J. Biochem. 126, 1144-1150 (1999)

Identification of the Hemidesmosomal 500 kDa Protein (HD1) as Plectin[1]

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Received July 19, 1999; accepted October 1, 1999

HD1 is a 500 kDa hemidesmosomal plaque protein recognized by monoclonal antibody mAb-121. Recent research on inherited skin disease has suggested that it might be identical to plectin or an isoform. To cast light on this question, we have prepared several monoclonal antibodies that recognize a 500 kDa protein in the hemidesmosome fraction. Unexpectedly, some staining pattern heterogeneity was observed on immunofluorescence microscopy. Attention was focused on two monoclonal antibodies which gave different localization in bovine skin and retinal pigment epithelial cells. Determination of the amino-terminal sequence of an antigenic 100 kDa polypeptide fragment derived from the 500 kDa component of an insoluble fraction of bovine hepatocytes revealed it was identical to that of plectin. Using the two antibodies, we screened a cDNA library derived from BMGE+H, a bovine mammary gland epithelial cell line. The isolated cDNA clones corresponded to the rod domain of bovine plectin, with two separate epitope regions for each of the antibodies. From these results we conclude that the hemidesmosomal 500 kDa component HD1 is identical to plectin. As judged on rough estimation of molar ratios on this basis, hemidesmosomes are composed of plectin, BP230, the integrin $\beta 4$ subunit, and $\alpha 6$ in a 1:1:1:1 ratio.

Key words: cell adhesion, hemidesmosome, HD1, mAb-121, plectin.

Hemidesmosomes are adhesion structures responsible for linking stratified and complex epithelia to extracellular matrix components of the basement membrane. They mechanically link the cytoplasmic intermediate filament (IF) network to collagen fibrils and other constituents of the connective tissue (*1, 2*). A hemidesmosome-rich fraction has been isolated from bovine corneal epithelial cells, and its major components, separated on SDS-PAGE, are 500, 230, 200, 180, and 120 kDa proteins (*3*), namely HD1, BP230 (BPAG1), the integrin $\beta 4$ subunit, BP180 (BPAG2, type XVII collagen), and the integrin $\alpha 6$ subunit, respectively (*4-9*). The 500 kDa protein HD1 has been detected on the most cytoplasmic side of hemidesmosomal plaques under an electron microscope (*4*), and has been thought to anchor intermediate filaments to other hemidesmosomal proteins. In contrast to the case of other components, its cDNA cloning has yet to be performed.

Plectin, first isolated from rat C6 glioma cells as a vimentin associated protein (*10*), is now known to be expressed in a variety of tissues and cell lines, including epithelia, muscles, and neural tissues (*11*). Within the cytoplasm, plectin associates with various elements of the cytoskeleton (IFs, microfilaments, and microtubules), also being present in membrane junctional complexes such as desmosomes, hemidesmosomes, and focal contacts (*12-14*). In line with this versatility, the ~500 kDa polypeptide deduced from a plectin cDNA exhibits a multidomain structure, including an actin binding domain and an intermediate filament binding domain (*15-17*).

Recent investigations have shown that epidermolysis bullosa simplex with muscular dystrophy (EBS-MD), a type of inherited skin disease, is caused by loss or deficient expression of plectin (*16, 18, 19*). Plectin appears essential for maintaining muscle integrity as well as dermal-epidermal adhesion. The skin of patients with EBS-MD also is not stained with an anti-HD1 monoclonal antibody, mAb-121 (*16, 19*). Considering similarities in size, localization, and expression, the two proteins might be identical or at least products of a single gene. However, it is unclear why mAb-121 only stains hemidesmosomes, whereas other antibodies against plectin detect not only hemidesmosomes, but also other intracellular structures, such as intermediate filaments and desmosomes (*12, 13*).

In our research we have noted that several monoclonal antibodies recognize the hemidesmosomal 500 kDa compo-

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: BMGE+H, bovine mammary gland epithelial cell; IF, intermediate filament; BPAG, bullous pemphigoid antigen; EBS-MD, epidermolysis bullosa simplex with muscular dystrophy; HD fraction, hemidesmosome-rich fraction; BMZ, basement membrane zone; AJ, adherens junction.

nent on immunoblotting, but some of them show heterogeneity in immunofluorescence staining. We therefore compared two of these monoclonal antibodies and analyzed their antigen molecules at the protein and cDNA levels.

