Identification of a New Hemidesmosomal Protein, HD1: A Major, High Molecular Mass Component of Isolated Hemidesmosomes

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Abstract. Hemidesmosomes (HDs) mediate cell adhesion to the extracellular matrix and have morphological association with intermediate-sized filaments (IFs) through cytoplasmic plaques. Though several proteins have been located in HDs, most of them have not been well characterized, with the exception of the 230-kD antigen of bullous pemphigoid (BP), an autoimmune skin blistering disease. Only recently we have succeeded in isolating HDs from bovine corneal epithelial cells and in identifying five major components on SDS-PAGE (Owaribe K., Y. Nishizawa, and W. W. Franke. 1991. *Exp. Cell Res.* 192:622–630). In this study we report on immunological characterization of one of the major components, termed HD1, with an apparent molecular mass of 500 kD. Immunofluorescence microscopy showed colocalization of HD1 with BP antigen at the basement membrane zone of those tissues that have typical HDs, including skin epidermis, corneal and tracheal epithelia, and myoepithelium. In cultured keratinocytes, HD1 demonstrated colocalization with BP antigen in the precise way, while being absent from focal adhesions. Immunoelectron microscopy revealed that an epitope of HD1 was located on the cytoplasmic side of HDs. Taking all these results together, we conclude that HD1 is a new hemidesmosomal component. Interestingly, HD1 also exists in endothelial and glial cells, which lack typical HDs.

HEMIDESMOSOMES (HDs)[1] and focal adhesions are cell junctions that mediate adhesion to the extracellular matrix (ECM). These two plasma membrane junction types have common ultrastructural features; e.g., both are associated with cytoskeletal elements through electron-dense cytoplasmic plaques. They thus provide us with models to explore the mechanisms of transmembrane connection between the ECM and the cytoskeleton. In focal adhesions associated with actin-containing microfilaments (for reviews see references 2 and 50), a number of proteins are known to be involved, including ECM receptors of the integrin family (5, 12, 17, 33) and plaque components such as talin (1) and vinculin (29). Yet our knowledge about the molecular architecture of the ECM-to-microfilaments junctions is still incomplete.

The HD, primarily found in the basement membrane zone (BMZ) of stratified and complex epithelia, has a complex structure: a cytoplasmic plaque associated with intermediate filaments (IFs), a subbasal dense plate with anchoring filaments in the basal lamina, and anchoring fibrils in collagenous connective tissues (6, 16, 39). The morphological appearance is essentially that of half of the desmosome of intercellular junctions. However, although several desmosomal proteins have been identified (e.g., references 4, 26; for review see reference 35) since the establishment of appropriate isolation procedures (e.g., reference 36), none of them have been found in HDs (13, 35). Thus, in marked contrast to other cell junctions, little information is available about the molecular components of HDs, with the exception of the well-characterized 230-kD antigen responsible for autoantibody production in patients with bullous pemphigoid (BP), an autoimmune skin blistering disease (27, 30, 32, 40, 45, 52).

Recently, however, we succeeded in isolating HDs from bovine corneal epithelial cells (31), allowing identification of five major bands, by SDS-PAGE analysis, with apparent molecular masses of 500 (formerly, referred to as 480), 230, 200, 180, and 120 kD, termed HD1 to HD5, respectively. HD2 and HD4 have been found to be, respectively, the 230- and 180-kD BP antigens, confirming enrichment of hemidesmosomal proteins in our HD fraction. Systematic analysis of our HD fraction should therefore provide important information not only on hemidesmosomal components but also on their functions. For that purpose we have been preparing monoclonal antibodies to molecules contained in the fraction.

In the present study we immunologically demonstrate a 500-kD component of HD1 to be actually a new hemidesmosomal protein. We also show the presence of HD1 in endothelial and glial cells, which lack typical HDs. Based on

1. *Abbreviations used in this paper*: BMZ, basement membrane zone; BP, bullous pemphigoid; ECM, extracellular matrix; GFAP, glial fibrillar acidic protein; HD, hemidesmosome; IF, intermediate filament.

these results, possible involvement of HD1 in IF anchorage to the plasma membrane is discussed.

Materials and Methods

Isolation of Hemidesmosomes

HDs were isolated from bovine corneal epithelium as described previously (31). Briefly, the epithelial cell layers of corneas were peeled off in PBS containing 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM PMSF as protease inhibitors, maintaining HD attachment to the stroma. Stromata were incubated in PBS containing 2 mM EDTA for 30 min at room temperature or treated with a glycerol solution (50% glycerol, 0.1 M KCl, 5 mM EDTA, 10 mM sodium phosphate, pH 7.4) for >2 d at 4°C, followed by removal and collection of HDs.

Antibodies

Monoclonal antibodies against HD1 were prepared as described previously (31), with the improvement that electrophoretically purified HD1 protein was used as the immunogen. 3 d after the final injection into mice, spleen cells were fused with mouse myeloma cells (line X63Ag8.653). Hybridomas producing antibodies to HD1 were selected through screening by immunofluorescence microscopy and immunoblotting, and cloned twice. Ascites was obtained by intraperitoneal injection of hybridomas producing anti-HD1 monoclonal antibodies and used at a 1:4,000 dilution.

A monoclonal BP autoantibody, named 5E, which was produced by Epstein–Barr virus transformation of lymphocytes from a BP patient (43), was the generous gift from Dr. T. Hashimoto of Keio University (Tokyo, Japan), while mouse antidesmoplakin monoclonal antibodies was from Dr. W. W. Franke of the German Cancer Research Center (Heidelberg, Germany). Mouse antivinculin monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies to laminin and neurofilaments were from Advance Co. (Tokyo, Japan), and rabbit polyclonal antibodies to glial fibrillar acidic protein (GFAP) were from Biomed. Technols. Inc. (Stoughton, MA).

Cell Culture

FRSK cells, keratinocytes derived from rat foreskin, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were grown in Eagle's MEM containing 10% FCS at 37°C.

Preparation of Cytoskeletal Fractions

Fresh bovine tissues were used, either immediately or after glycerination, for the preparation of cytoskeletal fractions. These tissues were homogenized in low- and high-salt buffers containing 1% Triton X-100 and protease inhibitors at 0°C, and the resultant nonextractable fractions were recovered as the cytoskeletal fractions, as described elsewhere (31).

FRSK cells were rinsed in PBS, scraped from dishes, and subjected to low- and high-salt buffer extraction as described above.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (25) with slight modifications.

