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Extracellular Cleavage of Bullous Pemphigoid Antigen 180/Type XVII Collagen and Its Involvement in Hemidesmosomal Disassembly

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Bullous pemphigoid antigen 180 (BP180)/type XVII collagen is a transmembrane hemidesmosomal protein. Previously, we demonstrated that the collagenous ectodomain of BP180 can be cleaved within the extracellular non-collagenous (NC) 16A domain adjacent to the cell membrane and released from the cell surface. Here, we report that the BP180 cleavage is mediated by a membrane-associated metalloprotease expressed in epithelial cells. A tissue inhibitor of metalloprotease 1 (TIMP-1), but not TIMP-2, like the synthetic metalloprotease inhibitor KB-R8301, significantly reduced the cleavage. Within epithelial cells cultured for more than 36 h past confluency, antibodies to BP180 showed a reduced hemidesmosomal staining. Observed for the first time, addition of KB-R8301 to the cell culture preserved this staining. To examine the effect of the extracellular cleavage of BP180 on molecular interactions among hemidesmosomal components, we eliminated its collagenous extracellular portion, except for the NC16A domain, by collagenase digestion. Interestingly, this collagenase treatment caused partial disassembly of hemidesmosomal components in cultured human keratinocytes. Moreover, a monoclonal antibody specific for the cleaved extracellular fragment detected a unique tissue distribution of the fragment that might reflect an association of the cleavage process with the mitotic activity of epithelial tissues. Our observations demonstrate that the cleavage of BP180 occurring within the NC16A domain is mediated by a membrane-associated metalloprotease and suggest a possible involvement of the cleavage in hemidesmosomal disassembly.

Key words: bullous pemphigoid, cell adhesion, hemidesmosome, shedding, transmembrane collagen.

Abbreviations: NC, non-collagenous; ECM, extracellular matrix; MoAb, monoclonal antibody; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; TIMP, tissue inhibitor of metalloproteases; CM fraction, cytoskeleton-membrane fraction; BMZ, basement membrane zone; MT-MMP, membrane-type matrix metalloprotease; ADAM, a disintegrin and metalloprotease.

Hemidesmosomes are membrane-associated supramolecular adhesion complexes linking cells to the extracellular matrix (ECM) in stratified and complex epithelia (*1*). They anchor intermediate filaments in the cytoplasmic plaque and are connected to the basement membrane by extracellular anchoring filaments. Bullous pemphigoid antigen 180 (BP180) and α6β4 integrin are the two major transmembrane components of hemidesmosomes (*2*). BP180 is also known as a major target antigen of the autoimmune blistering skin disease, bullous pemphigoid (BP) (*3*). cDNA cloning has demonstrated BP180 to be a unique type II-oriented transmembrane protein containing interrupted collagenous domains within its carboxyl-terminal extracellular portion (*4*). For this reason, BP180 was also referred to as type XVII collagen (*5*). BP180 interacts with the cytoplasmic bullous pemphigoid antigen 230 (BP230), a member of the plakin family of proteins that anchors keratin filaments to the hemidesmosomal plaque (*6*, *7*). In addition, BP180 binds to the cytoplasmic portion of the β4 (*8*) and the extracellular portion of the α6 subunit of α6β4 integrin (*9*, *10*). We previously demonstrated that BP180 forms a collagenous triple helix. In addition, by rotary shadowing of purified BP180, we showed that the molecule consists of an intracellular globular head and an extracellular rigid rod (60–70 nm) corresponding to the NC16A and the 15th collagenous domains. The remaining portion of the BP180 ectodomain forms a flexible tail (100–130 nm) (*2*, *11*). Subsequent immunoelectron microscopic studies suggested that the molecule is one of the major components of the anchoring filament (*12*, *13*), together with laminin-5, which is thought to be a major extracellular ligand for hemidesmosomal α6β4 integrin (*1*). The ECM component BP180 interacts with has not yet been identified.

We and others demonstrated that the extracellular portion of BP180 can be shed from the cell surface as a result of proteolytic cleavage (*14*, *15*). Deposition of the

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120-kDa cleaved fragment of BP180 at the dermal–epidermal junction was demonstrated by a fragment-specific monoclonal antibody, 1337, indicating the cleavage actually occurs in the skin (*14*). The cleavage process is thought to be mediated by a furin-like protease (*15*), but the enzyme(s) involved have not yet been identified. The cleaved 120-kDa extracellular fragment of BP180, also known as LAD-1, is a major autoantigen targeted by patients' sera with the subepidermal blistering disease, linear IgA disease (LAD) (*16*, *17*). The intriguing aspect of the autoimmune response in linear IgA disease is that sera preferentially react with LAD-1, but not with full-length BP180 (*17–20*). This suggests that the cleavage of the BP180 ectodomain may generate novel autoantigenic epitopes.

The present study aims at further characterizing the cleavage process of BP180 and exploring its physiological relevance. Our data strongly suggest that the cleavage of BP180 is mediated by a membrane-associated metalloprotease. Moreover, we present data suggesting a possible physiological role of the BP180 cleavage in hemidesmosomal disassembly.

EXPERIMENTAL PROCEDURES

Antibodies and Cultured Cells—Mouse monoclonal antibodies (MoAbs) against hemidesmosomal proteins had been prepared by immunizing mice with the hemidesmosome fraction isolated from bovine corneal epithelial cells as described (*21*). MoAb 233 and MoAb D20 are directed against the extracellular and MoAb 1A8c against the cytoplasmic portion of BP180 (*14*, *19*). MoAb 1337 specifically recognizes the cleaved extracellular fragment of BP180 (*14*), while MoAb R815 binds to BP230 (*21*). MoAb 855, MoAb 310, and MoAb 617 target the ectodomain of the bovine integrin β4 subunit, and MoAb 1A3 targets the cytoplasmic portion of the human integrin β4 subunit (*14*). MoAbs BML39 and BM515 are directed against type VII collagen and the laminin α3 chain, respectively (*22*). Polyclonal antibodies SA8009 and BOS6 were generated against the human BP180 NC16A domain (*23*) and the extracellular EC2 domain of desmoglein 3, respectively. MoAb K140 is directed against the laminin β3 (*24*) and polyclonal antibody SE144 against the laminin γ2 chain (*25*). Rat monoclonal antibody GoH3 binds to α6 integrin subunit (*26*). MoAb against the α6 integrin subunit was purchased from Serotec GmbH (Düsseldorf, Germany).

