Doctor thesis

Biochemical and ultrastructural analyses of BP180 (Type XVII collagen), a transmembrane collagenous protein of the hemidesmosome

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ABSTRACT

The hemidesmosome (HD) is a cell-to-substrate adhesion apparatus found in stratified and complex epithelia. One of the putative cell-matrix adhesion molecules present in the HD is the 180 kDa bullous pemphigoid antigen (BP180). BP180, also termed type XVII collagen, is a transmembrane glycoprotein comprising interrupted collagen domains in its extracellular part. But the question of whether it actually takes a collagen-like triple helical conformation *in vivo* has remained unanswered.

Using a monoclonal antibody, I found that a subpopulation of BP180 localizes at the lateral surfaces of corneal basal cells and cultured cells, in addition to the basal surface. This subpopulation of BP180 could be solubilized by 0.5% Triton X-100, and among examined cell lines, was found to be most abundant in BMGE+H, a bovine mammary gland epithelial cell line. The Triton-soluble fraction of BMGE+H cells was used for characterization. On sucrose gradient centrifugation, the soluble BP180 demonstrated a value of approximately 7 S, and chemical crosslinking experiments revealed a trimer form. The calculated frictional ratio, f/f_0= 2.8, suggests an asymmetric configuration. For further characterization, I purified the soluble human and bovine BP180 by immunoaffinity column chromatography using an anti-BP180 monoclonal antibody. Rotary shadowing images of the purified BP180 showed a quaver-like molecule consisting of a globular head, a central rod and a flexible tail. With regard to the primary structure and species comparisons, the central rod, 60–70 nm in length, probably corresponds to the largest collagenous region, forming a collagen-like triple helix. The globular head and the flexible tail seem to respectively correspond to the cytoplasmic and the interrupted collagenous region of the extracellular portions. The present demonstration of the entire configuration of BP180, with a collagen-like trimer in its extracellular part, suggests that BP180 is one of the major components of anchoring filaments which connect hemidesmosomes to the basement membrane.

In the previous study of our laboratory, using a monoclonal antibody, we have detected a

120 kDa collagenase-sensitive polypeptide in the HD fraction. The sensitivity suggests the presence of collagenous sequence in the polypeptide. Therefore the following study was undertaken to assess the relation of the 120 kDa polypeptide to BP180. Immunofluorescence microscopy of bovine skin revealed the basement membrane zone of skin to be stained clearly with mAb-1337, whereas the lateral surfaces of basal cells, which were decorated by typical antibodies against BP180, were not. The antibody did not detect HDs in cultured cells but rather in the culture medium. These results are quite different from those of other typical anti BP180 mAbs, and indicate a localization of mAb-1337 antigen distinct from BP180. However, the same polypeptide was also recognized by monoclonal antibodies to the extracellular but not the cytoplasmic part of BP180, and found to react with a polyclonal antibody against the non-collagenous (NC) 16A domain of BP180. Therefore, the 120 kDa polypeptide was identified as an extracellular fragment of BP180. This identification was supported by the detection of 60 kDa polypeptides on immunoblotting of cultured cell extract using monoclonal antibodies to the cytoplasmic but not to the extracellular part of BP180. The 60 kDa polypeptide appears to be the remnant BP180 after removal of its 120 kDa extracellular fragment. Trimer formation by the 120 kDa fragment was confirmed using non-boiled specimens. mAb-1337 immunoprecipitated the 120 kDa fragment from the medium, but not the 180 kDa molecule of BP180 extracted from cultured cells, indicating that the antibody specifically recognizes the fragment. The mAb-1337 apparently recognizes a unique epitope that is exposed or formed by the cleavage. Hence the staining pattern observed for bovine skin demonstrated the presence of the 120 kDa extracellular fragment. Rotary shadow electron microscopy of affinity-purified 120 kDa fragments demonstrated that they have the unique molecular shape consisting of a central rod and a flexible tail, without the globular head that is present in the BP180 molecule.

From these results, I conclude that mAb-1337 shows unique epitope specificity, recognizing only the 120 kDa extracellular fragment of BP180 which is constitutively cleaved on the cell surface as a 120 kDa fragment both in *in vivo* and *in vitro*.

BP180 is a transmembrane glycoprotein comprising collagen-like triple helical stretches

in its extracellular part, and its qualification to be hemidesmosomal anchoring filaments suggests this molecule to be an absolutely new type of adhesion receptor. Moreover, BP180 is a target molecule for some types of human skin autoimmune diseases and for a type of human inherited skin diseases. Thus presented results are thought to include significant contribution to pathology and biology of dermal-epidermal adhesion.

INTRODUCTION

In multicellular organisms, cell adhesion is indispensable for assembling cells together (1). During animal development, cell adhesion mechanisms have been thought to play essential roles to organize differentiated cells into tissues and organs specialized for the physiological function. In the adult body, cell adhesion systems connect cells stably and maintain the structure of fully developed tissues. The fact that one of the distinct characteristics of tumours is aberrant expression of cell adhesion molecules indicates the importance of cell adhesion in maintenance of tissue integrity. Recent studies have revealed that some constituents of adhesion complexes themselves are tumor suppressor gene products (2).

Adhesion apparatus are multi-protein complexes to connect cytoskeleton of a cell to the extracellular matrix or its neighbors (Table 1), and are made up from two classes of proteins (Fig. 1): (1) intracellular attachment proteins, which form cytoplasmic plaque and anchor actin filaments or intermediate filaments to the adhesion complex; and (2) transmembrane adhesion receptors, which associate with attachment proteins at their intracellular domains, and interact with either other adhesion receptors on neighboring cells or with proteins of extracellular matrix at their extracellular domains.

Hemidesmosomes are cell to matrix adhesion apparatus developed in stratified and complex epithelia which endure relatively harder mechanical stress than other epithelial tissues (3–6). Using an electron microscope, like desmosomes, hemidesmosomes are distinguishable by their electron dense cytoplasmic structure termed hemidesmosomal plaque (Fig. 2). The name of hemidesmosomes, which means half desmosomes, comes from their morphological resemblance to desmosomes. However, recent progress in studies of the hemidesmosome has revealed that constituents of the hemidesmosome are very different from those of the desmosome as describe below.

Hemidesmosomes, including anchoring filaments and fibrils are adhesion complexes mediating firm attachment of epithelial basal cells to the underlying basement membrane,

Table 1. Classification of adhesion apparatus.

	actin filaments	intermediate filaments
cell/cell	adherens (cell/cell)	desmosome
cell/ECM	adherens (cell/ECM)	hemidesmosome

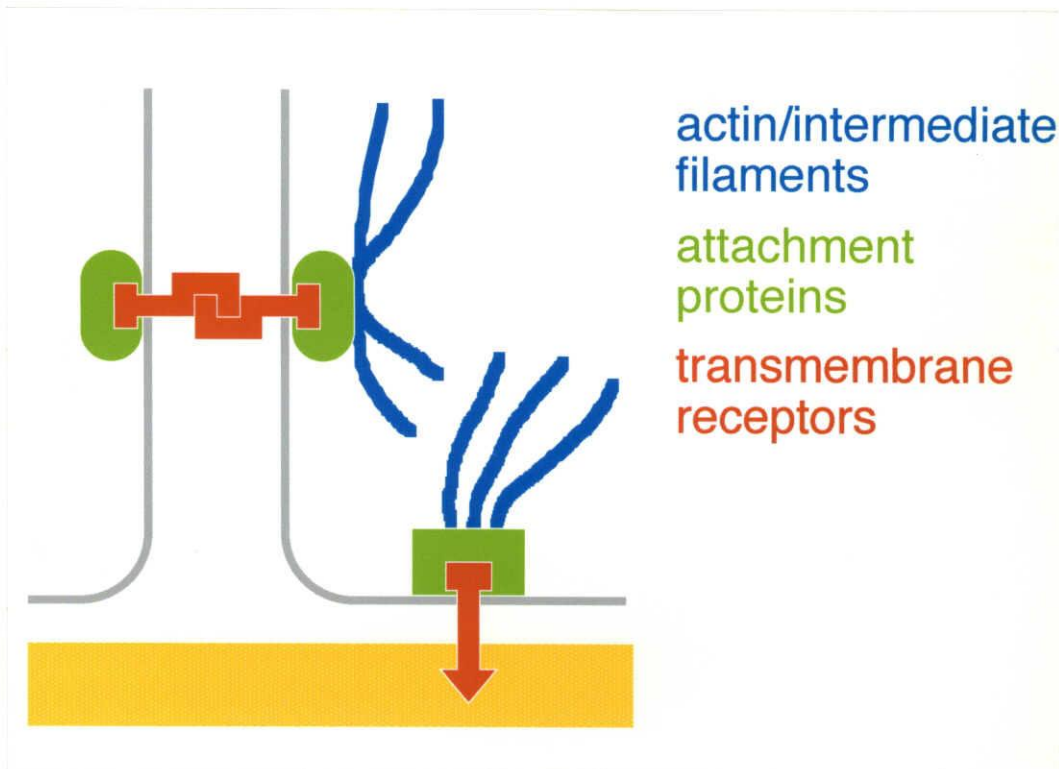


Figure 1. Construction of adhesion apparatus.

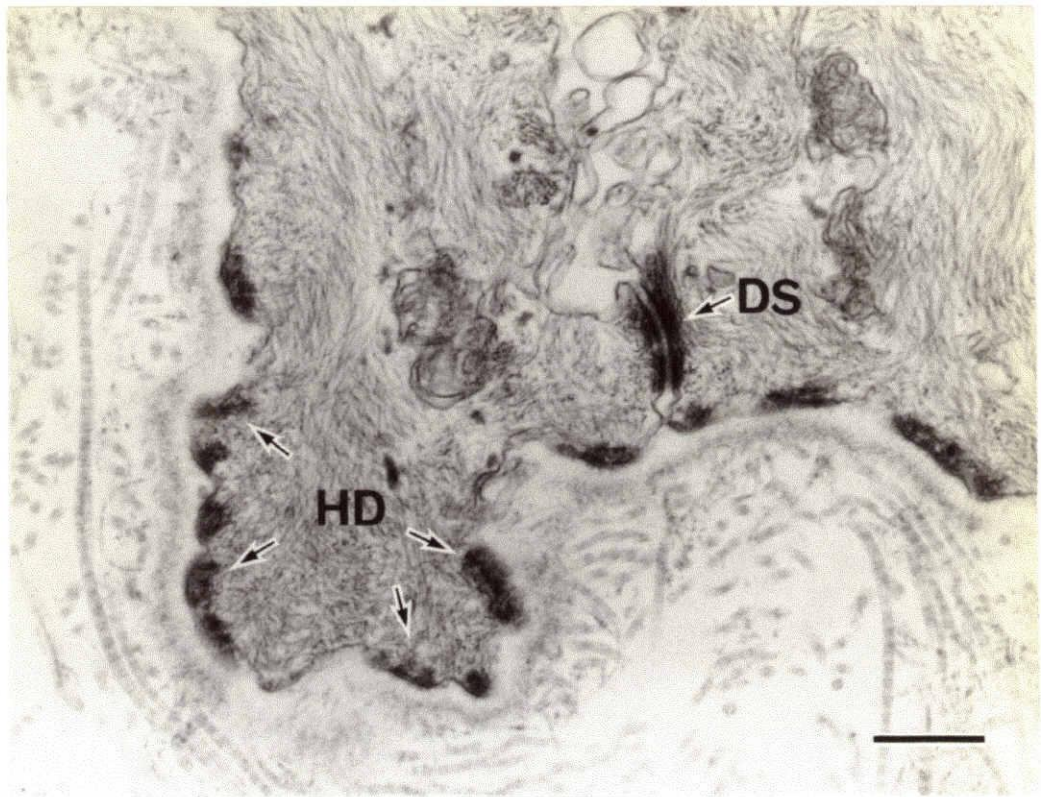


Figure 2. Electron microphotographic image of epithelial basal cells of bovine esophagus. Arrows indicate, DS: desmosome; HD: hemidesmosome. Bar: 1 μm.

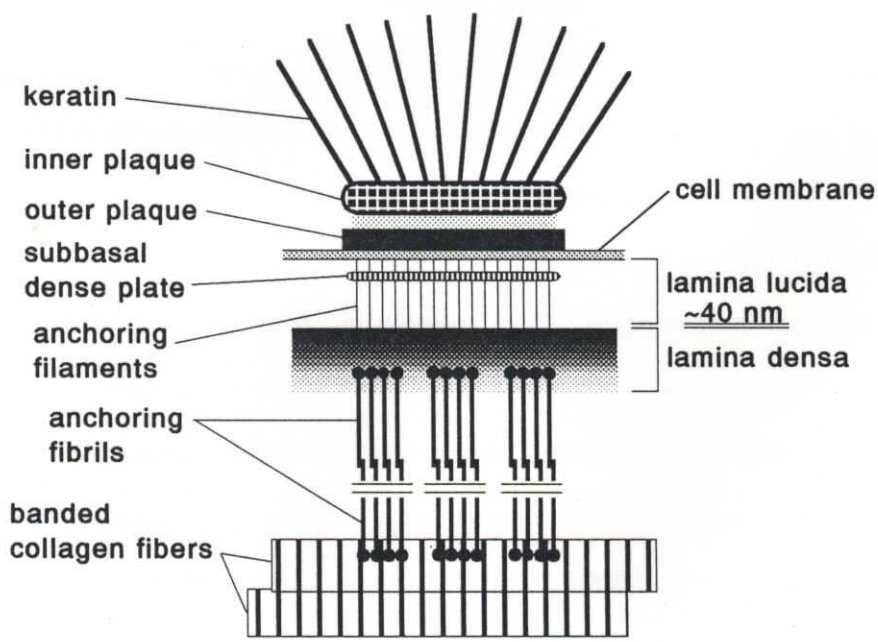


Figure 3. Schematic presentation of hemidesmosomal structure.

thereby mechanically linking the cytoplasmic keratin filament network to collagen fibrils and other extracellular matrix components of the connective tissue (Fig. 3).

In the process of epidermal organization and differentiation, cells dynamically regulate their hemidesmosomes during attachment to and detachment from the basement membrane. In most cases this involves new formation of hemidesmosomes but, during cell movement in wound healing and differentiation in stratification, hemidesmosomes must become detached from the basement membrane, with separation of hemidesmosomal transmembrane proteins from their ligands being an important step. However, the underlying mechanism is still poorly understood.

In the past decade, it has become clear that there are at least five hemidesmosomal proteins, including HD1/plectin (7–9) and BP230 (10–12) as cytoplasmic plaque proteins and integrin $\alpha 6\beta 4$ (13–15) and BP180 (16–19) as transmembrane proteins (Fig. 4). The serum from a patient with an autoimmune blistering skin disease named bullous pemphigoid was demonstrated to recognize antigenic 230 kDa (BP230) and/or 180 kDa (BP180) polypeptides of hemidesmosomes (20–22). Identification and characterization of these hemidesmosomal constituents have led to the new finding of type II hemidesmosomes, which have HD1/plectin and integrin $\alpha 6\beta 4$ but lack the two BP antigens and distinct plaques, and exist in certain cell types such as endothelial, Schwann and astroglial cells (7, 23).