Immunoblotting was performed by SDS-PAGE and subsequent electrophoretic transfer onto nitrocellulose sheets using a semi-dry system (18). The nitrocellulose sheets were incubated with 1% BSA in TBS (pH 8.0) containing 0.05% Tween 20 (Tween-TBS) and subsequently with primary antibodies for 90 min. After washing with Tween-TBS, they were incubated with alkaline phosphatase–conjugated secondary antibodies (Jackson ImmunoResearch Labs, Inc., West Grove, PA) for 90 min, followed by visualization with NBT (nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical Co.).

Immunofluorescence Microscopy

Freshly prepared tissues were snap-frozen in isopentane precooled in liquid nitrogen, cut into 5–6-μm-thick sections with a cryostat, mounted on glass slides, air dried, and fixed in 100% acetone at −20°C for 10 min. FRSK cells cultured on coverslips were briefly washed with PBS and fixed as above. The samples were incubated with primary antibodies for 30 min, followed by washing with PBS and incubation with appropriate secondary antibodies coupled with fluorescent dyes (Jackson ImmunoResearch Labs., Inc.) for 30 min.

Immunoelectron Microscopy

10-μm-thick frozen sections of bovine cornea and bovine optic nerve were fixed with cold acetone as for immunofluorescence microscopy. For the optic nerve, nonfixed sections were also examined. FRSK cells cultured on glass slides were fixed with cold acetone and subsequently treated with 0.5% Triton X-100 in PBS for 5 min at room temperature. The samples were incubated with 1% BSA in PBS for 30 min and subsequently with a mouse anti-HD1 monoclonal antibody (mAb HD-121) or with the human BP monoclonal antibody, 5E, at room temperature for 90 min. For the nonfixed sections of the optic nerve, the incubation with the primary antibodies was followed by PBS washing and fixation with 3.7% formaldehyde for 10 min. They were washed with PBS and incubated with anti-mouse IgG or protein A coupled to colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium) for 3 h at room temperature. 5-nm gold particles were used for the cornea, and 10-nm gold particles for the optic nerve and for FRSK cells. After washing with PBS, the samples were fixed with glutaraldehyde and osmium tetroxide, dehydrated in a series of ethanols, and embedded in Epon according to the conventional procedures for EM preparation. Ultrathin sections were examined with a JEM 100CX electron microscope at 80 kV.

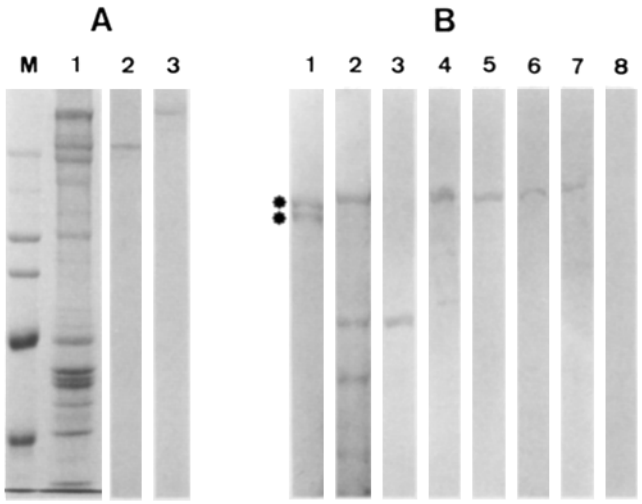

Figure 1. (*A*) Specificity of the anti-HD1 monoclonal antibody, mAb HD-121. HD fraction proteins were separated using a 7.5% gel and immunoblotted (lane *1*, Coomassie blue staining; lanes *2* and *3*, immunoblotting with the BP monoclonal antibody and mAb HD-121, respectively). mAb HD-121 specifically reacted with the highest molecular mass component of HD1 but not with other major HD components. The BP monoclonal autoantibody reacted with the 230-kD components. Molecular weight markers (lane *M*) are (*top* to *bottom*) myosin heavy chain, β-galactosidase, phosphorylase b, BSA, and ovalbumin. (*B*) (Lanes *1–4*) On a 4% gel, the specificity of mAb HD-121 was confirmed and the molecular mass of HD1 estimated. Of the three major bands of the HD fraction separated (lane *2*, Coomassie blue staining), mAb HD-121 specifically reacted with the largest major band (lane *4*; the minor bands most likely represent proteolytic products of HD1 proteins). HD1 showed slightly lower mobility than *Chlamydomonas* dynein heavy chains of 460–480 kD (asterisks in lane *1*, Coomassie blue staining), allowing estimation of the molecular mass of HD1 to be ~500 kD. The 230-kD BP antigen was manifested by immunoblotting with the BP monoclonal antibody (lane *3*). (Lanes *5–8*). Immunoblotting with mAb HD-121 demonstrated the presence of HD1 in the cytoskeletal fractions of bovine tracheal epithelium (lane *5*), bovine optic nerve (lane *6*), and cultured keratinocytes FRSK (lane *7*). Note that the reactive bands in all the above samples are similar in molecular mass to corneal HD1. Skeletal muscles were used as a negative control (lane *8*).

Results

Monoclonal Antibodies against HD1

The HD fraction isolated from bovine corneal epithelial cells included five major components, of 500, 230, 200, 180, and 120 kD (Fig. 1 *A*, lane *1*), as reported previously (31). We have been preparing monoclonal antibodies to characterize these components; the antibody to the 500-kD component—HD1—is described here.

By immunizing mice with electrophoretically purified HD1, we obtained three monoclonal antibodies to HD1, all of which belonged to the IgG1 subclass and showed the same results for immunoblotting and immunofluorescence staining. In this study, however, we used only one monoclonal antibody (mAb HD-121) because of its broad species cross-reactivity (see below). Immunoblotting data showed mAb HD-121 to specifically react with HD1 of the isolated HD fraction (Fig. 1 *A*, lane *3*). The 230-kD band, HD2, was recognized by the monoclonal BP autoantibody named 5E as reported previously (Fig. 1 *A*, lane *2*) (43; see also references 13, 20, 28, 30, 40). The specificity of mAb HD-121 was further confirmed by using a 4% gel. Under these conditions three major components of the HD fraction could be seen (Fig. 1 *B*, lane *3*). mAb HD2 was revealed by immunoblotting with BP autoantibody (Fig. 1 *B*, lane *3*). mAb HD-121 recognized the largest major band, HD1 (Fig. 1 *B*, lane *4*), which showed slightly lower mobility than *Chlamydomonas* dynein heavy chains of 460–480 kD (Fig. 1 *B*, lane *1*) (19). This allowed an estimate of the molecular mass of HD1 as ~500 kD although we previously described it as a 480-kD component (31). Using mAb HD-121, immunocytochemical studies were performed to demonstrate the localization of HD1 in HDs at both light and electron microscopic levels.

