# The Localization of Bullous Pemphigoid Antigen 180 (BP180) in Hemidesmosomes Is Mediated by Its Cytoplasmic Domain and Seems to be Regulated by the β4 Integrin Subunit

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***Abstract.*** Bullous pemphigoid antigen 180 (BP180) is a component of hemidesmosomes, i.e., cell-substrate adhesion complexes. To determine the function of specific sequences of BP180 to its incorporation in hemidesmosomes, we have transfected 804G cells with cDNA-constructs encoding wild-type and deletion mutant forms of human BP180. The results show that the cytoplasmic domain of BP180 contains sufficient information for the recruitment of the protein into hemidesmosomes because removal of the extracellular and transmembrane domains does not abolish targeting. Expression of chimeric proteins, which consist of the membrane targeting sequence of K-Ras fused to the cytoplasmic domain of BP180 with increasing internal deletions or lacking the $NH_2$ terminus, indicates that the localization of BP180 in hemidesmosomes is mediated by a segment that spans 265 amino acids. This segment comprises two important regions located within the central part and at the $NH_2$ terminus of the cytoplasmic domain of BP180.

To investigate the effect of the α6β4 integrin on the subcellular distribution of BP180, we have transfected COS-7 cells, which lack α6β4 and BP180, with cDNAs for BP180 as well as for human α6A and β4. We provide evidence that a mutant form of BP180 lacking the collagenous extracellular domain as well as a chimeric protein, which contains the entire cytoplasmic domain of BP180, are colocalized with α6β4. In contrast, when cells were transfected with cDNAs for α6A and mutant forms of β4, either lacking the cytoplasmic COOH-terminal half or carrying phenylalanine substitutions in the tyrosine activation motif of the cytoplasmic domain, the recombinant BP180 molecules were mostly not colocalized with α6β4, but remained diffusely distributed at the cell surface. Moreover, in cells transfected with cDNAs for α6A and a β4/β1 chimera, in which the cytoplasmic domain of β4 was replaced by that of the β1 integrin subunit, BP180 was not colocalized with the α6β4/β1 chimera in focal adhesions, but remained again diffusely distributed. These results indicate that sequences within the cytoplasmic domain of β4 determine the subcellular distribution of BP180.

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Hemidesmosomes (HD)[1] are multi-protein complexes, that mediate adhesion of epithelial cells to the underlying basement membrane, thereby linking elements of the cytoskeleton to the extracellular matrix in stratified epithelia (1, 12). Ultrastructurally, HD appear as tripartite membrane-associated electron dense plaques associated with the keratin intermediate filament (IF) network.

Several molecular components of HD have recently been identified. However, their exact interactions and their function in the formation of HD are not completely understood (1, 12). The cytoplasmic components of HD include the bullous pemphigoid antigen 230 (BP230, also termed bullous pemphigoid antigen 1) (49), IFAP300 (44), plectin/HD1 (6, 14, 57), and P200 (25). All these proteins have been implicated in the attachment of the IF to the hemidesmosomal plaque (1, 12). In BP230 knockout mice, HD lack the inner plaque and they are not associated with the IF, suggesting that BP230 is required to anchor the IF to the HD (13). It is possible that plectin and HD1 are the same protein, because of their similar molecular weight and tissue distribution (1, 6, 14, 57). The observation that both plectin and HD1 are absent in a subset of patients with epidermolysis bullosa (EB), which represents a group

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1. *Abbreviations used in this paper*: BP180, bullous pemphigoid antigen 180; BP230, bullous pemphigoid antigen 230; EB, epidermolysis bullosa; FNIII, type III fibronectin repeat; HD, hemidesmosomes; IF, intermediate filament; TAM, tyrosine activation motif.

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of hereditary disorders characterized by fragility of the skin and blistering in response to trauma, supports this contention (7, 33). However, comparison of the cDNA sequence of HD1 and of plectin is required to confirm their identity. Two transmembrane proteins have been characterized in HD, the α6β4 integrin (26, 45, 50) and the bullous pemphigoid antigen 180 (BP180, also termed bullous pemphigoid antigen 2 or type XVII collagen) (10, 19, 27, 29). The α6β4 integrin appears to be crucial for cell adhesion and the formation of HD; since (*1*) antibodies directed against the α6β4 integrin inhibit the assembly of HD and disrupt existing ones, and induce dermo-epidermal separation in vitro (26); (*2*) the formation of HD can be prevented by inhibition of the phosphorylation of β4 (9, 30, 31); (*3*) in humans, mutations of β4 cause junctional EB associated with pyloric atresia, a severe form of EB (36, 56); (*4*) finally, null-mutant mice for either β4 or α6 show widespread dermo-epidermal separation and lack HD (8, 55). α6β4-mediated functions are probably regulated by sequences within the large cytoplasmic domain of β4 (9, 30) which comprises two pairs of type III fibronectin repeats (FNIII) separated from each other by a connecting segment (17, 51). The other transmembrane protein, BP180, also contributes to the maintenance of dermo-epidermal integrity. Defects in the gene for BP180 have been described in generalized atrophic benign EB, a human disease in which dermo-epidermal cleavage occurs in the lamina lucida and, ultrastructurally, HD appear to be incompletely formed (21, 32). Furthermore, patients with the blistering skin disorder called bullous pemphigoid have frequently circulating autoantibodies against epitopes on the BP180 ectodomain, that may cause dermo-epidermal cleavage (11, 27). BP180 is a type II transmembrane protein. Its extracellular COOH-terminal amino acid sequence contains 15 interrupted collagenous domains, which probably form collagen-like triple helices (10, 16). The cytoplasmic amino acid sequence is highly basic and contains several potential phosphorylation sites (10, 19). The cytoplasmic domain of BP180 might play an important role in the organization of the cytoskeleton and the assembly of HD. Transfection studies with mutant forms of BP180 have identified two segments within BP180, one located in the intracellular $NH_2$-terminal and another in the extracellular membrane-proximal region, that may be important for the integration of this protein into HD (18).

We here report experiments to investigate (*1*) the function of specific regions of human BP180 in the recruitment of this protein into HD; and (*2*) the ability of BP180 to interact with α6β4. We have transfected 804G cells with wild-type and deletion mutants of the cDNA for BP180 as well as cDNA-constructs encoding chimeric proteins composed of different portions of the cytoplasmic tail of BP180 linked to the membrane localization sequence of the K-Ras protein. In contrast to the above-mentioned study (18) our results indicate that sequences within the cytoplasmic domain of BP180 are sufficient for HD targeting. Furthermore, by cotransfecting COS-7 cells, which do not form HD, with cDNAs for the human α6A and β4A integrin subunits, we demonstrated that these proteins form distinct junctional structures, in which recombinant BP180 molecules are colocalized with the α6β4 integrin. Our results suggest that the codistribution of BP180 with α6β4 is regulated by sequences contained within the COOH-terminal half of the β4 tail including the third and fourth FNIII and a part of the connecting segment. Specifically, signals mediated by the tyrosine activation motif (TAM) located within the connecting segment seem to be required to efficiently coordinate the codistribution of BP180 with α6β4.

# *Materials and Methods*

## *Cell Culture and Antibody Preparation*

The 804G cell line and the African monkey kidney cell line COS-7 were cultured in DMEM (Gibco BRL, Paisley, UK) supplemented with 10% (vol/vol) bovine FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. The 804G cell line derived from a rat bladder tumor has been described previously (40, 41). The cells were grown at 37°C in a humidified, 5% $CO_2$ atmosphere.

The following antibodies were used. The mouse IgG1 mAb anti-FLAG™M2 against the FLAG™ peptide (DYKDDDDK) was purchased (IBI, Eastman Kodak Company, New Haven, CT). The rabbit antiserum J17 directed against the intracellular portion of BP180 was kindly provided by Dr. J.C.R. Jones (Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL) (18, 19). The mouse IgG1 mAb 1A8c directed against the intracellular portion of BP180, and the mouse mAb 121 directed against HD1 were kindly donated by Dr. K. Owaribe (Department of Molecular Biology, Nagoya University, Nagoya, Japan) (14, 38). The mouse mAb 450-10D against the cytoplasmic domain of β4 was kindly supplied by Dr. S.J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN) (22). The rat mAb GoH3 against an extracellular epitope of human α6 (47), a rabbit antiserum against the cytoplasmic domain of β4 (34), a rabbit antiserum against the COOH-terminal portion of α6A (5) as well as a rabbit antiserum to rat IgG (46) have been described previously. The mAb against vinculin, clone VIN-11-5, was purchased (Sigma Chem. Co., St. Louis, MO). A rabbit antiserum against the COOH-terminal domain of BP230, was kindly provided by Dr. J.R. Stanley (Department of Dermatology, University of Pennsylvania, Philadelphia, PA) (52). Species specific FITC-conjugated goat anti–mouse IgG (Nordic Immunochemicals Laboratory, Tilburg, The Netherlands) and Texas red–conjugated donkey anti–rabbit IgG (Amersham International plc, Buckinghamshire, UK) were purchased.