BMGE+H cells, a bovine mammary gland epithelial cell line derived from lactating bovine udder, and DJM-1 cells, a human skin squamous carcinoma cell line, were cultured as described (*27*, *28*).

Protease Inhibitors—A hydroxamic acid-based metalloprotease inhibitor, [4-(*N*-hydoxyamino)-2*R*-isobutyl-3*S*-methysuccinyl]-L-3-(5,6,7,8-*tetra*-hydro-1-naphthyl)alanine-*N*-methylamide (KB-R8301/KB8301/OSU8-1), was provided by Nippon Organon, Osaka, and dissolved in dimethyl sulfoxide at 10 mM as stock solution (*29–31*). Other protease inhibitors included 1,10-phenanthroline, EDTA, phenylmethylsulfonyl fluoride (PMSF) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (all Wako Pure Chemical Industries, Osaka), pepstatin A, [L-3-*trans*-carboxyoxirane-2-carbonyl]-L-leucylagmatine (E-64), leupeptin, and elastatinal (all Peptide Institute, Osaka), decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (decanoyl-RVKR-chloromethyl ketone) (Bachem AG, Basel, Switzerland), and recombinant human TIMP-1 and TIMP-2 (Fuji Chemical Industries, Takaoka).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described (*11*). In some experiments, cultured cells, grown on glass cover slips, were treated with 0.5% Triton X-100 in PBS for 5 min before being fixed for immunofluorescence microscopy.

Preparation of Cellular and ECM Fractions and Immunoblotting—Extraction of cytoskeletal proteins, preparation of concentrated conditioned medium and ECM fractions, SDS-PAGE, electrophoretic transfer onto polyvinylidene fluoride (PVDF) or nitrocellulose membrane using a semi-dry system, and immunoblotting were performed as described (*14*, *22*, *32*). Polypeptides in the ECM fraction were identified by immunoblotting with MoAb 1A8c against BP180, MoAb 1A3 against integrin β4, and MoAb BM515 against laminin α3 as described (*22*), and also with MoAb against α6, MoAb K140 against laminin β3 chain and polyclonal antibody SE144 against the laminin γ2 chain.

Biotinylation of Cell Surface Proteins—BMGE+H cells were grown to semiconfluency in plastic culture dishes of 15 cm diameter, washed twice with PBS and once with 0.1 M Hepes buffer (pH 8.0) containing 50 mM NaCl, then labeled with 2 mg/ml sulfosuccinimidobiotin (sulfo-NHS-biotin) (Pierce), 5 ml of 0.1 M Hepes buffer (pH 8.0), 50 mM NaCl, 20 μg/ml leupeptin, and 5 μg/ml pepstatin A for 15 min at room temperature. To halt biotinylation, cells were washed with DMEM containing 10% FCS followed by PBS. Labeled cells were cultured for a further 48 h in fresh medium, and the resulting conditioned medium was used for immunoprecipitation as described (*14*).

Treatment of Cell Cultures with Metalloprotease Inhibitor or Collagenase—KB-R8301, dissolved in dimethyl sulfoxide at 10 mM as stock solution, was added to the medium of confluent cultured cells at a final concentration of 10 μM and incubated for different time periods. Addition of the inhibitor to the cells did not affect their shape and multiplication rate, as previously described for cultured keratinocytes in a juxtacrine growth factor assay (*30*). For collagenase digestion experiments, DJM-1 cells were cultured in Dulbecco's modified Eagle medium with 10% fetal calf serum supplemented with 0.4 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, and 84 ng/ml cholera toxin for 5 days, then collagenase from Clostridium histolyticum (Amano Enzyme, Aichi) was added to the medium at a final concentration of 200 U/ml.

In Vitro Cell-free Cleavage Assay—The *in vitro* cell-free cleavage assay was performed according to a published protocol with some modifications (*33*). Cells were rinsed in phosphate-buffered saline (PBS), scraped from dishes, and suspended in hypotonic lysis buffer (20 mM Hepes-NaOH, pH 7.5, 10 mM potassium acetate) supplemented with 20 mM EDTA. After extraction on ice for 5 min, cells were disrupted and centrifuged at 2,000 ×*g* for 5 min. Precipitates were resuspended in ice-cold buffer (30 mM Tris-HCl, pH 7.2) and centrifuged at 10,000 ×*g* for 30 min. Pellets were resuspended in ice-cold reaction buffer (30 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A) containing protease

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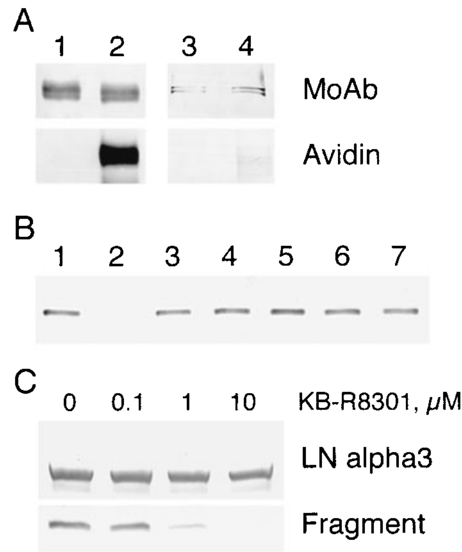

Fig. 1. **Cell surface cleavage of BP180 can be abolished by a metalloprotease inhibitor.** A: The 120-kDa fragment (lanes 1 and 2) and type VII collagen (lanes 3 and 4) were immunoprecipitated from the conditioned culture medium of non-labeled (lanes 1 and 3) or biotin-labeled (lanes 2 and 4) BMGE+H cells by MoAb 233 to the extracellular portion of BP180 (lanes 1 and 2) and MoAb BML39 to type VII collagen (lanes 3 and 4). The precipitated proteins were detected by immunoblotting with MoAb 233 (upper panel: lanes 1 and 2) and MoAb BML39 (upper panel: lanes 3 and 4) or avidin (bottom panels: lanes 1–4). B: BMGE+H cells were cultured for 24 h in the absence (lane 1) or presence of inhibitors for metalloprotease (lane 2, 10 μM KB-R8301), as well as serine (lane 3, 100 μM AEBSF; lane 4, 10 μg/ml aprotinin), aspartate (lane 5, 50 μM pepstatin A), and cysteine (lane 6, 50 μM E-64) proteases. Leupeptin (lane 7, 50 μM) is also effective to inhibit serine and cysteine proteases. The 120-kDa fragments were isolated from their conditioned media and examined by immunoblotting with the fragment-specific MoAb 1337. *C.* BMGE+H cells were cultured for 24 h in the presence of 0, 0.1, 1, and 10 μM KB-R8301. Laminin α3 (LN alpha3) and the 120-kDa fragment (Fragment) in the media were examined by immunoblotting with MoAbs BM515 and 1337, respectively.

inhibitors or Triton X-100. Reaction mixtures were incubated at 37°C for the time periods indicated in the results section. The reaction was stopped by the addition of SDS sample buffer.

