (Hopkinson et al., 1992; Li et al., 1993). The amino acid sequences (1–36 [MDVTKKNKRDGTEVTERIVTETVT-TRLTSLPPKGGT] and 494–520 [RKLKARVDELEIRRSIL-PYGDSMDRI]) were deleted from D1 to produce D1-36N and D1-27C, respectively. The c-myc epitope (shaded) is present at the NH<sub>2</sub> terminus of all three constructs.

purchased from Immunotech (Westbrook, ME). Affinity-purified rabbit antibodies against  $\alpha 6$  integrin were provided by Dr. Vito Quaranta (Scripps Research Institute). A  $\beta 4$  polyclonal antibody was a gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Human monoclonal antibodies (5E) against BP230, a plaque component of the hemidesmosome, were provided by Dr. T. Hashimoto, (Keio University, Tokyo, Japan) (Hashimoto et al., 1993).

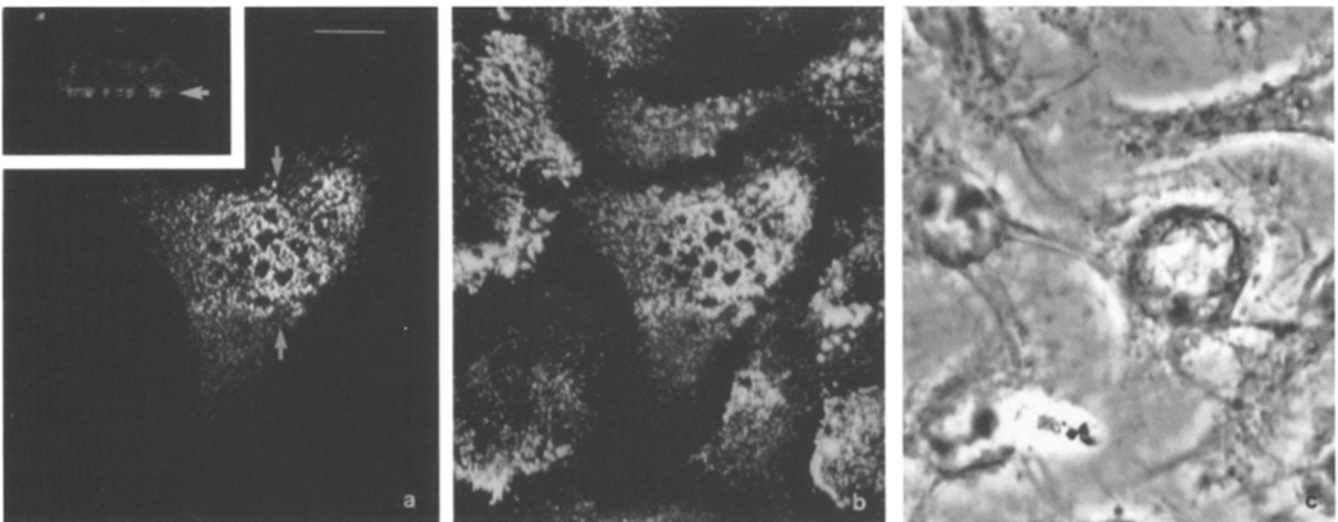
### DNA Transfections

804G, FG and HT1080 cells were maintained for 24 h on 22-mm glass coverslips. 804G cells were transfected with 10  $\mu$ g of plasmid DNA using the calcium phosphate protocol detailed in Sambrook et al. (1989). FG and HT1080 cells were transfected with 3  $\mu$ g of DNA using Lipofectin reagent (GIBCO BRL). At 24 h after transfection cells were harvested for immunoblotting or processed for immunofluorescence microscopy.

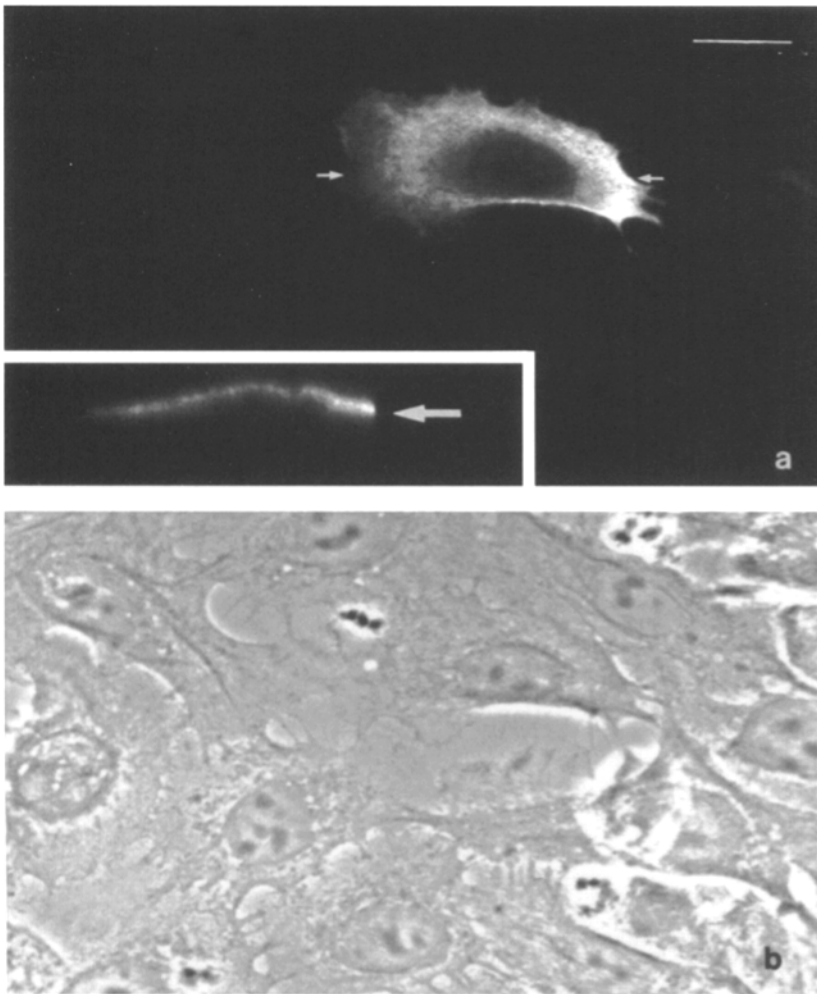
### Constructs for Transfection Analyses

The  $\beta 4$  integrin subunit cDNA, cloned into pBJ/neo plasmid vector was the kind gift of Dr. Vito Quaranta (Tamura et al., 1990).