Establishment of an isolation procedure of hemidesmosomes (24) and production of monoclonal antibodies against hemidesmosomal constituents have made it possible to screen patients with inherited skin disease. In addition, by the determination of complete nucleic acid sequences of hemidesmosomal constituents, it is also possible to detect mutations in their genes encoding the constituents. Mice with targeted genes encoding hemidesmosomal constituents have been generated and their deficiencies have been reported. These studies revealed some of the constituents to have unexpected novel function and significance in addition to the dermal-epidermal junction. It has been shown that patients with epidermolysis bullosa simplex with muscular dystrophy, a type of inherited skin diseases, have mutations in their genes of

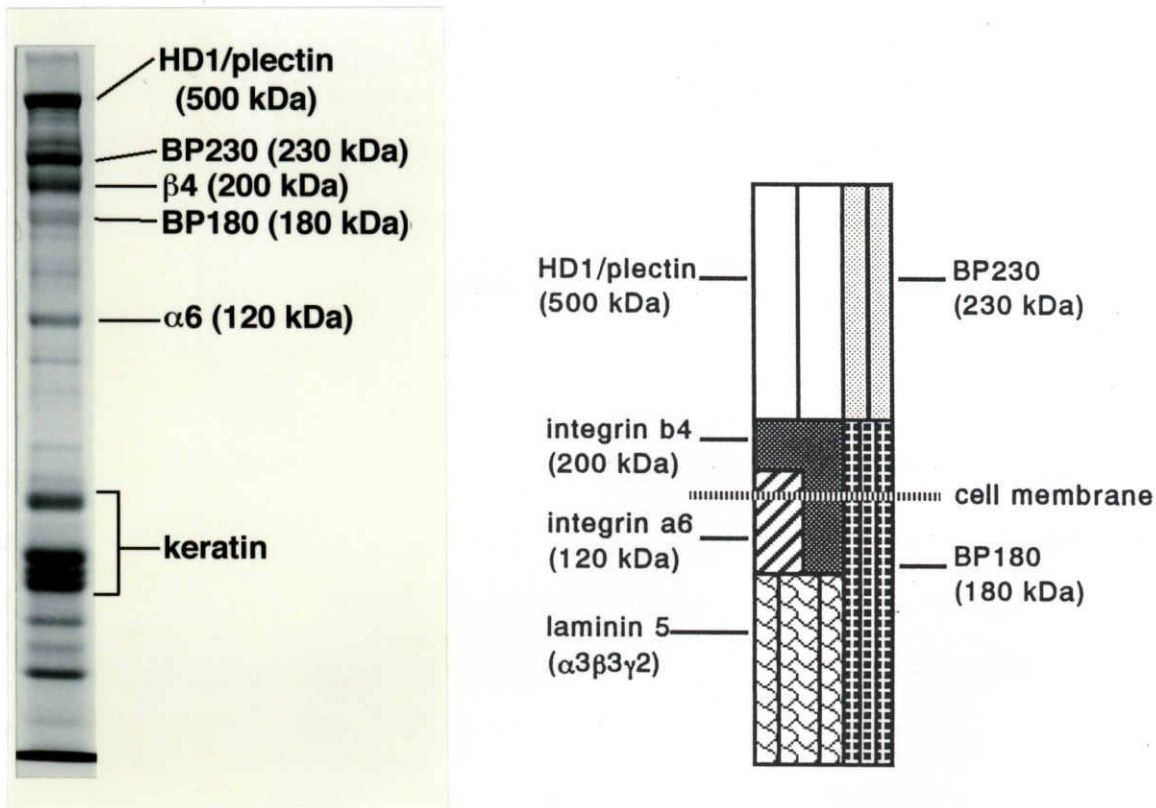


Figure 4. Constituents of the hemidesmosome.

SDS-PAGE of the HD fraction isolated from bovine cornea (left). Possible molecular construction of the hemidesmosome (right).

HD1/plectin (25–27). The result indicates that HD1/ plectin is responsible for muscle tissue integrity in addition to the dermal-epidermal adhesion. Studies on BP230 gene knock-out mouse have demonstrated the expression of specific isoforms of BP230 in some neural tissues and their crucial role in maintenance and survival of neuron during adult age (28–30).

The hemidesmosomal transmembrane proteins are considered to function as adhesion receptors. There are at least three hemidesmosomal transmembrane polypeptides, i.e. integrin α6 and β4 subunits and BP180, which is also called type XVII collagen. The integrin α6β4, an adhesion molecule whose ligands are laminins (31–33), especially laminin-5, plays an essential role in assembly and functioning of hemidesmosomes (33, 34). Mutations of the β4 integrin gene have been described in some forms of junctional epidermolysis bullosa with pyloric atresia (JEB-PA) (35, 36) and animals with targeted α6 integrin or β4 mutations show similar, but even more severe deficiencies (37–39). Recently, Hopkinson *et al.* have shown that integrin α6 subunit interacts with BP180 (40). On the other hand, Borradori *et al.* have reported the interaction between cytoplasmic domain of β4 and that of BP180 (41). These results suggest that the α6β4 complex plays a major role in epidermal cell-basement membrane adhesion. Moreover, studies of Giancotti and his coworkers have shown that laminin binding to the α6β4 integrin causes tyrosine phosphorylation of the β4 subunit and consequent activation of *ras*-MAP kinase pathway (42–43).

BP180, a 180 kDa bullous pemphigoid antigen, is a type II transmembrane glycoprotein with a collagenous carboxyl-terminal extracellular domain and a noncollagenous amino-terminal cytoplasmic domain (16–19). The extracellular domain is interrupted by non-triple-helical sequences, consisting of 15 separate triple-helical stretches in the human form and 13 in the mouse (Fig. 5). Autoantibodies to BP180 are thought to play a crucial role in skin blistering in patients with bullous pemphigoid, herpes gestationis, and cicatricial pemphigoid (20, 44, 45). This is supported by findings with a passive transfer model, featuring injection of rabbit polyclonal antibodies against a pathogenic epitope of BP180 into neonatal mice (46). In addition, mutations in the BP180 gene have been found in cases of generalized atrophic benign

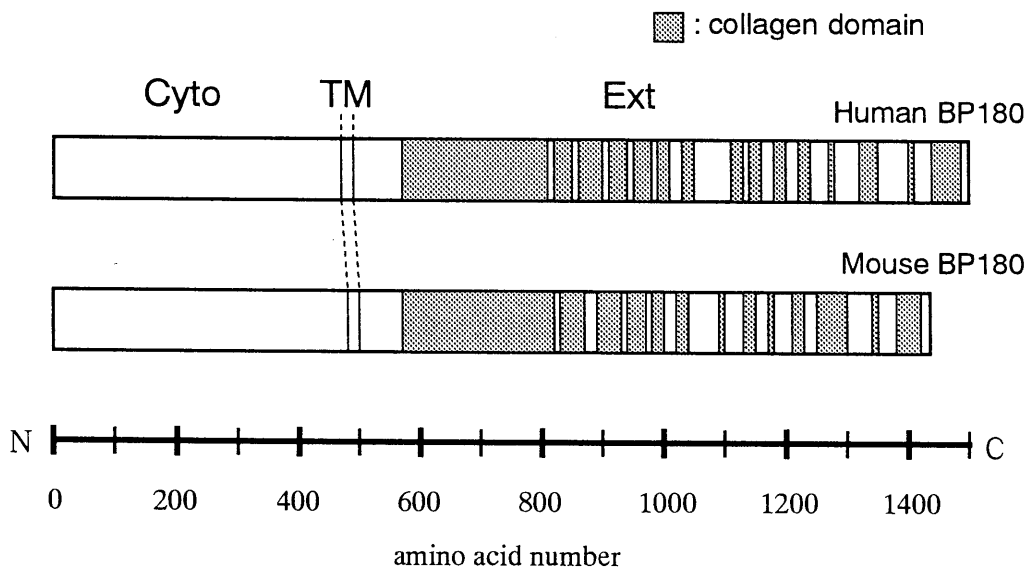


Figure 5. Schematics of human and mouse BP180.

The schematic domain organization of human BP180 is based on the primary structural analysis performed by Giudice *et al.* (16) and that of mouse BP180 is based on the analysis by Li *et al.* (18). Cyto, TM and Ext are the cytoplasmic, transmembrane and extracellular portions of BP180, respectively.

epidermolysis bullosa, which is characterized by universal alopecia and atrophy of the skin (47–50). However, while these results suggest an important role for BP180 in cell to extracellular matrix adhesion, in contrast to integrin $\alpha 6\beta 4$, our understanding of the molecular biology of BP180 is quite limited, and even its ligand(s) is unknown. One of the remained problems to be resolved concerns the molecular shape of BP180. For example, its extracellular collagenous sequence suggests collagen-like trimer formation, but biochemical confirmation of this has not been accomplished, because of the high insolubility and very low amounts of hemidesmosomes in tissues. Clearly, this problem must be overcome to facilitate further understanding of molecular architecture of hemidesmosomes.

In the first section of the results, I purified BP180 from the 0.5% Triton X-100 soluble fraction of cultured cells and analyzed its molecular configuration biochemically and ultrastructurally.

In the second, I present evidence that in both tissue and culture cells can sever the extracellular portion of the BP180 molecule from their surface. The biological significance of this finding will be considered with reference to detachment of hemidesmosomes from the basement membrane.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies (mAbs) against hemidesmosomal proteins were prepared by immunizing mice with the hemidesmosome fraction isolated from bovine corneal epithelial cells, as described previously (24). mAb-233, mAb-1D1, mAb-D20 and mAb-R223 are against the extracellular part and mAb-1A8c and mAb-1A6 are against the cytoplasmic part of BP180 (19). Epitopes of mAb-233 and mAb-1D1 are mapped on the carboxyl-terminal half, and that of mAb-D20 is mapped on the amino-terminal half of the extracellular part (unpublished data). mAb-1E5 binds specifically to BP230. mAb-855, mAb-310 and mAb-617 target the extracellular part and mAb-1A3 targets the cytoplasmic part of the integrin β4 subunit. For immunofluorescence microscopy, hybridoma supernatants of each mAb were diluted to ×50 their antibody titers, i.e. 50 fold the critical concentration to detect the antigen in skin BMZ by immunofluorescence microscopy. BP serum that recognizes both 230 and 180 kDa polypeptides was kindly provided by Dr. S. Fujiwara of Ooita Medical College (Ooita, Japan).

Purified ascites fluid of mAb-233 was biotinylated for some experiments. The purified immunoglobulin fraction of mAb-233 was mixed with sulfosuccinimidobiotin in 0.1 M HEPES buffer (pH 8.0) containing 50 mM NaCl to a biotin/mAb-233 molar ratio of 20. After incubation for 60 min at room temperature, the reaction was stopped by the addition of 1M Tris-HCl (pH7.5), and the fraction containing biotinylated antibody was dialyzed against Tris-buffered saline (TBS).

Mouse polyclonal anti NC16A antibody was prepared by immunizing a mouse with the human NC16A domain fused with glutathion S transferase (32).

Cells and cell cultures

BMGE+H and BMGE–H cells , mammary gland epithelial cell lines derived from a

lactating bovine udder, were the kind gift of Dr. W. W. Franke of the German Cancer Research Center (Heidelberg, Germany) (51). BMGE+H cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal calf serum supplemented with 1 μg/ml insulin, 1 μg/ml hydrocortisone, and 1 μg/ml prolactin (Sigma Chemical Co., St. Louis, MO), and BMGE–H cells were grown in DMEM containing 20% fetal calf serum as described by Schmid *et al.* (51). DJM-1 cells, a human skin squamous carcinoma cell line, were kindly provided by Dr. Y. Kitajima of Gifu University (Gifu, Japan) and grown in Eagle's medium containing 10% fetal calf serum supplemented with 0.4 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor and 84 ng/ml cholera toxin as described by Kitajima *et al.* (52). A431 cells, an epidermal cell carcinoma cell line derived from human vulva, and FRSK cells, a keratinocyte line derived from rat foreskin, were obtained from the Japanese Cancer Research Resources Bank (Tokyo,Japan). The A431 cells were grown in DMEM with high glucose containing 10% fetal calf serum and the FRSK cells were grown in Eagle's medium containing 10% fetal calf serum.

Immunofluorescence Microscopy

Freshly prepared tissues were snap-frozen in isopentane precooled in liquid nitrogen. The isolated epithelia of corneas from bovine were embedded in OCT Compound (Miles, Elkhart, IN) and immediately frozen. Sections from frozen specimens were cut at 5–6 μm with a cryostat, mounted on glass slides, air dried, and fixed in 100% acetone at –20 °C for 10 min. In some cases the sections were treated with 0.5% Triton X-100 (Tx-100) or 1.5 M KCl in PBS before fixation. The sections were then incubated with primary antibodies for 30 min at room temperature, washed in PBS, and incubated again with fluorescent dye-conjugated secondary antibodies. After washing in PBS, the specimens were mounted with PermaFluor (Lipshaw Immunon, Pittsburgh, PA). Cells grown on glass coverslips were fixed with acetone at –20 °C for 5 min and air-dried. For the Triton treatment, intact cells on coverslips were rinsed in PBS and incubated in Tx-PBS for 5 min on ice before fixation. These cells were stained with antibodies as described above.

Fractions prepared from cultured cells

For the preparation of Triton X-100 soluble and -insoluble fractions used in the first section of the results, cells were rinsed in phosphate-buffered saline (PBS), scraped from dishes, and homogenized on ice in a low-salt buffer solution (140 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 7.4) containing 0.5% non ionic detergent Triton X-100 (Tx) and 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. After extraction for 30 min on ice, cells were homogenized and centrifuged at 30,000×g for 30 min to yield Tx-soluble (supernatant) and -insoluble (pellet) fractions.

For the preparation of cytosolic, Tx-soluble membrane-bound and Tx-insoluble fractions, cells were rinsed in phosphate-buffered saline (PBS), scraped from dishes, and homogenized on ice in a lysis buffer (50 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. After extraction for 30 min on ice, cells were centrifuged at 30,000×g for 30 min, and the resultant supernatants were collected as cytosolic fractions. Then residual pellets were resuspended and extracted in a low-salt buffer solution (150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.4) containing nonionic detergent 0.5% Tx-100, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A, and were centrifuged to yield Tx-soluble membrane-bound (supernatant) and Tx-insoluble (pellet) fractions. Cytoskeletal fractions were recovered from pellets by resuspension of Tx-insoluble fractions in a high salt buffer solution (1.5 M KCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.4) containing 0.5% Tx-100.

Conditioned medium collected from 3 day cultures was centrifuged at 1,000 rpm for 10 min to remove unattached cells and 5 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin and 0.1 mM *N*-ethylmaleimide were added. Proteins were precipitated by the addition of saturated ammonium sulfate solution (half the volume of the medium). Precipitates were resuspended in TBS containing 1mM EDTA as the medium fractions. In some experiments, the spent medium was precipitated first with 50% ammonium sulfate for concentration, and subsequently the precipitant dissolved in TBS containing 1 mM EDTA was precipitated with 33% ammonium

sulfate.

For immunoprecipitation, the resuspension buffer for the medium fractions was changed to a low-salt buffer containing 0.5% Tx-100, to equalize the buffer conditions for the Tx-soluble membrane-bound fractions.

Isolation of hemidesmosomes

Hemidesmosomes were isolated from bovine corneal epithelial cells, as described previously (24).

Collagenase treatment of cultured cells

Collagenase from *Clostridium histolyticum* (Amano Pharmaceutical, Japan) was added at 50 Mandle units/ml final concentration to the culture medium of semiconfluent cultured BMGE+H cells. After treatment for 60 min at 37 °C, the culture medium containing collagenase was removed and cells were processed for the preparation of 0.5% Tx-soluble and -insoluble fractions as described above.

Collagenase digestion of the HD fraction

Collagenase from *Clostridium histolyticum* (Amano Pharmaceutical, Japan) was added at 50 Mandle units/ml final concentration to the HD fraction isolated from corneas of 30 bovine eyes. After incubation for 60 min at 37 °C, the enzymatic reaction was stopped by the addition of ×2 SDS-sample buffer.

Electrophoresis and immunoblotting

SDS-PAGE was performed according to the method of Laemmli with a slight modification (53). Immunoblotting was performed using SDS-PAGE and subsequent electrophoretic transfer onto nitrocellulose sheets using a semi-dry system as previously described (7).

from the bottom of the tubes. The fractions were analyzed by SDS-PAGE and immunoblotting. The frictional ratio of BP180, f/f_0 = 2.8, was calculated from $s_{20,w}$ and the expected molecular mass of trimeric BP180, assuming the usual values of 0.73 ml/g for partial specific volume and 0.4 g/g for hydration. Standards and corresponding $s_{20,w}$ values were thyroglobulin (19.3 S), bovine liver catalase (11.3 S), yeast alcohol dehydrogenase (7.6 S), and bovine serum albumin (4.6 S).

Low-angle rotary-shadowing electron microscopy

Affinity-purified specimens were dialyzed against 50% glycerol in TBS at 4 °C for 16 h. The samples were sprayed by using an air brush onto freshly cleaved mica. The droplets on the mica were dried at room temperature in a vacuum at 10^{-8} mmHg in newly developed freeze-etch equipment (Hitachi HR7000) for 10 min. Dried specimens were rotary-shadowed with platinum using an electron gun positioned at 2.5° to the mica surface, and then coated with a film of carbon generated by an electron gun positioned at 90° to the mica surface. The replica was floated on distilled water and collected on a grid covered with formvar-film. The specimens were observed with a JEM 100CX electron microscope.

PART I

Demonstration of the molecular shape of BP180 and its potential for trimer formation

RESULTS

Localization of Triton-soluble BP180 in corneal epithelium and cultured cells

The precise localization of BP180 in corneal epithelium was examined by immunofluorescence microscopy with a monoclonal antibody (mAb). In addition to the basal surface of the basal cells, immunofluorescence staining was observed on lateral surfaces where hemidesmosomes are not localized (Fig. 6A). However, in Triton X-100 treated specimens, the lateral staining was lost completely, while the basal one was still clearly observed (Fig. 6B). BP230 showed only basal localization with or without Triton X treatment (Fig. 6D). These results suggest that the lateral BP180 is free from the cytoskeleton and not incorporated into hemidesmosomes. I refer to this subpopulation as Tx-soluble BP180.