To remove surface proteins from the cell membrane, pellets were suspended in ice-cold 1 M NaCl containing 30 mM Tris-HCl, pH 7.2. After keeping them on ice for 15 min, samples were centrifuged at 10,000 ×*g* for 15 min at 4°C. Pellets were resuspended in ice-cold reaction buffer and assayed as described above.

RESULTS

Extracellular Cleavage of BP180 Mediated by a Metalloprotease—To determine whether the cleavage of the BP180 ectodomain occurs intracellulary or on the cell surface, surface proteins of BMGE+H cells were labeled with biotin. Labeled cells were cultured for 48 h, and the 120-kDa fragment was immunoprecipitated from the cultured medium using MoAb 233 against the extracellular portion of BP180. The precipitated fragment was detected by avidin, indicating that it was labeled with biotin (Fig. 1A, lane 2). On the other hand, type VII collagen, an ECM protein secreted directly into the medium, was not detected by avidin (lane 4). These results demonstrated that the 120-kDa fragment is derived from intact transmembrane BP180, *i.e.*, the cells cleave BP180 on their surface.

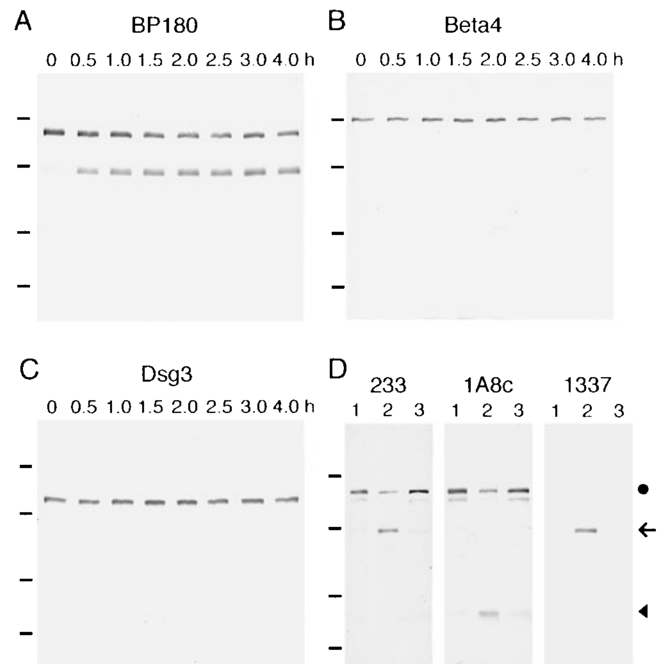

Fig. 2. **Detection of cleaved BP180 in *in vitro* cleavage assay.** A–C: A fraction containing constituents of cytoskeleton and cell membrane (CM fraction) was prepared from BMGE+H cells for an *in vitro* cleavage assay. The CM fractions were incubated at 37°C for the times indicated above the panels. Incubated fractions were immunoblotted with MoAb 233 against the extracellular portion of BP180 (A), a mixture of MoAb 855, MoAb 310 and MoAb 617 that recognize the extracellular portion of the β4 integrin subunit (B), and a polyclonal antibody against the extracellular portion of desmoglein 3 (C). D: CM fractions were incubated for 4 h in the absence (lane 2) or presence (lane 3) of 10 μM KB-R8301 and compared to the fraction obtained before the inhibitor was added (lane 1) by immunoblotting with MoAbs specific to the extracellular portion (233), cytoplasmic portion (1A8c) and the fragment (1337) of BP180. The intact 180-kDa band was recognized by MoAb 233 and MoAb 1A8c (dot). MoAb 233 and MoAb 1337 detected the 120-kDa fragment in the fraction incubated without the inhibitor (lanes 2, arrow), while addition of the inhibitor abolished detection of the fragment (lanes 3). MoAb 1A8c against the cytoplasmic portion of BP180 detected a 60-kDa instead of the 120-kDa fragment (lane 2, arrow head). Dashes at the left of each panel indicate molecular standards for the myosin heavy chain (205,000), β-galactosidase (116,000), BSA (66,000) and aldolase (42,000).

To establish the identity of the protease(s) mediating the cleavage process, several protease inhibitors were added to the medium (B and C). After a 24 h incubation, the amount of the 120-kDa fragment in the medium was determined by immunoblotting with the fragment-specific MoAb 1337. Inhibitors of serine, aspartate and cysteine proteases did not affect the cleavage, whereas a synthetic metalloprotease inhibitor, KB-R8301, markedly reduced the amount of the fragment when used at a final concentration of 10 μM. In contrast, processing of the laminin α3 chain from 200- to 165-kDa, mediated by

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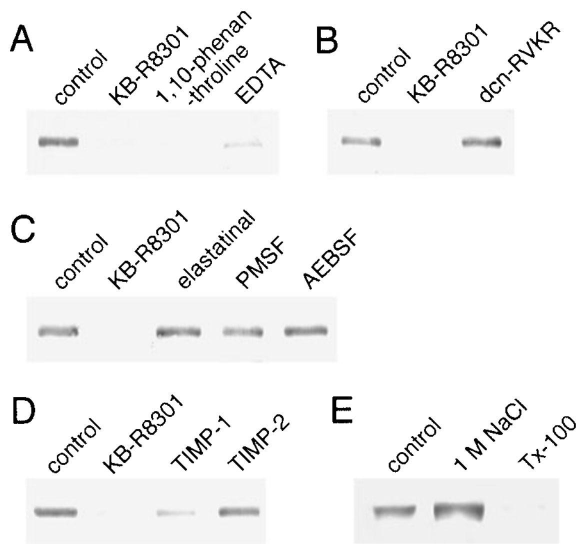