Distribution of HD1 in Stratified and Complex Epithelial Cells

Since typical HDs can be identified in basal cells of stratified epithelia, we first examined the distribution of HD1 in bovine tissues of this type by immunofluorescence microscopy. In corneal epithelium, from which the HD fraction was isolated, mAb HD-121 specifically stained the BMZ (Fig. 2, *A* and *B*); this is consistent with the distribution of HDs stained by BP autoantibodies (30, 31) (data not shown). In skin epidermis (Fig. 2, *C* and *D*) and tongue epithelium (data not shown), HD1 and the BP antigen showed very similar distributions along the BMZ. Similar staining with mAb HD-121

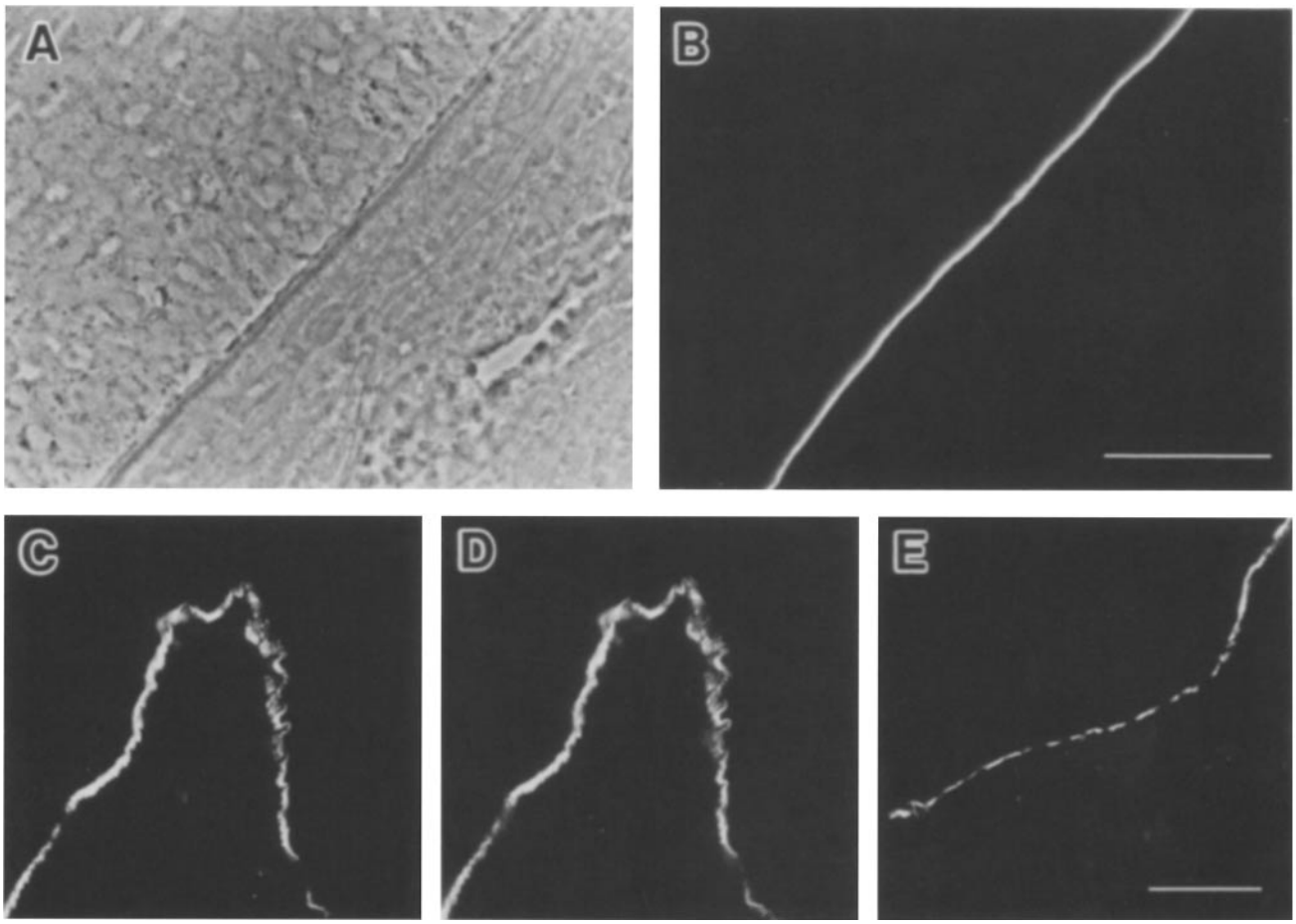

Figure 2. Immunofluorescence microscopy showing HD1 distribution in stratified epithelial tissues. In bovine cornea, HD1 was localized along the BMZ of the epithelium (compare *B* with the phase-contrast image in *A*). Double staining of bovine skin showed codistribution of HD1 (*C*) and BP antigen (*D*) at the basal epidermal surface. HD1 was also distributed at the basal surface of human skin epidermis (*E*). Bars: (*A* and *B*) 50 μm; (*C–E*) 40 μm.