To determine whether the Tx-soluble BP180 similarly exists also in cultured cells, BMGE+H cells and DJM-1 cells were examined by immunofluorescence microscopy. Hemidesmosomes were observed as dot-like staining in BMGE+H cells (Fig. 6E) and in an arc- or leopard skin-like pattern in DJM-1 cells (Fig. 6G), but additional non-ventral staining was also observed around cell-to-cell borders and what appeared to be the whole cell surfaces in both cell lines. In Triton-treated cells, hemidesmosomes were still observed clearly but the non-ventral staining was lost entirely, showing that non-ventral BP180 corresponds to the Tx-soluble BP180 of corneal epithelial cells (Fig. 6, F and H).

Isolation and detection of Tx-soluble BP180 in cultured cells by immunoblotting

Tx-soluble and -insoluble fractions of BMGE+H cells, DJM-1 cells, A431 cells and FRSK cells were examined by immunoblotting using mAb-233 and -1A8C, which recognize extracellular and cytoplasmic parts of BP180, respectively (Fig. 7, A and B). Among the four cell lines, Tx-soluble BP180 was found to be most abundant in BMGE+H cells, so that this cell line was chosen for further characterization of BP180. The ratio of soluble to insoluble BP180 in BMGE+H cells was roughly 2 to 1.

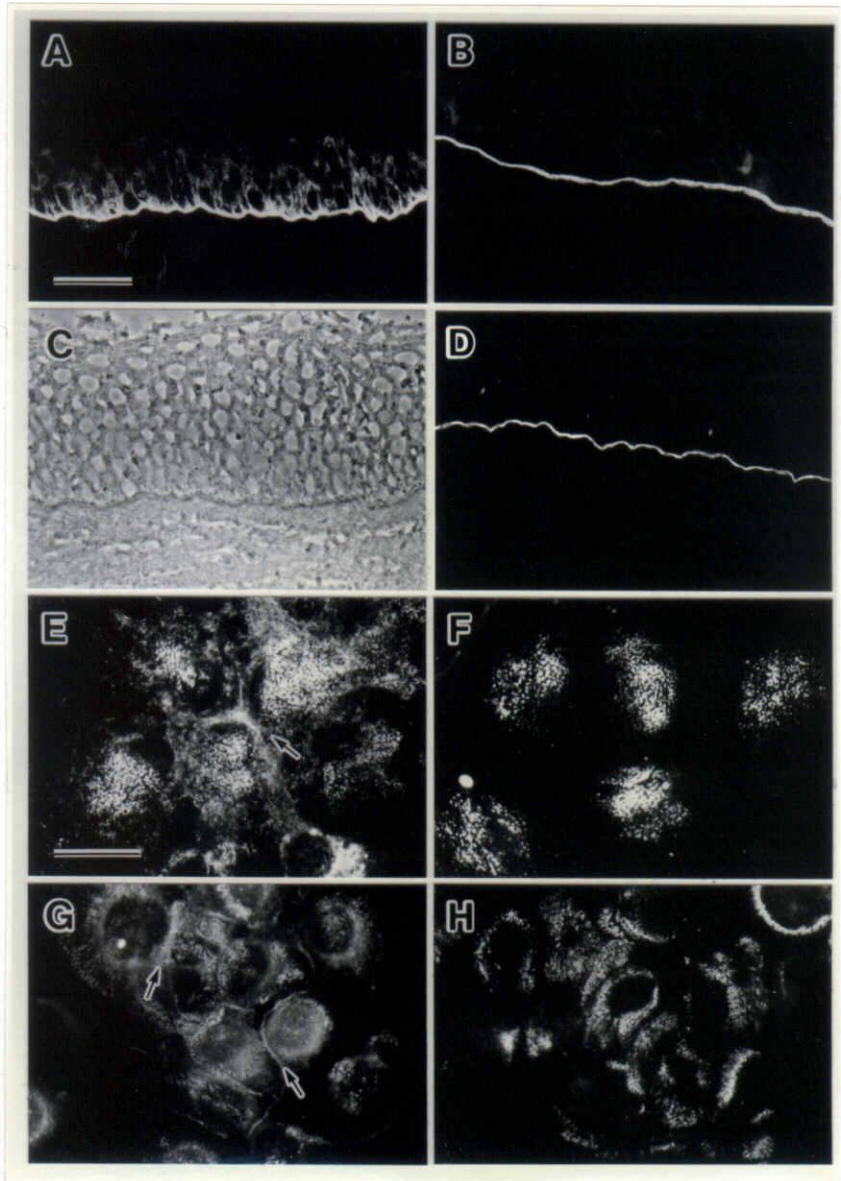


Figure 6. Immunofluorescence microscopy of corneal epithelia and cultured cells illustrating the locations of Triton-soluble BP180.

Corneal epithelia were stained with anti-BP180 mAb-233 (A and B) and anti-BP230 mAb-1E5 (D). A phase contrast image of A is shown in C. B was treated with 0.5% Triton X-100 before fixation. BMGE+H cells (E, F) and DJM-1 cells (G, H) were stained with mAb-233, in the F and H cases after treatment with Triton X-100. Arrows in E and G indicate cell-cell borders. Bars: (A–D) 50 μm; (E–F) 25 μm.

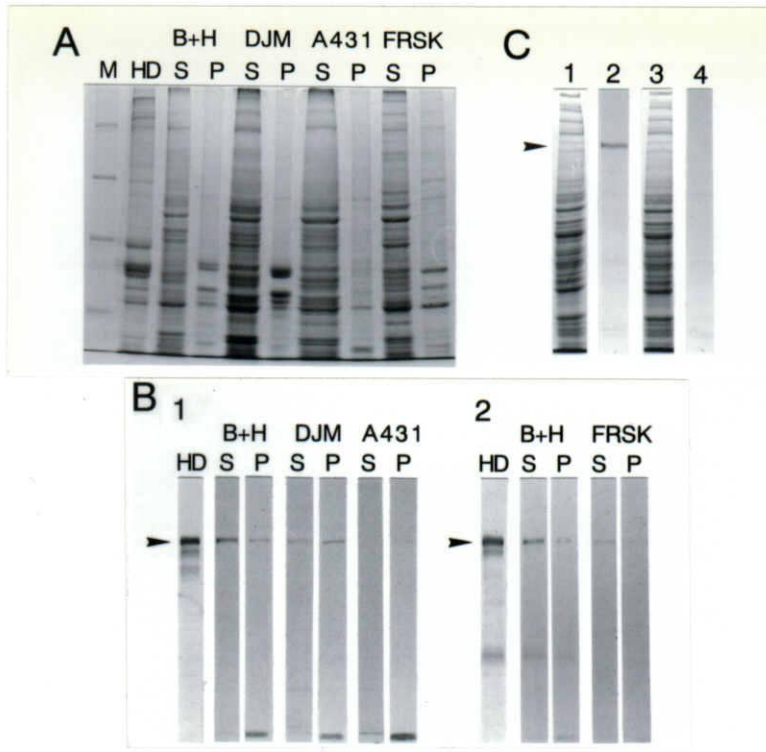


Figure 7. Detection of Triton-soluble BP180 in cultured cell lines by immunoblotting.

A; Coomassie blue staining of Triton X-100-soluble (S), -insoluble (P) and hemidesmosome (HD) fractions. The HD fraction was used as a control. To compare the content of BP180 in each fraction, 800 μl samples of soluble and insoluble fractions were prepared from each cell line, at confluence in 6 cm diameter dishes, and 40 μl aliquots were applied to SDS-PAGE. Molecular weight markers (M) are myosin heavy chain (205,000), β-galactosidase (116,000), BSA (66,000) and aldolase (42,000).

B; Immunoblotting of Triton X-100-soluble, -insoluble and HD fractions. Fractions were prepared as for A. 1; Immunoblotting with mAb-233. 2; Immunoblotting with mAb-1A8c. The arrowheads denote the 180 kDa polypeptide of BP180.

C; Collagenase sensitivity of BP180 in the Triton-soluble fraction. Triton X-100 soluble fractions prepared from BMGE+H cells cultured in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of collagenase were stained with Coomassie (lanes 1 and 3) and immunoblotted with mAb-233 (lanes 2 and 4). The 180 kDa polypeptide (arrow head) in lane 2 is not present in lane 3.

Surface localization of Tx-soluble BP180 was confirmed by its sensitivity to collagenase treatment of living cells (Fig. 7C). In intact cells, a 180 kDa band was recognized by mAb-233 whose epitope localizes to the collagenous extracellular region of BP180 (Fig. 7C, lane 2), but was completely absent in specimens prepared from cells treated with collagenase (Fig. 7C, lane 4).

Chemical crosslinking

The presence of collagenous sequences in the extracellular part of BP180 suggests that a collagen-like trimer conformation may exist. Chemical crosslinking experiments are effective for investigating this possibility. The Tx-soluble fraction was treated for 1 h on ice with 2.5 μM or 250 μM DSP, a homobifunctional reagent that crosslinks between amino bases, and analyzed by immunoblotting using mAb-233 (Fig. 8A). After crosslinking, the 180 kDa band detected in the intact fraction was reduced or disappeared, and instead two bands with higher molecular masses, about 400 and 600 kDa, newly appeared. Since the expected apparent molecular masses of dimers and trimers of BP180 are 360 kDa and 540 kDa, respectively, the 400 and 600 kDa bands might respectively correspond to such crosslinked forms. Alternatively, it is possible that crosslinking between BP180 and other associated proteins caused these higher molecular mass bands. In order to determine whether BP180 itself forms the same crosslinked products, I immunoprecipitated BP180 from the Tx-soluble fraction of biotinylated BMGE+H cells with mAb-233, and subsequently the immunoprecipitant was crosslinked with 0.5 mM *p*-PDM, a homobifunctional reagent that crosslinks between sulfide bases, for 1 h at room temperature (Fig. 8B, lane 3). The 180 kDa band in the intact fraction shifted to the higher molecular mass position at about 600 kDa in the crosslinked fraction (cf. lanes 1 and 3). Hence, together with our previous data that no co-immunoprecipitant with BP180 was detected under the same conditions used in these experiments (23), the results indicate that crosslinking between BP180 and associated protein(s) can be excluded. According to cDNA sequences of human and mouse BP180 (16, 18), cysteine residues are

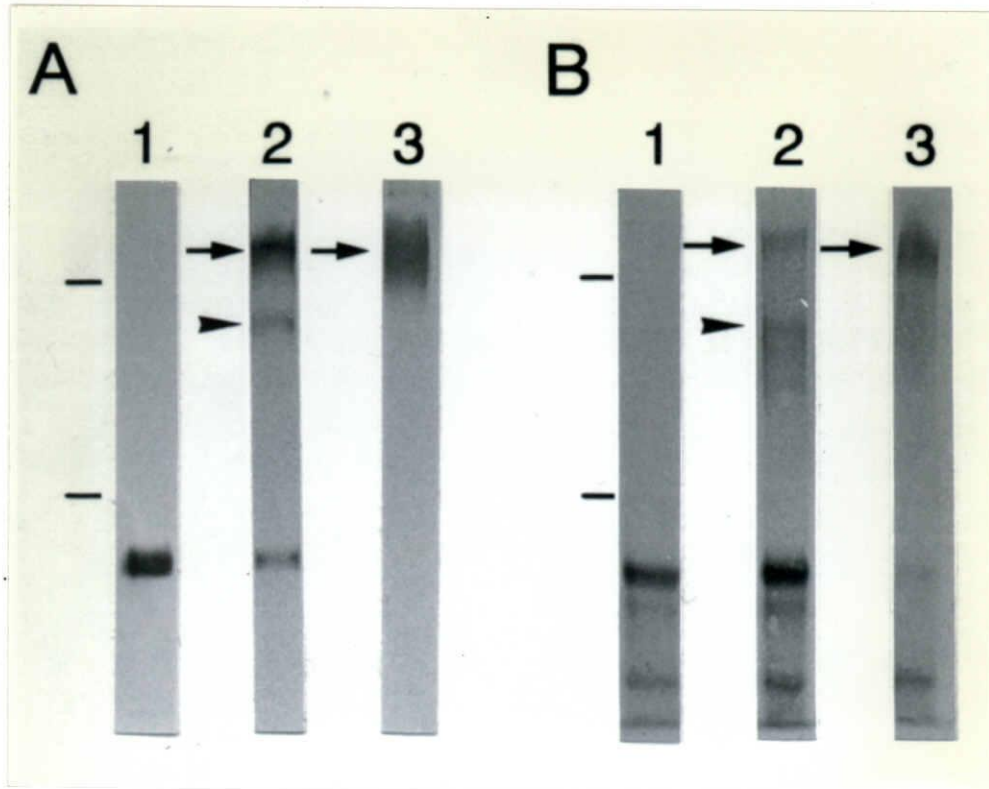


Figure 8. Chemical crosslinking of BP180.

A; Chemical crosslinking of the Triton-soluble fraction. The Triton X-100 soluble fraction from BMGE+H cells was treated with 0 μM (lane 1), 2.5 μM (lane 2) and 250 μM (lane 3) of DSP and immunoblotted with mAb-233 under reducing (lane 1) or non-reducing conditions (lane 2 and 3). Arrows and the arrowhead indicate the trimer and dimer of BP180, respectively. Note that the highly crosslinked specimen demonstrates only the band corresponding to the trimer (lane 3).

B; Chemical crosslinking of immunoprecipitated BP180. The Triton X-100 soluble fraction from BMGE+H cells with biotinylated surfaces was immunoprecipitated with mAb-233, and subjected to SDS-PAGE with (lane 1) or without (lane 2) reducing reagent. A sample crosslinked with 0.5 mM *p*-PDM as described in the Materials and Methods was also subjected to SDS-PAGE (lane 3). After samples were transferred onto nitrocellulose membranes, biotinylated proteins were detected by avidin-alkaline phosphatase. Arrows and arrowhead indicate the trimer and dimer of BP180, respectively. Note that the chemically crosslinked specimen demonstrates only the band corresponding to the trimer (lane 3). 4% gels were used for SDS-PAGE of the cross-linked products. Dashes at the left margin of A and B indicate positions of HD1 (500 kDa) and myosin heavy chain (200 kDa) applied as standards.

located exclusively in the cytoplasmic portion, and the epitope of mAb-233 used in the immunoprecipitation resides in the extracellular portion, so that the crosslinking by *p*-PDM can not be made between BP180 and immunoglobulin. For the reasons described above, the crosslinked products with higher molecular masses are concluded to be composed of BP180 oligomers. The mAb-233 immunoprecipitant resolved in SDS-PAGE without reducing reagent contained not only the 180 kDa polypeptide but also the 400 kDa and the 600 kDa polypeptides (lane 2). Since these higher molecular components were not detected under reducing conditions (lane 1), the 400 kDa and 600 kDa polypeptides, respectively, probably represent dimers and trimers of BP180 linked by disulfide bonds. Considering the fact that all the cysteine residues reside in the cytoplasmic portion where a reducing environment prevails, the reduction-sensitive oligomers appear to be formed during the experimental processes. The fact that the electrophoretic mobility of the disulfide bonded trimer (lane 2) was found to be approximately the same as that of the chemically crosslinked oligomers (lane 3) indicates the latter to also have a trimer nature. These chemical crosslinking experiments provide clear evidence of trimer formation by the Tx-soluble BP180.

Immunoaffinity purification of BP180

To further characterize BP180, I purified the Tx-soluble form from BMGE+H cells by immunoaffinity column chromatography using mAb-233 (Fig. 9). Eluted fractions were analyzed by SDS-PAGE using silver staining, and demonstrated only a 180 kDa band (lane 4). The band was also recognized by both mAb-233 (lane 8) and BP serum (lane 9), showing that the Tx-soluble BP180 was purified. Chemical crosslinking of purified BP180 with DSP showed its trimer formation (Fig. 10A). The purified BP180 was treated with 250 μM DSP and analyzed by SDS-PAGE. After crosslinking, a higher molecular mass band of 600–500 kDa was recognized by silver staining (lane 2). The molecular mass of the crosslinked product was approximately equivalent to that of the BP180 trimer described above. The relatively broad banding pattern of the crosslinked trimer indicates some heterogeneity in the

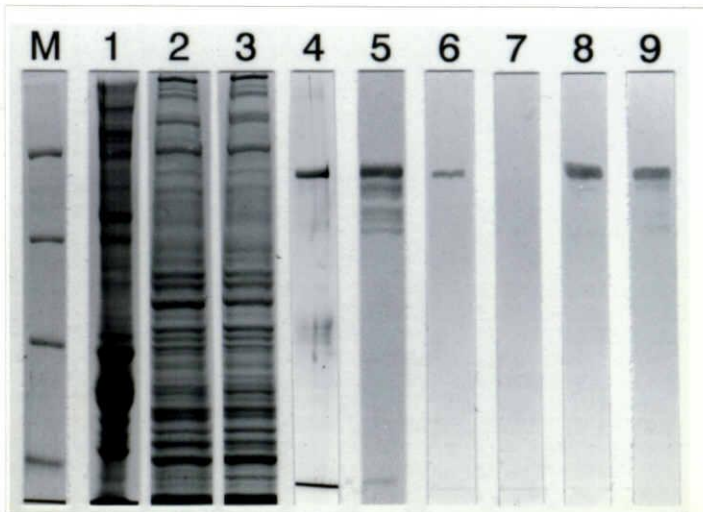


Figure 9. Immunoaffinity purification of BP180 from the Triton-soluble fraction.
Coomassie blue (lanes 1–3) and silver (lane 4) stained gels and corresponding immunoblots stained with mAb-233 (lanes 5–8) and BP serum (lane 9). HD fraction (lanes 1 and 5), the Triton X-100 soluble fraction prepared for affinity chromatography (lanes 2 and 6), the Triton X-100 soluble fraction flowing through the immunoaffinity column (lanes 3 and 7) and the eluted fraction (lanes 4, 8 and 9) were analyzed. Molecular markers (M) are the myosin heavy chain (205,000), β-galactosidase (116,000), BSA (66,000) and aldolase (42,000).