Fig. 3. **Effects of various protease inhibitors on the cleavage of BP180 in *in vitro* cleavage assay.** A–D: CM fractions were incubated at 37°C for 4 h in the absence (control) or presence of metalloprotease inhibitors (A), a furin inhibitor (B), serine protease inhibitors (C), and tissue inhibitors of metalloproteases (TIMPs) (D). Cleaved fragments were detected by immunoblotting with MoAb 1337. Final concentrations of reagents added were as follows: KB-R8301, 10 μM; 1,10-phenanthroline, 10 mM; EDTA, 10 mM; decanoyl-RVKR-chloromethyl ketone (dcn-RVKR), 0.1 mM; elastatinal, 0.2 mM; PMSF, 1 mM; AEBSF, 1mM; TIMP-1, 10 μg/ml; TIMP-2, 10 μg/ml. E: Effects of 1 M NaCl wash (1 M NaCl) and 0.5% Triton X-100 (Tx-100) on in vtro cleavage. The CM fraction was washed in ice-cold buffer containing 1 M NaCl before incubation. Triton X-100 at a final concentration of 0.5% was added to the fraction, followed by incubation at 37°C for 4 h.

plasmin (*34*), was not affected under these conditions. In DJM-1 cells, a human squamous carcinoma cell line, the metalloprotease inhibitor, used at a concentration of 10 μM, also abolished the cleavage of BP180 (data not shown).

In Vitro Cleavage Assay—For further characterization of the putative metalloprotease mediating the cleavage of BP180, we developed an *in vitro* cell-free cleavage assay. BMGE+H cells were chosen for the experiment, because they had been demonstrated to abundantly contain BP180 (*11*). Cultured BMGE+H cells were lysed in a hypotonic buffer. After extraction on ice, the lysed fraction was centrifuged, yielding a pellet containing constituents of the cytoskeleton and cytoskeleton-associated cell membrane, designated the cytoskeleton-membrane (CM) fraction. This fraction was resuspended in reaction buffer at 37°C for 0.5 to 4 h and analyzed by immunoblotting with MoAb 233 against the extracellular portion of BP180 (Fig. 2A). At time 0, only the intact 180-kDa band was detected. However, after 30 min, the MoAb detected the 120-kDa fragment in addition to the 180-kDa polypeptide. With longer incubation times, the amount of the 120-kDa fragment slightly increased (and that of intact 180-kDa polypeptide decreased) and reached its peak at 2.5 to 3 h. Antibodies against the β4 integrin subunit (B) and desmoglein 3 (C) did not detect any cleaved or degraded fragments of their antigens in fractions incubated for up to 4 h. These results demonstrate the presence of active and specific protease(s) cleaving BP180 in the CM fraction. To examine the effect of KB-R8301 on the in vitro cleavage of BP180 (D), CM fractions were incubated for 4 h in the absence (lanes 2) or presence (lanes 3) of the reagent, analyzed by immunoblotting using monoclonal antibodies against the extracellular portion (MoAb 233), cytoplasmic domain (MoAb 1A8c), and the fragment (MoAb 1337) of BP180, and their reactivity was compared with that of the fraction obtained before the protease inhibitor was added (lanes 1). In the fraction incubated omitting the inhibitor (233, lane 2), MoAb 233 detected the 120-kDa fragment (arrow), in contrast to the fraction containing the inhibitor (233, lane 3), indicating the involvement of a metalloprotease in the in vitro cleavage of BP180. The cleaved fragment was also recognized by fragment-specific MoAb 1337 (1337, lane 2), indicating that is has the identical antigenic property to the one detected in the skin and conditioned culture medium. MoAb 1A8c labeled, instead of the 120-kDa fragment, a 60-kDa fragment in the fraction incubated without the inhibitor (1A8c, lane 2, arrow head). This 60-kDa fragment corresponded to the cytoplasmic remnant of the cleavage that we described previously (*14*) and was only faintly detected in inhibitor-supplemented fractions or prior to adding the inhibitor.

Using the *in vitro* cleavage assay, we further characterized the cleavage process of BP180 using MoAb 1337 (Fig. 3). To compare the quantities of cleaved 120-kDa fragments under different conditions, equal amounts of sample were taken from a cell extract (CM fraction) in each set of experiments. Therefore, each sample contained the same amount of BP180 before incubation. Addition of 1,10-phenanthroline, which chelates cations associated with metalloprotease, to the cell-free fraction affected the cleavage to the same extent as KB-R8301 (A). EDTA was also effective to reduce the amount of the 120-kDa fragment. Previously, it had been shown that a synthetic inhibitor of furin proprotein convertase inhibited the cleavage of BP180 in cultured keratinocytes (*15*). To examine the role of furin-like proteases in the cleavage in our *in vitro* cleavage assay, we added decanoyl-RVKR-chloromethyl ketone, an inhibitor of furin, at a final concentration of 0.1 mM to the CM fraction (B). However, the reagent did not inhibit the cleavage. It had also been suggested that blister formation in the skin of BP patients is associated with the cleavage of the extracellular portion of BP180 by elastase, a serine protease, secreted by neutrophils (*35*). However, in the *in vitro* cleavage assay (C), elastatinal, a specific inhibitor for elastase, and general serine protease inhibitors did not affect the cleavage. Our findings using protease inhibitors indicate that only reagents able to compromise the activity of metalloproteases can inhibit the cleavage of BP180. Moreover, addition of tissue inhibitor of metalloprotease (TIMP) 1, a natural inhibitor of known matrix metalloproteases (MMPs), to the CM fraction significantly suppressed the cleavage, while TIMP-2 had no major effect (D). The CM fraction does not contain soluble proteins, indicating that the cleaving enzyme is a membrane-associated protein. To determine whether the protease is a cell surface-attached or transmembrane protein, the CM fraction was washed with 1 M NaCl to remove surface proteins from the cell membrane (E). Interestingly, this procedure did not