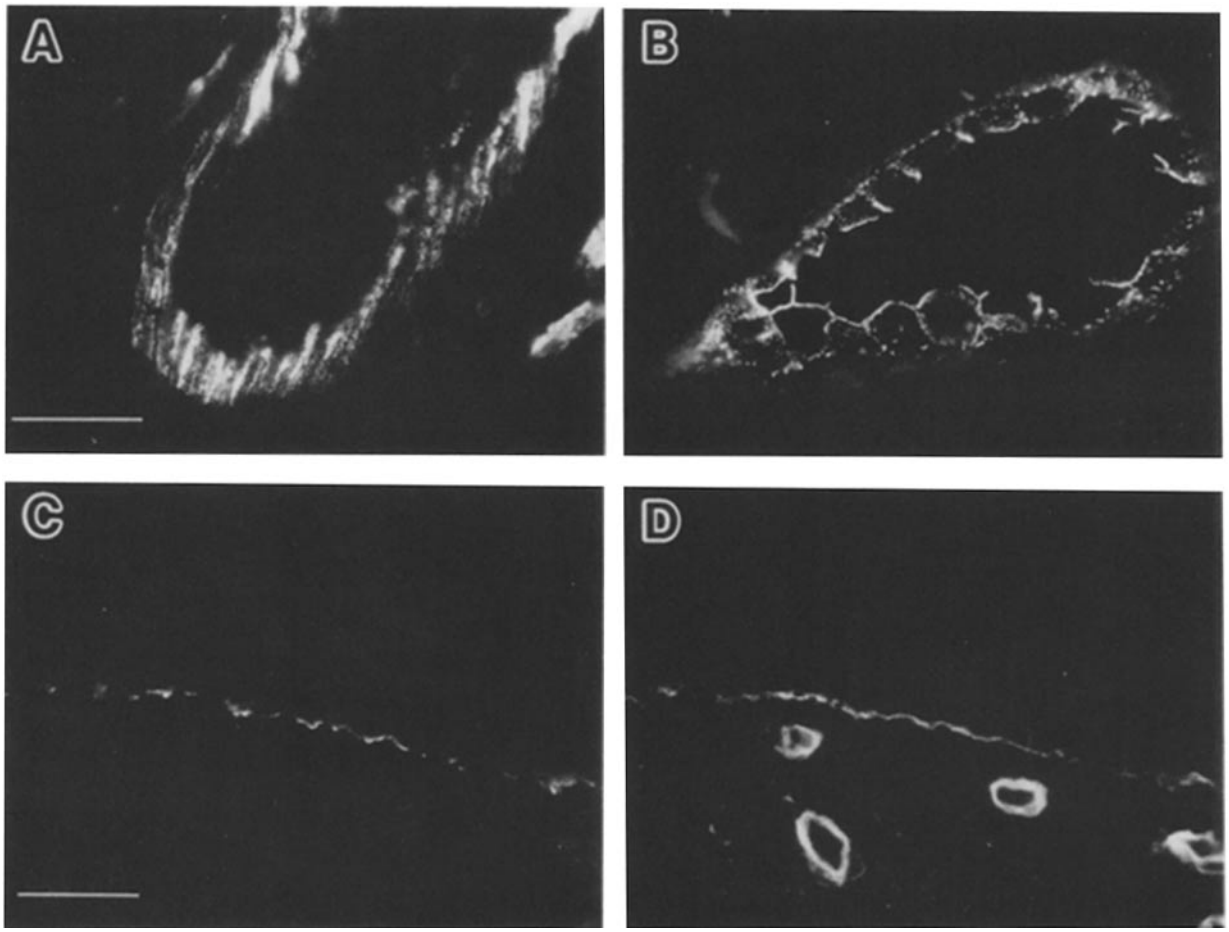

Figure 3. Immunofluorescence microscopy of bovine skin apocrine glands (*A* and *B*) and tracheas (*C* and *D*). In apocrine glands HD1 was distributed in linear punctate patters, typical of HDs in myoepithelial cells surrounding glandular epithelial cells (*A*). This was contrasted to the distribution of desmoplakins at the intercellular region between glandular epithelial cells, and between glandular epithelial and myoepithelial cells (*B*). In trachea HD1 was distributed along the BMZ of the epithelium (*C*) where antilaminin staining was also observed (*D*). Bars, 30 μm.

was observed in a broad spectrum of species including man (Fig. 2 *E*), rabbit, rat, and chick (data not shown). These results taken together suggested predominant localization of HD1 to HDs in stratified epithelial cells, although some positive staining by mAb HD-121 but not by BP autoantibodies was also noted in dermis (see below).

Next, HD1 distribution was examined in complex epithelial cells, such as myoepithelial and tracheal cells, which are known to have HDs along the BMZ (30, 31, 35; and for electron microscopy see reference 6). In apocrine sweat glands present in skin dermis, which have myoepithelial cells surrounding the glandular epithelial cells, mAb HD-121 gave a staining pattern of finely punctate arrays in myoepithelial cells but not in glandular cells (Fig. 3 *A*). The distribution pattern of HD1 was very similar to that of the BP autoantigen (data not shown), but was quite distinct from those of desmoplakins which are marker proteins of the desmosome (Fig. 3 *B*).

HD1 in tracheal epithelial cells was also observed in the BMZ labeled with antilaminin (Fig. 3, *C* and *D*), and colocalized with the BP antigen (30, 31) (data not shown). Immunoblotting of the cytoskeletal fraction of tracheal basal cells demonstrated that mAb HD-121 reacted with a band similar in molecular mass to corneal HD1 (Fig. 1 *B*, lane *5*). These data indicated the occurrence of HD1 in HDs of complex epithelial cells.

Localization of HD1 in Hemidesmosomes at the Electron Microscopic Level

To obtain further evidence of HD1 localization in HDs, frozen sections of bovine cornea were subjected to immunoelectron microscopy using secondary antibodies coupled to colloidal gold particles. Immunolabeling was exclusively observed in HDs (Fig. 4 *A*), confirming the results of immunofluorescence microscopy. Close inspection revealed that the gold particles were mostly present in the innermost region of the hemidesmosomal plaque (Fig. 4, *B* and *C*), where linkage to keratin IFs was apparent. In contrast the BP antigen was present throughout the entire plaque as reported previously (data not shown; 13, 20, 28, 30, 32, 47, 52). These observations indicated that at least an epitope of the HD1 molecule is to be found on the cytoplasmic side of HDs.