## *cDNA-Constructs*

The BP180 nucleotide and protein sequences are numbered according to Giudice et al. (10) and Hopkinson et al. (19), respectively. Full-length BP180 was obtained from human keratinocyte RNA by reverse transcriptase-PCR with primers based on published sequence of human BP180 (GenBank accession number M91669) (10) using the Riboclone cDNA Synthesis Kit (Promega, Madison, WI), the RNA-PCR Kit (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NY), and the PCR Reagent System (GIBCO-BRL). The PCR reaction consisted of 35 cycles of 15 s at 95°C, 30 s at 55°C and 90 s at 72°C. PCR products corresponding to overlapping portions of BP180 were first cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and ligated together to obtain full-length BP180 using distinct restriction sites: PflmI, StuI, SacII, ClaI and EcoRI (at nucleotide positions 566, 1307, 1694, 2548 and 3619, respectively). A PCR site-directed mutagenesis system was subsequently used to prepare the various expression plasmids (Fig. 1 *A*). To generate clone A, B, C, and D, a 5′ end primer was used that contained an EcoRI and a NotI site for cloning, sequences for optimal initiation of translation (bold) (24), sequences coding for the FLAG™ peptide (underlined) (IBI, Eastman Kodak Company) for immunodetection, as well as nucleotides corresponding to sequences between 109 and 131 of human BP180 (italic) located immediately downstream from the predicted methionine start site of BP180 (nucleotide 106-109) (18) (5′-GCCGGAATTCGCGGCCGC**CGCC**ATGGACTACAAGGACGACGATGACAAG*GATGTAACCAAGAAAAACAAAACG* 3′-). The 3′ primer of clone A contained a NotI and a XbaI restriction site and the nucleotides corresponding to sequences between 4579 and 4599 of BP180 (italic) including the stop codon (5′-CGAT GCGGCCGCTCTAGA*TCACGGCTTGACAGCAATACT* 3′-). The 3′ end primer of clone B contained a NotI and a XbaI restriction site, a stop codon, and nucleotides corresponding to sequences 1641 and 1662 of BP

180 (italic) (5′-CGATGCGGCCGC TCTAGATTA*TATTCTATCCATGCTGTCCCCA* 3′-) The 3′ primer used to generate clone C contained a NotI and a XbaI site restriction site, a stop codon, and nucleotides corresponding to sequences between 1480 and 1503 of BP180(italic) (5′-CGATGCGGCCGCTCTAGATTA*CTTCCACCAGCTGCAGCAGGAGCC* 3′-). The 3′ primer used for clone D was designed in order to generate a chimeric protein composed of the cytoplasmic domain of BP180 fused to the membrane localization sequences of K-Ras (KMSKDGKKKKKKSKTKCVIM) (GenBank accession number M54968 and M38506). This primer contained a NotI and a XbaI restriction site, a stop codon, nucleotides encoding the membrane localization sequences of the K-Ras (bold) as well as the sequence between 1479-1503 of BP180 (italic) (5′-CGATGCGGCCGCTCTAGATTA**CATAATTACACACTTTGTCTTTGACTTCTTTTTCTTCTTTTTACCATCTTTGCTCATCTT***CTTCCACCAGCTGCAGCAGGAGCC* 3′-). The clones E, F, and G, which encoded chimeric proteins with increasing internal truncations of the cytoplasmic portion of BP180 starting from amino acid 244, 201 and 113, respectively, to 402, were generated by partial digestion of clone D with Ecl136 II (nucleotide sites 440, 701, and 830) and complete digestion with StuI (nucleotide 1307). The construct H lacking the sequences encoding amino acids 1 to 36 was generated using a 5′ primer that contained a XbaI site, sequences for optimal initiation of translation (bold) and for the FLAG™ tag (underlined), as well as nucleotides corresponding to sequences between 214 and 230 of BP180 (5′ GCCGTCTAGA**CGCC**ATGGACTACAAGGACGACGATGACAAG*AGCAATGGCTATGCTAAAACAGC* 3′-). Combined alanine substitutions at positions $Ser^{169}$, $Ser^{175}$, and $Ser^{180}$ were introduced in clone B using the overlap extension method for site directed mutagenesis as previously described (5).

The correctness of all clones was verified by sequence analysis, the various constructs were subsequently cloned using the XbaI and/or a NotI restriction site in the eukaryotic expression vector pCI-neo (Promega, Madison, WI). This vector carries a CMV enhancer/promotor for strong constitutive expression, an intron located upstream from the multiple cloning site as well as a SV40 origin for episomal replication in cell lines expressing the SV large T antigen, such as COS-7 cells. Transfection studies have indicated that an intron flanking the cDNA of interest may significantly increase the gene expression level (2, 20).

For generation of the wild-type α6A cDNA, two overlapping cDNA clones, A33 and A84, isolated from a λgt11 human keratinocyte library were used as previously described (5). The full-length α6A was inserted into the HindIII site of the pRc/CMV expression vector (Invitrogen, San Diego, CA) (Fig. 1 *B*) (5).

For generation of full-length β4A cDNA, a cDNA clone isolated from a λgt11 human keratinocyte library was used that encodes β4A from position 1880 to the 3′ terminus. The λ DNA was digested with SfiI and the resulting 2.2-kb fragment was exchanged for the 2.35-kb SfiI fragment of β4B (35, 37). The cDNA was then ligated into the XbaI site of the pRc/CMV vector (Invitrogen). The cDNA-construct encoding a truncated β4 molecule, clone $\beta4^{1382}$, was obtained by exchanging a PCR fragment with a wild-type fragment of β4 and will be described in detail elsewhere. After sequence analysis, the $\beta4^{1382}$ cDNA construct was subcloned into pcDNA3 (Invitrogen) (Fig. 1 *B*). The cDNA plasmid encoding a β4 with combined phenylalanine substitutions of the tyrosine activation motif (30) was kindly provided by Dr. F.G. Giancotti (Department of Pathology, New York University School of Medicine, New York, NY). The construct was assembled into pcDNA3. Construction of the β4/β1 cDNA encoding a chimeric protein, in which the cytoplasmic domain of β4, with the exception of a 19–amino acid stretch immediately close to the transmembrane domain, was replaced with that of β1, has been recently described. The β4/β1 cDNA was inserted into the XbaI site of the pcDNA-1Hyg expression vector (43).

The following cDNA constructs were used as a control for transfection. A full-length murine BP180 cDNA (29), provided by Dr. J. Uitto (Department of Dermatology, Thomas Jefferson University, Philadelphia, PA), was subcloned into the EcoRI site of pcDNA3. A full-length CD31 cDNA, provided by Dr. B. Seed (Massachusetts General Hospital, Boston, MA), was subcloned into the HindIII/NotI sites of pRc/CMV. A full-length CD8 cDNA, provided by Dr. A. Kelly, Guy's and St. Thomas's Medical and Dental School, London, UK), was subcloned into the HindIII and BamH1 site of pcDNA3.

## Transfection Experiments

For transfection, cells were first grown to 50–80% confluency in six-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). The rat bladder carcinoma cell line 804G was transfected using a cationic lipid, Lipofectin® (1:1-N-[1-(2,3-dioleyloxy) propyl]-n,n,n-trimethylammonium chloride and dioleoyl phosphatidylethanolamine (GIBCO-BRL). The DNA:Lipofectin® mixture was prepared using serum free medium (OPTI-MEM®, GIBCO-BRL). The final concentration of plasmid DNA and Lipofectin® in serum free transfection medium was 5 μg/ml and 20 μg/ml, respectively. 1 ml of transfection medium was added to each monolayer that had been previously washed with serum free medium and cells were incubated with the transfection medium for 12–18 h at 37°C with 5% $CO_2$. The transfection medium was then replaced with normal growth medium and cells were incubated for additional 24–36 h and then assayed for gene expression. COS-7 cells were transfected using the DEAE-dextran method (4) and assayed for gene expression after 36 h.

## Western Blot Analysis

Cells were lysed with 1% SDS in 25 mM Tris-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. Protein concentration in the cell lysates was determined with the BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein were loaded on a 8.5% or 13.5% SDS polyacrylamide gel, separated, and electrophoretically transferred to nitrocellulose sheets for 1 h at 240 mA in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (vol/vol) methanol and 0.0375% SDS as previously described (53). The filters were incubated in TBST (10 mM Tris-HCl, pH 7.6, and 150 mM Nacl, 0.1% Tween-20) containing 2% (wt/vol) BSA and 2% (wt/vol) baby milk powder for 12 h at 4°C. Subsequently the filters were probed with the primary antibody for 90 min, after which they were washed four times for 5 min in TBST. The filters were subsequently incubated with horseradish peroxidase–linked sheep anti–mouse IgG or horseradish peroxidase–linked goat anti–rabbit IgG (Amersham International plc), diluted to 1:5,000 and 1:2,000, respectively, for 1 h and then washed with TBST. Proteins were visualized using enhanced chemiluminescence (Amersham International plc).

## Immunofluorescence Microscopy

Cells grown on glass coverslips in six-well tissue culture plates were fixed with 1% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 5 min. After rinsing with PBS and blocking with 2% (wt/vol) BSA in PBS for 30 min, the cells were incubated with primary antibody for 30 min at 37°C, then washed three times with PBS. The cells were stained with fluorescein- or Texas red–labeled anti–mouse IgG, anti–rat IgG or rabbit IgG for 30 min at 37°C. The coverslips were subsequently washed, mounted with Vectashield (Vector, Burlingame, CA), and viewed under an MRC-600 confocal scanning laser microscope (Bio-Rad Laboratories, Richmond, CA).

## Cell Labeling and Immunoprecipitation

Transfected COS-7 cells were washed twice with PBS and incubated with DMEM without methionine and cysteine (ICN Biomedicals Inc., Costa Mesa, CA) for 1 h at 37°C. Cells were then labeled with 100 μCi/ml [$^{35}S$]methionine/cysteine (Amersham International plc) for 4 h, then washed and lysed with 1% NP-40 in 25 mM Tris-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Cells were also lysed with 1% Triton X-100, 2 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor or 10 mM CHAPS in HBSS. The lysates were clarified by centrifugation at 10,000 *g* and precleared with protein A–Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Samples of precleared lysates were immunoprecipitated with antibodies previously bound to protein A–Sepharose or to protein A–Sepharose to which rabbit anti–mouse IgG was attached. The immunoprecipitates were subsequently analyzed by SDS-PAGE.