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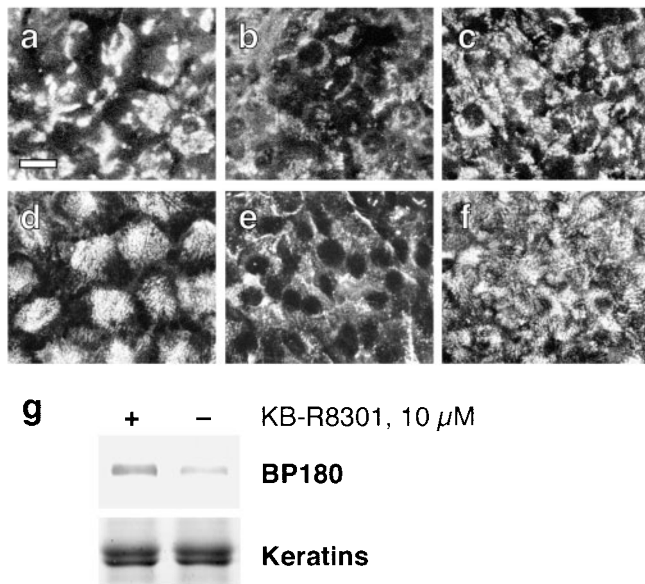

Fig. 4. **The metalloprotease inhibitor KB-R8301 affected hemidesmosomal localization of BP180.** To study the effect of the metalloprotease inhibitor KB-R8301 on hemidesmosomal localization of BP180 in DJM-1 (a–c) and BMGE+H cells (d–f), cells were cultured to confluency (a and d) and subsequently for 36 h in the absence (b and e) or presence (c and f) of KB-R8301 at a final concentration of 10 μM. Membrane staining was removed by treating the cells with 0.5% Triton X-100. Cells were fixed in methanol, followed by detection of BP180 using MoAb 233. Without the inhibitor, hemidesmosomal staining was decreased and mainly found at the cell periphery (b and e). However, in the presence of the inhibitor (c and f), hemidesmosomal staining was preserved or became even stronger. Immunoblotting of the cytoskeletal fraction prepared from BMGE+H cells cultured with (+) or without (–) the inhibitor confirmed an abundance of BP180 in cells cultured in the presence of the inhibitor (g). Bar, 20 μm.

inhibit and even increased the cleavage of BP180. In contrast, treatment of the CM fraction with 0.5% Triton X-100 (which dissolved the cell membrane) abolished the cleavage process. These results suggest that the metalloprotease involved is a transmembrane protein of epithelial cells.

Effect of the Metalloprotease Inhibitor KB-R8301 on the Cellular Localization of Hemidesmosomes—In a next set of experiments, we examined the effect of the metalloprotease inhibitor KB-R8301 on the localization of hemidesmosomes in DJM-1 cells (a–c) and BMGE+H (d–f) by immunofluorescence microscopy using the BP180-specific MoAb 233 (Fig. 4). Hemidesmosomes of cultured cells that had just reached confluency, revealed arc- or leopard skin-like patterns in DJM-1 (a) and a dot-like staining in BMGE+H cells (d). After further culturing for 36 h, cells became smaller and their hemidesmosomes reduced, localizing to the cell periphery (b and e). However, when the cells were cultured in the presence of KB-R8301, hemidesmosomes were larger in number and still showed a dot- or arc-like pattern (c and f). The preservation of hemidesmosomes in the cells treated with the inhibitor was also confirmed by immunoblotting (g). As expected, the cytoskeletal fraction prepared from BMGE+H cells cultured with the inhibitor contained more BP180 than the one prepared from untreated cells. These results indicate that KB-R8301 affects both the cleavage of BP180 and the re-localization of hemidesmosomes.

Collagenase Digestion of the Extracellular Portion of BP180 in Cultured Cells—The NC16A domain of BP180 has been demonstrated to be involved in interaction with the α6 subunit of α6β4 integrin (*10*). Interestingly, the extracellular cleavage of BP180 occurs within the NC16A domain (*14*). To examine the effect of the extracellular cleavage of BP180 on the cellular localization of the cytoplasmic domain of BP180 and other hemidesmosomal components, the physiologically occurring cleavage was reproduced by the addition of collagenase to the medium of cultured epithelial cells (Fig. 5). Most of the extracellular portion of BP180 is composed of interrupted collagenous domains, so that digestion with collagenase eliminates the extracellular portion except for the NC16A domain, which locates next to the transmembrane region. Though not identical, the amino-terminal digestion product is therefore similar to the membrane-associated remnant of BP180 resulting from the physiological cleavage occurring within the NC16A domain. After addition of collagenase, the fate of this remnant and of other hemidesmosomal constituents was studied using MoAbs to the cytoplasmic (a–d) and extracellular portions (e and f) of BP180, BP230 (g–i), β4 subunit of α6β4 integrin (j–l), and laminin α3 chain (m and n). This collagenase treatment did not affect the shape of the cells or their attachment to the dish. To confirm the elimination of the extracellular portion of BP180 by collagenase digestion, DJM-1 cells were stained with MoAb D20, which recognizes the 15th collagenous domain locating next to the NC16A domain. Before the addition of collagenase, MoAb D20 stained hemidesmosomes (arc- or leopard skin-like staining pattern) (e). After incubation of the cells with collagenase, the hemidesmosomal staining was lost within 1 h, indicating that the majority of the BP180 ectodomain was digested (f). The epitope for MoAb 233 was also lost after 1 h of incubation with collagenase (*11*). To determine the fate of the cell-associated remnant, cells treated with collagenase were stained with MoAb 1A8C to the cytoplasmic portion of BP180. Before addition of the enzyme, the antibody revealed a hemidesmosomal staining pattern (a). Interestingly, after a 2 h incubation with collagenase, in addition to the arc-like pattern, the cells showed larger dots (b). After a 4 h incubation, the arc-like pattern had disappeared, while the dot-like staining persisted (c). Further incubation for up to 18 h resulted in disappearance of staining for BP180 (d). The cytoplasmic localization of the collagenase digestion product of BP180, represented by the dot-like staining, was confirmed using polyclonal antibody SA8009 to the extracellular NC16A domain (Fig. 6). Cultured DJM-1 cells were stained with the polyclonal antibody (a and c) and then fixed for subsequent double immunostaining with MoAb 1A8c (b and d). In cells cultured without collagenase (a and b), both antibodies demonstrated the typical hemidesmosomal localization of BP180 in an almost identical pattern. However, in cells treated with collagenase for 4 h (c and d), MoAb 1A8c detected bright larger dots (d), while the polyclonal antibody was negative (c), demonstrating the internalization of the cell-