MATERIALS AND METHODS

Isolation of Hemidesmosomes—The hemidesmosome-rich fraction (HD fraction) was isolated from bovine corneal epithelial cells, as described previously (*3*).

Cells and Cell Cultures—Retinal pigment epithelia were isolated from bovine eye cups as cell sheets after treatment with a glycerol solution (50% glycerol, 10 mM phosphate buffer, pH 7.2, 5 mM EDTA) as described previously (*20*).

BMGE+H cells, a mammary gland epithelial cell line derived from a lactating bovine udder, were a kind gift from Dr. W.W. Franke of the German Cancer Research Center (Heidelberg, Germany). The cell culture conditions were as described previously (*21*).

Antibodies and Immunofluorescence Microscopy—Mouse monoclonal antibodies (mAbs) against hemidesmosomal proteins were prepared by immunizing mice with the HD fraction or a 500 kDa component isolated from the HD fraction electrophoretically, as described previously (*3*).

Freshly prepared tissues were snap-frozen in isopentane precooled in liquid nitrogen. Frozen specimens were cut at 5–6 μm with a cryostat, mounted on glass slides, air dried, and then fixed in 100% acetone at −20°C for 10 min. In some cases the sections were treated with 1% Triton X-100 (Tx-100), 0.5% sodium deoxycholate, or 1.5 M NaCl in PBS before fixation. Cells grown on glass coverslips were also fixed with acetone. The fixed tissue sections and cells were processed for immunofluorescence staining with monoclonal antibody mAb-121 or -156 followed by a fluorescein-conjugated secondary antibody as described previously (*21*).

Electrophoresis and Immunoblotting—SDS-PAGE was performed according to the method of Laemmli with a slight modification (*22*), with immunoblotting after electrophoretic transfer to nitrocellulose sheets using a semi-dry system (*4*).

Aminoterminal Sequencing—Fresh bovine liver cells were homogenized and extracted in a low salt buffer and further extracted in a high salt buffer as described previously (*23*). After rinsing with the low salt buffer, the resultant cytoskeletal fraction was extracted with a urea solution (6 M urea, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM DTT) for 30 min on ice, and then centrifuged for 30 min at 30,000×g. The supernatant obtained was analyzed by SDS-PAGE on a 7.5% gel, and the 500 kDa band was cut out and washed 3 times with an acetonitrile solution (50% acetonitrile, 62.5 mM Tris-HCl, pH 6.8, 0.05% SDS) and air dried. The gels were soaked in a digestion solution (100 μg/ml α-chymotrypsin, 125 mM Tris-HCl, pH 6.8, 0.1% SDS) and incubated for 60 min at 37°C. The cleaved proteins were separated by SDS-PAGE (7.5% gel), and the 100 kDa band was cut out, purified, electrophoresed and transferred to a nitrocellulose membrane for immunoblotting or a PVDF (polyvinylidene fluoride) membrane for amino acid sequencing. The PVDF membrane was stained with Ponceau S, the band cut out and the N-terminal amino acid residues of the polypeptide sequenced with a peptide sequencer (Applied Biosystems, USA).

Construction of a cDNA Library, Screening for Partial cDNA Cloning, Estimation of Epitope Regions, and DNA Sequencing—Total RNA of cultured BMGE+H cells was isolated by the guanidine thiocyanate method (*24*) and mRNA was purified with an oligo(dT) column. cDNA was synthesized using random primers, ligated with a linker, and then inserted into the *Eco*RI site of expression vector λgt11.

Polypeptides induced by 10 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) were transferred to nitrocellulose membranes, and positive plaques were detected in the same manner by immunoblotting using mAb-121 and -156. The isolated cDNA clones and their fragments obtained on digesting with the restriction enzymes, *Hin*dIII, *Bal*I, *Esp*I, *Sph*I, *Sma*I, *Pst*I, and *Aor51*HI, were subcloned into a plasmid vector, pBluescriptSK(−) (Stratagene, La Jolla, CA), for sequencing, or pGEX vectors (Amersham Pharmacia Biotech AB, Uppsala) for expression of fusion proteins with GST (glutathione-S-transferase). DNA sequencing was performed using a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) and a LI-COR dNAsequencer model 4000L (LI-COR, Lincoln, NE). The DNA sequences were analyzed with the DNASIS program (Hitachi Software Engineering, Tokyo), and the GenBank database was searched with the BLAST program (*25*) at the National Center for Biotechnology Information (Bethesda, MD). Fusion proteins were expressed in *Escherichia coli* XL1-Blue by 1 mM IPTG induction, and whole cells were sonicated in PBS and then subjected to SDS-PAGE.