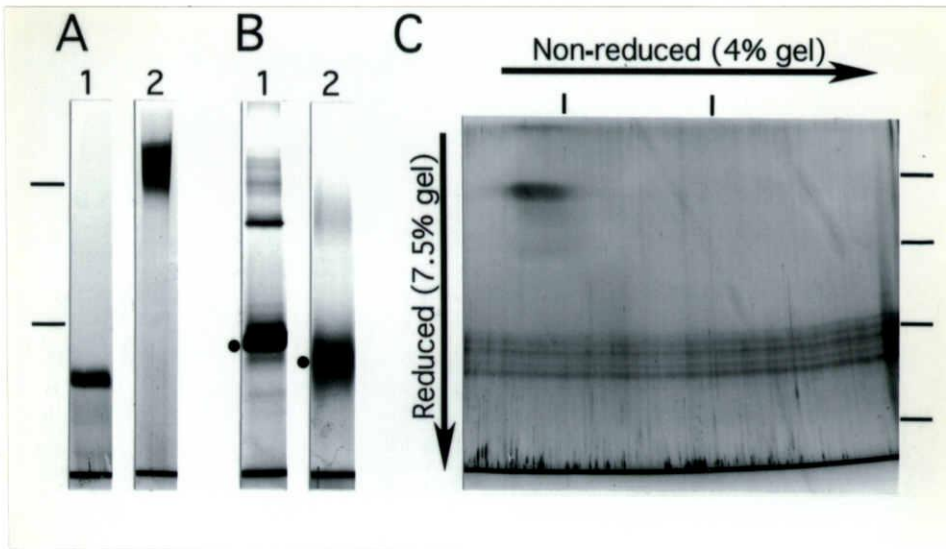


Figure 10. Chemical crosslinking of affinity-purified BP180.

A; Eluted fractions containing purified BP180 were treated with 0 μM (lane 1) or 250 μM (lane 2) of DSP, and subjected to SDS-PAGE with (lane 1) or without (lane 2) reducing agent. Dashes on the left indicate positions of HD1 (500 kDa) and myosin heavy chain (200 kDa) applied as standards.

B; 25 μg/ml BSA in PBS was treated with 0 μM (lane 1) or 250 μM DSP (lane 2), and subjected to SDS-PAGE with (lane 1) or without (lane 2) reducing agent. Dots indicate the position of BSA.

C; Two dimensional SDS-PAGE of purified BP180 crosslinked with DSP. The specimen was crosslinked with 250 μM DSP and subjected to electrophoresis on 4% gel under non-reducing conditions for the first dimension. The arrow at the top indicates the direction of migration, and dashes indicate the positions of 500 kDa and 200 kDa. The gel was then subjected to electrophoresis on a 7.5% gel under reducing conditions for the second dimension. The arrow at the left indicates the direction, and dashes on the right indicate positions of standards of 205 kDa, 116 kDa, 66 kDa and 42 kDa. All gels were silver stained.

intramolecular crosslinking. Under the same conditions, the molecular mass of BSA was not changed after the crosslinking reaction, but broad banding was observed (Fig. 10B). The DSP used in this experiment contains a disulfide bond between the *n*-hydroxysuccinimidate reactive groups, so that crosslinked oligomers can be dissociated under reducing conditions. The crosslinked specimen was subjected to two dimensional SDS-PAGE (Fig. 10C), the first dimension (4% separating gel) being run under non-reducing conditions, and the subsequent second dimension (7.5% separating gel) under reducing conditions. As expected, under reducing conditions the crosslinked product of 600–500 kDa observed in the first dimension showed a spot at about 180 kDa corresponding to the BP180 monomer.

Hydrodynamic properties of BP180

The Tx-soluble fraction of BMGE+H cells and immunoaffinity purified BP180 were analyzed by 5–20% sucrose gradient centrifugation (Fig. 11). Fractions obtained were examined by immunoblotting or silver staining. The peak positions proved to be almost the same in the two cases, with similar or identical sedimentation velocities. Svedberg constant of the peak position was estimated to be about 7 S. Since BP180 demonstrated the same hydrodynamic properties before and after immunoaffinity purification, I conclude that the purified BP180 essentially maintained its native soluble form.

Morphology of BP180

The rotary shadowed images of purified bovine BP180 showed a characteristic molecular shape resembling a quaver, a musical note, consisting of three domains, a linear rod (rod domain) with a globule (head domain) at one end and a flexible or amorphous part (tail domain) at the opposite end (Fig. 12). Molecules exhibited variation in length, depending on the configuration of the third domain. High power images of the flexible/amorphous region allowed tracing along its entire length, and this part was found very changeable like a winding rope or a wet noodle (Fig. 13). The three distinct domains were designated as the globular

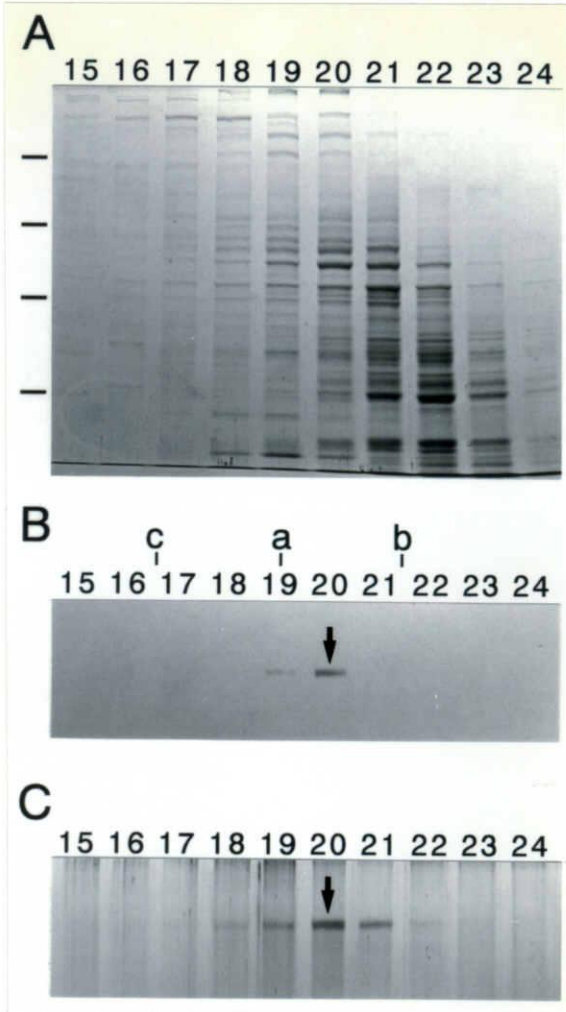


Figure 11. Hydrodynamic properties of soluble BP180 assessed by sucrose gradient centrifugation.

Proteins contained in the Triton X-100 soluble fraction of BMGE+H cells were fractionated by 5–20% sucrose gradient centrifugation. Then the fractions obtained were subjected to SDS-PAGE followed by coomassie blue staining (A) or immunoblotting with mAb-233 (B). The eluted fraction containing purified BP180 was also analyzed by sucrose gradient centrifugation at the same time, and silver stained (C). Note the peak fractions (arrows) at 20 in both B and C. The relative positions of standard proteins in a parallel gradient are indicated at the top of B: c, bovine liver catalase (11.3 S); a, yeast alcohol dehydrogenase (7.6 S); b, bovine serum albumin (4.6 S). The position of thyroglobulin (19.3 S) was at 11. Fraction numbers are given at the top of all figures. Dashes on the left of A indicate standards of 205 kDa, 116 kDa, 66 kDa and 42 kDa.

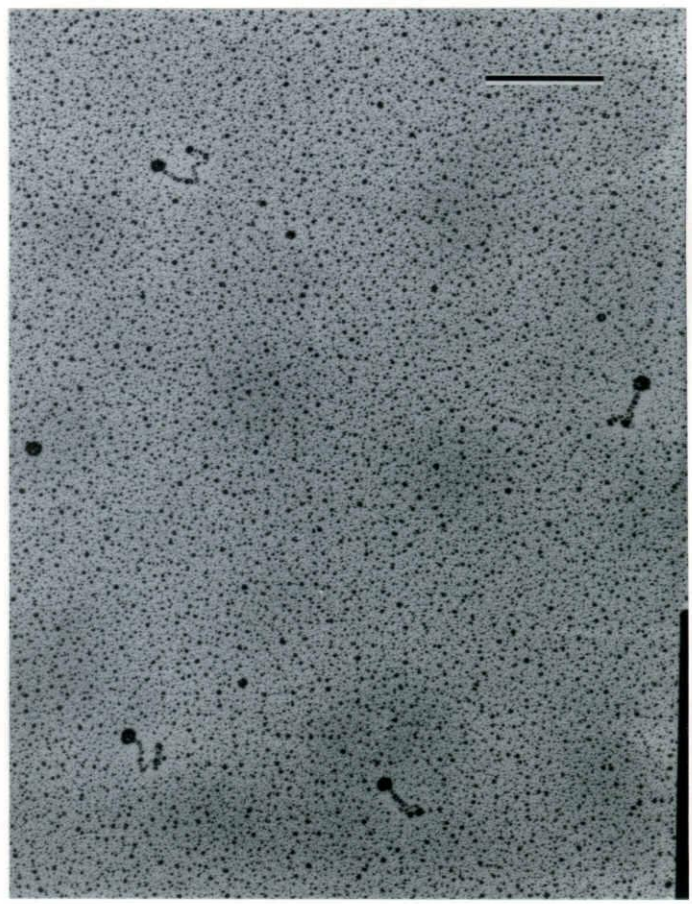


Figure 12. Rotary shadow-images of BP180.

The eluted fraction containing affinity-purified bovine BP180 was dialyzed against 50% glycerol in TBS and rotary shadowed as described in the Materials and Methods. A low magnification field is shown. Bar: 200 nm.

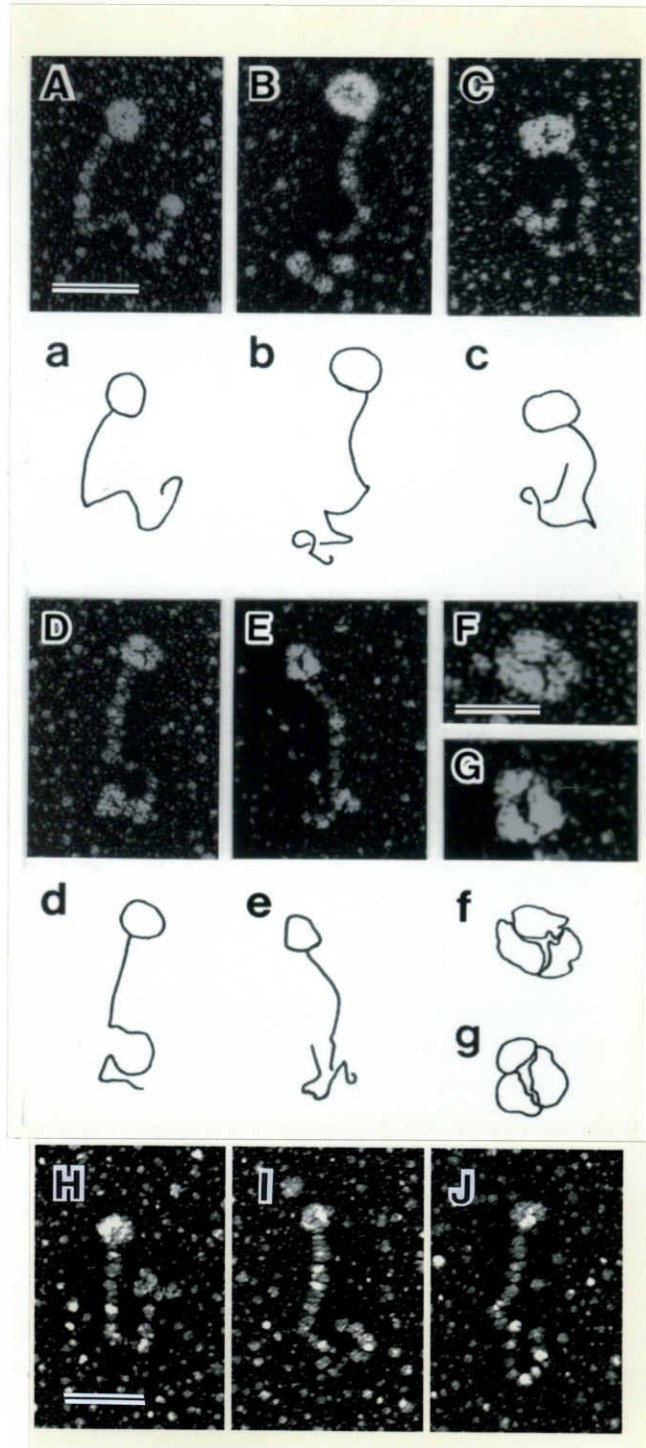


Figure 13. Typical examples of BP180.

Several representative molecules (bovine BP180: A–E, human BP180: H–J) are shown. F and G are enlargements of the globular regions of D and E, respectively. Images (A–G) are respectively interpreted in drawings (a–g). Bars: (A–E; H–J) 50 nm; (F and G) 25 nm.

head, the central rod and the flexible tail, respectively.

The round shaped globular heads demonstrated a relatively uniform diameter of 25–35 nm, although probable overestimation because of the contribution of the platinum coating should be born in mind. In optimally shadowed specimens, three subdomains of equal size were recognized in such globule heads, suggesting trimer formation (Fig. 13, F and G).

The central rod was revealed to be of relatively uniform dimensions and apparently straight and rigid appearance. The length of individual rods was 60–70 nm, when the distance between the periphery of the globular head and the first bending point was measured.

The flexible tail appeared very variable in shape. Fifteen selected images that were easy to trace and relatively extended were measured (Fig. 13, A–E), the distance between the first bending point and the far end averaging 115.3 nm.

Intact human BP180 molecules were also purified from the Tx-soluble membrane-bound fraction by immunoaffinity column chromatography using mAb-1A8c. Fractions were prepared from DJM-1 cells. The molecular dimensions of the observed molecules were essentially as same as those of its bovine counterpart described above (Fig. 13, H–J).

PART II

Cleavage of BP180 yields a 120 kDa collagenous extracellular polypeptide

RESULTS

Identification of the mAb-1337 antigen

The mAb-1337 is a monoclonal antibody obtained by immunizing a mouse with the HD fraction, and stained basement membrane zone (BMZ) of bovine epidermis in immunofluorescence microscopy. Immunoblot analysis of the HD fraction showed that the antibody recognized a 120 kDa polypeptide as described previously in our preliminary report (54). To confirm the previous results and further characterize the antigen, the HD fraction was treated with collagenase and examined by immunoblotting (Fig. 14). The 120 kDa band was completely lost by collagenase digestion as reported previously, showing that the polypeptide has a collagenous domain(s) as BP180 do. Hence, I investigated the 120 kDa polypeptide with special reference to BP180, a hemidesmosomal transmembrane collagen. As shown in Fig. 14, immunoblotting with mAbs against extracellular parts of BP180 showed the electrophoretic mobility of the antigen to be equal to that of the smallest polypeptide among a group of proteolytic fragments appearing in the HD fraction. On two-dimensional SDS-PAGE and subsequent immunoblotting, the antigen and the BP180 fragment showed an identical spot (data not shown). From these results, I conclude that mAb-1337 recognizes primarily the 120 kDa fragment of BP180 on immunoblotting.