Truncated BP180 cDNAs were constructed using sequences encoding amino acids 1–520 of the published BP180 sequence (Hopkinson et al., 1992; Li et al., 1993). The methionine start site of BP180 that was predicted by Hopkinson et al. (1992) has been confirmed by Li et al. (1993). To generate fragments D1 and D1-36N, PCR was used to amplify sequences contained within a phage clone ( $\lambda$ 180) containing a 1620-bp cDNA



**Figure 2.** D1-transfected 804G cells were processed for double label immunofluorescence using 9E10 antibodies against a c-myc epitope tagged to D1 protein (a) and anti-BP230 5E antibodies (b). 9E10 antibodies produce a cat-paw, punctate stain concentrated along the basal aspect of the transfected cell in a. There is colocalization of this stain with that generated by the 5E antibodies in the same cell shown in b. The z-section in the inset in a was prepared from the region indicated by the large white arrows. The arrow in the inset indicates the position of the substrate. The phase image of the cells is shown in c. Bar, 25  $\mu$ m.



**Figure 3.** An 804G cell population expressing D1-36N was processed for immunofluorescence using 9E10 antibodies (*a*). A z-section of the region between the arrows in one of the transfected cells in *a* is shown in the inset. The arrow in the inset marks the substrate. Note that the 9E10 antibodies primarily stain the apical surface of the cell. The phase image of the cells is shown in *b*. Bar, 25  $\mu\text{m}$ .

that encodes these 520 amino acids as well as some of the 5' BP180 non-translated region. D1 and D1-36N had the same 3' end generated by PCR using primer (P1) corresponding to sequences between bp 1539 and 1561 of  $\lambda$ 180. The 3' end of the P1 primer was modified to contain a BamHI site to facilitate subcloning. To prepare the D1 construct that encodes amino acids 1–520, a 5' primer, corresponding to –20 to –1 bp of  $\lambda$ 180 and the common 3' primer P1 were used to generate a 1581-bp PCR product. To prepare D1-36N, a 5' primer corresponding to bp 110 to 130 of  $\lambda$ 180 and primer P1 were used to generate a PCR product lacking the sequence that encodes amino acids 1–36 of BP180. All fragments were subcloned into Bluescript KS+ (Stratagene, La Jolla, CA) that contained sequences encoding 11 residues of the c-myc polypeptide recognized by the monoclonal antibody 9E10. This plasmid was obtained from Dr. Norman Karin (Department of Physiology, University of Texas, Houston, TX).

D1-27C was prepared from the c-myc/D1 plasmid by PCR, using the T3 primer that recognizes vector sequences 5' to the insert and a 3' primer corresponding to bp 1454 to 1479 of the coding sequence of the D1 cDNA. After amplification, the fragment was digested with HindIII and subcloned into pRC/CMV (Invitrogen). The vector was prepared by first digesting with XbaI, followed by treatment with Klenow to produce blunt ends, and then digesting with HindIII.

All constructs were sequenced according to standard procedures to ensure that reading frame and orientation were correct.

### Immunofluorescence

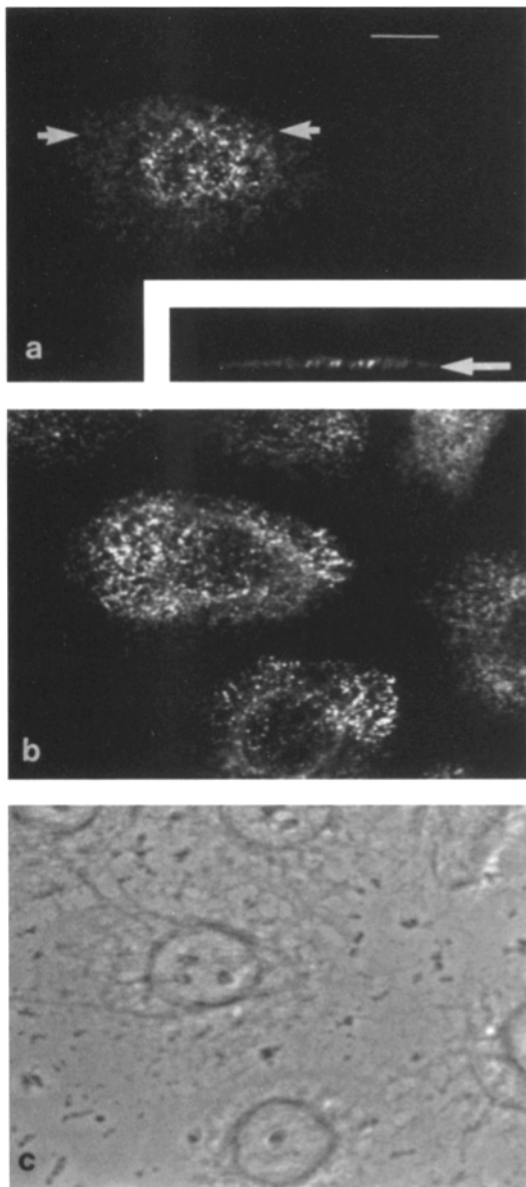
Cells on coverslips were fixed for 1 min in 3.7% formaldehyde in PBS and then extracted for 2 min in –20°C acetone. After air drying the cells were processed for single and double label immunofluorescence as detailed elsewhere (Jones and Goldman, 1985; Riddelle et al., 1991). Some coverslip preparations were rinsed in PBS and subsequently incubated in anti-

body at 37°C for 30 min. After extensive washing the cells were fixed in 3.7% formaldehyde in PBS and then incubated with the appropriate conjugated secondary antibody. Cells on coverslips were viewed in an LSM10 confocal microscope fitted with lasers and filters appropriate for the imaging of fluorescein and rhodamine (Carl Zeiss, Thornwood, NY). Images were stored on optical disc.