Densitometry—For molar ratio analysis of hemidesmosomal components, the CBB stained SDS-gels of five different HD fraction preparations were scanned with a Scanmaster 3+scanner and the gel images were analyzed with the Scanalytics program (M&S Instruments Trading, Osaka). The following apparent molecular weights were used for estimation of the molar amounts in the protein bands: HD1/plectin, 500 kDa; BP230, 230 kDa; integrin β4 subunit, 200 kDa; BP180, 180 kDa; and integrin α6 subunit, 125 kDa.

RESULTS

Characterization of Monoclonal Antibodies That Recognize the 500 kDa Band of the HD Fraction—We prepared a panel of mAbs recognizing a 500 kDa polypeptide present in the HD fraction. Immunoblot analyses of the HD fraction using these antibodies gave an identical pattern (Fig. 1). However, immunofluorescence staining of hemidesmosomes, adherens junctions (AJ), and the IF network exhibited heterogeneity. Desmosomes were not stained by any of the monoclonal antibodies prepared. Two antibodies, mAb-121 (IgG1) and -156 (IgM), which showed the most striking contrast were selected for a detailed study, to determine whether the 500 kDa band of the HD fraction on SDS-PAGE contains a single polypeptide, HD1.

As shown in Fig. 2, A and C, mAb-121 gave linear fluorescence staining along the basement membrane zone (BMZ) of the bovine epidermis and a dot array on myoepithelial BMZ, identical to the typical localization of hemidesmosomes, as reported previously (*4*). In contrast, while mAb-156 gave a similar fluorescent pattern for myoepithelial BMZ, little staining of epidermal BMZ was

observed. Furthermore, it gave an AJ-like staining pattern for apocrine glandular cells (Fig. 2, B and D). The staining patterns with the two mAbs were not dependent on fixation (with acetone, or no fixation), or affected by extraction with 1% Triton X-100, 0.5% sodium deoxycholate, or 1.5 M NaCl before fixation (data not shown).

To confirm that mAb-156 stains AJ-like structures, we also examined bovine retinal pigment epithelial cells, which have no hemidesmosomes but conspicuous AJ. mAb-121 clearly revealed a filamentous staining pattern similar to a keratin network (Fig. 3A), whereas mAb-156 gave an AJ-like polygonal staining pattern (Fig. 3B, *cf.* Ref. *20*).

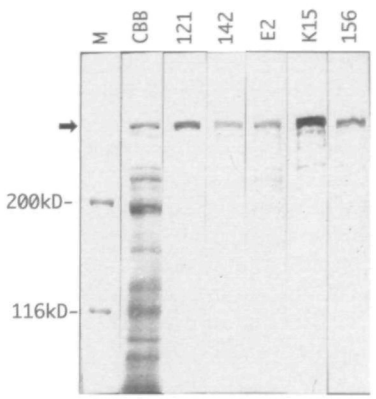


Fig. 1. **Immunoblotting of the HD fraction.** The HD fraction was separated on a 7.5% acrylamide gel, and then stained with the monoclonal antibodies indicated above the lanes. M, markers; CBB, Coomassie Blue staining. The arrow indicates the position of the 500 kDa polypeptide.

Reactivity to Skin of an EBS-MD Patient—Epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) is a distinct recessively inherited variant of EB associated with late-onset muscular dystrophy (*26*). Recent studies revealed that this disease is caused by a homozygous mutation in the plectin gene and its defective expression, mAb-121 reactivity being completely absent in the epidermis and muscle of EBS-MD patients (*16, 18, 19*). We examined immunofluorescence staining of human skin using the other mAbs applied in Fig. 1, except for mAb-142, which does not cross-react with human (Table I). In normal human skin, the epidermal BMZ was recognized in all cases, including mAb-156, which does not recognize the bovine epidermal BMZ, but in the skin of an EBS-MD patient whose plectin gene mutations were previously reported (*27*), none of the antibodies gave BMZ staining.