Immunofluorescence microscopy of bovine skin and cultured cells

To examine whether the mAb-1337 specifically recognizes the 120 kDa fragment of BP180 by immunofluorescence microscopy, the staining pattern of mAb-1337 was compared with those of mAbs against BP180. The titers among the mAbs were equalized to allow comparative observations. The mAb-1337 clearly stained the BMZ of bovine skin, as did the other mAbs to BP180, but, in contrast, not the lateral surfaces of the basal cells (Fig. 16, a–d). Thus, mAb-1337 did not recognize Tx-soluble BP180, which is free from the cytoskeleton and not incorporated into HDs. Immunofluorescence microscopy of BMGE+H cells and primary

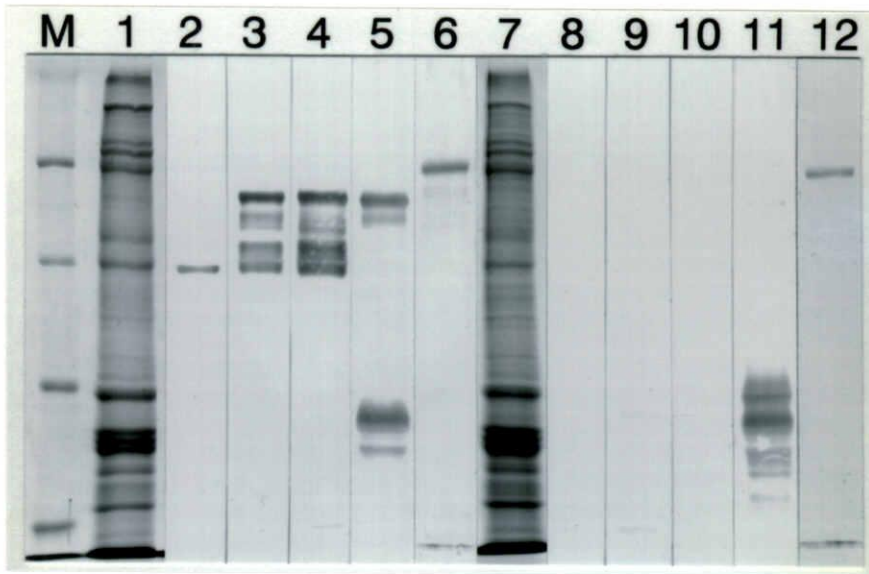


Figure 14. Comparison of the mAb-1337 antigen with BP180 by immunoblotting.
HD fractions treated with (lanes 7–12) or without (lane 1–6) collagenase were stained with Coomassie blue (lanes 1 and 7) or immunoblotted with mAb-1337 (lanes 2 and 8), mAb-233 (lanes 3 and 9), mAb-1D1 (lanes 4 and 10), mAb-1A8c (lanes 5 and 11) and anti integrin β4 mAb-1A3 (lane 6 and 12). The locations of epitopes for mAbs against BP180 are presented in Fig. 15. The 120 kDa polypeptide recognized by mAb-1337 and the 180 kDa polypeptide of BP180 were digested by the treatment, but the 200 kDa polypeptide of the integrin β4 subunit was not. Molecular weight markers (M) are myosin heavy chain (205,000), β-galactosidase (116,000), BSA (66,000) and aldolase (42,000).

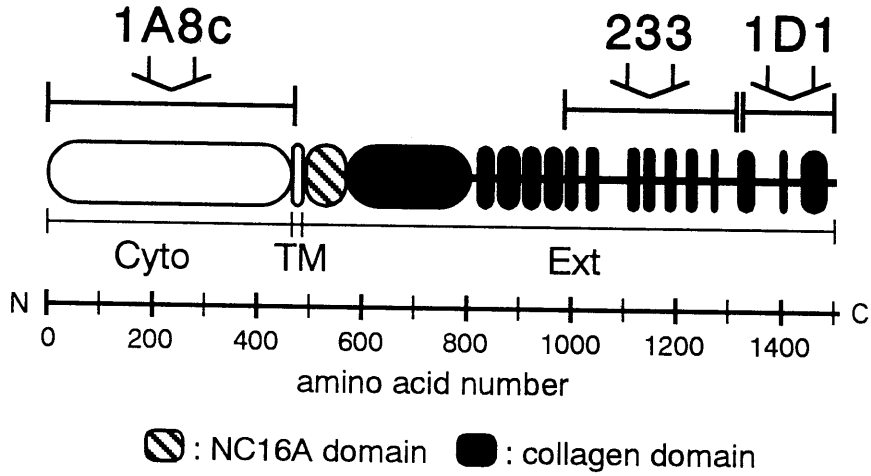


Figure 15. Schematic diagram of human BP180, with the locations of epitopes for mAbs. Arrows indicate the portions including epitopes for mAbs. The epitope of mAb-1A8c is mapped on the cytoplasmic part, and those of mAb-233 and -1D1 are mapped on the carboxyl-terminal half of the extracellular part of BP180 (Nishizawa, Y. *et al.*(19)., Hirako, Y. and Owaribe, K. unpublished data). In this study, the epitope of mAb-1D1 is localized within the most carboxyl-terminal 20 kDa portion, which is not recognized by mAb-233. Recently, Borradori *et al.* (41) have suggested the probable location of the epitope for mAb-1A8c within the region of 113–201th amino acids of BP180. Cyto, TM and Ext are the cytoplasmic, transmembrane and extracellular portions of BP180, respectively. The schematic domain organization of human BP180 is based on the primary structural analysis performed by Giudice *et al.*(16).

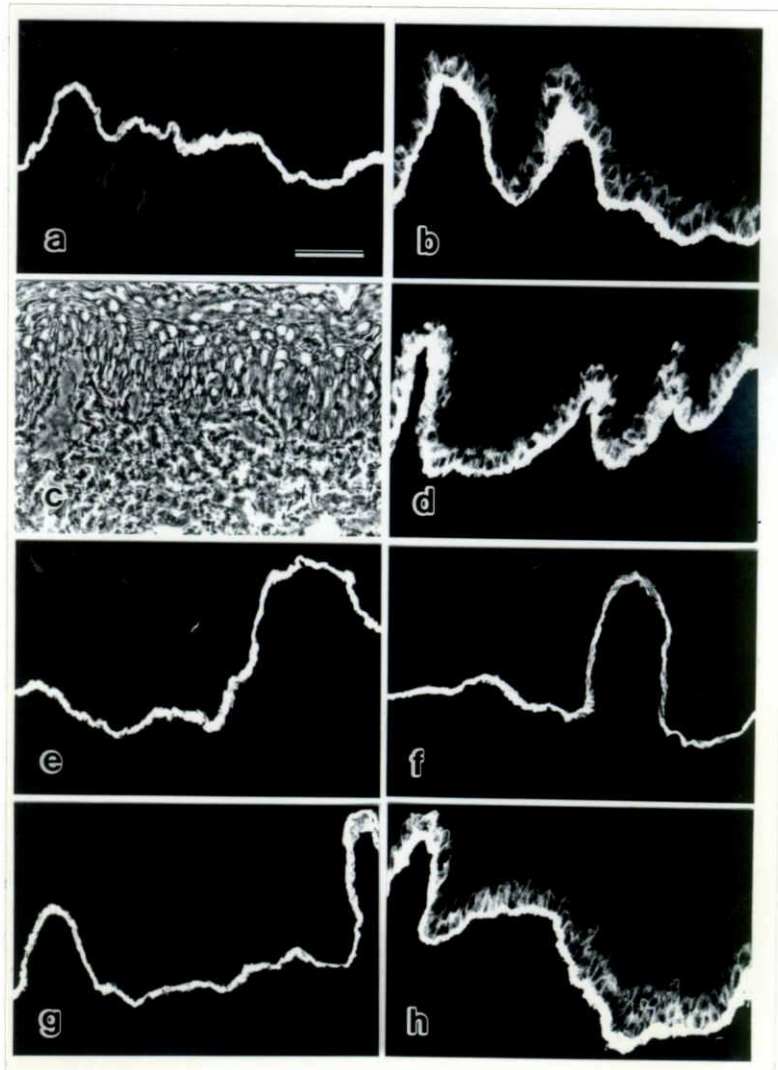


Figure 16. Immunofluorescence microscopy of bovine skin.

Sections of bovine skin were stained with mAb-1337 (a, e, g), mAb-233 (b, f, h) and mAb-1A8c (d). A phase contrast image of a is shown in c. For e and f prior treatment with 0.5% Tx-100, and for g and h with 1.5 M KCl before fixation was performed. Note that the lateral staining pattern of basal cells in b and d is not apparent in a. See the loss of lateral stainings by the Tx-treatment in f. Bar: 50 μm.

cultured bovine conjunctival epithelial cells (data not shown) showed that mAb-1337 did not detect HDs (Fig. 17), pointing to a lack of binding to insoluble BP180. These results are quite different from those with other typical anti-BP180 mAbs, and suggest that mAb-1337 does not recognize intact BP180 in either soluble or insoluble states. Therefore, its staining pattern observed in bovine skin indicates the presence of the 120 kDa fragment. When frozen sections of bovine skin were treated with 0.5% Tx-100 or 1.5 M KCl and stained with mAb-1337, the immunofluorescent staining was essentially the same as in control sections, showing that the 120 kDa fragments are largely insolubilized in the tissue (Fig. 16, e–h).

Detection of 120 kDa fragments in culture medium

Immunofluorescence microscopy with mAb-1337 showed the antigenic fragment to be lacking or present at only a very low level on BMGE+H cells (Fig. 17). If the fragment does not have a transmembrane domain nor cytoplasmic part, diffusion into the culture medium would be expected. To examine this possibility, spent media from BMGE+H cells were analyzed by immunoblotting using mAb-1D1, a monoclonal antibody against the extracellular part of BP180, and a specific band of 120 kDa was detected (Fig. 18A). This polypeptide was concentrated in the fraction precipitated with 33% ammonium sulfate, and was also recognized by mAb-1337 and mAb-233, but not by mAb-1A8c that recognizes the cytoplasmic part of BP180 (Fig. 18B). The electrophoretic mobility of the 120 kDa polypeptide proved equal to that of the fragment from the HD fraction. Therefore, the polypeptide in the spent medium was concluded to be the 120 kDa fragment of the HD fraction. The 120 kDa polypeptide appeared prone to degradation to a 100 kDa polypeptide when the spent medium was precipitated first with 50% ammonium sulfate for concentration, and consequently the precipitant dissolved in TBS containing 1 mM EDTA was precipitated with 33% ammonium sulfate. The 100 kDa polypeptide was recognized by mAb-1337 and -233, but not by mAb-1D1, the epitope of which appeared to be removed (Fig. 18C).

In accordance with these results, no polypeptide was recognized in the Tx-insoluble

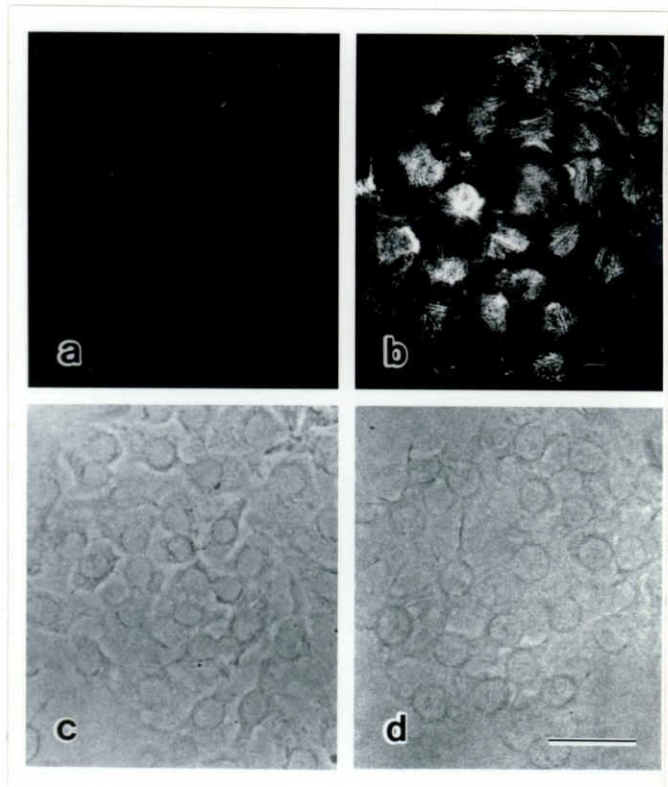


Figure 17. Immunofluorescence microscopy of cultured cells.

BMGE+H cells were stained with mAb-1337 (a) and mAb-233(b). Phase contrast images of a and b, respectively, are illustrated in c and d. HDs recognized by mAb-233 (b) were not detected by mAb-1337 (a). Bar: 50 μm.

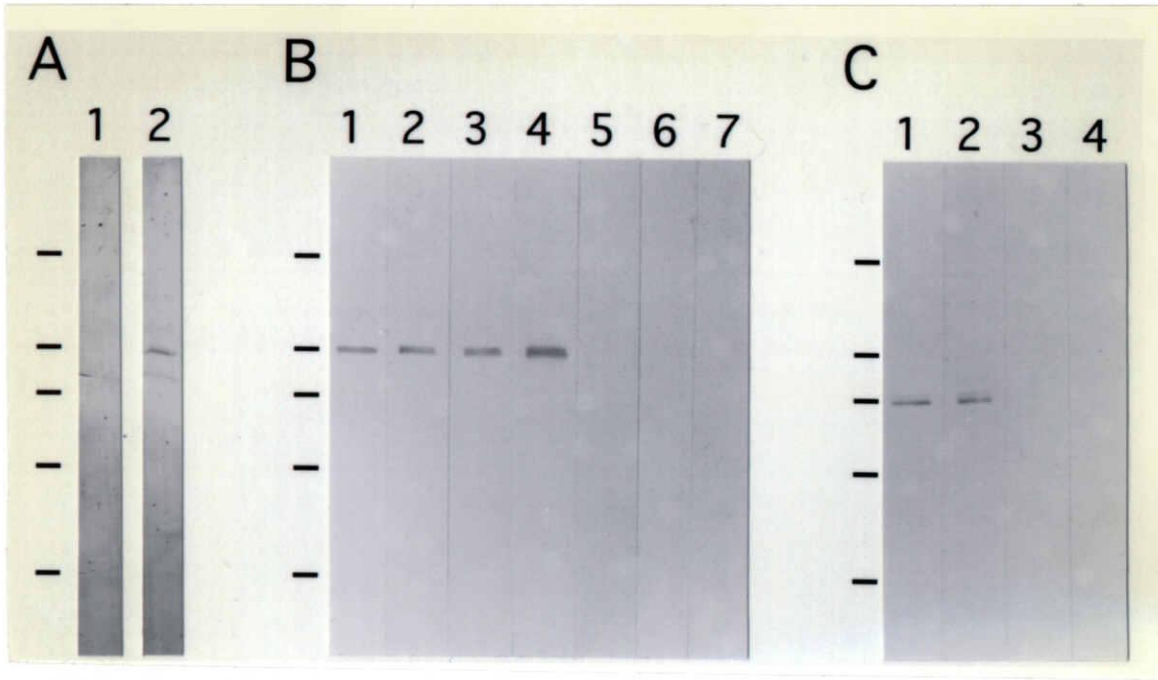


Figure 18. Detection of a 120 kDa fragment in spent culture medium from BMGE+H cells.

A; Control medium (lane 1) and crude spent medium from BMGE+H cells (lane 2) were immunoblotted with mAb-1D1.

B; The HD (lane 1) and medium fractions (lanes 2–7) were immunoblotted with mAb-1337 (lanes 1 and 2), mAb-233 (lane 3), mAb-1D1 (lane 4), mAb-1A8c (lane 5), mAb-1A6 (lane 6) and a mixture of mAb-855, mAb-310 and mAb-617, which recognizes the extracellular part of the integrin β4 subunit (lane 7). For concentration, proteins were precipitated from the crude spent medium by the addition of saturated ammonium sulfate solution (half the volume of the medium). Precipitates were resuspended in TBS containing 1mM EDTA as the medium fractions. 120 kDa polypeptides of medium fractions were recognized by mAb-1337 (lane 2) and mAbs against the extracellular part of BP180 (lanes 3 and 4), but not by mAbs against the cytoplasmic part of BP180 (lanes 5 and 6).

C; Immunoblotting of the medium fraction pre-precipitated with 50% ammonium sulfate. Fractions were immunoblotted with mAb-1337 (lanes 1), mAb-233 (lane 2), mAb-1D1 (lane 3) and mAb-1A8c (lane 4). 100 kDa polypeptides were not recognized by mAb-1D1 (lane 3). Dashes indicate standards of myosin heavy chain (205,000), β-galactosidase (116,000), phospholipase B (97,400), BSA (66,000) and ovalbumin (45,000).

fraction by immunoblot analysis with mAb-1337 (Fig. 19B). Both mAb-233 and -1D1 recognized the 120 kDa polypeptide of the medium fraction as well as the 180 kDa polypeptide in the Tx-insoluble fraction, while mAb-1A8c did not recognize the 120 kDa polypeptide (Fig. 19, C–E). The molar ratio of the 120 kDa polypeptide, Tx-soluble BP180 and Tx-insoluble BP180 was roughly 1:6:3 in BMGE+H cells. The approximately 60 kDa component recognized by mAb-1A8c should be noted (Fig. 19E). This 60 kDa polypeptide was not recognized by either mAb-233 or -1D1 and its apparent molecular mass and specific recognition by mAb-1A8c indicate that it mainly comprises the cytoplasmic part of BP180. Since the 60 kDa polypeptide was not detected in the cytosolic fraction but in both Tx-soluble membrane-bound and insoluble fractions, it would appear to have a transmembrane domain. On the other hand, soluble form of BP230, a hemidesmosomal plaque component, was detected in the cytosolic fraction but not in Tx-soluble membrane-bound fraction (Fig. 19F). It is reasonable to assume that the 60 kDa polypeptide is the fragment which remains after cleavage of the 120 kDa extracellular part from BP180. Therefore, considering the apparent molecular mass of the two fragments, the cleavage site(s) is probably on the extracellular side near the cell membrane.