### Gel Electrophoresis, Immunoblotting, and Immunoprecipitations

Cell populations were solubilized in 8 M urea, 1% SDS, 10 mM Tris-HCl, pH 6.8, 10%  $\beta$ -mercaptoethanol. Protein concentrations were determined using a Bio-Rad assay kit (Bio-Rad Labs., Richmond, CA). Some samples were processed for SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide slab gels according to Laemmli (1974). For analyses of low molecular mass proteins, samples were run on 15% slab gels. Approximately 10  $\mu\text{g}$  of protein was loaded per gel lane. Separated proteins were transferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose sheets were processed for immunoblotting as detailed in Zackroff et al. (1984). In immunoblotting analyses of immunoprecipitates, nitrocellulose bound antibody was detected with protein A conjugated horse radish peroxidase (Zymed Labs., San Francisco, CA).

For immunoprecipitations, HT1080 cells were metabolically labeled with [ $^{35}\text{S}$ ]methionine for 3 h at 37°C, washed in PBS, then lysed in TBS, pH 7.4, containing 1% Triton X-100, 2 mM  $\text{CaCl}_2$ , 1 mM PMSF, 100 M leupeptin, and 1 M pepstatin. The lysate was centrifuged to remove cell debris and the supernatant was precleared with anti-rat IgG agarose beads (Sigma) for 1 h at 4°C. After centrifugation to remove the beads, the supernatant was incubated with the  $\alpha 6$  mAb GoH3 at 4°C with rotation for 2 h. Anti-rat IgG agarose beads were added and the incubation continued for 2 h more. After several washes with washing buffer (TBS with 1% Tri-



**Figure 4.** 804G cells were transfected with D1-27C and then prepared for double label immunofluorescence using 9E10 (*a*) and 5E (*b*) antibodies. There is a punctate basal stain in the transfected cell in *a* (a z-section of the region between the arrows is shown in the inset in *a* where the substrate is marked by an arrow). 5E antibodies also produce a punctate stain concentrated along the basal aspect of the cells (*b*). However, 9E10 staining in *a* is concentrated in the perinuclear region where there is minimal 5E antibody reactivity (*b*). (*c*) Phase image of the cells. Bar, 25  $\mu\text{m}$ .

ton X-100) the pelleted beads were resuspended in SDS-PAGE sample buffer, under reducing conditions, and boiled for 3 min. Samples were electrophoresed on a 7.5% polyacrylamide gel and processed either for immunoblot analysis or dried on a slab dryer and then exposed overnight to X-OMAT AR film (Kodak, Rochester, NY).

## Results

### BP180 Deletion Constructs for Transfection

We have prepared a series of constructs encoding various

portions of BP180. Those described in this study are shown diagrammatically in Fig. 1. One construct (D1) encodes the entire cytoplasmic domain of BP180, its membrane spanning region as well as an additional 30 extracellular amino acids (residues 1–520) (Li et al., 1993). D1 lacks the collagen domains of the wild-type molecule that begin at residue 568 (Li et al., 1993). Two other constructs have been used. D1-36N cDNA is identical to D1 except that it is missing the region that encodes residues 1 through 36 at the NH<sub>2</sub> terminus of BP180. D1-27C lacks 27 amino acids that are located at the COOH terminus of D1 (residues 494–520). All constructs were tagged at their NH<sub>2</sub> terminus with sequence encoding the c-myc epitope.

It should be noted that we have tested constructs with other COOH- and NH<sub>2</sub>-terminal deletions of D1 (for example, D1-133N) in the transfection assays to be detailed below. However, constructs with more extensive deletions in the 5' or 3' end produce protein that either remains diffusely localized in the cytoplasm or failed to polarize correctly in the membrane (not shown). D1-27C and D1-36N represent the minimal deletions in each terminus of D1 that give obvious phenotypes in our *in vivo* experiments.

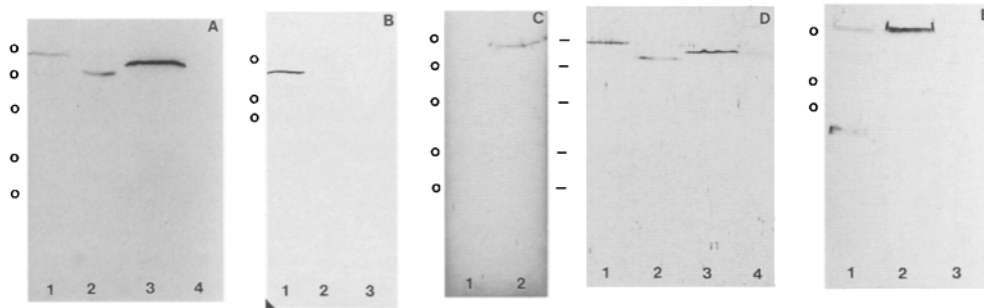
### Identification of Two Functionally Important Domains in BP180

For our initial studies we used 804G cells that assemble hemidesmosomes *in vitro* (Riddelle et al., 1991). Since 804G cells possess endogenous BP180 protein, we used 9E10 antibody that recognizes the c-myc epitope tag on transfected protein to distinguish transiently expressed protein from wild-type BP180. The fate of the transfected protein was assessed by confocal laser scanning microscopy and its localization compared with a 230-kD protein component of the hemidesmosomal plaque that is recognized by the human monoclonal antibody 5E (Hashimoto et al., 1993). All images show focal planes close to the site of cell–substrate interaction. In some instances, computer generated z-sections of cells are presented.