# Results

## Expression of Recombinant Forms of Human BP180

The role of specific sequences of BP180 in the localization of the protein in HD was investigated by introducing a series of cDNA-constructs into the epithelial cell line 804G. The 804G cells, which have previously been used to study the assembly and formation of HD, contain the structural

components of HD, i.e., proteins that may interact with BP180, including the α6β4 integrin, BP230, and HD1 (40, 41, 48). As illustrated in Fig. 1 *A*, we have generated cDNA-constructs encoding full-length (clone A) or two truncated forms of BP180, that consist of either the entire cytoplasmic domain, the transmembrane region and a segment of 30 amino acids located adjacent to the transmembrane region (clone B) or of the cytoplasmic domain alone (clone C). Clone B was used because a previous study had indicated that the protein encoded by this construct contains the minimal sequence of the ectodomain of BP180 essential for incorporation into HD (18). To further investigate the contribution of distinct cytoplasmic domains in the recruitment of BP180 to HD, we generated cDNA clones encoding chimeric proteins, in which the membrane localization sequence of K-Ras (28, 39) had been linked to various parts of the cytoplasmic region of BP180 (clones D to H). To identify proteins encoded by the transfected plasmids a 24-bp sequence encoding the FLAG™ peptide was added at the 5′ end of the cDNAs. In addition to DNA sequencing of the various constructs, the proteins encoded by the clones were analyzed by in vitro transcription/translation followed by SDS-PAGE and found to be of the predicted mol wt (not shown). Finally, to assess whether the transiently transfected 804G cells expressed the appropriately sized recombinant BP180 molecules, we performed immunoblot analysis of cell extracts (Fig. 2) using mAbs directed against the FLAG™ peptide as well as against the cytoplasmic domain of BP180. In transfected cells, the apparent mol wt of the recombinant proteins were close to the sizes predicted on the basis of the corresponding cDNA sequence, i.e., 151.6 kD, 55.7 kD, 49.4 kD, 51.7 kD, 35.3 kD, 30.9 kD, 21.4 kD, and 47.9 for clones A, B, C, D, E, F, G, and H, respectively. The size of the protein encoded by clone A, corresponding to full-length human BP180, was slightly smaller than that of endogenous rat BP180, possibly due to insufficient posttranslational processing of this protein and/or a species difference. In extracts from COS-7 cells transfected with cDNAs for either human or mouse full-length BP180, the mol wt of the expressed proteins were similar (not shown).

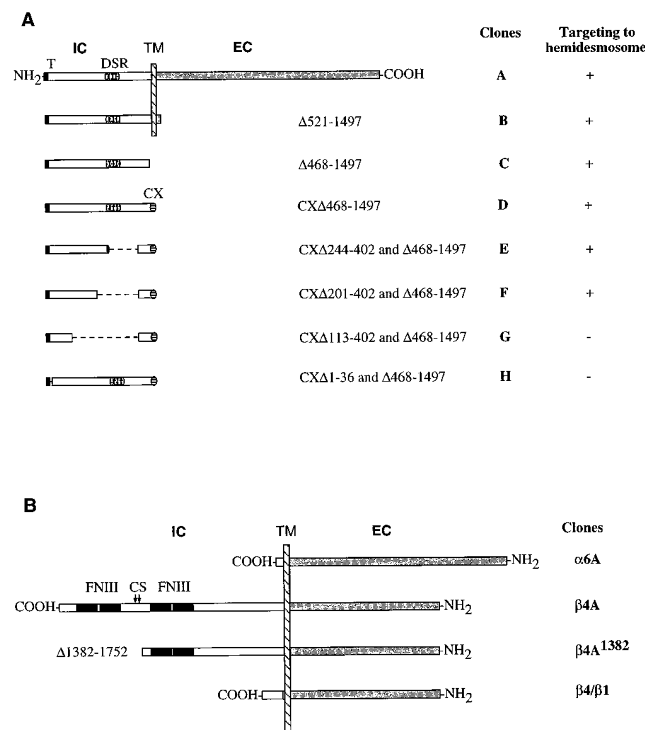



*Figure 1.* (*A*) Schematic representation of wild-type and mutant forms of BP180 with summary of their localization in 804G cells. The upper three cDNA-constructs represent wild-type (clone A) and COOH-terminal truncations of BP180 (clones B and C), while the lower five (clones D, E, F, G, and H) represent chimeric cDNA-constructs encoding the membrane localization sequence of K-Ras fused with cDNAs encoding various cytoplasmic regions of BP180. IC, intracellular domain; EC, extracellular domain; T, FLAG™ tag; DSR, degenerate set of four 24-26 residue tandem repeats; TM, membrane-spanning domain; CX, membrane localization sequences of K-Ras. Truncations introduced by cloning procedures are indicated by the segment of amino acids that were deleted (Δ). The protein sequence of BP180 is numbered according to Hopkinson et al. (18). (*B*) Schematic representation of α6A and β4A integrin subunits, of mutant form $\beta4A^{1382}$, and of the β4/β1 chimera. The mutant form $\beta4A^{1382}$ lacks the COOH-terminal half of the cytoplasmic domain, including the third and fourth type III fibronectin repeats (FNIII) and a portion of the connecting segment (CS). The β4/β1 chimera consists of the extracellular and the transmembrane domain of β4 fused to the entire cytoplasmic domain of β1. The position of the two tyrosine residues, $Tyr^{1422}$ and $Tyr^{1440}$, in the cytoplasmic domain of β4, which are part of the tyrosine activation motif, is indicated (*arrows*). The COOH-terminal truncation (Δ) is indicated by the stretch of amino acids that was removed. The protein sequence of β4A is numbered according to Suzuki and Naitoh (51).

### *Wild-Type and Mutant Forms of BP180 Lacking the Extracellular and the Transmembrane Domains Are Recruited into Hemidesmosomes of 804G Cells*

As shown by confocal laser immunofluorescence microscopy, the hemidesmosomal proteins of 804G cells are concentrated at sites of cell-substrate contact in structures appearing as dots and patches arranged in a characteristic "Swiss cheese" pattern (40, 41, 48). To investigate the distribution of the recombinant BP180 molecules, transiently transfected cells were subjected to double immunofluorescence microscopy. The mAb anti-FLAG™M2 directed against the FLAG™ peptide was used to distinguish the recombinant, tagged proteins from endogenous wild-type BP180. When 804G cells were transfected with clones A and B, mutant proteins were found at the basal side of the cells, colocalized with α6 (Fig. 3) and β4, and with BP230 (not shown) producing the typical Swiss cheese–like staining pattern. However, the clone C encoded protein, which lacks the transmembrane and extracellular domains, was not only localized in HD-like structures, but was also diffusely distributed in the cytoplasm (Fig. 3, *E* and *F*). The localization of this mutant protein was more obvious in cells with low transgene expression levels, as estimated by fluorescent staining intensities. These results indicate that the full-length (clone A) as well as the two truncated forms of BP180 (clones B and C), that lack either the whole collagenous extracellular domain or both the transmembrane and extracellular domain, are normally incorporated into HD of 804G cells. The peculiar localization of clone C en-

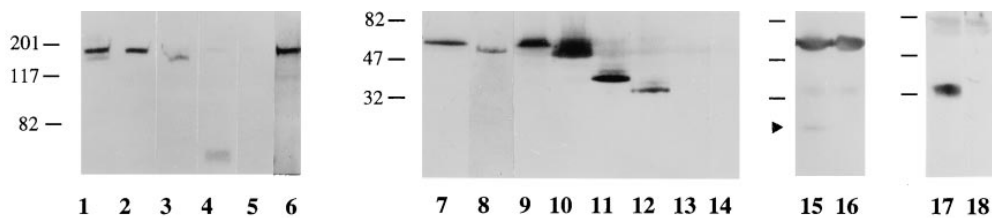



*Figure 2.* Identification of mutant forms of BP180 expressed in 804G cells by immunoblotting. Extracts of cells transfected with clones A (lanes *1* and *3*), B (lane *7*), C (lane *8*), D (lanes *4* and *9*), H (lane *10*), E (lane *11*), F (lane *12*) or G (lanes *13* and *15*) and of mock-transfected controls (lanes *2*, *5*, *14*, and *16–18*) were processed using the BP180 antiserum J17 (lanes *1* and *2*) and the mAb 1A8c (lanes *3–5*, and *7* to *14*), that are both directed against the intracellular portion of human BP180, as well as the mAb anti-FLAG™M2 against the FLAG™ tag (lanes *15* and *16*). Samples were separated by 8.5% (lanes *1–6*) and 13% (lanes *7–18*) SDS-PAGE under reducing conditions. Note that the rabbit antiserum J17 clearly recognizes a protein corresponding to endogenous wild-type BP180 (lanes *1* and *2*) with a slightly slower electrophoretic mobility than full-length human BP180 (lane *1*). The mutant protein encoded by clone G, which carried the largest deletion of the cytoplasmic domain, is not recognized by mAb 1A8c (lane *13*), but is recognized by the mAb anti-FLAG™M2 (lane *15*) (*arrow head*). Since mAb anti-FLAG generated high unspecific background (lanes *15* and *16*), only short time exposure is depicted. Mock-transfected cells were also processed using an antiserum against the cytoplasmic domain of β4 (lane *6*), an anti-α6A antiserum recognizing the light chain of the endogenous α6 (lane *17*), and a normal rabbit serum (lane *18*). Molecular weight markers are indicated in kD.

coded protein in both HD and the cytoplasm suggests that not only the cytoplasmic domain but also the transmembrane region and/or portions of the extracellular domain are required for efficient recruitment of the protein into HD; or that the amount of the recombinant protein is too large to be completely incorporated into HD. The second possibility is supported by the observation that in cells with high levels of the mutant proteins encoded by clone A or B, these proteins were not found uniquely in HD, but also localized diffusely over the cell surface (not shown).

### *A 265–Amino Acid Segment Mediates the Incorporation of BP180 into Hemidesmosomes*

To further define the function of distinct cytoplasmic domains, cDNA-constructs encoding the plasma membrane targeting sequence of K-Ras fused to various cytoplasmic parts of BP180, were expressed in 804G cells (clones D to H, Fig. 4). The K-Ras membrane localization signal, which consists of a CAAX motif (in which A means aliphatic -, and X refers to any amino acid) and a lysine-rich polybasic domain, targets proteins with high efficiency to the inner surface of the plasma membrane (28, 39). We assumed that because of the presence of the the K-ras sequence, the truncated BP180 proteins would become localized at the plasma membrane where interactions with hemidesmosomal proteins take place. Transient expression of clone D, which contained the sequence for the entire cytoplasmic domain of BP180, resulted in the incorporation of the chimeric protein in HD-like structures, where it was codistributed with endogenous α6 (Fig. 4, *A* and *B*) and β4, and with BP230 (not shown). Computer generated z-sections demonstrated that the mutant protein was concentrated at the basal cell surface (Fig. 4). Recombinant proteins encoded by clones E and F with increasing internal deletions of 159 and 202 amino acids in the cytoplasmic domain of BP180, showed also the ability to be correctly localized in HD (Fig. 4, *C* and *E*). However, in many cells these mutants were also distributed at the apicolateral cell membrane, suggesting that the amount of the recombinant proteins was too large to be completely incorporated into HD or, alternatively, that their incorporation into HDs was partially defective. These recombinant proteins lacked the degenerate set of four 24-26 residue tandem repeats (10). In contrast, the protein encoded by clone G, in which, compared to clone F, there is an additional deletion of 88 amino acids towards the $NH_2$ terminus, was never found to be codistributed with α6, but remained diffusely distributed at the cell surface (Fig. 4, *G* and *H*). Finally, the same diffuse cell membrane localization was observed with the chimeric protein encoded by clone H lacking the first 36 amino acids at the $NH_2$ terminus (Fig. 4, *I* and *J*). This short segment has previously been reported to be required for the localization of a truncated BP180 protein at the ventral surface of 804G cells (18). These results show that the chimeric protein consisting of a 265–amino acid stretch of the cytoplasmic tail of BP180 (clone F) contains sequences sufficient for the localization of the protein in HD. Furthermore, deletion of specific segments located at the $NH_2$ terminus (clone H) and within the central portion of the cytoplasmic domain of BP180 (clone G) completely prevents the recruitment of mutant forms of BP180 into HD.