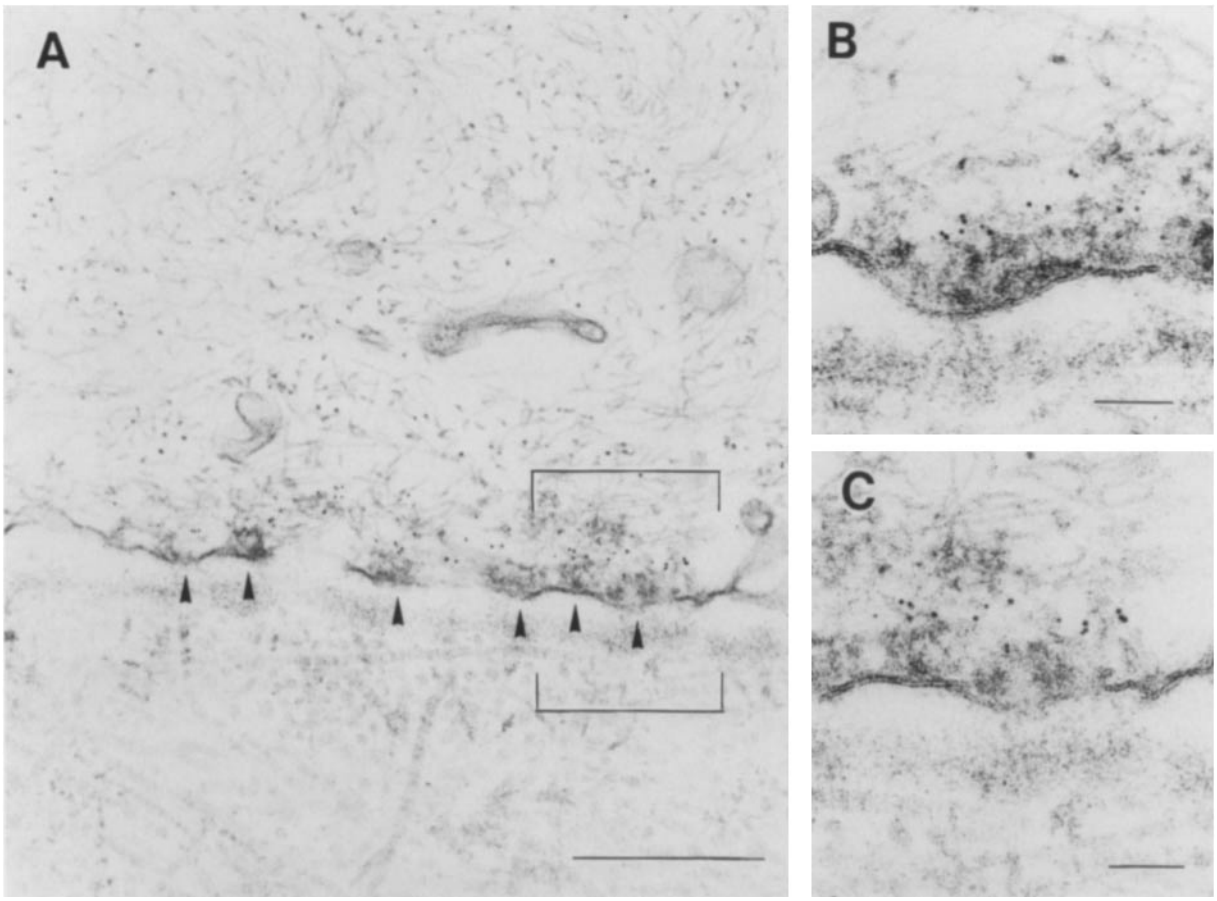

Figure 4. Immunoelectron microscopic localization of HD1 in bovine corneal HDs using secondary antibodies coupled to 5-nm colloidal gold particles. (*A*) Specific immunolabeling observed in HDs (*arrowheads*) of the epithelial cells, but not in the cytoplasm or in the extracellular region. An enlarged micrograph of the bracketed region is shown in *C*. (*B* and *C*) At higher magnification gold particles were apparent in the innermost region of the HD plaque, where linkage with IFs was apparent. Bars: (*A*) 0.5 μm; (*B* and *C*) 0.1 μm.

Distribution of HD1 in Cultured Keratinocytes

Overall distribution of HD1 in cells became evident from immunofluorescence microscopic observation of cultured keratinocytes, FRSK cells, derived from rat foreskin. FRSK cells demonstrated numerous dots labeled by mAb HD-121 (Fig. 5 *A*), whose distribution was consistent with that of the BP antigen in a precise way (Fig. 5 *B*). In contrast, HD1 was absent from focal adhesions labeled by antivinculin (data not shown) and from cell–cell junctions (see Fig. 5 *A*). Immunoblotting data also provided evidence of the occurrence of HD1 in FRSK cells (Fig. 1 *B*, lane 7). By electron microscopic observation we found structures similar to HDs at the basal surface of FRSK cells. As shown in Fig. 6, these possessed electron-dense cytoplasmic plaques associated with IFs. Immunoelectron microscopy revealed labeling by mAb HD-121 of the cytoplasmic region in these structures, consistent with the distribution in HDs of corneal epithelial cells.

Distribution of HD1 in Nerves and Blood Vessels

As described above, HD1 as well as the BP antigen was located in HDs of stratified and complex epithelial tissues. However, in the course of immunofluorescence microscopy of skin and trachea, we noted that nerves and blood vessels were also positively stained by mAb HD-121 but not by BP autoantibodies.

Positive staining of blood vessels was evident from comparison with phase-contrast images (Fig. 7 *A*). mAb HD-121 clearly stained endothelial cells, while the smooth muscles were negative (Fig. 7 *B*). We detected no positive staining by BP autoantibodies in blood vessels (Fig. 7 *C*). Thus far, however, we have failed to uncover the ultrastructural localization of HD1 in the endothelial cells; we have not been able to find HD-like structures, nor could we correlate the distribution of HD1 with that of IFs. This might be because the cells are very thin and because IFs are less abundant than in epithelial cells. Experiments using cultured endothelial cells are under study as another project.

The presence of HD1 in nerves also became evident from double-label immunofluorescence microscopy of bovine skin using anti-neurofilaments (data not shown). To identify which types of cells in nerves were HD1-positive, we examined the bovine optic nerve. Immunofluorescence microscopy revealed staining by mAb HD-121 mainly in the periphery of each nerve fiber bundle (Fig. 7, *D* and *E*), where anti-GFAP staining was also observed (Fig. 7 *F*). Since the