In 804G cells transfected with the construct coding D1 cDNA, mutant BP180 protein shows a polarized basal distribution in a cat-paw pattern (Fig. 2). Moreover, the mutant protein is polarized and codistributes with BP230 along regions of cell–substrate interaction (Fig. 2, *inset*). This cat-paw pattern for BP230 has been described in 804G cells by several groups (Riddelle et al., 1991; Spinardi et al., 1993; Jones et al., 1994). In contrast, D1-36N mutant protein that lacks the 36 NH<sub>2</sub>-terminal most amino acids of the cytoplasmic domain of BP180 polarizes to the apical surface of transfectants (Fig. 3). Furthermore, it fails to associate with BP230 (result not shown).

In 804G cells transfected with the D1-27C construct, mutant BP180 protein localizes at the basal aspect of transfected cells in a punctate manner (Fig. 4 *b*, *inset*). However, it fails to codistribute with BP230 (Fig. 4).

As controls for the above studies, we have confirmed that the transfected 804G cell populations are expressing the appropriately sized tagged mutant proteins by western blotting using 9E10. Proteins close to their predicted mass of 58, 53, and 54 kD are recognized by 9E10 antibodies in whole cell extracts of D1, D1-36N, and D1-27C transfected 804G cell populations respectively (Fig. 5 A).



**Figure 5.** (A) Extracts of 804G cell populations transfected with D1 (lane 1), D1-36N (lane 2), D1-27C (lane 3), and mock transfected controls (lane 4) were processed for immunoblotting after 15% SDS-PAGE using 9E10 antibodies. Note that the 9E10 antibodies recognize tagged polypeptides close to their predicted masses of 58, 53, and

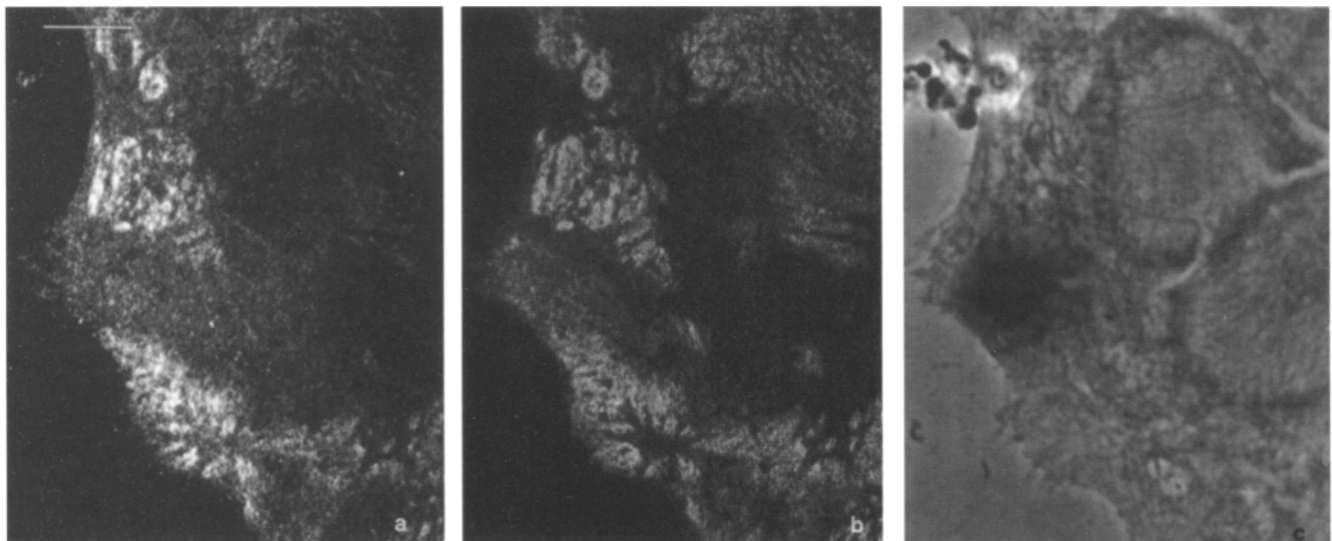
54 kD in lanes 1–3, respectively, but fail to recognize any proteins in lane 4. (B) Extracts of 804G (lane 1), FG (lane 2), and HT1080 (lane 3) cells were processed for immunoblotting using J17 rabbit antibodies after 7.5% SDS-PAGE. The antibodies recognize only a 180-kD polypeptide in lane 1. (C) Mock transfected (lane 1) and D1 transfected (lane 2) FG cells were processed for immunoblotting using J17 antibodies after 15% SDS-PAGE. Note the 58-kD reactive polypeptide in lane 2. (D) Extracts of HT1080 cell populations transfected with D1 (lane 1), D1-36N (lane 2), D1-27C (lane 3), and mock transfected controls (lane 4) were processed for immunoblotting after 15% SDS-PAGE using J17 antibodies. Note that the antibodies recognize polypeptides close to their predicted weights of 58, 53, and 54 kD in lanes 1–3, respectively, but fail to recognize any proteins in lane 4. (E) Extracts of FG (lane 1),  $\beta 4$  integrin transfected HT1080 (lane 2) and mock transfected HT1080 (lane 3) cells were processed for immunoblotting using a  $\beta 4$  rabbit antiserum. Note that the  $\beta 4$  integrin antibodies recognize a 200-kD protein in lanes 1 and 2 while they fail to recognize polypeptides in lane 3. Circles on A and C and dashes on D denote standards of 66, 45, 31, 21.5, and 14.5 kD. Circles on B and E indicate 200, 116, and 97 kD.

Since amino acid deletions in the cytoplasmic domain of BP180 might change its membrane topology from a type II to a type III orientation, as is the case for NH<sub>2</sub>-terminally mutated paramyxovirus HN polypeptide, we also assessed whether mutant BP180 NH<sub>2</sub>-terminal domains could be detected extracellularly by incubating fixed but unpermeabilized cells with 9E10 (Parks and Lamb, 1993). Unpermeabilized D1, D1-27C, and D1-36N 804G transfectants were not recognized by 9E10 antibodies indicating that the deletions that we made had no impact on membrane topology of the transfected proteins (result not shown).