Partial Amino Acid Sequencing of an Antigenic Polypeptide Fragment—Since all the antibodies caused conspicuous outlining of bile canaliculi of bovine hepatocytes on immunofluorescence microscopy and recognized a 500 kDa protein on immunoblotting of the cytoskeletal fraction of liver cells (data not shown), the 500 kDa antigenic polypeptide was isolated from hepatocytes by preparative SDS-PAGE and partially cleaved with α-chymotrypsin. The resultant ~100 kDa polypeptide, which was still recognized by both mAb-121 and -156 (Fig. 4), was processed for N-terminal amino acid sequencing, the sequence KAQLE-PVASP, in a one-letter expression, being obtained. This sequence is identical to that found for the central rod domain of human keratinocyte plectin (a.a.1317-1326, GenBank accession no. U53204, see Ref. *16*), and the fragment contained most of the rod domain judging from its molecular mass of ~100 kDa (Fig. 5A).

Partial cDNA Cloning for Estimation of Epitope Sites—To identify antigenic regions of the 500 kDa polypeptide,

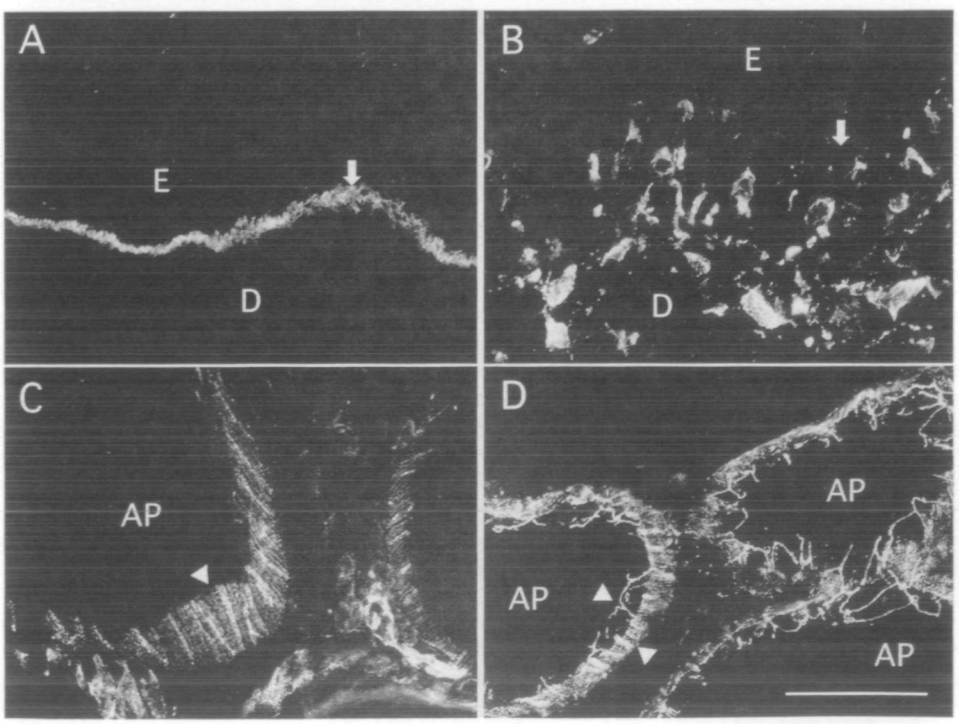


Fig. 2. **Immunofluorescence microscopy of bovine skin.** Frozen sections were stained with mAb-121 (A, C), and mAb-156 (B, D). A and B show the boundary between the epidermis and dermis. Arrows indicate the basement membrane zone (BMZ). C and D show myoepithelial cells around apocrine glands. Arrowheads indicate the BMZ of myoepithelial cells. E, epidermis; D, dermis; AP, apocrine gland. Bar, 50 μm.

we screened a cDNA expression library constructed from BMGE+H cells using mAb-121 and -156, and isolated 10 and 13 clones, respectively. Sequence analysis revealed that some clones contained the same inserts, probably because the cDNA library was amplified. In addition, 2 common clones were isolated for both mAb-121 and -156. We finally isolated 2 clones (designated here as B6-3 and B8-2) recognized by both mAb-121 and -156, and 1 clone (designated as B130) recognized by only mAb-156. These 3 clones partially overlap, as shown in Fig. 5B.