### *Localization of BP180 in Hemidesmosomes Is Not Affected by Mutations at Serine Residues within the Central Portion of its Cytoplasmic Domain*

A recent study has suggested that phosphorylation at serine residues of BP180 may regulate its localization into HD (23). Analysis of clone F encoded protein, which contains the minimal sequences required for the targeting of the protein to HD, reveals the presence of several potential phosphorylation sites ($Thr^{28}$, $Ser^{41}$, $Thr^{157}$, $Ser^{169}$, $Ser^{180}$, and $Thr^{191}$) as well as a recognition sequence for $p34^{cdc2}$ kinase ($Ser^{175}$) (19). Notably, the deletion of three serine residues ($Ser^{169}$, $Ser^{175}$, and $Ser^{180}$) located in the central portion of the cytoplasmic domain of the BP180 recombinant protein encoded by clone G, prevents its incorporation into HD. To examine the role of these serine residues, we mutated the recombinant protein encoded by clone D by substituting the three $Ser^{169}$, $Ser^{175}$, and $Ser^{180}$ by alanines. As shown in Fig. 5, by immunofluorescence, this mutated BP180 molecule was distributed in a Swiss cheese–like pattern, which indicates that this recombinant protein was correctly localized in HD. Thus, phosphorylation of the

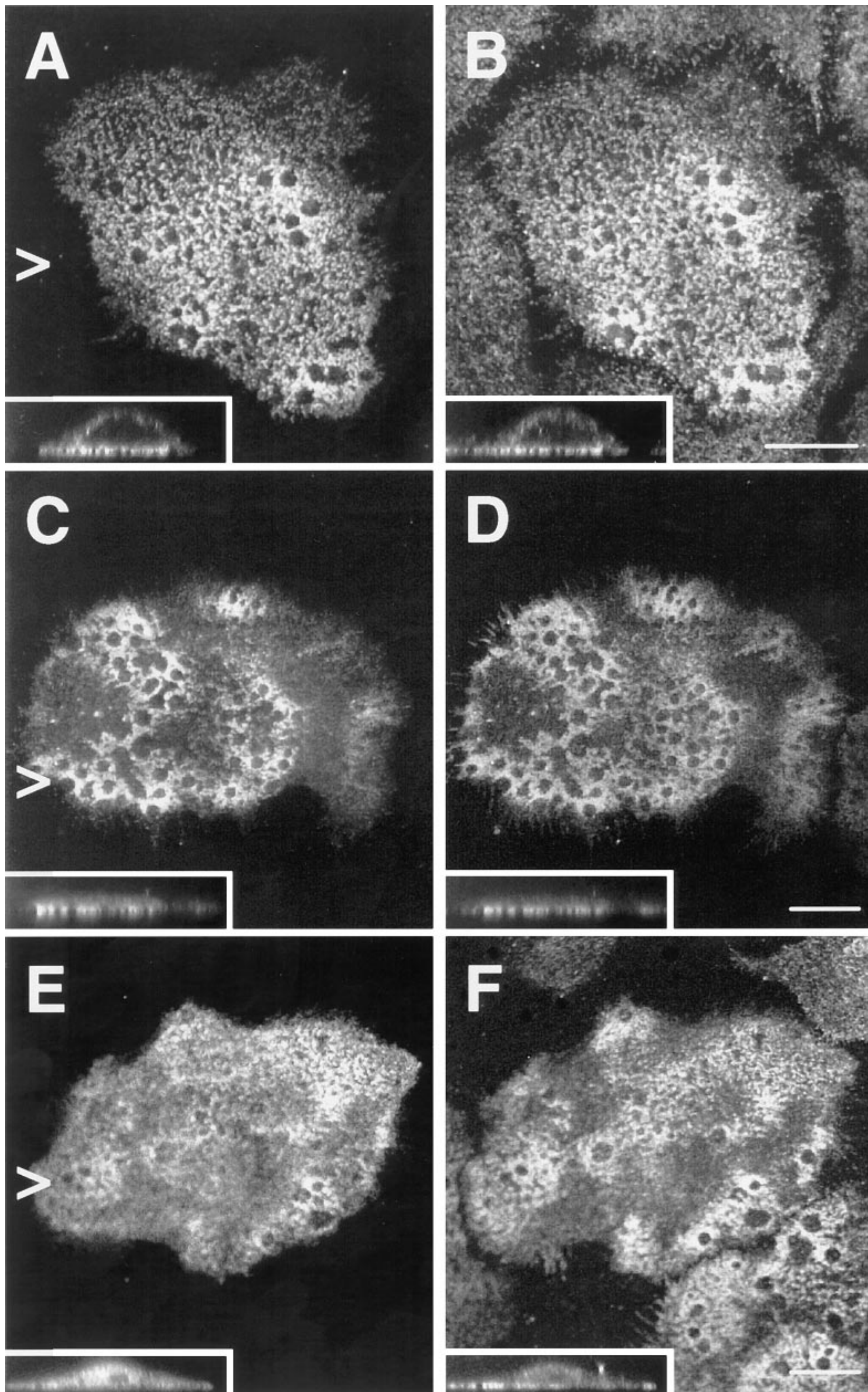



*Figure 3.* Immunolocalization of mutant forms of BP180 in hemidesmosomes of transfected 804G cells by confocal laser microscopy. Cells were grown on glass coverslips and transfected with clones A (*A* and *B*), B (*C* and *D*) or C (*E* and *F*). After 36 h, cells were fixed with 1% formaldehyde, permeabilized with 0.5% Triton X-100, and subjected to double immunofluorescence using the mAb anti-FLAG™M2 (*A*, *C*, and *E*) and an anti-α6A antiserum (*B*, *D*, and *F*). FITC-conjugated goat anti–mouse IgG (*left*) and Texas red–conjugated donkey anti–rabbit IgG (*right*). The recombinant forms of BP180 were concentrated in a Swiss cheese–like pattern characteristic for hemidesmosome-like structures, where they were colocalized with α6 along the basal cell surface as demonstrated by z-sections of individual cells (*insets*). Note that the mutant form expressed by clone C showed, in addition to its localization in hemidesmosomes, a diffuse cytoplasmic distribution (*E*). Bar, 10 μm.

above Ser residues does not seem to be required for the localization of BP180 in HD.

### *The Cytoplasmic Tail of BP180 Is Colocalized with the α6β4 Integrin and HD1 in COS-7 Cells*

Previous studies have shown that the α6β4 integrin plays an essential role in the assembly of HD (8, 26, 30, 31, 55). We next investigated the effect of the α6β4 integrin on the subcellular distribution of wild-type or mutant forms of BP180 by expressing cDNAs for these different components in COS-7 cells. COS-7 cells express HD1, the α3β1 integrin, and a small amount of α6β4, but lack BP230, BP180, and α6β4 (37) (see Fig. 9 *A*). In COS-7 cells, vincu-