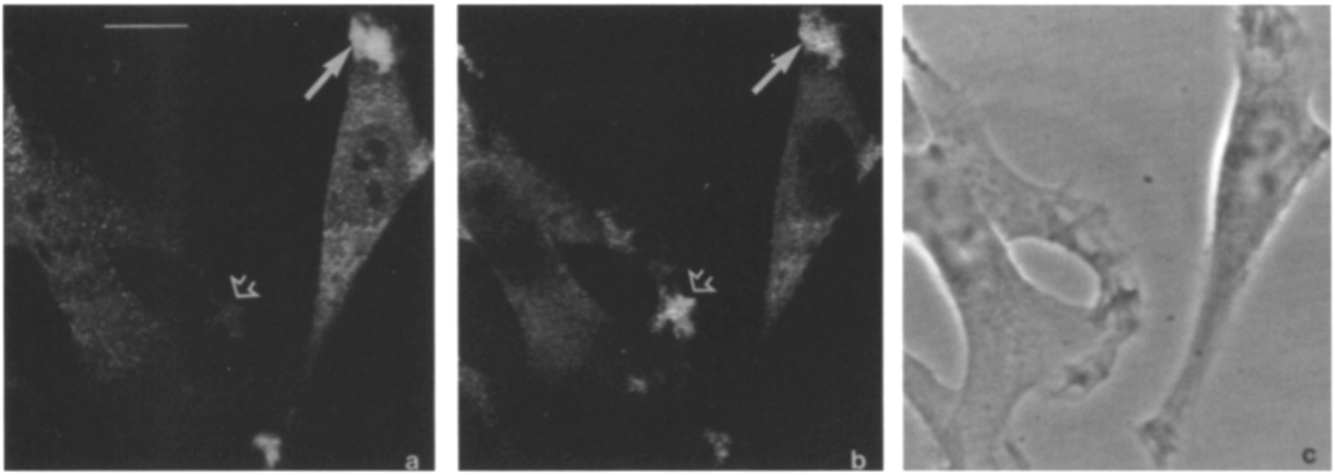
#### **D1 Protein Colocalizes with $\alpha 6\beta 4$ Integrin in FG cells**

FG cells are derived from a human pancreatic carcinoma and possess the hemidesmosome associated integrin  $\alpha 6\beta 4$  (Tamura et al., 1990). This integrin heterodimer shows

basal polarization and is concentrated in streaks and in rows of spots towards the edge of the cells (Tamura et al., 1990). Unlike 804G cells, FG cells do not express BP180 or BP230 as determined both by immunoblotting and by immunofluorescence (Hopkinson and Jones, 1994; Fig. 5 B). To determine whether D1 protein is able to associate with  $\alpha 6\beta 4$  integrin in the absence of BP230, we transfected them with the construct containing the D1 cDNA. Since 9E10 recognizes endogenous c-myc in FG cells, we used an antiserum (J17) that we have generated against the NH<sub>2</sub> terminus of BP180 to assess expression of D1 protein in the transfected cells by immunoblotting and immunofluorescence (Hopkinson et al., 1992). J17 antibodies recognize a 58-kD polypeptide in the transfected FG cells but fail to recognize any proteins in vector alone transfected cells (Fig. 5 C). Furthermore, D1 polarizes in FG cells and appears concentrated along the basal surface of FG cells



**Figure 6.** FG cells transfected with D1 were processed for double label immunofluorescence using an NH<sub>2</sub>-terminal monoclonal BP180 antibody (Hopkinson et al., 1992)(a) and a  $\beta 4$  integrin rabbit antiserum (b). Note that both antibody preparations generate similar staining patterns. (c) Phase contrast image. Bar, 25  $\mu$ m.



**Figure 7.** Double label immunofluorescence was performed on HT1080 cells transfected with D1 using J17 antiserum against the NH<sub>2</sub> terminus of BP180 (a) and rat mAb against the  $\alpha 6$  integrin subunit (b). The solid arrows indicate colocalization of D1 protein and  $\alpha 6$  while the open arrow indicates a region of  $\alpha 6$  staining in a nontransfected cell. (c) Phase contrast image. Bar, 25  $\mu\text{m}$ .

towards the cell periphery. Indeed, the D1 protein primarily codistributes with  $\beta 4$  integrin (Fig. 6).

#### ***D1 Protein Colocalizes with $\alpha 6\beta 1$ Integrin in HT1080 Cells***

HT1080 cells express the integrin heterodimer  $\alpha 6\beta 1$  but not  $\alpha 6\beta 4$  and fail to express endogenous BP180 (Lin et al., 1993; Fig. 5, B and E). In HT1080 cells,  $\alpha 6$  localizes in patches towards the edges of cells (Fig. 7 b). We have observed this same pattern of distribution using two rat monoclonal (GoH3 and 135-13C) and affinity-purified rabbit antibodies against  $\alpha 6$ .