120 kDa fragment is present as a trimer form

Trimer formation by the 120 kDa fragment was confirmed by immunoblotting (Fig. 20). When the membrane-bound fraction in SDS-PAGE sample solution was not boiled, a 600 kDa component was detected for BP180. The apparent molecular mass is equal to that of the chemically cross-linked trimers of BP180 as described in the first section of the results. Therefore, in the non-boiled condition, BP180 molecules exist in a trimer form, probably due to the collagen links. In the non-boiled condition, the 120 kDa fragment in the medium fraction was detected as a 350 kDa component. Since this molecular mass is consistent with a trimer of the fragment, it is concluded that the fragment exists as trimer form. mAb-1337 recognized the fragment in both monomer and trimer forms, but did not the intact with the 180 kDa polypeptide.

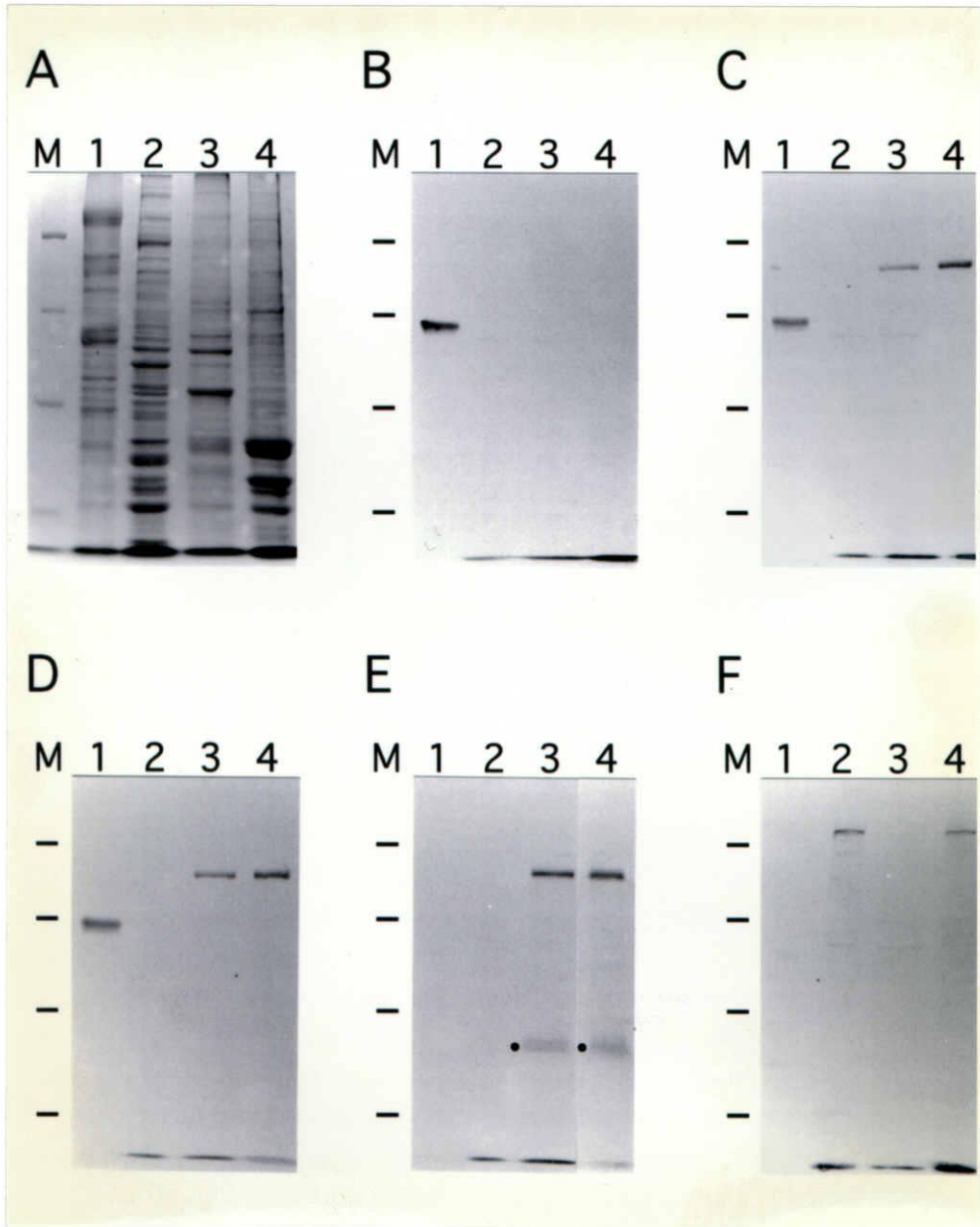


Figure 19. Immunoblotting of fractions prepared from cultured cells.

Medium (lane 1), cytosolic (lane 2), Tx-soluble membrane-bound (lane 3), and Tx-insoluble (lane 4) fractions prepared from BMGE+H cells were stained with Coomassie blue (A) and immunoblotted with mAb-1337 (B), mAb-233 (C), mAb-1D1 (D), mAb-1A8c (E) and mAb-1E5 (F). Dots in E indicate the position of a 60 kDa polypeptide recognized by mAb-1A8c. Molecular weight markers (M) are myosin heavy chain (205,000), β-galactosidase (116,000), BSA (66,000) and aldolase (42,000).

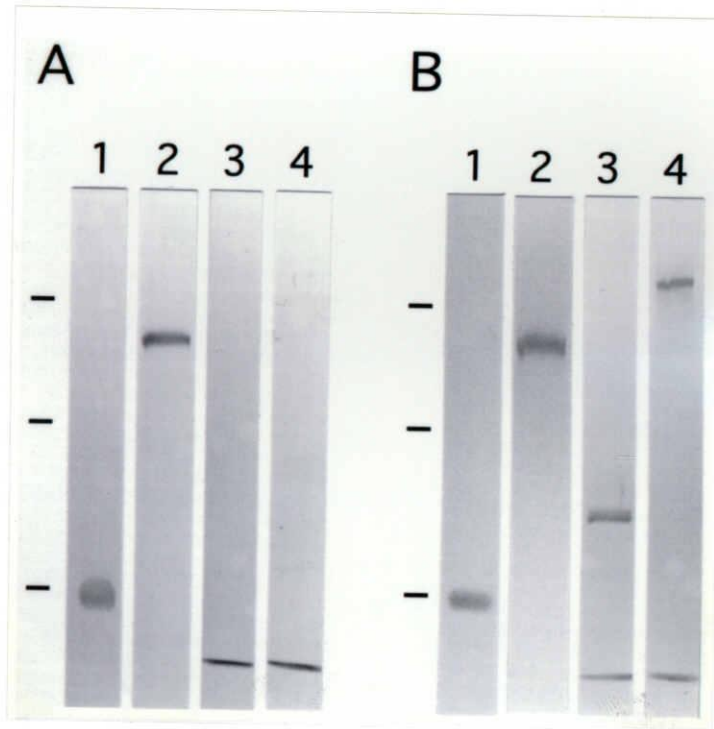


Figure 20. Examination of trimer formation by the 120 kDa fragment.

Medium (lanes 1 and 2) and Tx-soluble membrane-bound (lanes 3 and 4) fractions from BMGE+H cells were immunoblotted with mAb-1337 (A) and mAb-1D1 (B) under boiled (lanes 1 and 3) or non-boiled conditions (lanes 2 and 4). mAb-1337 (A) did not recognize the band corresponding to the trimer of 180 kDa polypeptide (lane 4) or the monomer (lane 3). Dashes indicate positions of HD1 (500 kDa), myosin heavy chain (200 kDa) and β-galactosidase (116 kDa) applied as standards.

Specificity of mAb-1337

The immunoreactivity of mAb-1337 against the 120 kDa fragment and the intact BP180 molecule in their native soluble forms was examined by immunoprecipitation. Immunoprecipitants were also analyzed by immunoblotting with mAb-233. While mAb-233 precipitated both the intact molecule and the fragment, mAb-1337 precipitated only the latter (Fig. 21A). The result demonstrated that mAb-1337 specifically recognizes the fragment, offering an explanation for the unique staining pattern observed on immunofluorescence, and supports the idea that the fragment actually exists in tissues. The recognition of the mAb-1337-immunoprecipitant with mAb-233 clearly demonstrated that the 120 kDa fragment is identical to the extracellular part of BP180.

The 120 kDa fragment was also detected in several cultured cells derived from epithelial tissues. When medium fractions of BMGE+H, DJM-1, A431 and BMGE–H cells were examined by immunoblotting using mAb-233 (Fig. 21B), the BMGE–H cell line lacking BP antigens was also found to be only one without the 120 kDa fragment in its medium fraction. Thus cleavage of BP180 to give the 120 kDa fragment is a common phenomenon in cells expressing the bullous pemphigoid antigen.

Purification of the 120 kDa fragment and determination of its morphology

120 kDa fragments were purified from the medium fraction by immunoaffinity column chromatography using mAb-233 (Fig. 22). Intact BP180 molecules were purified from the Tx-soluble membrane-bound fraction by immunoaffinity column chromatography using mAb-1A8c. Fractions were prepared from DJM-1 cells, a human skin squamous carcinoma cell line. The purity of the eluted fractions was assessed by SDS-PAGE using silver staining (Fig. 22, lanes 1 and 4). The eluted fractions from the mAb-233 column showed the 120 kDa band, recognized by mAb-233 (Fig. 22, lane 2) and other monoclonal antibodies (mAb-1D1, mAb-D20 and mAb-R223) that binding to the extracellular part of BP180 (data not shown). The eluted fraction of the mAb-1A8c column demonstrated the 180 kDa band, and, on

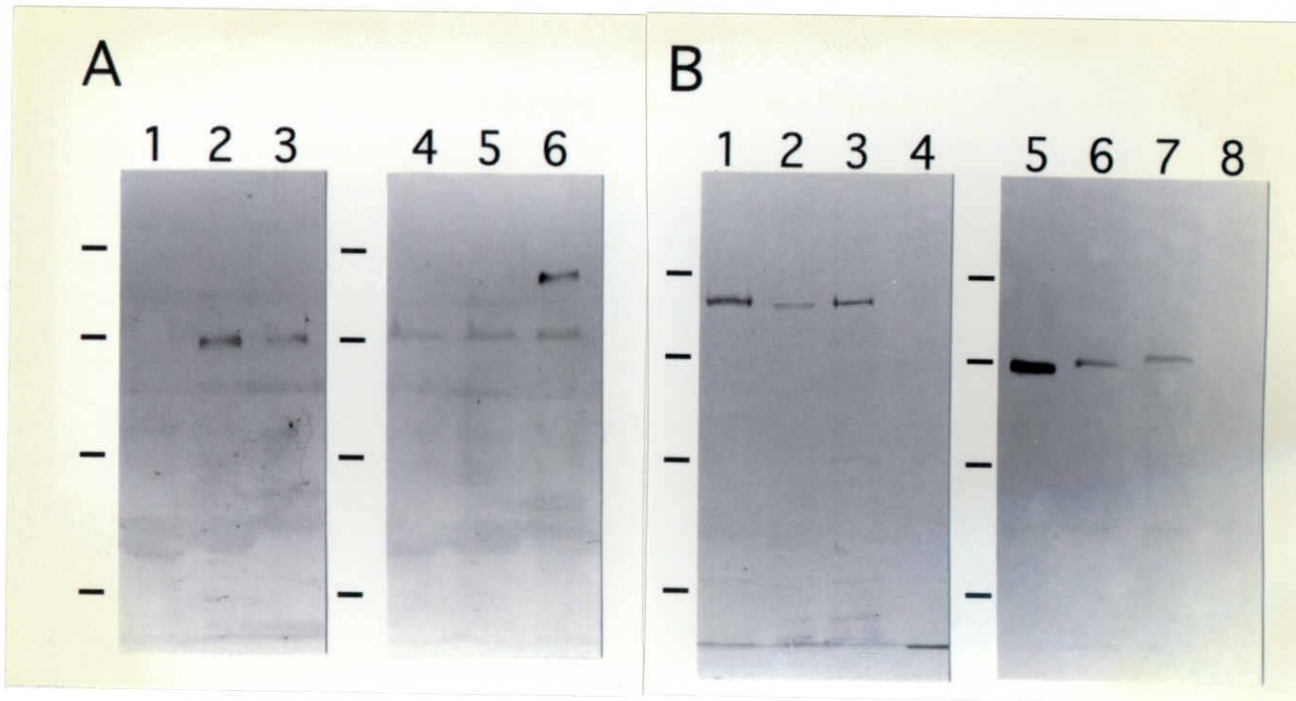


Figure 21. Immunoprecipitation of the 120 kDa fragment and the intact BP180 molecule.
A; Medium (lanes 1–3) and Tx-soluble membrane-bound (lanes 4–6) fractions were immunoprecipitated with mAb-R223 (lanes 1 and 4), mAb-1337 (lanes 2 and 5) and mAb-233 (lanes 3 and 6). Immunoprecipitants were immunoblotted with biotinylated mAb-233. mAb-R223 does not recognize bovine BP180 and was used as a negative control. mAb-1337 immunoprecipitated the 120 kDa fragment (lane 2) but not the 180 kDa molecule (lane 5). Dashes (M) indicate standards of 205 kDa, 116 kDa, 66 kDa and 42 kDa.
B; Detection of the 120 kDa fragment in spent media from several cultured cell lines. Cytoskeletal fractions (lanes 1–4) and medium fractions (lanes 5–8) prepared from BMGE+H (lanes 1 and 5), DJM-1 (lanes 2 and 6), A431 (lanes 3 and 7) and BMGE–H (lanes 4 and 8) cells were stained with mAb-233. Dashes (M) indicate standards of 205 kDa, 116 kDa, 66 kDa and 42 kDa.

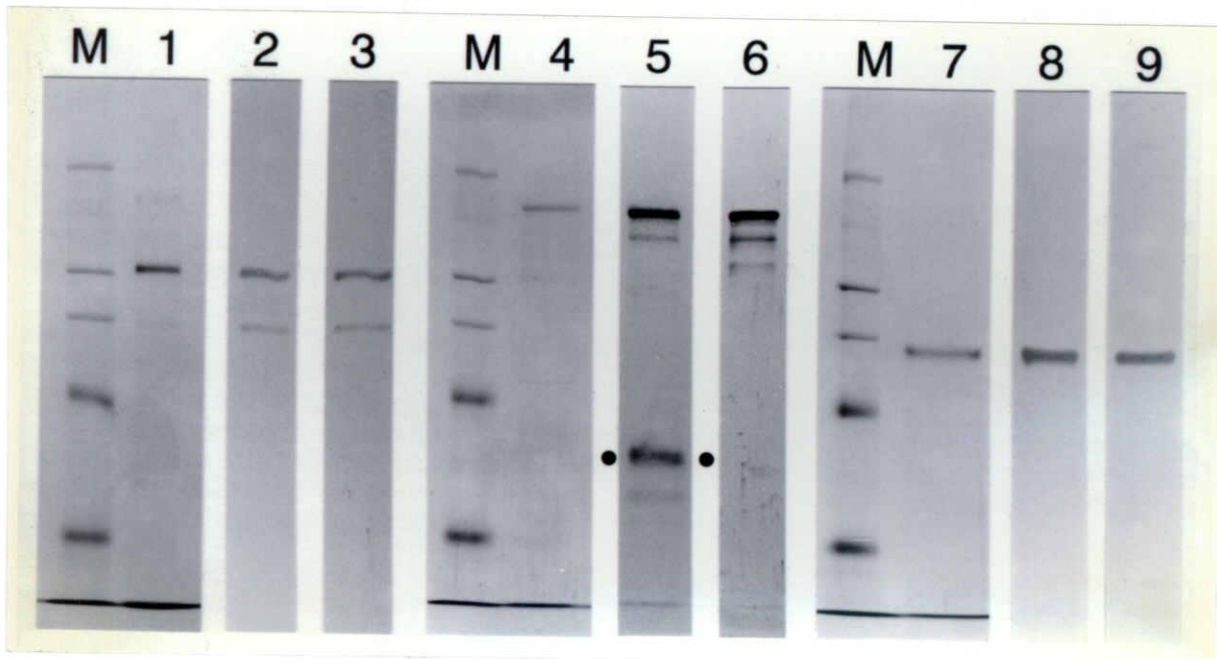


Figure 22. Immunoaffinity purification of 120 and 100 kDa fragments from the medium fraction.