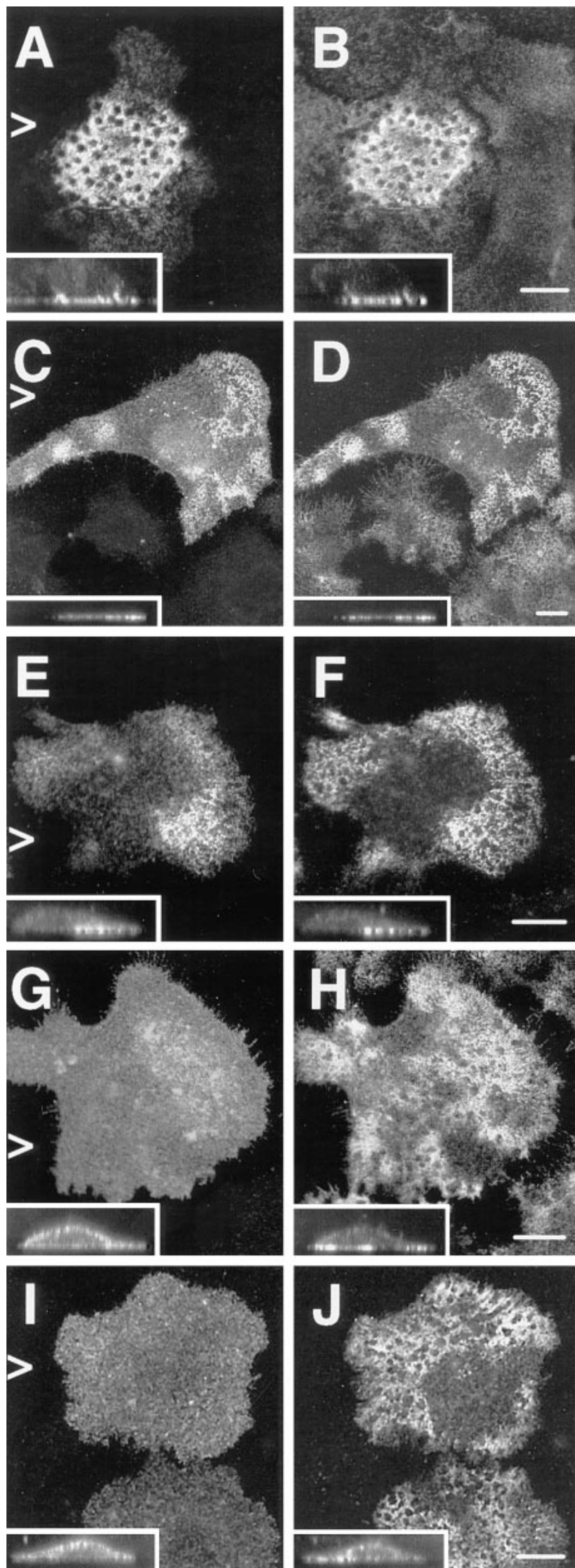



*Figure 4.* Immunolocalization of chimeric forms of BP180 composed of the membrane targeting sequence of K-Ras combined with various cytoplasmic portions of BP180 in 804G cells. Cells transfected with clones D (*A* and *B*), E (*C* and *D*), F (*E* and *F*), G (*G* and *H*), or H (*I* and *J*) were fixed, permeabilized, and subjected to double immunofluorescence using the mAb anti-FLAG™M2 (*A*, *C*, *E*, *G*, and *I*) and an anti-α6A antiserum (*B*, *D*, *F*, *H*, and *J*). FITC-conjugated goat anti–mouse IgG (*left*) and Texas red–conjugated donkey anti–rabbit IgG (*right*). In cells transfected with clones D, E, or F, the chimeric proteins are clearly codistributed with α6 and are concentrated along the basal cell surface (z-sections in the insets). In contrast, the chimeric proteins expressed by clone G (*G*) or H (*I*) are diffusely distributed at the cell surface and are not colocalized with α6. Bar, 10 μm.

lin (Fig. 6 *B*) and β1 are localized in punctate and streaky arrays at sites of cell-substrate contact which represent focal adhesions, while a similar localization of α6 was observed in only a few cells. In contrast, HD1 is found throughout the cytoplasm in a dense and delicate cytoskeletal network, similar to that previously described for plectin (Fig. 6 *D*) (37, 57).

In COS-7 cells transfected with clone A encoding wild-type BP180, the recombinant molecule remained diffusely distributed in the cytoplasm, where it was localized in granules and tubular structures, particularly prominent in perinuclear regions (not shown). Because the recombinant protein was synthesized but not expressed on the cell surface, as shown by immunoprecipitation (Fig. 9) and FACS analysis (not shown), the full-length BP180 was probably retained in the ER and/or Golgi apparatus. This was most likely due to incorrect folding and/or impaired trimer formation of this collagenous protein (16). In contrast, the mutant proteins encoded by clones B or D were properly expressed at the plasma membrane. Both proteins were distributed predominantly at the apicolateral cell surface. By double immunofluorescence microscopy, the localization of these mutant forms of BP180 was clearly different from that of vinculin (Fig. 6, *A* and *B*), β1, and HD1 (Fig. 6, *C* and *D*).

Transfection of COS-7 cells with cDNAs for α6A and β4A resulted in the expression of the α6β4 integrin at sites of cell-substrate contact and, concomitantly, in the redistribution of HD1 from the cytoskeleton into junctional complexes containing α6β4 (37) (Fig. 7, *B* and *D*, and Fig. 8 *B*). When COS-7 cells were transfected with cDNAs for α6A and a β4/β1 chimera encoding the extracellular domain of β4 fused to the cytoplasmic domain of β1, α6 was associated with β4/β1 as assessed by immunoprecipitation (not shown) and was found together with vinculin in focal contacts (see Fig. 8 *F*) (43). In these cells, HD1 remained diffusely distributed in a pattern similar to that observed in untransfected COS-7 cells.

When clone B or D cDNA was cotransfected with α6A and β4A cDNAs, the mutant BP180 proteins were concentrated at the basal cell surface and were codistributed with the α6β4 integrin (Figs. 7 and 8, *A* and *B*) and HD1 (Fig. 7, *C* and *D*), although in some cells staining of the apicolateral cell surface was still observed, in particular of the clone D encoded protein (Fig. 7, *A* and *C*). In contrast, after identical transfections with the β4/β1 chimera instead of β4, the two mutant BP180 proteins were not codistributed with α6β4/β1 in focal adhesions, but remained diffusely distributed at the cell surface (Fig. 8, *E* and *F*). These results show that the BP180 mutants encoded by clones B and D contain sequences necessary and sufficient for the codistribution of BP180 with α6β4 and HD1, and that the β4 cytoplasmic domain is involved in determining

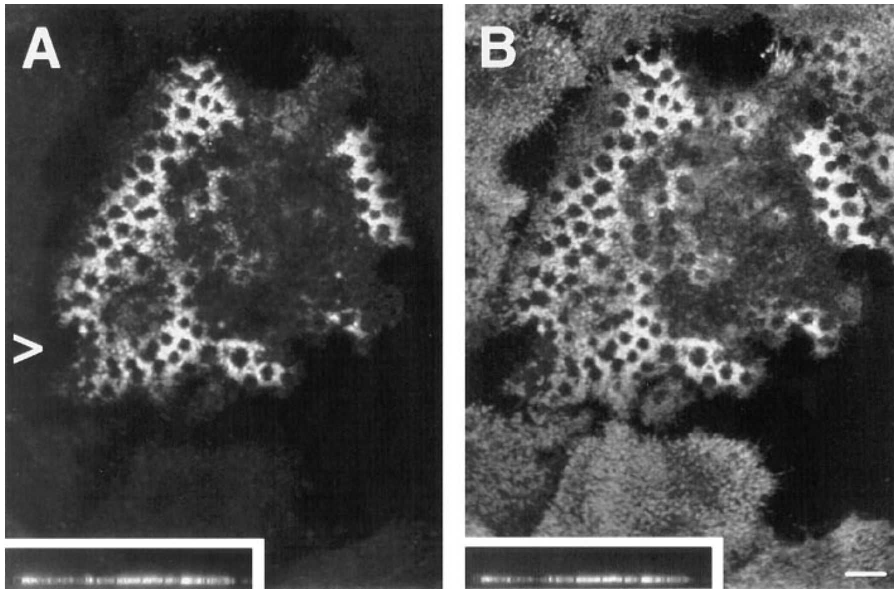



*Figure 5*. Immunolocalization of clone B encoded protein, in which combined alanine substitutions of three serine residues ($Ser^{169}$, $Ser^{175}$, and $Ser^{180}$) were introduced. 804G cells were grown on glass coverslips, transfected, fixed with 1% formaldehyde, permeabilized with 0.5% Triton X-100, and subjected to double immunofluorescence using the mAb anti-FLAG™M2 (*A*) and an anti-α6A antiserum (*B*). FITC-conjugated goat anti–mouse IgG (*left*) and Texas red–conjugated donkey anti–rabbit IgG (*right*). The recombinant form of BP180 was distributed in a Swiss cheese–like pattern characteristic for hemidesmosome-like structures, where it was colocalized with α6. Bar, 10 μm.

the subcellular distribution of the BP180 recombinants and HD1. The colocalization of BP180, α6β4, and HD1 along the basal aspect of the transfected cells is unlikely to be coincidental. First, BP180 recombinants were not concentrated at the basal cell side in the absence of α6β4. Second, when cells were cotransfected with cDNAs for either CD8 or CD31, these two transmembrane proteins were distributed diffusely at the cell membrane and did not codistribute with α6β4 (not shown).

Finally, COS-7 cells were also cotransfected with clone G or H with more extensive deletions: in this case, the staining of the plasma membrane was too intense, due to the high level of expression, to assess the colocalization with α6β4.

### *Mutant Forms of BP180 Do Not Coprecipitate with α6β4 in COS-7 Cells*

A recent study has provided evidence for an interaction between BP180 and α6 (18). To determine the ability of mutant forms of BP180 to associate directly with α6 and β4, we performed immunoprecipitation experiments using extracts from radiolabeled COS-7 cells that were transiently transfected with either of the clones A, B, or D together with cDNAs encoding α6A and β4. The SDS-PAGE analysis of these immunoprecipitates is shown in Fig. 9 *A*. In all cases, the mol wt of the detected proteins corresponded with the sizes predicted on the basis of the cDNA sequence. Neither α6 nor β4 were precipitated in association with BP180 recombinant polypeptides encoded by clones A, B, or D by the mAb anti-FLAG™M2. Furthermore, the recombinant forms of BP180 encoded by these clones could not be coimmunoprecipitated with either α6 or β4 by an anti-α6 or anti-β4 antiserum, respectively. Identical results were obtained when COS-7 cells were transfected with clones A, B, or D together with cDNA for either α6A or β4 (Fig. 9 *B*). When the samples immunoprecipitated with the anti-α6 or anti-β4 antiserum were subjected to immunoblotting with the mAb anti-FLAG™M2, this mAb did not show any reactivity with the various precipitates. Moreover, no coprecipitation of recombinant forms of BP180 with either α6A or β4A was observed when cells were lysed with different detergents, such as Triton X-100 or CHAPS (not shown). In contrast, immunoprecipitation with either a rabbit anti-α6 or a rabbit anti-β4 antiserum of lysates from cells transfected with cDNAs for the α6A and β4 yielded, as expected, a heterodimer complex consisting of α6 and β4. Together, these data do not provide evidence for a physical association between mutant forms of BP180 and the α6β4 integrin in transfected COS-7 cells.