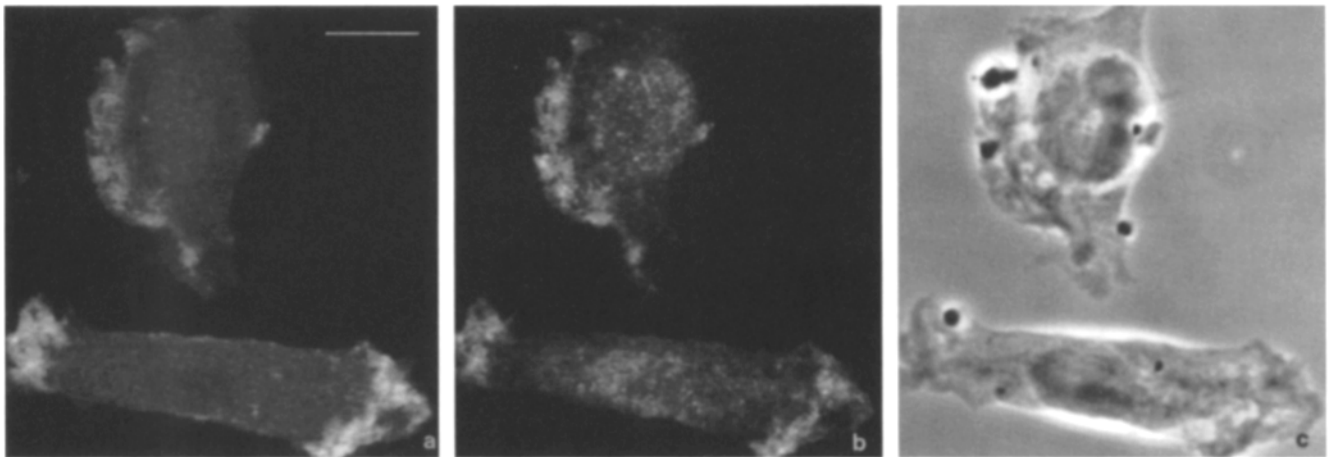
The fate of D1, D1-36N, and D1-27C mutant BP180 proteins was evaluated in HT1080 transfected cells by immunofluorescence. Since HT1080 cells fail to express endogenous BP180, we could use our antibodies directed against the NH<sub>2</sub> terminus of BP180 to detect transfected protein. In D1 HT1080 transfectants processed for double labelling, D1 protein colocalizes with the  $\alpha 6$  (Fig. 7). To satisfy ourselves that the localization of D1 was consistent

with its interacting with  $\alpha 6$  integrin we also transfected HT1080 cells with a vector encoding full length human  $\beta 4$  integrin. The latter is known to preferentially bind  $\alpha 6$  integrin in  $\beta 1$  integrin-containing cells (Giancotti et al., 1992). In  $\beta 4$  integrin HT1080 transfectants, the transfected protein associates with  $\alpha 6$  integrin in an identical manner to that observed in the D1 transfected cells (Fig. 8).

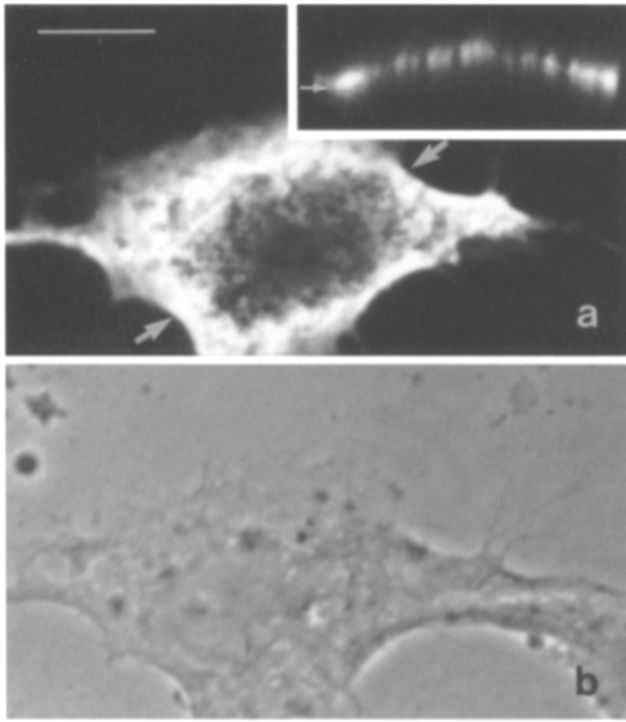
In contrast to both D1 and  $\beta 4$  integrin, D1-36N and D1-27C proteins do not associate with  $\alpha 6$  integrin in HT1080 cell transfectants (Figs. 9 and 10). Rather, as is the case in 804G cells, D1-36N protein polarizes apically in HT1080 cells while D1-27C shows a punctate stain along the entire basal aspect of transfected cells (Figs. 9 and 10). To confirm expression of mutant protein in the transfected HT1080 cells, immunoblotting was undertaken (Figs. 5, D and E).

#### ***D1 Protein Coimmunoprecipitates with $\alpha 6$ in HT1080 Cells***

To more rigorously examine the possibility of an association between BP180 and  $\alpha 6$  containing integrin hetero-



**Figure 8.** HT1080 cells, transfected with the  $\beta 4$  integrin subunit, were processed for double label immunofluorescence using 3E1 antibodies against  $\beta 4$  integrin (a) and rat monoclonal antibodies against  $\alpha 6$  (b). Note the colocalization of the two proteins. (c) Phase contrast image. Bar, 25  $\mu\text{m}$ .



**Figure 9.** D1-36N transfected HT1080 cells were processed for immunofluorescence using J17 antibodies (a). A z-section (between solid arrows) illustrating apical polarization of the transfected protein is shown in the inset in a. In the inset the substrate is marked by the arrow. (b) Phase contrast image. Bar, 25  $\mu$ m.

dimers, coprecipitation analyses using HT1080 transfected cells were undertaken. HT1080 cells transiently transfected with D1 and D1-27C were incubated in [<sup>35</sup>S]methionine-supplemented media. After 3 h, cells were lysed and processed for immunoprecipitation using the  $\alpha$ 6 rat mAb GoH3 as described in Materials and Methods. Samples were separated by SDS-PAGE and either transferred to nitrocellulose, or dried and exposed to radiographic film. As shown in Fig. 11, GoH3 precipitates the heavy chain of  $\alpha$ 6 integrin migrating at 125 kD from reduced extracts of both D1 and D1-27C HT1080 transfectants. When these samples were analyzed by Western blotting using the anti-BP180 serum J17, a single polypeptide of 58 kD is detected in the immunoprecipitate of the D1 transfected cells whereas J17 antibodies show no reactivity with the precipitate prepared from D1-27C transfectants.