Eluted fractions containing 120 kDa fragments (lanes 1–3), intact BP180 molecules (lanes 4–6) and 100 kDa fragments (lanes 7–9) were silver-stained (lanes 1,4 and 7) and immunoblotted with mAb-233 (lanes 2 and 8), mAb-1A8c (lane 5) and the anti-NC16A domain polyclonal antibody (lanes 3, 6 and 9). The 60 kDa polypeptide was not recognized by the latter (lane 6), while the 120 kDa polypeptide was recognized by the polyclonal antibody (lane 3). Dots indicate the position of the 60 kDa polypeptide. Molecular markers (M) are the myosin heavy chain (205,000), β-galactosidase (116,000), phospholipase B (97,400), BSA (66,000) and ovalbumin (45,000).

immunoblotting with mAb-1A8c, an additional 60 kDa band was detected (Fig. 22, lane 5). As described above, the 60 kDa polypeptide appears to be the remnant BP180 after removal of its 120 kDa extracellular fragment. To identify the cleavage site more precisely, the 120 kDa fragment and the 60 kDa fragment were immunoblotted with the polyclonal antibody against the human NC16A domain comprising the 76 amino acid stretches positioned between the transmembrane domain and the first extracellular collagenous domain (cf. Fig. 15). The 120 kDa fragment was recognized by the polyclonal antibody, while the 60 kDa fragment was hardly detected (Fig. 22, lanes 3 and 6). This demonstrates most of the NC16A domain to be within the 120 kDa fragment. Therefore the cleavage site(s) is localized within the NC16A domain near the cell membrane. 100 kDa fragments, degradation products of 120 kDa fragments, were also purified using the mAb-233 column and found to be recognized by the polyclonal antibody against the NC16A domain. Thus this fragment is produced by cleavage at the carboxyl-terminal side of the 120 kDa fragment (Fig. 22, lane 9). The 100 kDa fragment was recognized by mAb-233 (Fig. 22, lane 8), -D20 and -R223, but not by mAb-1D1 (data not shown). Amino-terminal sequencing of the purified 120 kDa fragment failed, probably due to the low amount of the protein.

Molecular shapes of the 120 kDa fragment were examined by rotary shadowing electron microscopy (Fig. 23) and compared with those of intact BP180 (Fig. 24). The rotary shadowed images of the 120 kDa fragment showed the molecule to be composed of the central rod and the flexible tail, but lack the globular head of the intact BP180 (compare Figs. 24A and 24B). Twelve selected images of the 120 kDa fragment were measured, and the averaged length of the rod and the tail were 68.2 nm and 116.3 nm, respectively. Since the dimensions of the central rod and the flexible tail of the fragment were found to be almost equal to those of the intact molecule, the amino-terminal side of the intact molecule consisting of the cytoplasmic and presumably the transmembrane domains must be within the globular head. The molecular shape of the 100 kDa fragment was similar to that of the 120 kDa fragment, having a little shorter tail (~80 nm), consistent with the results of immunoblot analyses (Fig. 24C).

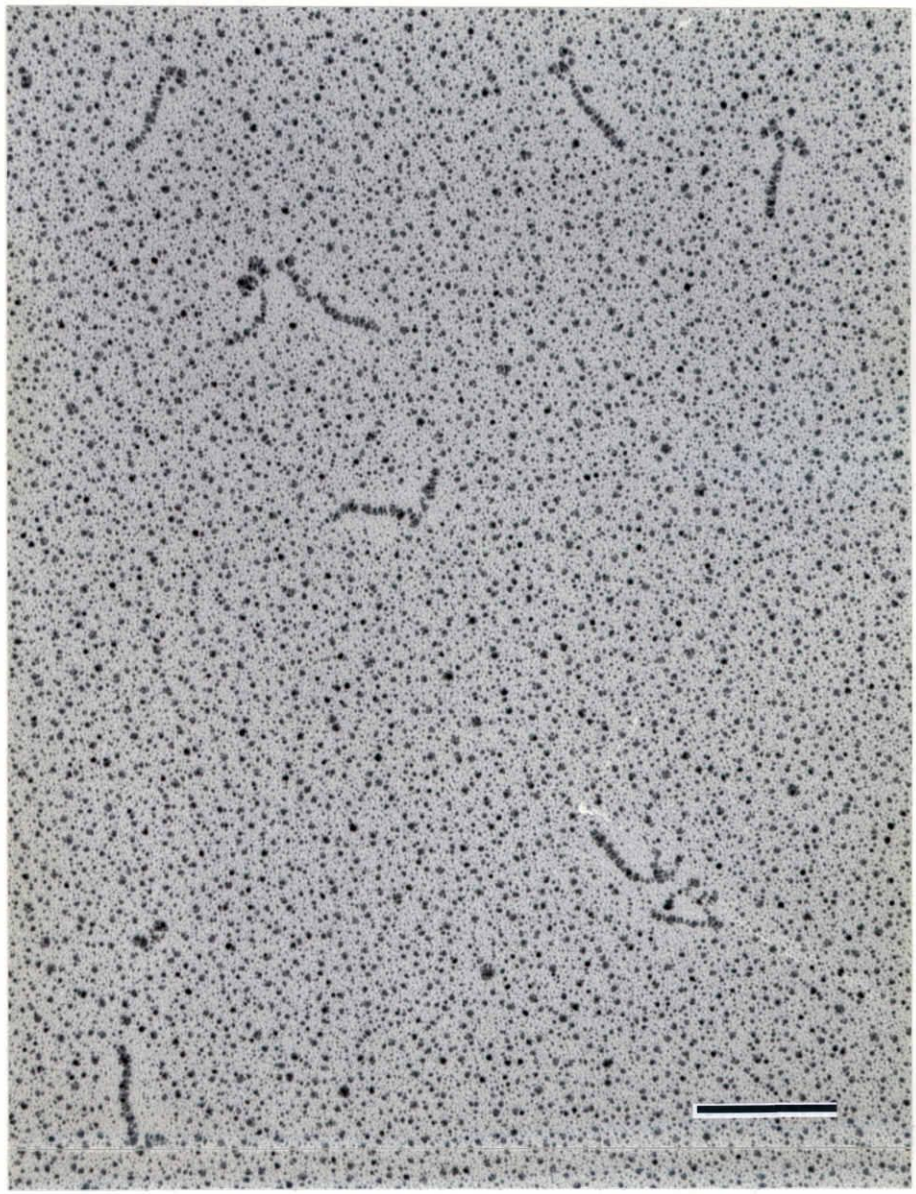
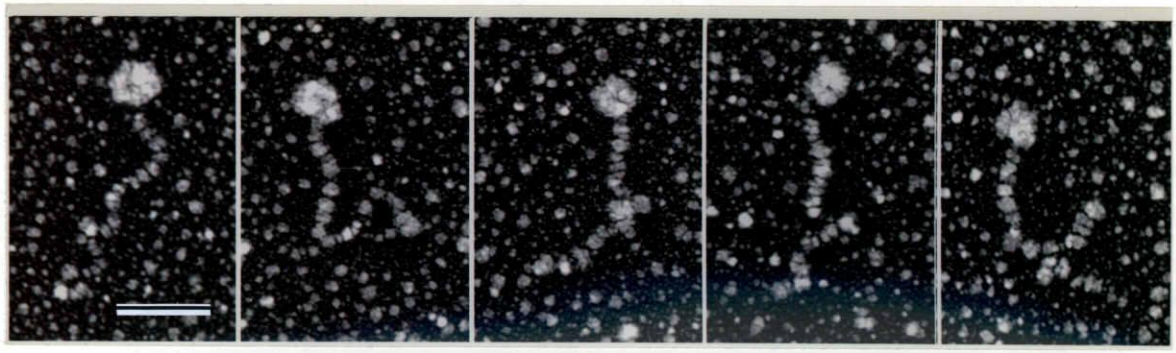


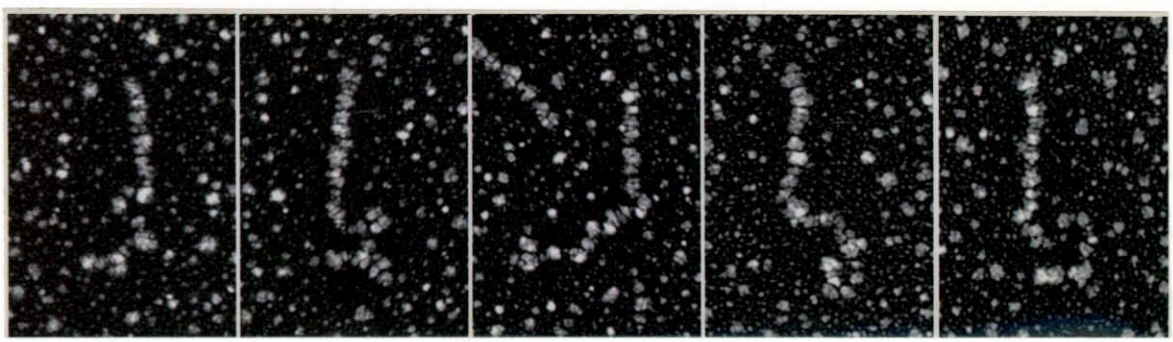
Figure 23. Rotary shadow-images of 120 kDa fragments.

A low magnification field illustrates rotary shadowed 120 kDa fragments. Bar: 150 nm.

A



B



C

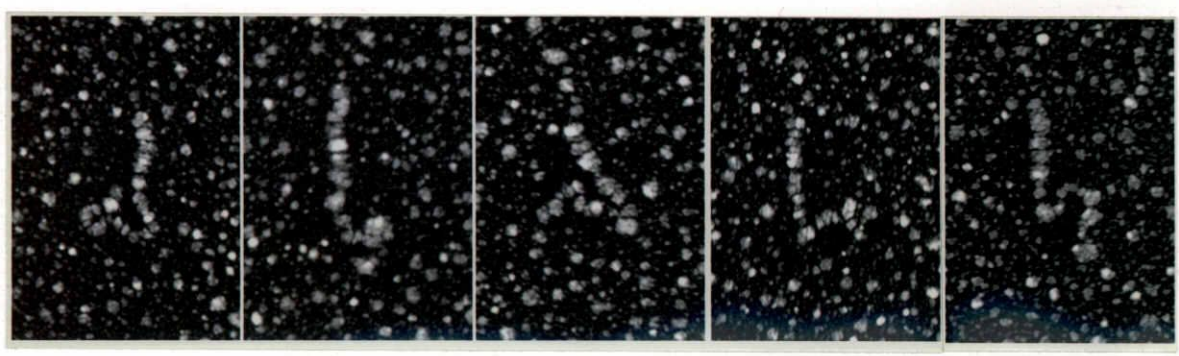


Figure 24. Rotary shadow-images of purified human specimens.

Several representative intact BP180 molecules (A), 120 kDa fragments (B) and 100 kDa fragments (C) are shown. Bar: (A–C) 50 nm.

DISCUSSION

Tx-soluble BP180

In the first section of the results, I showed that a subpopulation of the hemidesmosomal transmembrane protein, BP180, localized on the lateral surface of basal cells of corneal epithelia is solubilized by Triton treatment. Such a localization and the Triton solubility suggest that the subpopulation is neither incorporated into hemidesmosomes nor connected to the cytoskeleton. Integrin $\alpha 6\beta 4$ also exhibited a similar staining pattern (data not shown), while HD1/plectin (7) and BP230 were found to be localized exclusively on the basal surface. Therefore, the lateral staining pattern seem to be a common characteristic of hemidesmosomal transmembrane proteins. Moreover, in cultured cells, soluble BP180 and BP230 were extracted separately in the membrane-bound and in the cytosolic fraction, respectively (compare Figs. 19C and 19F). The biological meaning of the laterally localized proteins is not clear, but one possible hypothesis is that such proteins are stock for formation of new hemidesmosomes (Fig. 25). We have shown that antibody binding to BP180 at the lateral cell surface causes its internalization and, as a result, inhibit new formation of hemidesmosomes induced by a Ca^{2+}-switch in DJM-1 cells, and have speculated that similar effects cause skin blistering in BP patients (55, 56). Our most recent study has proved an actual internalization of BP180 as immune complexes with autoantibodies in lesional skin of BP patients (57).

Molecular shape of BP180

BP180 is a type II oriented transmembrane protein and comprises interrupted collagen domains (15 in man and 13 in the mouse) in its carboxy terminal extracellular part, but it has not been demonstrated that BP180 exists as a collagenous trimer *in vivo* (16–19). I purified the soluble BP180 and demonstrated its trimer formation and entire molecular configuration using chemical crosslinking and rotary shadowing. The frictional ratio of the soluble BP180 ($f/f_0 = 2.8$), calculated from Svedberg constant of 7 S and the presumed molecular mass of

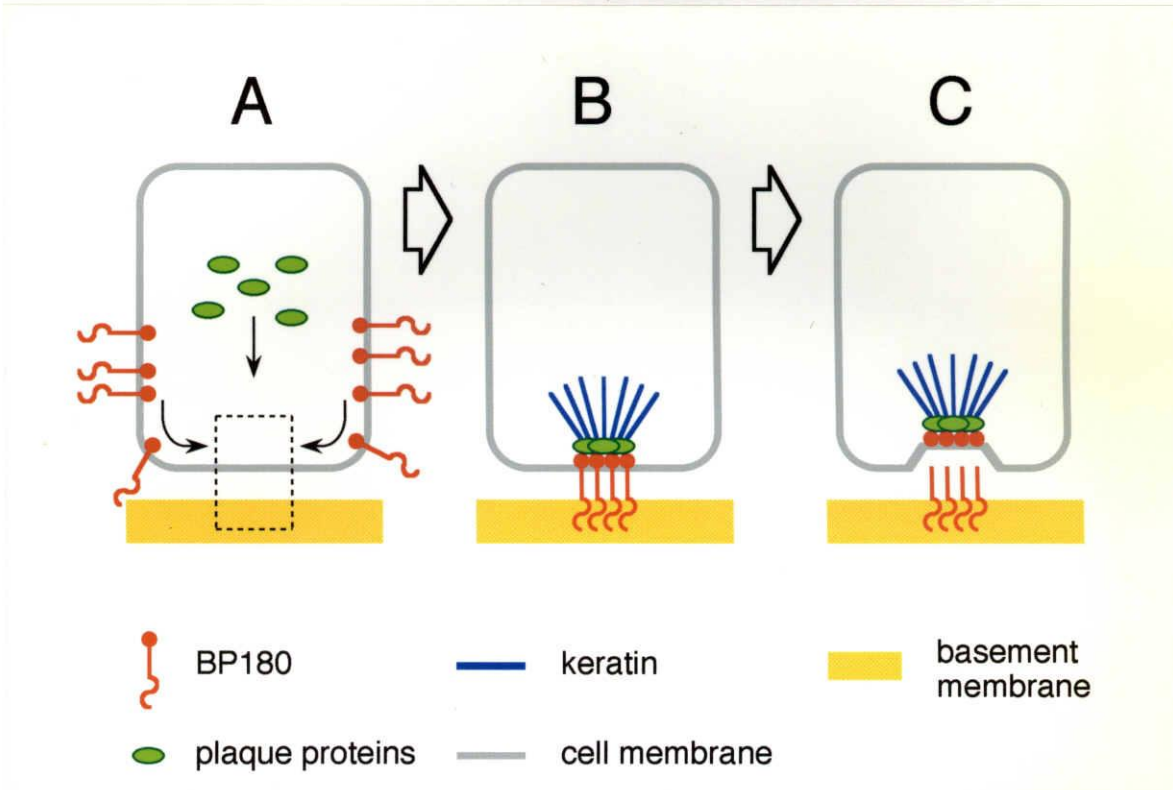


Figure 25. Hypothetical model for the dynamics of BP180 during epidermal cell migration or differentiation.

New formation of hemidesmosomes is an indispensable event to achieve epidermal organization and differentiation (A to B) but, during cell migration in wound healing and differentiation in stratification, hemidesmosomes must be detached from the basement membrane (B to C). In the former aspect, soluble BP180 molecules are recruited from the basolateral surface of basal cells to basal adhesion sites, and then meet cytoplasmic plaque proteins to form hemidesmosomes. In the latter aspect, BP180 molecules involved in hemidesmosomes are cleaved at their specific extracellular portion in order to allow cells to move away from the underlying basement membrane.

the trimer (540 kDa), is consistent with the observed asymmetric images. On the basis of the observations and the measurements of the shadowing images, a possible molecular configuration of BP180 is shown in Fig. 26, with division into three distinct molecular domains named the globular head, central rod and flexible tail.