Sequence comparisons revealed the obtained cDNA sequence (total 3,333 bp, data not shown) exhibits 88.2% identity with the coiled-coil rod domain of human plectin (GenBank acc. no. U53204), and 85.7% with rat plectin (X59601). The deduced amino acid sequence exhibits 93.2% identity with the human and 92.2% with the rat form. The human and rat plectins are 87.1% identical in cDNA and 93.4% in amino acid sequence in this region. This indicated that the isolated clones correspond to the rod domain of bovine plectin, and that this molecule has been highly conserved during evolution, at least in this region (*15, 16, 28*). According to exon-intron organization of the

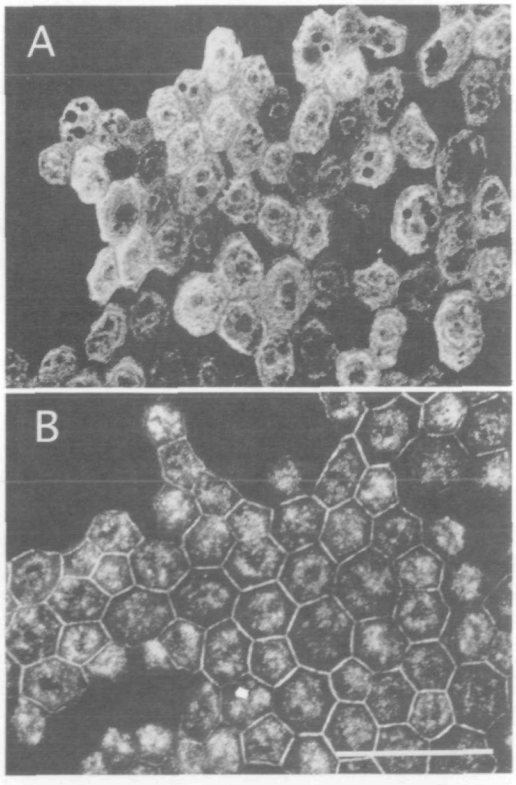


Fig. 3. **Immunofluorescence microscopy of primary cultured bovine retinal pigment epithelial cells stained with mAb-121 (A), and mAb-156 (B). Bar, 50 μm.**

TABLE I. **Immunoreactivity of the human skin BMZ with monoclonal antibodies.**

	mAb-121	mAb-156	mAb-E2	mAb-K15
NHS	+	+	+	+
EBS-MD	−	−	−	−

NHS, normal human skin; EBS-MD, skin of an epidermolysis bullosa simplex with muscular dystrophy patient.

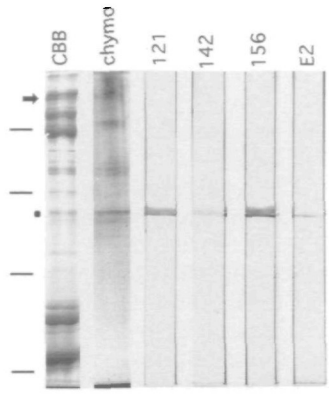


Fig. 4. **Preparation of the ~100 kDa polypeptide.** A 6 M urea-extract of bovine liver cells was separated on a 7.5% gel, and then stained with CBB (lane, CBB). The 500 kDa band (arrow) was cut out and digested with α-chymotrypsin as described under "MATERIALS AND METHODS" (lane, chymo). The dot indicates the resultant ~100 kDa band. This ~100 kDa polypeptide was immunoblotted using the monoclonal antibodies indicated. Note the strong signals with mAb-121 and -156 but not mAb-142 or -E2.