## Discussion

BP180 possesses the classic properties of a cell adhesion molecule. It localizes to the cell surface and antibodies against its external domain perturb epidermal cell adhesion (Klatte et al., 1989; Hopkinson et al., 1992; Liu et al., 1994). BP180 is, however, an unusual protein not only in that it is a hybrid collagen but also because of its type II orientation in the plasma membrane (Giudice et al., 1991, 1992; Hopkinson et al., 1992).

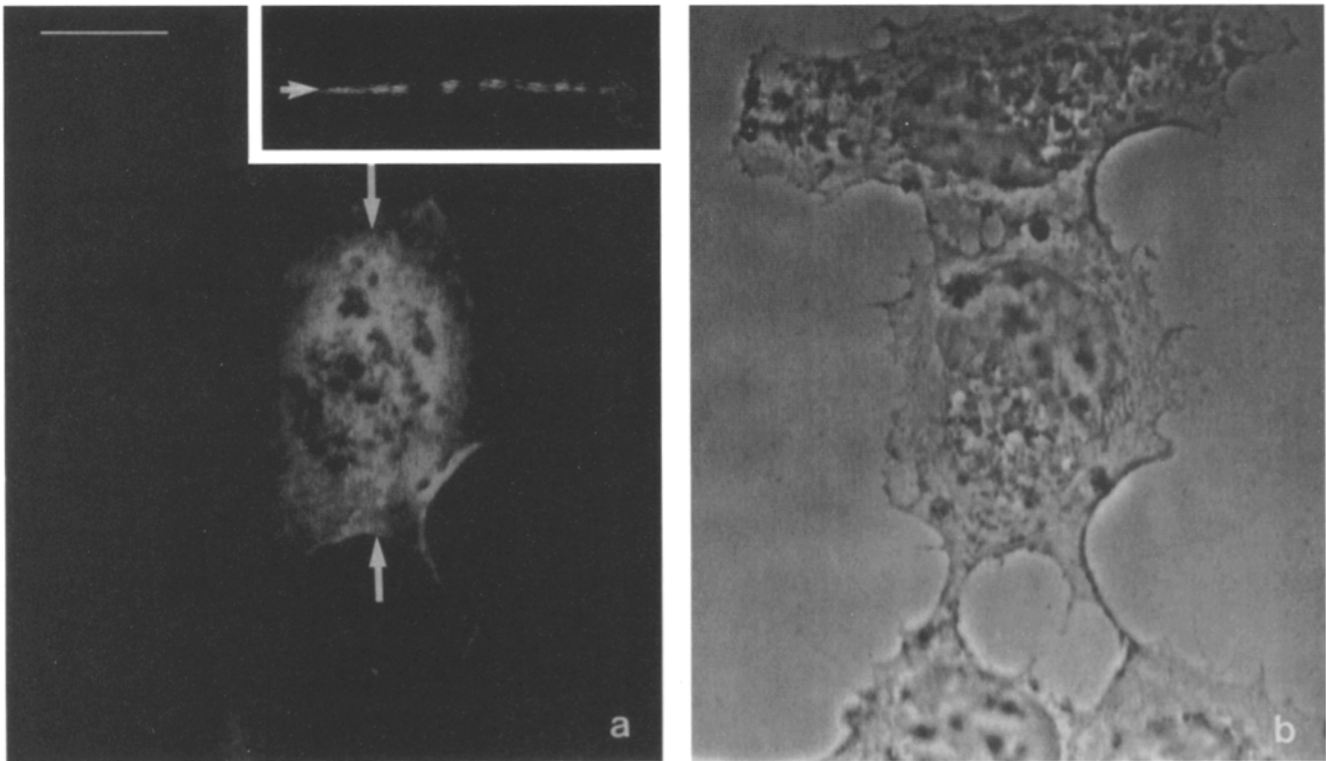
We have used molecular genetics to characterize important functional domains within BP180. 804G cell transfection data reveal that there is sufficient "information" en-

coded in the sequence of a polypeptide we term D1, comprised of the cytoplasmic and transmembrane domains as well as a short extracellular stretch of 30 residues of BP180, for its targeting to membranes, for its polarization within the cell surface and for its association with other hemidesmosomal elements such as BP230 and the  $\alpha$ 6 $\beta$ 4 integrin heterodimer. The collagen-like sequences of BP180 are not required for these properties of BP180.

To map potential membrane targeting domains and protein-protein interaction sites within D1, we made a series of deletions and expressed them in 804G cells. When the NH<sub>2</sub>-terminal 36 residues of D1 are removed, D1-36N protein becomes concentrated in the apical cell membrane in both 804G and HT1080 transfectants. This indicates that the NH<sub>2</sub>-terminal 36-amino acid cytoplasmic tail of BP180 is necessary for its correct basal polarization. In this regard, it is interesting to note that Kundu and Nayak (1994) have recently shown that the cytoplasmic tail of another type II protein, namely, the human transferrin receptor, also possesses a membrane baso-lateral targeting signal. The importance of the NH<sub>2</sub> terminus of type II proteins in membrane localization is also seen in a recent study by Parks and Lamb (1993) who have shown that the cytoplasmic domain of the type II membrane protein, paramyxovirus HN polypeptide, determines protein topography in the membrane. However, we emphasize that BP180 is quite distinct from these other type II proteins. Firstly BP180 possesses an extensive cytoplasmic domain of 400 amino acids while the paramyxovirus HN polypeptide and the transferrin receptor have only short cytoplasmic domains consisting of 17 and 61 amino acids, respectively. Also, BP180 is also a component of a large macromolecular entity that shows basal membrane polarization. Thus, there are far more restraints on BP180 than on both the paramyxovirus HN polypeptide and transferrin receptor with regard to its membrane localization.

In contrast to D1-36N, D1-27C protein, where the extracellular noncollagenous COOH-terminal most 27 residues of D1 are removed, can be detected along sites of cell-substrate interaction in 804G cells i.e., is correctly polarized. However, D1-27C fails to colocalize with other hemidesmosomal elements such as BP230. Indeed, these 27 extracellular amino acids of BP180, a highly charged domain containing eight basic and six acidic residues, would appear to be an important site of extracellular interaction between BP180 and other components of the hemidesmosome.