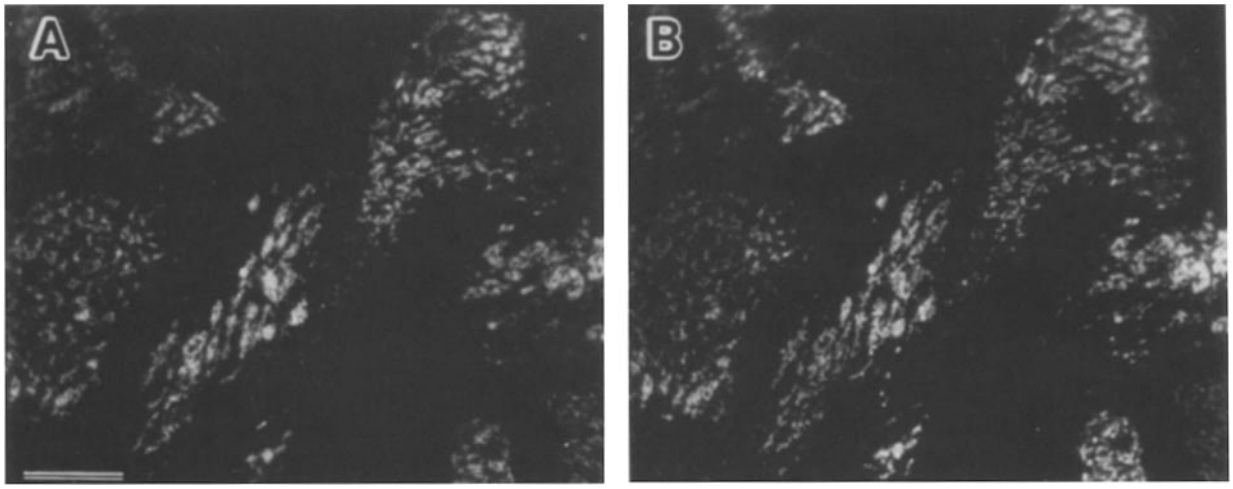

Figure 5. Immunofluorescence microscopy showing HD1 distribution in cultured FRSK keratinocytes. (*A* and *B*) Double-label immunofluorescence microscopy using mAb HD-121 (*A*) and the BP monoclonal antibody. Both antibodies demonstrate similar patterns of numerous dots. Note the absence of immunostaining from intercellular regions. Bar, 10 μm.

glial cells surrounding the bundles are astrocytes, these observations indicated that HD1-positive nerve cells are astrocytes and not neurons. No staining by BP autoantibodies was observed (data not shown). Immunoblot analyses confirmed the presence of HD1 in the cytoskeletal fraction of the optic nerve (Fig. 1 *B*, lane *6*). HD1 distribution in astrocytes was further explored by immunoelectron microscopy of the bovine optic nerve. In the optic nerve, the collagenous connective tissue surrounds the nerve fiber bundles to form septa. Astrocytes exist between each nerve bundle and the septum, sending processes into the bundle (10). Therefore, when the edge of the nerve bundle was examined, astrocytes that had basal lamina and large amounts of glial filaments could be easily found between myelinated axons and the collagenous matrix. We reproducibly observed labeling by mAb HD-121 in cells with basal lamina, overlaying the collagenous matrix and nearby myelin sheaths, of which the latter, however, was partly destroyed (Fig. 8, *A* and *B*). Furthermore, gold particles were specifically seen in the cytoplasmic region just beneath the plasma membrane in obvious association with glial IFs (Fig. 8, *B–D*), although typical HDs could not be seen in the region.

Discussion

The hemidesmosome, which mediates cell adhesion to the ECM, has a distinctive structure consisting of an IF-associated cytoplasmic plaque just beneath the plasma membrane, a subbasal dense plate and anchoring filaments at the basal lamina, and anchoring fibrils in the connective tissue. There have been reports concerning several proteins and antigens located in the specific regions described above: the 230-kD BP antigen (13, 20, 28, 30, 32, 47, 52); a 200-kD polypeptide (23); a 180-kD BP antigen (8, 20); α6β4 integrin as an inte-

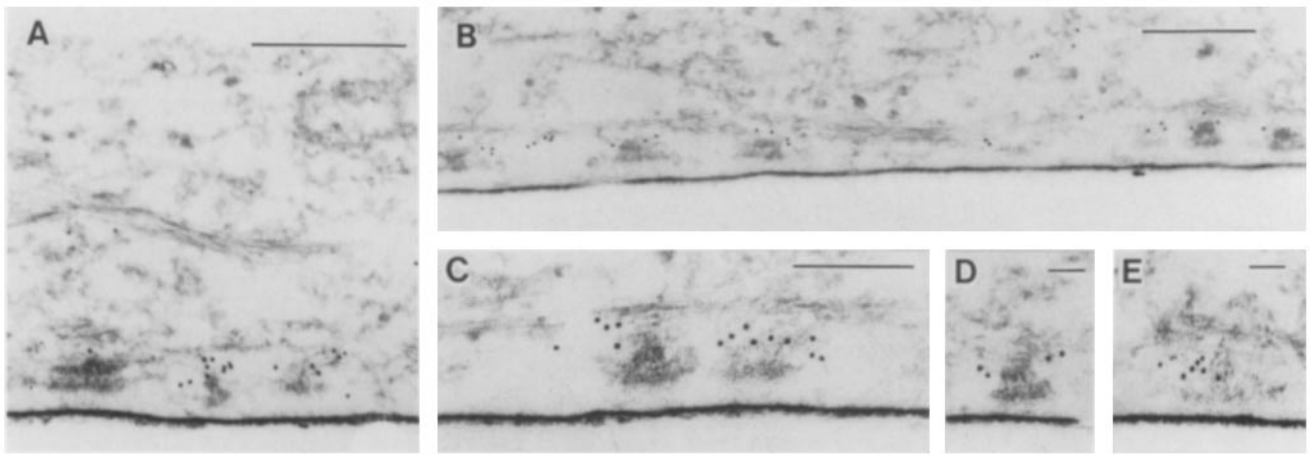

Figure 6. Immunoelectron microscopic localization of HD1 in FRSK cells using secondary antibodies coupled to 10-nm colloidal gold particles. At the basal surfaces of FRSK cells, we observe structures that are similar to HDs in that they consist of electron-dense plaques associated with IFs. Note immunolabeling in the cytoplasmic regions of the structures. Bars: (*A* and *B*) 0.5 μm; (*C*) 0.25 μm; (*D* and *E*) 0.1 μm.