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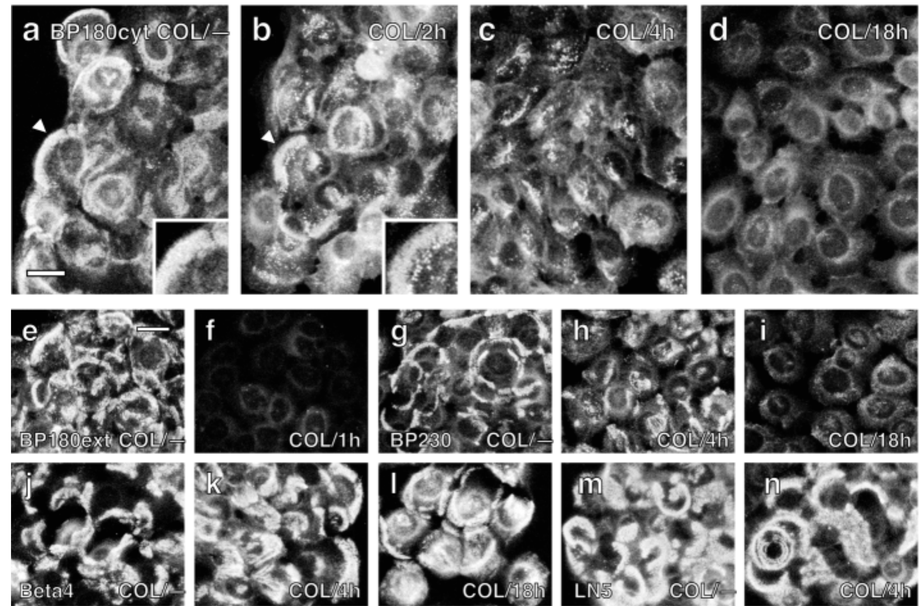

Fig. 5. Elimination of the collagenous ectodomain of BP180 caused internalization of cell-associated remnant of BP180 and partial disassembly of hemidesmosomes. DJM-1 cells were cultured in the absence (a, e, g, j, and m) or presence (b–d, f, h, i, k, l, and n) of collagenase for up to 18 h. The cells were stained with MoAb 1A8c against the cytoplasmic region of BP180 (a–d), MoAb D20 against the 15th collagen domain of BP180 next to the NC16A domain (e and f), MoAb R815 against BP230 (g–i), MoAb 1A3 against integrin β4 subunit (j–l) and MoAb BM515 against laminin α3 chain (m and n). Arrowheads (a and b) indicate areas that were enlarged in insets. Hemidesmosomes were observed as small speckles that often clustered into arc-like structures (a, e, g, j, and m) before the addition of collagenase. Staining for the epitope recognized by the D20 antibody was lost within 1 h of collagenase treatment (f). Interestingly, cells cultured with collagenase for 2 h started to internalize the remaining cell-associated portion of BP180 in larger dots (b) (see also Fig. 6). On higher magnification, in contrast to untreated cells (a, inset), the internalized dots were observed along the hemidesmosomal arc-like staining (b, inset). Cytoplasmic dots were most frequently found in cells cultured with collagenase for about 4 h (c). Further incubation for 18 h resulted in disappearance of staining for BP180 (d). After addition of collagenase, hemidesmosomal staining with the BP230-specific antibody decreased (h: 4 h culture with collagenase), and finally, after an 18 h incubation, became almost negative (i). During the collagenase treatment for 4 h (k) and 18 h (l), the integrin β4 subunit produced local clusters. However, after 18 h, the staining pattern was more diffuse. Localization of extracellular laminin 5 was not affected by the collagenase treatment for 4 h (n). Bars, 20 μm.

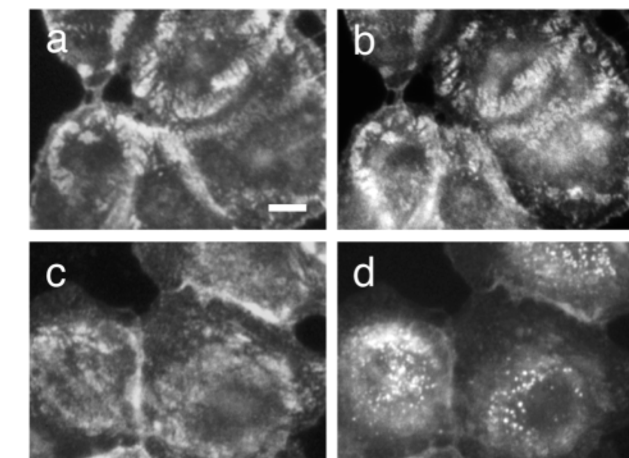

Fig. 6. **Cytoplasmic localization of the cell-associated remnant of BP180 in collagenase-treated cells.** DJM-1 cells cultured in the absence (a and b) or presence of collagenase for 4 h (c and d) were stained with polyclonal antibody SA8009 to the BP180 extracellular NC16A domain (a and c). Cells were then fixed for subsequent double immunostaining with MoAb 1A8c to the cytoplasmic portion of BP180 (b and d). In cells cultured without collagenase, the polyclonal antibody showed an almost identical staining pattern (a) to the staining of MoAb 1A8c (b). However, in cells cultured in the presence of collagenase, the polyclonal antibody did not stain the larger dots (c) that were detected by MoAb 1A8c (d), indicating the cytoplasmic localization of the dots composed of the cell-associated remnant of BP180. Bar, 10 μm.

associated remnant of BP180. In addition, the distribution of BP230 was studied using MoAb R815 (Fig. 5). Before addition of collagenase, staining with this antibody showed a typical arc-like hemidesmosomal pattern (g), but it became more fragmented during collagenase digestion (h; 4 h incubation) and finally disappeared almost completely (i; 18 h incubation). In contrast to BP180 and BP230, the cellular distribution of the hemidesmosomal β4 integrin was not much affected by the collagenase digestion (j, k, l). During the treatment, MoAb 1A3 to the cytoplasmic portion of the β4 subunit of integrin α6β4 produced an arc-like hemidesmosomal staining (j, k and l; 0, 4 and 18 h incubations, respectively). In cells cultured with collagenase for 18 h, the staining pattern was more diffuse. The extracellular localization of laminin 5 was not affected by the collagenase treatment (m and n; 0 and 4 h incubations, respectively).