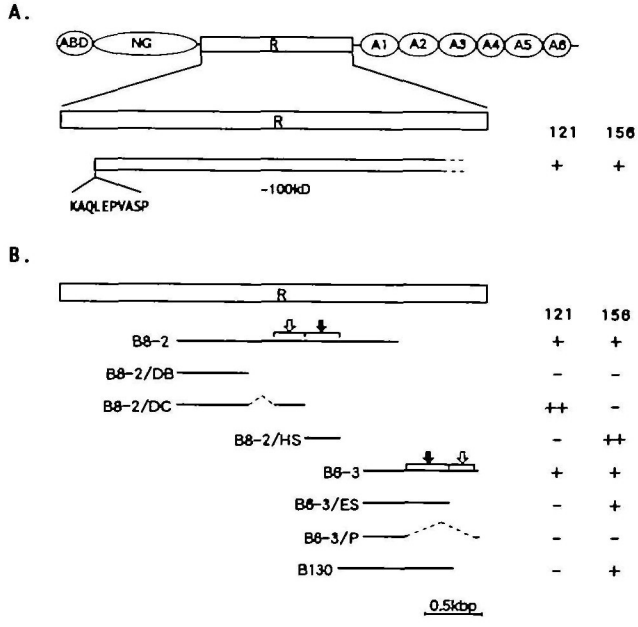


Fig. 5. **Schematic illustration of the structure of human plectin and corresponding alignment of bovine fragments.** (A) Basic structure of human plectin essentially according to the domain structure suggested by McLean *et al.* (1996). ABD, actin binding domain; NG, N-terminal globular domain; R, rod domain; A1-A6, tandem repeats in the C-terminal globular domain. The rod domain is expanded and indicated by an open box, and the ~100 kDa polypeptide is also indicated below with the N-terminal 10 amino acid sequence. The C-terminal of this polypeptide remains to be determined, as indicated by the dotted lines. The immunoreactivity with each monoclonal antibody is shown on the right. (B) cDNA clones and their truncated fragments. The expanded rod domain of plectin is indicated by an open box. cDNA clones isolated on screening, B6-3, B8-2, and B130, and their truncated fragments are indicated by lines at the corresponding positions under the rod domain. The immunoreactivity of the GST fusion protein with each monoclonal antibody is shown on the right. The estimated antigenic regions are indicated by open arrows for mAb-121 and closed ones for mAb-156.

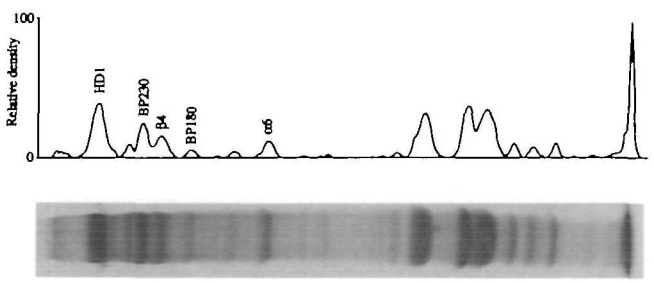
human plectin gene, all these clones belong to an unusually large exon that encodes most of the rod domain (*28*). Here, we obtained no evidence defining HD1 as a novel plectin isoform.

The isolated clones other than plectin were supposed to be coiled-coil regions of bovine p160^{ROCK} and periplakin from mAb-121 and the bovine kinesin heavy chain from mAb-156. We thought that the immunoreactivities observed here could not be due to them, judging from their reported molecular weights and localization patterns, together with the EBS-MD skin immunoreactivities (*29-31*).

To determine the antigenic regions for each antibody in more detail, the cDNA inserts and their truncated fragments were subcloned into pGEX expression vectors (Amersham Pharmacia Biotech AB), and fusion proteins with glutathione-S-transferase (GST) were obtained. Unexpectedly, the results of the fusion protein-assay indicated that both mAb-121 and -156 each have two distinct antigenic regions on the rod domain of bovine plectin. The estimated antigenic regions for each mAb are indicated by arrows in Fig. 5B.

Molar Composition of Hemidesmosomal Constituents— The SDS-PAGE polypeptide patterns of the HD fraction were traced by densitometry, and the amounts of each component were determined (Fig. 6A). Since the hemidesmosomal 500 kDa component appeared to be homogeneous in this study, the relative molar ratios of each component were roughly estimated from the densitometry data for 5 different preparations (Fig. 6B). For calculation of the molar ratios, we assumed that hemidesmosomes were isolated in an intact state, and that each component has similar dye-binding ability. Furthermore, the preparations would be expected to contain an equal molar ratio of the integrin $\alpha 6$ and $\beta 4$ subunits because it has been revealed that they form 1:1 heterodimers. Although the apparent molecular masses of components on SDS-PAGE sometimes differ from those deduced from cDNA sequences, we used the former for the present purpose because they well fitted the above conditions. From densitometry data, the relative molar ratio of components was roughly estimated to be 1:1:1:0.3:1 for plectin, BP230, $\beta 4$, BP180, and $\alpha 6$, respectively (Fig. 6B). Only BP180 showed a low molar composition, but underestimation would be expected because BP180 always gives strong signals on silver staining and immunoblotting, in spite of weak staining with CBB (data not shown).