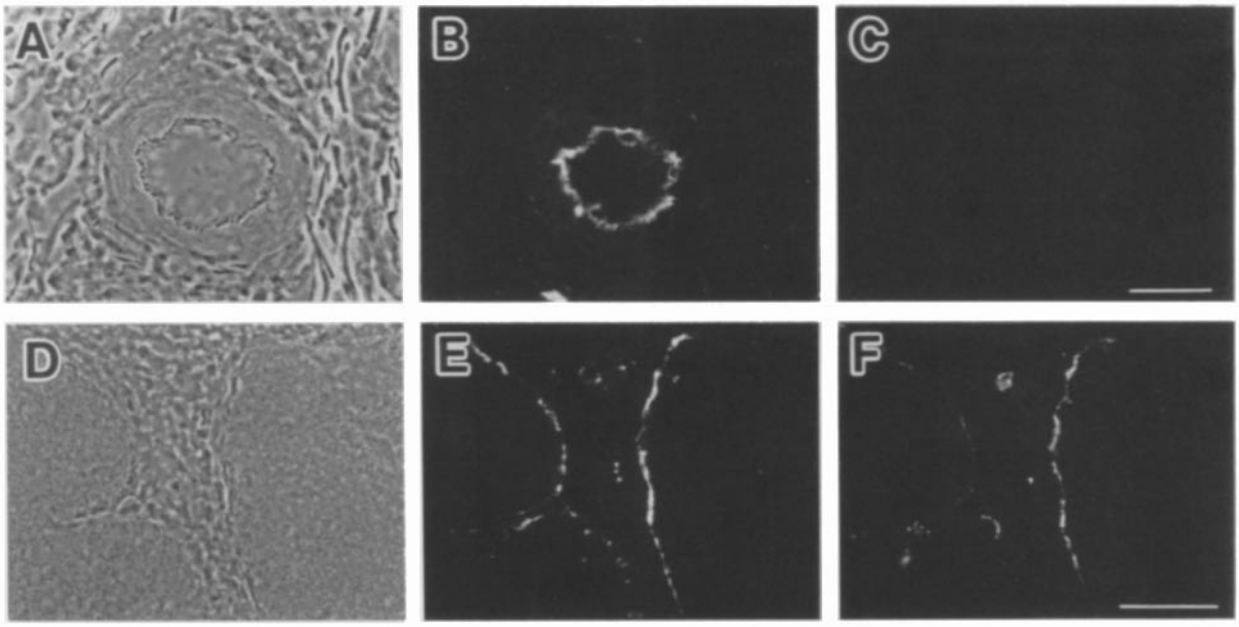

Figure 7. Immunofluorescence microscopy showing the occurrence of HD1 in cells lacking typical HDs. (*Top*) Bovine blood vessels stained with mAb HD-121 (*B*) or the BP monoclonal antibody (*C*). As is evident from comparison with the phase-contrast image (*A*), anti-HD1 staining is apparent in endothelial cells but not the smooth muscles. No staining was seen with the BP antibody. (*Bottom*) Bovine optic nerve stained with mAb HD-121 (*E*) or anti-GFAP (*F*). Anti-HD1 staining was observed mainly in the peripheries of nerve bundles (compare with the phase-contrast image in *D*), where anti-GFAP staining was also observed. These observations indicate the occurrence of HD1 in astrocytes but not in neurons. Bars, 10 μm.

gral membrane protein (3, 14, 38, 42); a 125-kD polypeptide (24); and GB3 antigen (51) as possible components of the anchoring filaments and/or the subbasal dense plate; and type VII collagen as a component of the anchoring fibrils (15, 34, 44). Since these data were obtained fragmentarily, we established an HD fraction isolation procedure, allowing identification of five major components by SDS-PAGE analysis (31).

In this study we described the immunological characterization of a 500-kD protein termed HD1, one of the five major components, and confirmed it to be a new hemidesmosomal protein. Immunofluorescence microscopy of stratified and complex epithelia, all of which have bona fide HDs, revealed HD1 to be localized on the basal cells along the BMZ, as is the case for the 230-kD BP antigen. Also in cultured keratinocytes (FRSK cells), which had obvious plaque-bearing HDs at the electron microscopic level, HD1 was found to be distributed in a punctate pattern indistinguishable from that of the BP antigen, while it was absent from focal adhesions and cell–cell adhesion sites. Moreover, immunoelectron microscopy of corneal epithelium and FRSK cells indicated that at least a portion of the HD1 molecule is located in the innermost region of the HD plaque, where linkage with IFs is apparent. These observations offer convincing that HD1 is a new structural protein present in typical HDs, though we cannot exclude the possibility that the 500-kD component in our HD fraction includes a protein(s) besides the antigen to anti-HD1.

An unexpected and interesting result obtained in this study was the presence of the HD1 molecule in astrocytes of the optic nerve and in endothelial cells of blood vessels, which lack typical HDs. In addition, anti-HD1 staining was observed in the nerves of skin and trachea, suggesting the presence of HD1 also in Schwann cells of peripheral nerves. Immunoelectron microscopic data demonstrated the HD1 molecule to be located near the plasma membrane of astrocytes in association with IFs, whereas we could not demonstrate this in endothelial cells. Although most hemidesmosomal proteins identified so far show tissue distributions restricted to those with typical HDs, HD1 is not the only HD-associated protein present in tissues having no typical HDs. We have some evidence that a 200-kD protein of HD3 and a 55-kD protein in the isolated HD fraction also demonstrate similar distributions (Nishizawa, Y., and K. Owaribe, manuscripts in preparation).

Furthermore, Sonnenberg and Linders (37) have demonstrated expression in Schwann cells of peripheral nerve of the $\alpha 6\beta 4$ complex of integrin, which has only recently been shown to occur in HDs and has been implicated in cell adhesion to the ECM (3, 14, 38, 42). Though visual confirmation is lacking, the occurrence of $\alpha 6\beta 4$ in central nerves is likely since the possible existence of multiple forms of $\beta 4$ has been demonstrated (46). Sequence analysis of the $\beta 4$ subunit has revealed a unique cytoplasmic domain not seen in other β subunits (11, 44, 46), as is the case with desmoglein, a desmosomal cell adhesion molecule of the cadherin family (21). For both $\beta 4$ and desmoglein, their additional cytoplasmic domains are thought to be responsible for the interactions with IFs. Considering the HD1 localization in astrocytes described above, astrocytes and probably endothelial cells may possess simple or primitive forms of cell adhesion systems associated with IFs, where HD1 protein together with the $\alpha 6\beta 4$ integrin may play roles in IF anchorage to the plasma membrane. If so, bona fide HDs could be interpreted as