As the cDNA sequences of BP180 demonstrate a high amino acid homology of 86.0% between the human and mouse forms (18), it is reasonable to suppose that the bovine counterpart is also quite similar. Actually, rotary shadowed images of human BP180 molecules demonstrate indistinguishable configuration from the bovine form. Thus molecular dimensions of human BP180 molecule can be expected to be quite similar to the bovine BP180 molecule.

It is clear from the rotary shadowed images of the 120 kDa fragments together with biochemical results that they have no globular head, showing that the globular head corresponds to the amino-terminal cytoplasmic part of the molecule. This domain with a common globular image of 25–35 nm in diameter, was moreover revealed to have three subdomains in some specimens. I speculate that each corresponds to the cytoplasmic part of a BP180 monomer.

In comparison with the primary structure of human BP180 (16), the central rod would correspond to the longest, continuous collagen domain, which is closest to the cell membrane, considering its central location in the molecule and apparent rigid image with constant length. In addition, the averaged length of the rod, 64.2 nm, is nearly equal to the predicted one of the corresponding collagen domain (70 nm). According to the published sequences, there is a non-collagen domain termed NC16A between the transmembrane domain and the longest collagen domain, which has been supposed to form an alpha helical coiled-coil of about 8 nm length from its eight heptad repeats. In the presented model, this non-collagen domain is assumed to be included in the central rod region. The putative transmembrane domain is placed on the border between the head and the rod.

The flexible tail would correspond to the remaining carboxy terminal part consisting of

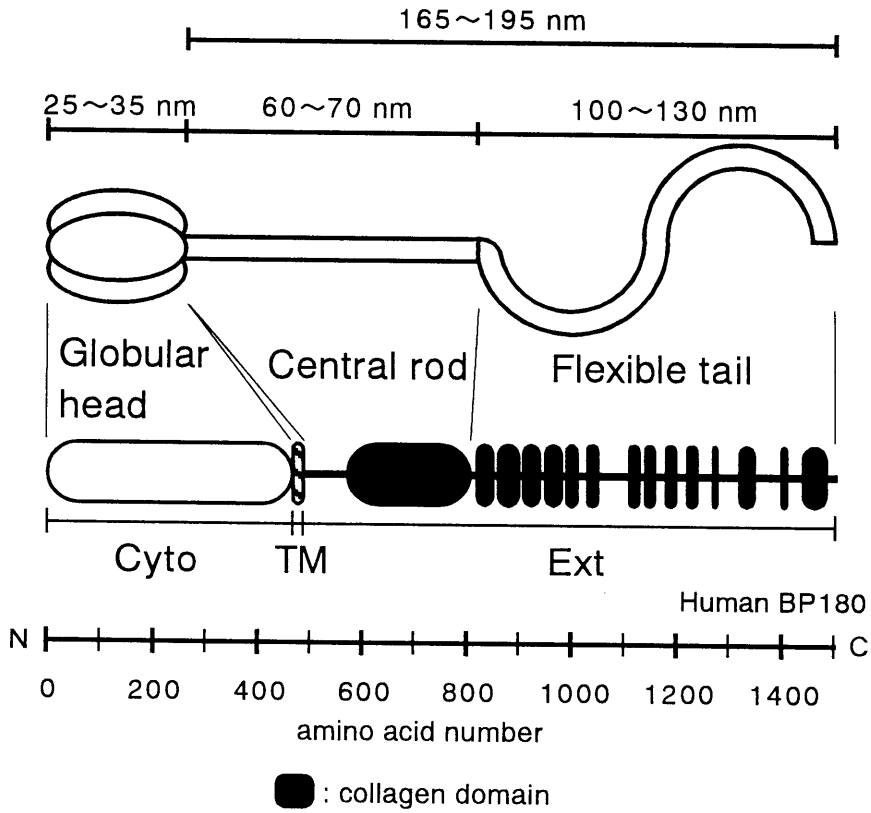


Figure 26. Model of the BP180 molecule.

The shape and dimensions of the represented model (upper) are based on the observations and the measurements of rotary shadow images. The schematic domain organization of human BP180 (lower) is based on the primary structural analysis performed by Giudice *et al.* (16). Cyto, TM and Ext are the cytoplasmic, transmembrane and extracellular portions of BP180, respectively.

interrupted short collagen domains. The flexibility indicated by the various configuration observed can be explained by the non-collagenous interruptions. The dimensions of the extended form, in the range of 100–130 nm, are in line with the amount of the predicted length of the collagen domains of the corresponding part in the human form (100 nm).

Molecular architecture of BP180 in the hemidesmosome

Based on the proposed model described above, I can address the molecular architecture of BP180 in the hemidesmosome. The cytoplasmic globular head of BP180 is located right under the cytoplasmic membrane, and is concluded to be a constituent of the hemidesmosomal outer plaque from its globular size and position. Hemidesmosomes from BP230 gene knock-out mice lack inner plaques and keratin connection, but have otherwise normal outer plaques and BP180 (28). On the other hand, type II hemidesmosomes, which have HD1/plectin and integrin $\alpha 6\beta 4$ but no BP antigens, do not have distinct plaques (neither inner nor outer ones) (7, 23). From these observations, it is suggested that BP180 is responsible for the formation of the outer plaque through some protein-protein interaction. The molecular interaction is probably regulated by phosphorylation. Recently, we showed that phosphorylation of BP180 on serine residues and destruction of hemidesmosomes occurred simultaneously on 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation of DJM-1 cells (58). Hopkinson *et al.* have shown an association between the integrin $\alpha 6$ subunit and truncated BP180 that lacks the entire collagen domain (40). Borradori *et al.* have reported the interaction between cytoplasmic domain of $\beta 4$ and that of BP180 (41). In addition, cells from patients with generalized atrophic benign epidermolysis bullosa, in which BP180 is deficient, have rudimentary hemidesmosomes (47–50).

The rod and tail regions, the putative extracellular portions of BP180, have enough length, the most extended specimens measuring 193 nm, to reach the lamina densa through the lamina lucida (Fig. 27). This configuration remarkably resembles that of anchoring filaments, which are located within the lamina lucida and are responsible for attachment of the

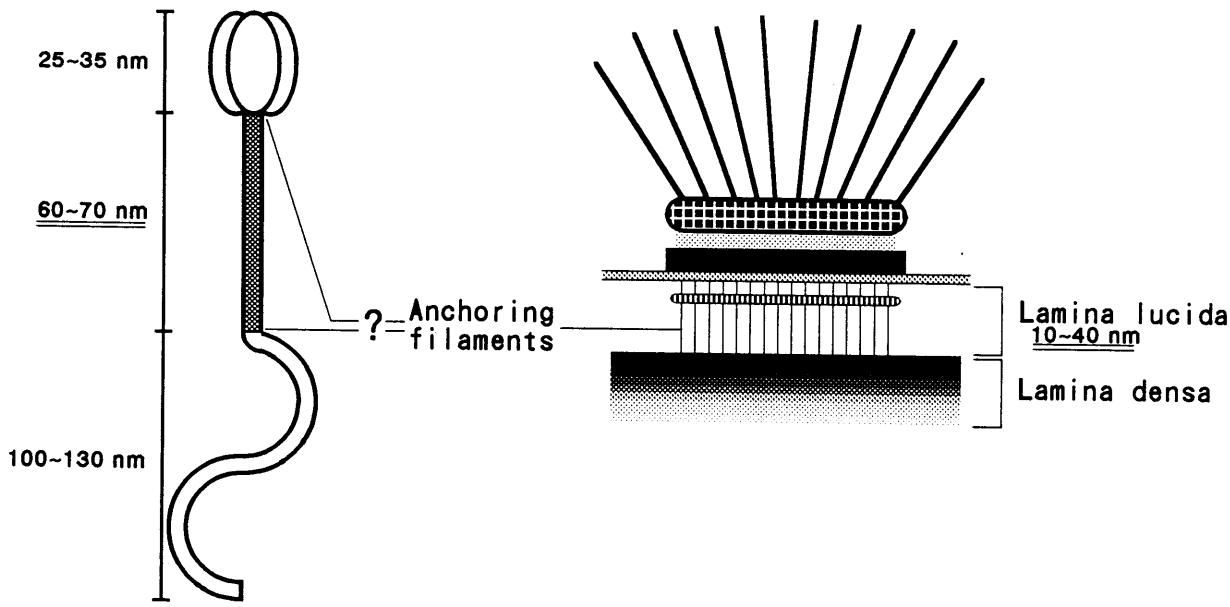


Figure 27. Comparison between the hemidesmosomal anchoring filament and the extracellular part of BP180.

hemidesmosome to the lamina densa (59). Therefore I assume that the extracellular part of BP180 is a major cellular component of anchoring filaments, together with the laminin-5 (kalinin/nicein) that was previously suggested as such an extracellular component (60–62). Since the rod and tail are so different in apparent flexibility, although this is highly speculative, they may also be functionally distinct from each other. For instance, one might form a rigid bridge across the lamina lucida and the other play an important role in the lamina densa, such as in ligand binding. In fact, even the length of the rod itself, 60–70 nm, is enough to cross over the lamina lucida, which is usually within 40 nm in thickness (59, 63). This speculation is well consistent with the fact that BP180 is also a target molecule of cicatricial pemphigoid, an autoimmune subepidermal disease different from BP which is characterized by primary involvement of oral and ocular mucous membranes and scar formation (64, 66). CP autoantibodies recognize the lamina densa as well as the lamina lucida of the basement membrane. Most recently, Masunaga *et al.* (66) have demonstrated that the extracellular domain of BP180 extends to the lamina densa of skin BMZ by immunoelectron microscopy.

In conclusion, considering the characteristic molecular configuration of BP180 demonstrated in this study, it is strongly suggested that BP180 is an absolutely new type of transmembrane adhesion receptor.

120 kDa fragment of BP180

In the second section of the results, I demonstrated the existence of a 120 kDa extracellular fragment of BP180, which can be further degraded to a 100 kDa form, in culture medium of keratinocytes and skin BMZ, suggesting that the cells cleave the BP180 molecule at their surfaces. The mAb-1337 was found to be very useful to distinguish the fragment from intact BP180, apparently recognizing a unique epitope that is exposed or formed by the cleavage (Fig. 28). I could not determine the cleavage site exactly, but it is located in the NC16A domain near to the cell membrane. The epitope may be present in the NC16A domain, which is probably more affected by the cleavage than other stable collagenous parts.

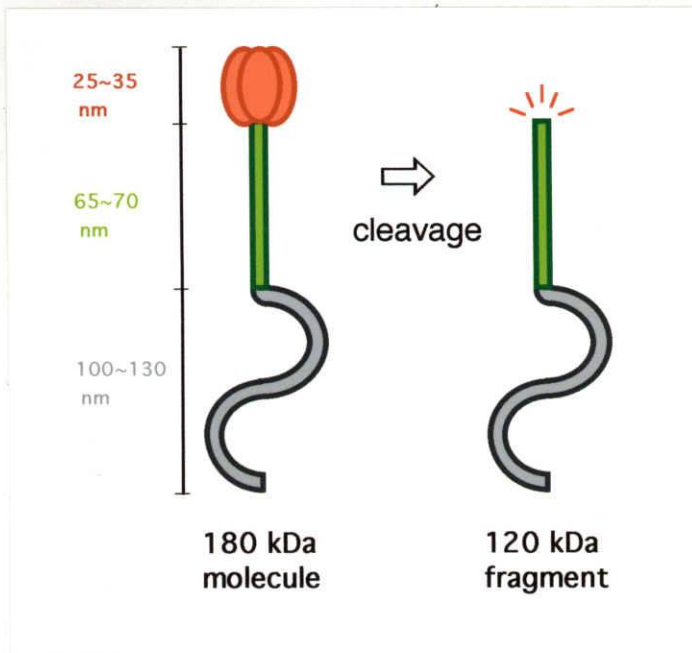


Figure 27. Cleavage of BP180.

Cleavage of BP180 exposes or forms an epitope for mAb-1337.

Rotary shadowing of the 120 kDa fragments confirmed that the fragments have no globular head corresponding to the amino-terminal cytoplasmic part of the molecule.

The immunofluorescent staining pattern of mAb-1337, showing the distribution of the 120 kDa fragments, differed between tissues and cultured cells. The fragments were localized exclusively in the BMZ in skin, whereas, in cultured cells, they are detected in the culture medium. Since the staining of skin BMZ with mAb-1337 proved resistant to 0.5% Tx-100 or 1.5 M KCl treatments, the fragments in the tissue appear to be insoluble, whereas the intact BP180 molecules present in the lateral surfaces of basal cells are solubilized by 0.5% Tx-100 treatment. The insolubility of the fragments in the tissue is probably due to their anchorage to or interaction with other molecule(s). Since the fragments lack the transmembrane domain and seem to be no longer anchored to the cell body directly, they are probably fixed extracellularly. Hemidesmosomal transmembrane proteins such as integrin $\alpha 6\beta 4$ and BP180 are candidates for the extracellular anchorage of the fragment. In fact, Hopkinson *et al.* (40) have shown that truncated BP180 lacking the entire collagen domain, in other words consisting of only the cytoplasmic and non-collagenous extracellular domains, is incorporated into hemidesmosomes and associates with integrin $\alpha 6$ subunit extracellularly. However, these candidates cannot explain the absence of the 120 kDa fragment on the basal sides of cultured cells, because they do have hemidesmosomes including integrin $\alpha 6\beta 4$ and BP180. Another possibility is that an unidentified extracellular ligand(s) of BP180 exists in tissue but not in cultured cells.

Speculation on the biological meaning of the cleavage

The presence of the 120 kDa fragment in skin suggests that the cleavage has some biological meaning. In wound healing or stratification, hemidesmosomes must be detached from the basement membrane, with transmembrane proteins becoming separated from their ligands.

Like other integrins, $\alpha 6\beta 4$ appears highly regulative and its recycling has been shown during cell locomotion (67). During epithelial wound healing of cornea, the integrin $\alpha 6\beta 4$

redistributes from their location within hemidesmosomes to more evenly in the basal cell membrane, and this event occurs without any measurable change in the synthesis of $\alpha6\beta4$ and with no indication of proteolytic cleavage of either $\alpha6$ or $\beta4$ chain (68). Recent studies have demonstrated that the ligand ligation of integrin $\alpha6\beta4$ causes tyrosine phosphorylation on its cytoplasmic domain (69). Considering these results, it is reasonable to suppose that integrin $\alpha6\beta4$ controls ligand detachment by some structural change in the cytoplasmic domain.

My structural analyses of BP180 have demonstrated collagen-like trimer formation. Considering the stable collagenous extracellular rod of BP180, it might be expected that BP180 is not as regulative as integrin $\alpha6\beta4$. Thus if structural change in the cytoplasmic domain cannot cause detachment of its extracellular domain from its ligand, a capacity for cleavage would be necessary to allow differentiation and movement away from the basement membrane (Fig. 25). Kitajima *et al.* (55, 58) observed disappearance of BP180 in DJM-1 cells during rearrangement of hemidesmosomes induced by the treatment with 12-*O*-tetradecanoylphorbol-13-acetate. However, the possibility that the fragment plays a more direct role in cell-matrix adhesion distinct from the intact molecule, can not be excluded. The fact that significant amounts of the 60 kDa polypeptide could be detected on immunoblotting of cell extracts using mAb-1A8c but not -233 or 1D1, is suggestive of constitutive cleavage and removal of the 120 kDa extracellular part rather than generation of alternatively spliced products of BP180 gene.

The fragment and diseases

Although this is highly speculative, cleavage of the extracellular part of BP180 might have some clinical, in addition to biological significance. Thus alteration in BP180 fragments might cause autoimmune diseases like bullous pemphigoid and other skin blistering disorders. Linear IgA bullous dermatosis (LAD) is an autoimmune subepidermal blistering disease characterized by linear deposits of IgA autoantibodies at the dermal-epidermal basement membrane. Immunoblot analyses of patients' sera have demonstrated that the target antigens are heterogeneous, although a 97 kDa antigen is most frequently detected (70, 71). Recent studies have shown that the autoantibodies recognize a 97 kDa and a 120 kDa polypeptide in

skin extracts and a 120 kDa form in keratinocyte culture medium (72). The available data suggest that the 97 kDa peptide is a degradation rather than a specifically processed product. Immunoelectron microscopy has indicated that the 97 kDa polypeptide is localized to lamina lucida, suggesting a novel anchoring filament protein (73). Very recently, Pas *et al.* (74) have found that both BP IgG and LAD-IgA recognize the same 120 kDa protein in keratinocyte conditioned medium and in cell extracts. They also showed that the 120 kDa protein is glycosylated and collagenous, and lacking in culture medium and in skin from GABEB patients. These results suggest close relationship between LAD-antigen and BP180, though LAD sera have so far not been found to recognize BP180.

I have no direct evidence concerning the relationship between the LAD-antigen(s) and the 120 kDa BP180 fragment at present. However, further characterization of the latter appears warranted to clarify the molecular nature and function of BP180, under physiological and pathological conditions.

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