### *The Subcellular Distribution of BP180 Is Affected by the Cytoplasmic Domain of β4*

As an alternative approach to identify domains involved in the association of BP180 and α6β4, we generated a cDNA construct encoding a β4 molecule, $\beta4^{1382}$, with a truncated cytoplasmic domain, which contains the first pair of FNIII and a segment of 64 amino acids at the $NH_2$ terminus of the connecting segment, but which lacks the tyrosine activation motif (TAM) (30) of the connecting segment as well as the third and fourth FNIII. In COS-7 cells transfected with cDNAs encoding α6A and this truncated β4, the β4 mutant protein was distributed together with α6 and HD1 along the basal cell surface in a pattern indistinguishable from that observed in cells transfected with wild-type α6A and β4A, as assessed by double immunofluorescence staining (Fig. 7 *F*). Strikingly, in COS-7 cells expressing α6 and $\beta4^{1382}$ the mutant forms of BP180 encoded by clone B (not shown) and D were not colocalized with α6β4 and HD1 in these junctional complexes, but in most cells remained diffusely distributed at the apicolateral cell surface (Fig. 7, *E* and *F*). Since recent studies (30, 31) have demonstrated that phosphorylation of the TAM of β4 is required for the incorporation of α6β4 into HD and for their assembly, we then investigated if the TAM of β4 plays a role in the subcellular distribution of BP180 by using a β4 subunit carrying a mutated TAM. In COS-7 cells trans-

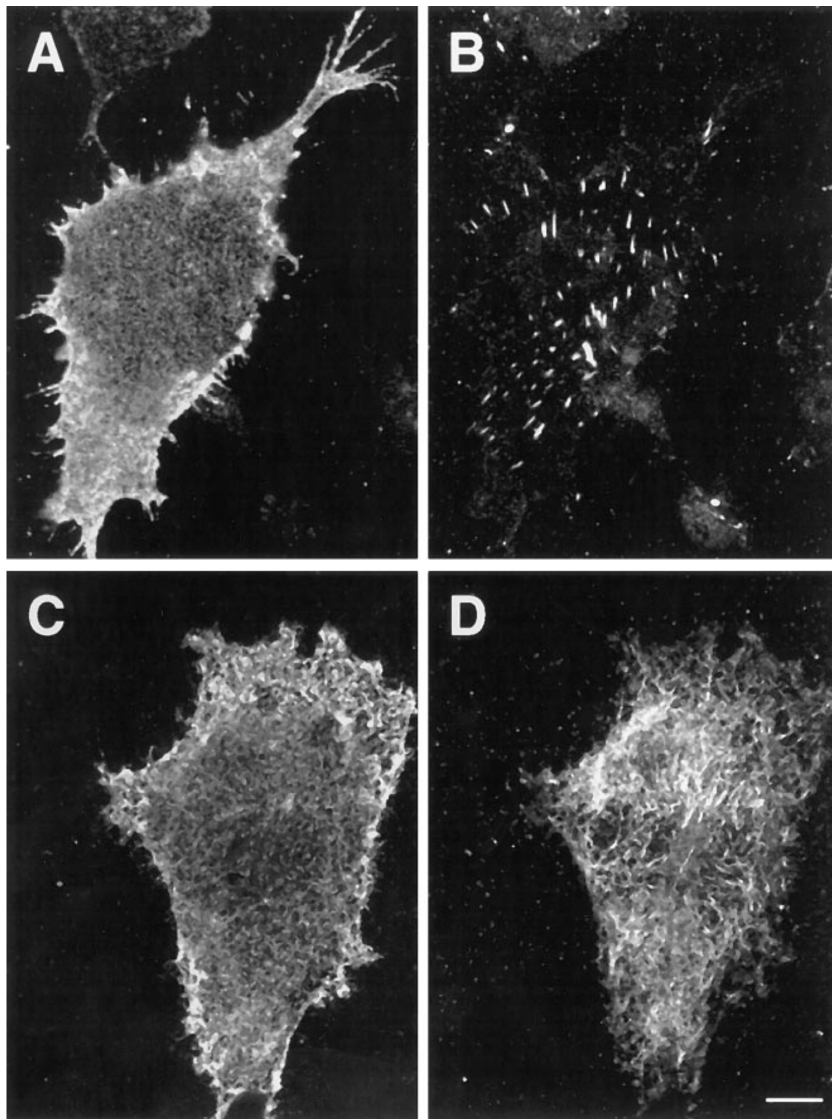



*Figure 6.* Confocal double immunofluorescence microscopy of transfected COS-7 cells showing the subcellular distribution of the chimeric protein composed of the membrane targeting sequence of K-Ras combined with the cytoplasmic domain of BP180 (clone D). Cells grown on glass coverslips were transfected with clone D. After 36 h, cells were fixed, permeabilized, and subjected to double labeling using the BP180 antiserum J17 (*A* and *C*) and the mAb VIN 11-5 against vinculin (*B*), or the mAb 121 against HD1 (*D*). Texas red–conjugated donkey anti–rabbit IgG (*left*) and FITC-conjugated goat anti–mouse IgG (*right*). The chimeric BP180 protein is distributed diffusely at the cell surface and is not colocalized with endogenous vinculin or HD1. Bar, 10 μm.

fected with cDNAs for α6A and a β4 subunit with combined phenylalanine substitutions at the TAM, the colocalization potential of the clone B encoded protein with the mutated β4 was impaired as compared to that of wild-type β4. In most cells expressing α6 and the mutated β4, clone B encoded protein was diffusely distributed over the plasma membrane (Fig. 8, *C* and *D*), and in only a few transfected cells it was concentrated at the basal cell surface together with α6β4 (not shown). Notably, in cells expressing α6 and β4 with a mutated TAM, HD1 was found to be codistributed with α6β4 in a pattern indistinguishable from that observed with α6 associated with wild-type β4 (not shown). Taken together, these results indicate, first, that sequences contained in the COOH-terminal half of the cytoplasmic tail of β4 are involved in determining the subcellular distribution of BP180; second, that mutations of the β4 TAM interfere with the efficient colocalization of BP180 with α6β4; finally, that colocalization of HD1 with α6β4 is, in contrast to that of BP180, mediated by sequences within the $NH_2$-terminal half of the cytoplasmic domain of β4.

## Discussion

We have studied the contribution of various segments of BP180 to its incorporation into HD by transfecting 804G cells with cDNA-constructs encoding wild-type, deletion mutant forms of BP180, and chimeric proteins, in which the membrane targeting sequence of K-Ras had been fused to segments of the cytoplasmic domain of BP180. The results indicate that the cytoplasmic domain of BP180 contains sequences sufficient for the incorporation of mutant forms of BP180 into HD-like structures in 804G cells. The localization of BP180 in HD appears to be mediated by a region that spans 265 amino acids (clone F) comprising two important domains, that are located at the $NH_2$ terminus and within the central portion of the BP180 cytoplasmic domain.

Based on transient transfections of 804G cells, it was recently reported that a 36–amino acid stretch located at the $NH_2$ terminus determines the localization of BP180 at the basal side of the cell, whereas the extracellular segment close to the membrane-spanning domain is required for its

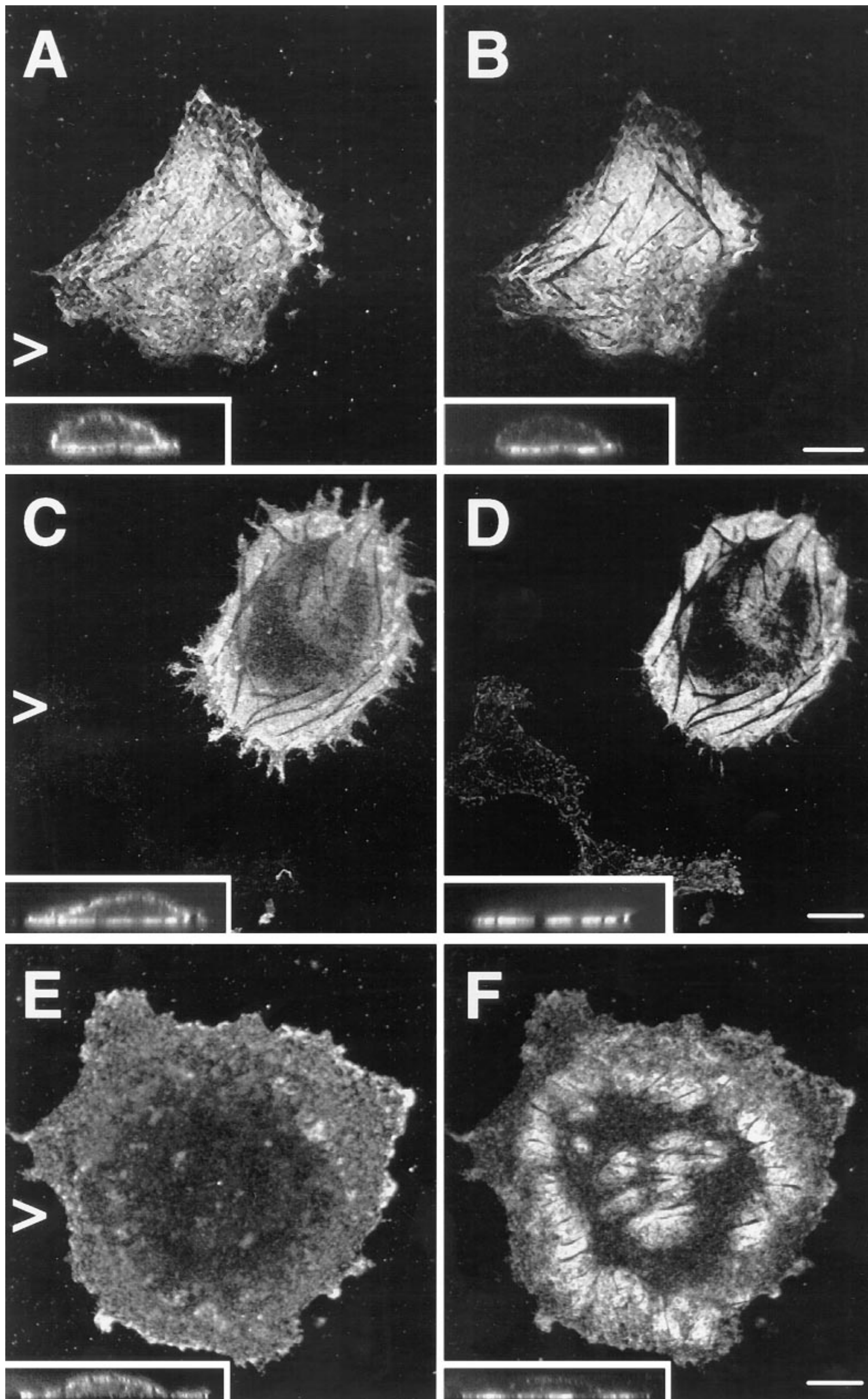
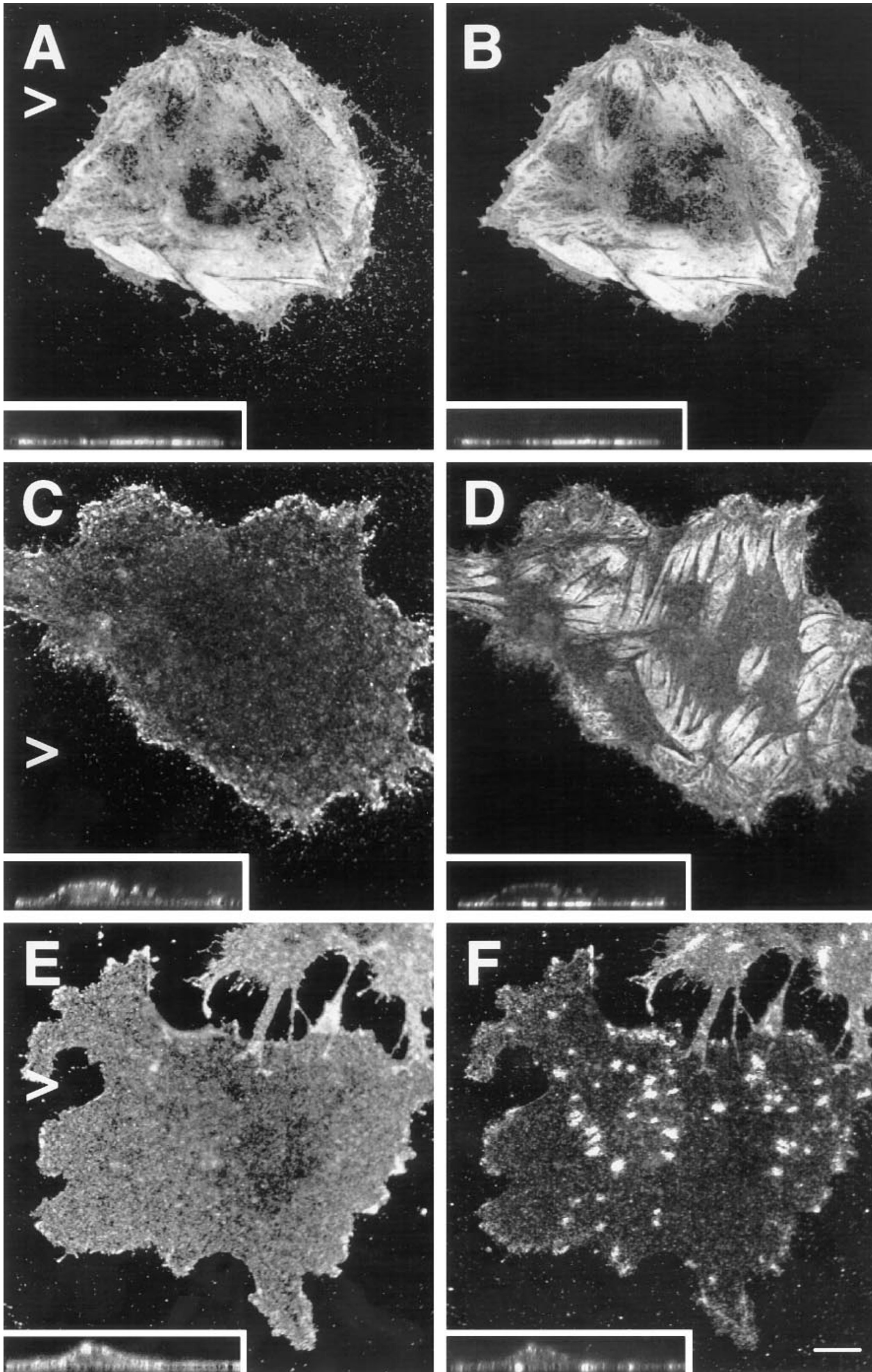