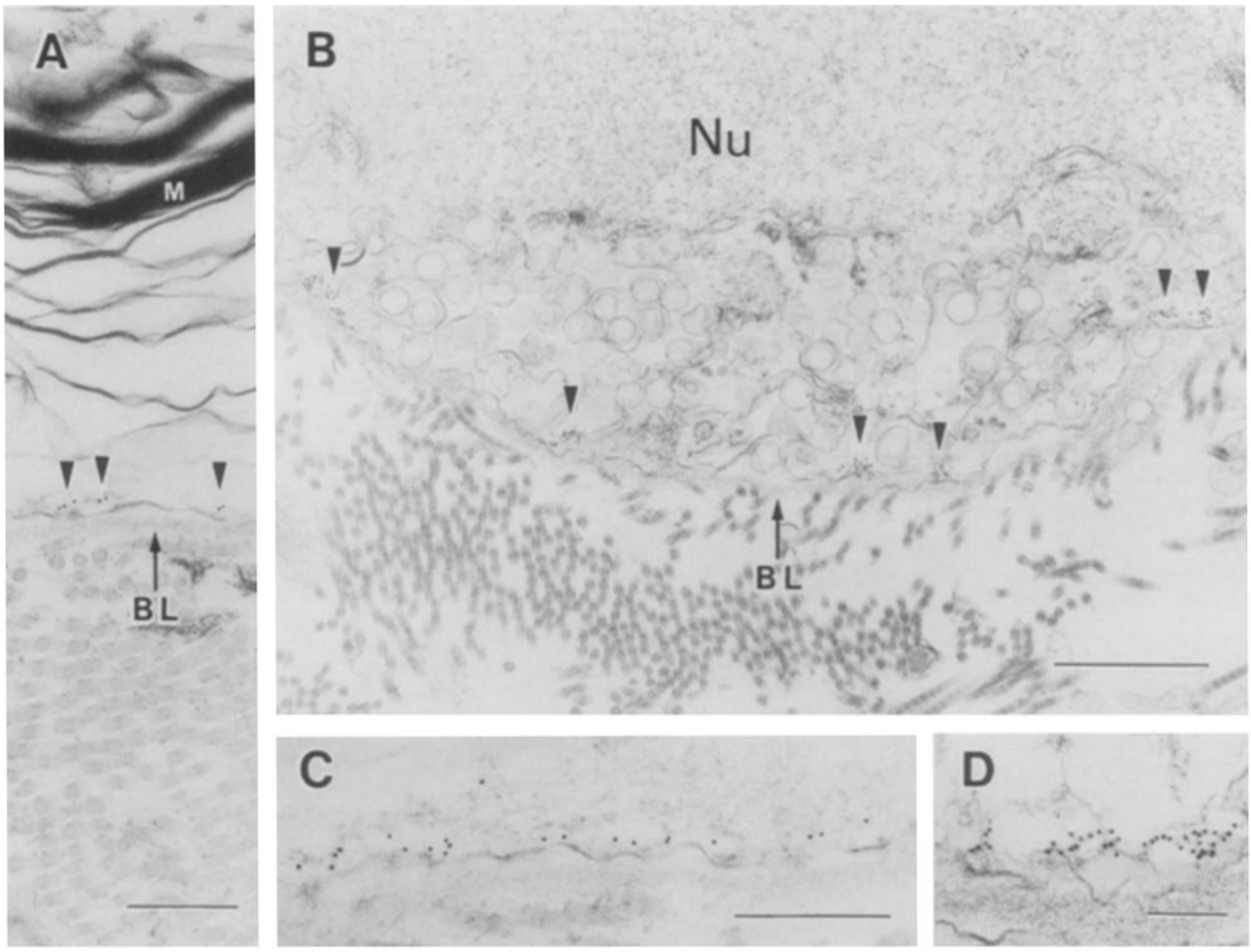

Figure 8. Immunoelectron microscopic localization of HD1 in astrocytes using secondary antibodies coupled to 10-nm colloidal gold particles. (*A*) Immunolabeling (*arrowheads*) is apparent in the cells with the basal lamina (*BL*), existing between axons with myelin sheaths (*M*) and the collagenous matrix. (*B*) Gold particles (*arrowheads*) observed in the cytoplasmic region near the plasma membrane facing the basal lamina (*BL*), but not in the nucleus (*Nu*) nor in the extracellular region. (*C* and *D*) Observations at higher magnification reveal the gold particles in association with IFs. Bars: (*A* and *C*) 0.5 μm; (*B*) 1 μm; (*D*) 0.25 μm.

more advanced and complex forms of cell-to-ECM adhesion sites associated with IFs. In addition to proteins such as HD1 and the $\alpha 6\beta 4$ integrin, many other proteins specific for the typical HD may take part in forming the complicated structure. Taking into consideration these situations, we propose the terms type I HD and type II HD. Type I HDs (i.e., bona fide HDs) can be defined as the IF-associated cell–substrate adhesion device with the BP antigens, while type II HDs can be defined as the BP antigens-negative device such as those found in astrocytes. The absence from astrocytes and endothelial cells of the anchoring filament and the subbasal dense plate, which are thought to contain ligands for cell adhesion receptors in the HDs, may indicate that these cells use a ligand(s) different from that in bona fide HDs.

Concerning a prominent feature of HDs, i.e., interactions of IFs with the plasma membrane, the 230-kD BP antigen has been suspected to play a part in IF anchorage because of its localization within the HD plaque (20, 52) and of similarity in amino acid sequence to desmoplakins that are thought to play a role in linking IFs to desmosomes (9, 35, 41). However, no biochemical evidence for this idea has been available. Considering the ultrastructural localization of HD1 in HDs of corneal epithelial and FRSK cells, we suppose that this protein could be also involved in anchoring IFs to the hemidesmosomal plaque. Furthermore, the presence of HD1 but not the 230-kD BP antigen in astrocytes and endothelial cells, and the apparent association of HD1 with IFs in astrocytes, may suggest that HD1 could function as an IF-anchoring protein to the plasma membrane even without the 230-kD BP antigen. Biochemical studies, however, on the interaction of HD1 protein with IFs and/or with other hemidesmosomal proteins await their purification.

It has been reported that HD formation requires three stages: the first stage is the extension of anchoring filaments from the plasma membrane to the BMZ; the second stage is the appearance of an electron-dense cytoplasmic plaque; and the last stage is the appearance of a subbasal dense plate between the plasma membrane and the BMZ and the attachment of intermediate-sized filaments to the cytoplasmic plaque (7, 22). In addition, Trinkaus-Randall and Gipson (48) described HD formation to be dependent on extracellular Ca^{++} and regulated through the action of calmodulin.

A study of which stage of the HD formation needs HD1 and an examination of whether HD1 or other hemidesmosomal components, like desmocalmin of desmosomes (49), can bind calmodulin are of obvious interest. The present findings suggested that FRSK cells may provide an excellent in vitro model to explore the molecular mechanisms of HD formation.

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