To examine the effect of collagenase digestion on other hemidesmosomal components, ECM fractions were prepared from cultured DJM-1 cells treated with collagenase for 4 h (Fig. 7). At this time point, internalization of the BP180 remnant was most prominent. ECM fractions treated with collagenase were subjected to SDS–PAGE and compared with ECM fractions from untreated cells, contained integrin β4 subunit, BP180, the processed form of laminin α3, the unprocessed form of laminin γ2, laminin β3, integrin α6 subunit and the processed form of γ2 (A, band 1–7) as major high-molecular-weight polypeptides. BP180 was completely lost from the fraction isolated from the treated cells in silver staining (A, band 2) and immunoblotting (B, arrow head), whereas integrin α6β4 and laminin 5 were not affected, showing that the collagenase digestion was specific for BP180, at least among the hemidesmosomal proteins studied here. Type VII collagen, which interacts with laminin 5 and plays an important role in dermal-epidermal adhesion, is not present in the ECM of cultured cells (*36*).

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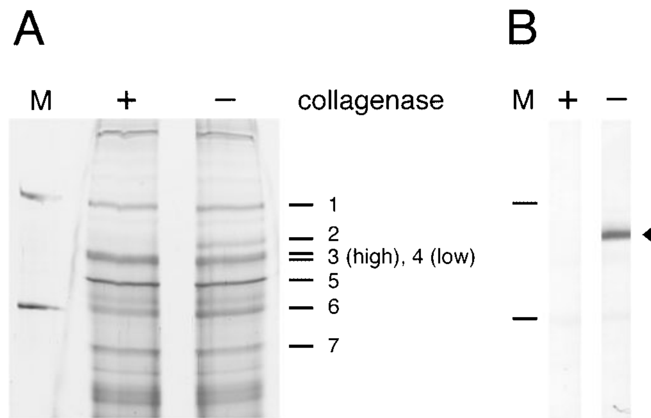

Fig. 7. **Influence of collagenase digestion on hemidesmosomal transmembrane proteins and laminin 5.** ECM fractions containing hemidesmosomal transmembrane proteins and laminin 5 were prepared from DJM-1 cells cultured in the absence (–) or presence (+) of collagenase for 4 h. The prepared fractions were subjected to SDS-PAGE and visualized by silver staining (A) or immunoblotted by BP180-specific MoAb 1A8c (B). The visualized bands of 1–7 are integrin β4 subunit, BP180, processed laminin α3, unprocessed laminin γ2, laminin β3, integrin α6 subunit and processed γ2. The band for BP180 disappeared (A, band 2; B, arrow head), but others were not affected by the collagenase treatment. Polypeptides in the ECM fractions were identified by immunoblotting (*22*). Dashes at the left are for molecular standards of 205-, and 116-kDa.

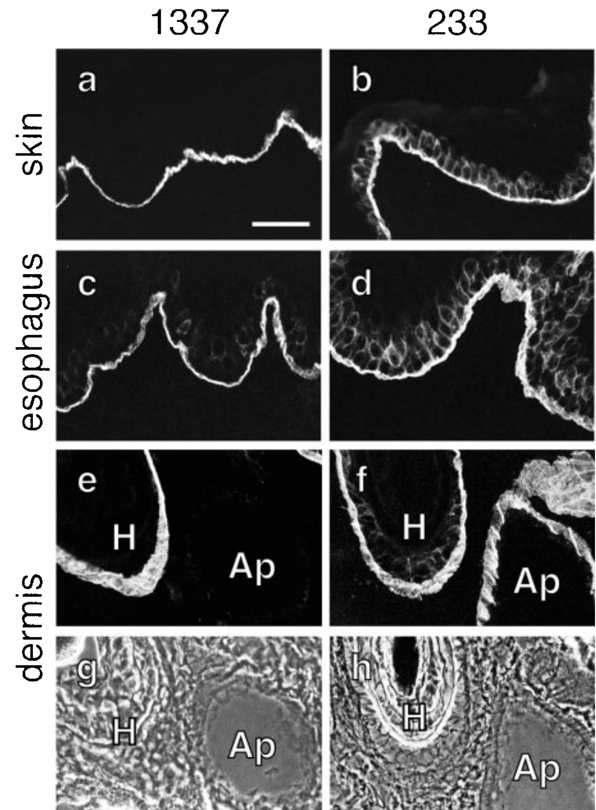

Fig. 8. **Distribution of the 120-kDa fragment in different epithelial tissues.** Sections of bovine epidermis (a and b), esophagus (c and d), and dermis (e–h) were stained with fragment-specific MoAb 1337 (a, c, and e) and MoAb 233 to the extracellular portion of BP180 (b, d, and f). Phase contrast images of e and f are shown in g and h, respectively. H: hair follicles; Ap: apocrine gland. Positive staining for the fragment was found at BMZ of epidermis (a), mucous membrane of esophagus (c), and hair follicles (H in e), but not in myoepithelium of the apocrine gland (Ap in e), although they were all stained by MoAb 233, which recognizes both the fragment and full-length BP180 (b, d, and f). Bar, 20 µm.

Distribution of the 120-kDa Fragment in Epithelial Tissues—We previously demonstrated that after cleavage, the 120-kDa fragment is retained in tissue as an insoluble component of the basement membrane zone (BMZ) (*14*). The distribution of the fragment in different tissues was studied by immunofluorescence microscopy using the fragment-specific MoAb 1337 (Fig. 8). The intensity of staining with MoAb 1337 was compared with that of a MoAb also staining full-length BP180 (MoAb 233). To facilitate comparison between the staining intensities of MoAbs 233 and 1337, supernatants of hybridoma cells were diluted 20-fold the minimal concentration necessary to stain the dermal-epidermal junction by immunofluorescence microscopy. MoAb 233 clearly stained BMZ of epidermis (b), mucous membrane of esophagus (d), hair follicles (H in f), and myoepithelium of apocrine glands (Ap in f). However, fragment-specific MoAb 1337 only labeled BMZ of epidermis (a), mucous membrane of esophagus (c) and hair follicles (H in e), while no staining for the fragment was seen in BMZ of myoepithelium (Ap in e). BMZ staining of sebaceous glands was also detected using MoAb 1337, but the staining intensity of this antibody was much weaker than that of MoAb 233 (data not shown).