*Figure 7.* Double immunofluorescence microscopy of transfected COS-7 showing that the chimeric protein composed of the membrane targeting sequence of K-Ras combined with the entire cytoplasmic domain of BP180 (clone D) is codistributed with the α6β4 integrin. Cells grown on glass coverslips were transfected with clone D (*A–F*) together with cDNAs for human α6A and β4A (*A–D*), or with cDNAs for human α6A and $\beta4A^{1382}$ (*E* and *F*). After 36 h cells were fixed, permeabilized, and subjected to double labeling using the mAb anti-FLAG™M2 (*A* and *E*) and the rat mAb GoH3 (*B* and *F*) as well as the BP180 antiserum J17 (*C*) and the mAb 121 against HD1(*D*). FITC-conjugated goat anti–mouse IgG (*A*, *D*, and *E*), Texas red–conjugated donkey anti–rabbit IgG (*C*). The rat mAb GoH3 was stained using a rabbit anti–rat IgG (absorbed against mouse IgG) and Texas red–conjugated donkey anti–rabbit IgG (*B* and *F*). In cells transfected with α6A and β4A cDNAs, the BP180 chimeric protein is found concentrated along the basal cell surface, where it is codistributed with α6 and HD1. In contrast, in cells cotransfected with cDNAs for α6A and $\beta4A^{1382}$, the mutant form of BP180 displayed uniquely an apicolateral cell surface distribution and is not colocalized with α6. Z-sections of transfected cells are shown in the insets. The optical plane is the same as in Fig. 6. Bar, 10 μm.

*Figure 8.* Double immunofluorescence microscopy of transfected COS-7 showing that the subcellular distribution of clone B encoded protein is affected by the cytoplasmic domain of the β4 integrin subunit. Cells were transfected with clone B (*A–F*) together with cDNAs for human α6A and β4A (*A* and *B*), or with cDNAs for human α6A and a mutated β4 carrying phenylalanine substitutions of the TAM (*C* and *D*), or, finally, with cDNAs for α6A and a β4/β1 chimera, in which the cytoplasmic domain of β4 was replaced by that of β1 (*E* and *F*). After 36 h cells were fixed, permeabilized, and subjected to double labeling using the mAb anti-FLAG™M2 (*A*, *C*, and *E*) and the rat mAb GoH3 (*B*, *D*, and *F*). FITC-conjugated goat anti–mouse IgG (*A*, *C*, and *E*). The rat mAb GoH3 was stained using a rabbit anti–rat IgG (absorbed against mouse IgG) and Texas red–conjugated donkey anti–rabbit IgG (*B*, *D*, and *F*). In cells transfected




with α6A and β4A cDNAs, the clone B encoded protein was localized along the basal cell surface codistributing with α6β4. In contrast, in cells cotransfected with cDNAs for α6A and a β4 carrying a mutated TAM or a β4/β1 chimera, the BP180 recombinant molecule displayed predominantly an apicolateral cell surface distribution and is not colocalized with α6β4 or α6β4/β1, respectively. Z-sections of transfected cells are shown in the insets. The optical plane is the same as in Fig. 6. Bar, 10 μm.

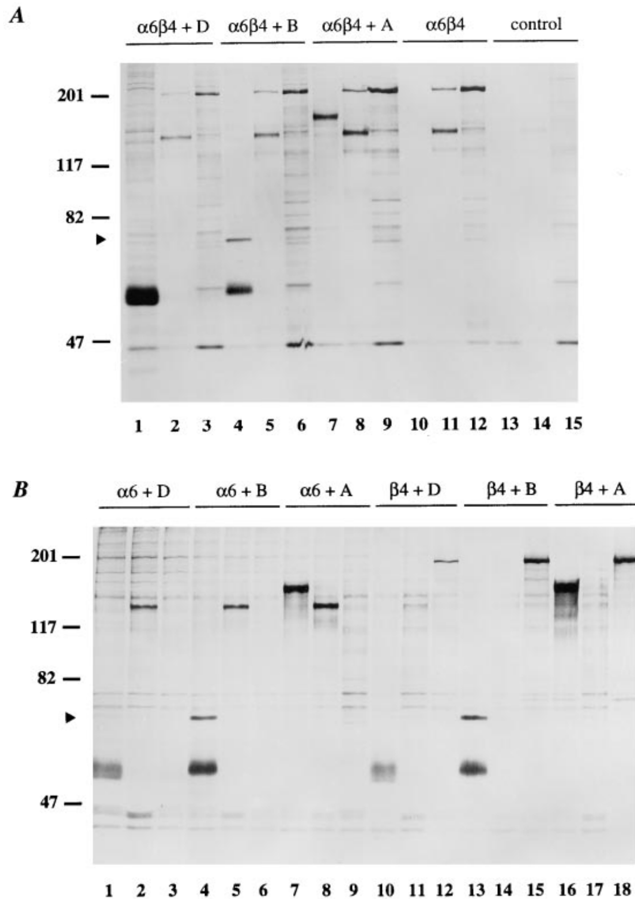



*Figure 9. (A)* Immunoprecipitation analysis of transfected COS-7 cells. Lysates of $^{35}$S-radiolabeled cells cotransfected with cDNAs for α6A and β4A as well as clone D (lanes *1–3*), clone B (lanes *4–6*), or clone A (lanes *7–9*), with cDNAs for α6A and β4A alone (lanes *10–12*), and of mock-transfected cells (lanes *13–15*), were immunoprecipitated with the mAb anti-FLAG™M2 (lanes *1*, *4*, *7*, *10*, and *13*), an anti-α6 antiserum (lanes *2*, *5*, *8*, *11*, and *14*), and an anti-β4 antiserum (lanes *3*, *6*, *9*, *12*, and *15*). Note that immunoprecipitation of cells transfected with cDNAs for α6A and β4A with either an anti-α6 and an anti-β4A antiserum yielded a heterodimer complex of α6A and β4A, while no coprecipitation of BP180 is observed. No coprecipitation of the α6A and β4A is detected by using the mAb anti-FLAG™M2, even after longer exposure of the gel. (*B*) Lysates of $^{35}$S-radiolabeled cells cotransfected with cDNA for α6A as well as with clone D (lanes *1–3*), clone B (lanes *4–6*), or clone A (lanes *7– 9*), and of cells cotransfected with cDNA for β4A and clone D (lanes *10–12*), clone B (lanes *13–15*), or clone A (lanes *16–18*). Immunoprecipitation was performed with the mAb anti-FLAG™M2 (lanes *1*, *4*, *7*, *10*, *13*, and *16*), an anti-α6 antiserum (lanes *2*, *5*, *8*, *11*, *14*, and *17*), and an anti-β4 antiserum (lanes *3*, *6*, *9*, *12*, *15*, and *18*). No coprecipitation of the α6A and β4A is found by using the mAb anti-FLAG™M2. In addition, no coprecipitation of mutant BP180 proteins is detected with either an anti-α6 or an anti-β4A antiserum. Note that one radiolabeled polypeptide (*arrow heads*) is coprecipitated by the mAb anti-FLAG™M2 from extracts of cells transfected with clone B. The identity of this protein, which was not precipitated when cells were lysed with 1% Triton X-100 (not shown), is unclear. Samples were analyzed by 8% SDS-PAGE under reducing conditions. Molecular mass markers are indicated in kD.