To identify candidate molecules that could interact with BP180, we made use of two cell lines that fail to express one or more of the known components of hemidesmosomes. In particular, we used FG cells, which lack BP230 and BP180, and HT1080 cells, a fibrosarcoma line, which lack not only BP230 and BP180, but also the  $\beta$ 4 integrin subunit (Lin et al., 1993; Hopkinson and Jones, 1994). Yet despite these deficiencies, in both FG and HT1080 cells, D1 protein clearly colocalizes with the  $\alpha$ 6 integrin regardless of its  $\beta$  partner. Moreover, D1 protein coprecipitates with the  $\alpha$ 6 integrin subunit in HT1080 transfectants suggesting the possibility that BP180 may bind  $\alpha$ 6 integrin. Since D1-27C fails to colocalize with  $\alpha$ 6 integrin in all the cell types we have analyzed even though it polarizes correctly, we further speculate that BP180- $\alpha$ 6 interaction occurs extracellularly. Of course, bear in mind that we cannot



**Figure 10.** HT1080 cells were transfected with D1-27C and processed for immunofluorescence using J17 antibodies (a). The inset shows a z-section generated between the arrows. The arrow in the inset shows the plane of the substrate. Note the basal concentration of fluorescence in the z-section. (b) Phase contrast image. Bar, 25  $\mu\text{m}$ .

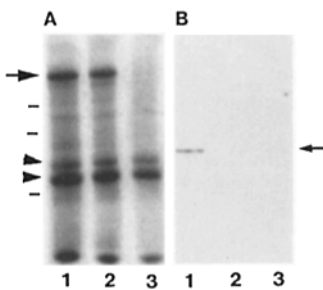
rule out that this interaction may involve other proteins. Nonetheless, to our knowledge this would be the first described extracellular association between an integrin heterodimer and another membrane protein. Whether this in some way modulates  $\alpha 6\beta 4$  integrin–ligand binding is the object of our current studies.

There is some circumstantial support for an association between  $\alpha 6$  and BP180 in another experimental system. In normal mouse skin  $\alpha 6\beta 4$  integrin, BP180 and BP230 localize to the basal cell epidermal–dermal border (unpub-

lished). When treated with retinoic acid, both  $\alpha 6$  and BP180 dramatically reorganize in mouse epidermis and become localized suprabasally. This is independent of other hemidesmosome elements such as  $\beta 4$  integrin and BP230 that remain restricted to the epidermal–dermal border (Tennenbaum T., A. Mroz, A. Belanger, T. Wildnauer, J. C. R. Jones, and J. S. Gordon. 1994. *J. Invest. Dermatol.* 102:555.).

Spinardi et al. (1993), have also used 804G cells as a molecular genetic system for studying hemidesmosomal protein interactions. Their results suggest some interesting parallels between BP180 and the  $\beta 4$  integrin subunit with regard to both targeting of protein to the hemidesmosome as well as  $\beta 4$  association with  $\alpha 6$  integrin. They analyzed sequences of  $\beta 4$  integrin that are required for its incorporation into the 804G cell hemidesmosomes. As is the case for BP180, a specific stretch of 303 amino acids in the cytoplasmic domain of  $\beta 4$  integrin is necessary for its incorporation into hemidesmosomes while the extracellular domain of  $\beta 4$  is apparently required for interaction with its obligate partner  $\alpha 6$  (Spinardi et al., 1993).

It is intriguing to relate our results with the studies of Liu et al. (1993). These workers generated antibodies against 14 amino acids (residues 508–521) in the extracellular noncollagenous region (NCE domain) of BP180 (Giudice et al., 1993). Such antibodies induce disruption in epidermal cell–dermis interactions in neonatal mice. All but one of these amino acids (RSILPYGDSMDRI) are contained within the 27–amino acid stretch of D1 that appear necessary for a BP180– $\alpha 6$  interaction. Furthermore, over 60% of BP sera contain human autoantibodies that



**Figure 11.** An autoradiograph of GoH3 immunoprecipitates of radiolabeled HT1080 cells transfected with D1 or D1-27C is shown in panel A (lanes 1 and 2, respectively). Lane 3 shows a control immunoprecipitate of radiolabeled HT1080 D1 transfectants in which the primary antibody, GoH3, was omitted. A 125-kD labeled species, corresponding to  $\alpha 6$ , is observed in lanes 1 and 2 only (arrow). Note that two radiolabeled polypeptides (arrow heads) are nonspecifically precipitated in all three samples. The same three immunoprecipitates were processed for immunoblotting using antiserum J17, followed by horse radish peroxidase conjugated goat anti-rabbit secondary antibody (B). Reactive species were visualized using chloro-L-naphthol substrate (Zackroff et al., 1984). An arrow marks a single reactive species of 58 kD in the D1 precipitate (lane 1). This species is absent in lanes 2 and 3. Dashed lines indicate molecular masses of 97, 66, and 45 kD.

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also recognize epitopes within this same series of residues (Giudice et al., 1993). Such autoantibodies are considered to be pathogenic i.e., capable of inducing blistering that characterizes the disease. We speculate that such antibodies, when bound to BP180, perturb the interaction between BP180 and  $\alpha 6$ , thereby inducing the hemidesmosome to "fall apart." This would parallel the disruption of hemidesmosomes seen in various model systems where epithelial cells have been treated with antibodies that recognize external domains of either the  $\alpha 6$  or the  $\beta 4$  integrin subunits (Kurpakus et al., 1991).

In summary, we have used molecular genetics to identify two functionally important sequences within the noncollagenous domains of the BP180 molecule. The  $\text{NH}_2$ -terminal sequence is involved in protein polarization while the COOH-terminal noncollagenous 27 amino acids is necessary for colocalization with other hemidesmosomal elements. Our studies have not only provided new insights into potential protein-protein interactions in the hemidesmosome but also indicate a potential mechanism for the pathogenesis of autoantibodies in a blistering skin disease.

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