DISCUSSION

Our data strongly suggest that a membrane-associated metalloprotease, which can be inhibited by both TIMP-1 and the synthetic inhibitor KB-R8301, is involved in the proteolytic cleavage of the BP180 ectodomain. Membrane-associated metalloproteases include membrane-type metalloproteases (MT-MMPs) (*37*) and ADAMs (a disintegrin and metalloproteases) (*38*). MT-MMPs can be inhibited by TIMP-2, which is not effective to abolish the cleavage of BP180 (*39–42*). Therefore, the metalloprotease cleaving BP180 is unlikely to belong to the group of MT-MMPs, although we cannot exclude the possibility of cleavage of BP180 by an unknown MT-MMP. Over 30 ADAMs have been identified and approximately half of them have a catalytic consensus sequence (required for enzymatic activity) in their metalloprotease domain (*34*). Only a few ADAMs have been characterized with regard to their sensitivity to TIMPs: ADAM10, in contrast to ADAM12 and 17, was reported to be inhibited by TIMP-1, while TIMP-2 did not affect these ADAMs (*44–46*). These findings suggest that the enzyme cleaving the BP180 ectodomain belongs to the group of ADAMs. It has previously been reported that a synthetic inhibitor for furin proprotein convertases affects this cleavage in cultured keratinocytes (*15*). However, this inhibitor did not show any effect on the cleavage of BP180 in our *in vitro* assay, indicating that a furin-like protease is not likely to cleave BP180 directly. Furin-like proteases are known to activate precursor forms of MT-MMPs and ADAMs by removing their prodomains that attach to the catalytic domain

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(*37*, *38*). The furin inhibitor used in the previous study (*15*) may have affected this activation process in cultured keratinocytes.

In the next set of experiments, we studied the role of metalloproteases on the localization of hemidesmosomes in cultured cells. When epithelial cells were cultured more than 36 h past confluency, their hemidesmosomes were reduced and relocalized to the cell periphery. However, addition of the metalloprotease inhibitor KB-R8301 to the culture medium resulted in preservation of hemidesmosomes. These observations suggest that metalloprotease(s) contribute to hemidesmosomal turnover. In addition to cleavage of BP180, metalloproteases were shown to mediate extracellular processing of the γ2 chain of laminin 5 (*47*, *48*). Therefore, the effect of the metalloprotease inhibitor on hemidesmosomal turnover may be due to a synergistic inhibition of different protein cleavage processes.

BP180 interacts with BP230 (*6*, *7*), the cytoplasmic portion of the β4 (*8*), and the extracellular portion of the α6 subunit of α6β4 integrin (*9*, *10*). To examine the effect of the cleavage of the BP180 ectodomain on interaction between hemidesmosomal constituents, we added collagenase to the medium of cultured cells. Though this did not completely reproduce the physiological cleavage occurring within the NC16A domain, the amino-terminal collagenase-digestion product was similar to the membrane-associated remnant of BP180 resulting from the physiological cleavage. Addition of collagenase to the culture medium caused internalization of the cell-associated remnant of BP180, resulting in a dot-like staining pattern, the disappearance of BP230 from hemidesmosomes, and a diffuse redistribution of α6β4 integrin. The disappearance of BP230 may be due to the loss of its major interacting partner, BP180. However, we did not detect any staining for BP230 co-localizing with the cytoplasmic dots, indicating that the 2 BP antigens do not associate with each other when they dissociate from hemidesmosomes. Such partial disassembly of hemidesmosomal components suggests that digestion of the collagenous extracellular portion of BP180 destabilizes not only the extracellular interaction with the α6 integrin subunit, but also cytoplasmic interactions with BP230 and the β4 integrin subunit. The effect of elimination of the collagenous ectodomain on the molecular interactions of hemidesmosomal components may be due to an alteration of the trimeric structure of the cytoplasmic portion of BP180. One may speculate that in epithelial tissues, the physiological cleavage of BP180 within the NC16A domain may also lead to a disassembly of hemidesmosomes. Collagenase treatment eliminates the extracellular portion of BP180 in a short time (1 h), and the digestion occurs on all BP180 molecules of all cells in a dish. Therefore, hemidesmosomal disassembly triggered by this treatment proceeds simultaneously in most of the cells, making it easy to trace the fate of the remnant by immunofluorescence microscopy. On the other hand, the metalloprotease-mediated reduction and redistribution of BP180 were observed in cells cultured for more than 36 h after confluency. We speculate that metalloprotease-mediated cleavage of BP180 does not occur in all cultured cells at the same time but proceeds gradually over the 36 h culture. This might be why we could not trace the fate of the remnant in this condition.

Tissues with a higher cleavage activity would be expected to deposit larger amounts of the processed 120-kDa fragments in their BMZ. The fragment-specific MoAb 1337 detected the cleaved extracellular portion of BP180 at the BMZ of epidermis, hair follicles, and mucous membranes of esophagus. In contrast, in basal cells of sebaceous glands, the fragment was only weakly detected, and in myoepithelium of apocrine glands, it was completely absent, suggesting a higher rate of BP180 cleavage in the epidermis, hair follicles and mucous membranes of esophagus. These three are examples of stratified epithelia exposed to strong mechanical stress, necessitating a rapid regeneration of damaged cells. In contrast, myoepithelium of secretory glands is characterized by a lower mitotic activity (*49*). In addition, basal cells of sebaceous glands have a slower migration rate to suprabasal layers compared with epidermal cells (*50*). One may speculate that the distribution pattern of the BP180 extracellular fragment may reflect the rate of basal cell renewal accompanied by hemidesmosomal disassembly in different epithelial tissues.

Metalloprotease-mediated shedding has been shown in transmembrane adhesion receptors such as E-cadherin (*51*), CD44 (*52*), L-selectin (*53*), and L1 adhesion molecule (*54*). Shedding of these molecules is thought to be involved in cell migration. Recent studies demonstrated that cleavage of E-cadherin at the membrane-cytoplasm interface, mediated by presenilin-1/γ-secretase, causes dissociation of both the cleaved cytoplasmic remnant and catenins from the cytoskeleton and subsequent disruption of adherens junction (*55*). These observations show that the proteolytic cleavage of transmembrane adhesion receptors is one of the regulatory mechanisms for cell adhesion activity. From the data presented in this study, we speculate that the physiological role of the extracellular cleavage of BP180 may also be a mechanism by which the release of epithelial cells from basement membrane anchorage is regulated.

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