recruitment into HD (18). Our results confirm the functional importance of the $NH_2$-terminal domain of BP180. However, our observation that a chimeric protein composed of the membrane targeting signal of K-Ras and a cytoplasmic tail of BP180 lacking this $NH_2$-terminal region (clone H) is not only localized at the apical, but also often at the basolateral cell surface, indicates that this segment is not uniquely involved in sorting the protein to the basal cell surface, but rather is required for targeting BP180 to HD. The membrane targeting signal of K-Ras may be responsible for the diffuse localization of the protein (28, 39), but this possibility seems less likely, because recombinant molecules encoded by clones B or D, that lack or contain the K-Ras membrane localization sequence, respectively, were frequently similarly distributed. Alternatively, it is conceivable that the localization of the mutant protein is dynamic and dependent on its relative expression level in transfectants.

The results of the subcellular localization of chimeric proteins with deletions of increasing size within the BP180 cytoplasmic domain indicate the existence of an additional, as yet unrecognized, functionally important region. Whereas a truncated BP180 molecule consisting of a 265–amino acid stretch was localized in HD (clone F), a recombinant protein with an additional internal deletion of 88 amino acids towards the $NH_2$ terminus (clone G) was not.

What are the mechanisms by which the truncations of the cytoplasmic domain of BP180 interfere with its incorporation in HD? First, the deleted regions may contain the sequences responsible for the interaction of BP180 with elements of the hemidesmosomal cytoskeleton. Second, conformational changes in protein architecture may be responsible for the failure of clone G and clone F encoded proteins to be incorporated into HD. PHD secondary structural analysis (42) predicts that the protein encoded by clone F, containing the minimal sequences for recruitment into HD, has several β-sheet regions as well as one α–helical domain, which are absent in the recombinant protein encoded by clone G. Moreover, the functionally important domain at the $NH_2$ terminus of BP180 contains a β-sheet. The above two regions at the $NH_2$ terminus and within the central portion of the cytoplasmic domain may thus be involved in the proper folding of BP180, which may be essential for its assembly in HD. Finally, the truncations may interfere with the transmission of an intracellular signal required to render BP180 competent to interact with other components of HD. Phosphorylation pathways appear to critically regulate the assembly of HD (9). Inhibition of the phosphorylation of the β4 integrin subunit was found to prevent localization of the α6β4 integrin in HD (9). In addition, a recent study suggests that a protein kinase C is involved in the phosphorylation of BP180 at serine residues, which affects its localization at the basal cell membrane of squamous carcinoma cells (23). Although combined alanine substitutions at three serine residues located in the central portion of the cytoplasmic domain of BP180 did not affect its localization in HD, it is conceivable that the truncations in clone G and H encoded proteins G might interfere with signaling pathways that control the association of BP180 with HD.

Our results demonstrate that removal of the extracellular and transmembrane regions of BP180 or their substitu-

tion by the membrane localization sequence of K-Ras does not abolish the localization of BP180 in HD of 804G cells. This was unexpected, because in a previous study, deletion of a segment of 27 amino acids located immediately after the transmembrane domain prevented the incorporation of the BP180 protein into HD, suggesting that this region is required for targeting (18). However, in this latter study (18) it has not been ascertained whether the truncated protein was correctly inserted in the plasma membrane. BP180 is a type II transmembrane protein, whose insertion in the membrane depends on both the "signal-anchor" sequence of the transmembrane domain and the number and type of flanking charged amino acids (15). Therefore, deletion of the region proximal to the transmembrane domain may have a deleterious effect on the translocation and insertion of the truncated protein into the plasma membrane, preventing its interaction with the hemidesmosomal cytoskeleton. Since our transfection studies only assess the ability of mutant proteins to be incorporated, together with endogenous components, into HD (48), our findings do not exclude the possibility that the ectodomain of BP180 is also involved in the localization of the protein in HD as well as in their nucleation and formation by binding to an extracellular ligand(s).

To analyze the potential of BP180 to interact with α6β4, we have performed transfection experiments using COS-7 cells. In COS-7 cells, transfected with cDNAs for the α6A and β4 integrin subunits, expression of the α6β4 integrin results in the generation of distinct structures at sites of cell-substrate contact (37). These junctional complexes, containing α6β4 and HD1, may correspond to the recently described type II HD, which are found in some cell types and which lack the two hemidesmosomal components BP230 and BP180 present in HD (54). When COS-7 cells were cotransfected with clones B or D, the recombinant BP180 molecules were present in the same complexes as α6β4. These findings confirm the previously reported colocalization of a mutant form of BP180 (identical to that encoded by clone B) with α6β4 in FG cells which, like COS-7 cells, lack endogenous BP180 and BP230 (18). However, the observation that codistribution also occurred with the mutant form of BP180 lacking the entire extracellular region (clone D), suggests that the cytoplasmic domain of BP180 contains sufficient elements for its recruitment into complexes containing α6β4 and HD1.

Our mutagenesis experiments indicate that the COOH-terminal half of the β4 subunit contains sequences required for the colocalization of mutant forms of BP180 with α6β4 in transfected COS-7 cells. This region of β4 comprises the third and fourth FNIII as well as part of the connecting segment including the TAM, that consists of two tyrosine residues at position 1422 and 1440 of the β4 subunit (30). Recent studies have demonstrated that phosphorylation of this TAM is required for the incorporation of α6β4 into HD as well as their assembly (30). Combined phenylalanine substitutions of the TAM impaired the capacity of BP180 to codistribute with α6β4, suggesting that the TAM is involved in transducing signals that critically regulate the subcellular distribution of BP180 in cos-7 cells. However, the observation that in some cells the expression of a mutated β4 TAM did not completely suppress this codistribution suggests that additional factors also contribute. Since type III fibronectin homology modules have been implicated in protein–protein interactions (3, 48), it is conceivable that the third and fourth FNIII within the COOH-terminal half of the cytoplasmic domain of β4 also coordinate the localization of recombinant BP180 molecules with the α6β4 integrin by providing a binding site for interaction. However, coimmunoprecipitation experiments failed to provide evidence for a direct association between mutant BP180 proteins and α6β4, but a weak association between these proteins which is lost after cell lysis cannot be ruled out.

The results of our immunofluorescence analysis indicate that neither the elimination of the COOH-terminal half of β4 nor the mutation of the β4 TAM prevent the subcellular redistribution of HD1 with α6β4, which occurs after transfection of COS-7 cells with cDNAs for α6 and the mutated β4 molecules. The colocalization of HD1 with α6β4 may thus be mediated by distinct sequences in the $NH_2$-terminal half of the cytoplasmic domain of β4. Recent in vitro binding and transfection studies using β4 recombinant proteins have provided evidence that HD1 associates with the cytoplasmic tail of β4 (37). Based on our findings in COS-7 cells, it is tempting to speculate that α6β4 and HD1 form a complex that serves as a core for the assembly of HD to which BP180 and BP230 aggregate in a later step, possibly regulated by TAM-dependent signals. Clearly, further experiments are needed to elucidate the mechanisms controlling the association of the α6β4 integrin with the other hemidesmosomal cytoskeletal molecules.

It is difficult to reconcile some of our findings with a previous report stating that a mutant form of BP180, identical to that encoded by clone B, coprecipitated with α6 from HT1080 cells transfected with this mutant, which contain α6β1 but not α6β4. This interaction appears to rely on the extracellular segment of BP180 close to the transmembrane domain (18). It is possible that the reported association between α6 and BP180 only occurs in some cell types. In fact, in cultured keratinocytes derived from a patient with junctional EB associated with pyloric atresia, who was completely deficient for β4, α6 was associated with β1 and was localized in focal contacts, whereas BP180 was present in HD (36). This latter observation is consistent with our results in transfected COS-7 cells, in which BP180 recombinants were not codistributed with α6, associated with β4/β1, in focal contacts. Alternatively, coprecipitation of α6 with a recombinant form of BP180 may have been due to incomplete solubilization of BP180 and/or aggregation of molecules after cell lysis.

In conclusion, the studies reported here show that the cytoplasmic tail of BP180 contains sequences critical and sufficient for mediating the localization of BP180 in HD. Upon expression of both the α6 and β4 integrin subunits in cells that do not form HD, mutant forms of BP180 are codistributed with α6β4 and HD1 in complexes along the basal cell surface. In these complexes, the localization of recombinant BP180 molecules with α6β4 appears to be regulated by sequences within the COOH-terminal half of the β4 tail, including the third and fourth FNIII and part of the connecting segment containing the TAM. These observations provide new insights relevant for the understanding of the molecular interactions and regulatory elements involved in the organization and assembly of HD.

We are indebted to Dr. K. B. Yancey, in whose laboratory at the Dermatology Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD), part of the cloning of BP180 was performed during a postdoctoral fellowship (L. Borradori). We thank Drs. K. Owaribe, J. Kennel, J.R. Stanley, and J.C.R. Jones for kindly providing antibodies. We are grateful to Drs. E. Roos, J. Neefjes, P. Engelfriet, C. Gimond, and C. Baudoin for critical reading of the manuscript and to Dr. T. Sixma for helpful discussion about structural analysis predictions. Dr. L. Oomen is thanked for excellent assistance with the confocal laser microscope and N. Ong for photographic work.

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO 902-11-060), the Dutch Cancer Society (NKI 91-260), and from the Swiss National Foundation for Scientific Research (L. Borradori).

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