A.



B.

	MW	PN 1.	2.	3.	4.	5.	Ave.
HD1	500	1	1	1	1	1	1
BP230	230	1.042	0.966	1.124	0.973	1.059	1.033
β4	200	0.925	1.019	1.236	1.234	0.801	1.043
BP180	180	0.266	0.334	0.343	0.384	0.249	0.315
α6	125	0.986	1.222	1.393	0.921	0.923	1.089

Fig. 6. **Densitometry of the HD fraction.** (A) A typical SDS-PAGE pattern and its densitometric tracing. The top of the gel is on the left side. The relative density of the staining traced by densitometry is shown above. The peaks correspond to the five major constituents, HD1, BP230, integrin $\beta 4$, BP180, and integrin $\alpha 6$, as indicated. (B) Relative molar amounts of protein bands. Densitometry analysis was performed for five different HD fraction preparations. The molar ratios of the proteins were estimated from the molecular weights and staining density, averages being calculated. The relative amount of HD1, 500 kDa, was used as a standard. MW, molecular weights used here (kDa); PN, preparation number; Ave., averages.

DISCUSSION

HD1, a hemidesmosomal 500 kDa protein recognized by mAb-121 (*4*) and present in the innermost part of the hemidesmosomal plaque, linking the plaque and intermediate filaments, was here demonstrated to be identical to plectin, a widely expressed high molecular weight protein that is involved in cytoskeleton-membrane attachment in a variety of cells (*12, 13*). This is in line with the complete absence of both HD1 and plectin immunoreactivities in tissues of EBS-MD patients (*16, 18, 19*).

It has been unclear why mAb-121 only stains hemidesmosomes (*4*), whereas other antibodies against plectin stain hemidesmosomes as well as other intracellular structures, such as intermediate filaments and desmosomes (*11*). In the view of the recent discovery of multiple plectin transcripts arising from a single gene (*32*), although of unknown biological significance, the recognition of alternative isoforms is suggested.

The present focusing on two monoclonal antibodies, mAb-121 and -156, revealed that both recognized a 500 kDa polypeptide concentrated in the HD fraction, and the same ~100 kDa fragment derived on α-chymotrypsin digestion. Furthermore, they both stained the BMZ of normal but not EBS-MD patient skin, and cDNA clones isolated with both mAbs contained sequences of the same molecule, plectin.

The reason why the two antibodies show heterogeneous patterns on immunofluorescence microscopy remains to be determined. In this context it is of interest that, on cDNA screening of the expression library, we found two antigenic regions for mAb-121 and also two for mAb-156 on bovine plectin. Unexpectedly, the epitopes for the two mAbs are very closely aligned on the rod domain of the plectin molecule. The two epitope regions for mAb-121 do not show much similarity, and only contain, as a common sequence, LxExxAExE or RxxxEEIxA. On the other hand, the two antigenic regions for mAb-156 have AxQEAARxRQLAxE in common. The epitope of mAb-156 may reside somewhere in this sequence. However, we are not sure whether both the pair of epitopes for mAb-121 or mAb-156 are functional with the intact plectin molecule, and it is possible that native full length molecules have limited antibody-accessibility. Supporting this possibility, both mAb-121 and -156 reacted more strongly with epitopes on fragmented B8-2 than the full expressed B8-2 clone (Fig.

5B). Our data simply show that mAbs can bind small fragments of plectin expressed as GST fusion proteins.

There are several possibilities explaining the unique and heterogeneous patterns observed on immunofluorescence microscopy with the monoclonal antibodies. First, access of the mAbs to the epitope sites might be hampered by other proteins depending on the functional state of the plectin molecule. In spite of the closeness of the epitope sites for mAb-121 and -156 determined this study, delicate or complicated protein interactions could influence the accessibility or sensitivity of mAbs. A second possible explanation is that modifications occur within epitope regions, like phosphorylation. Plectin is known to be a substrate of several protein kinases, including Ca^{2+}/calmodulin-dependent kinase, PKA, PKC, and $p34^{cdc2}$ kinase (*33-36*), a phosphorylation target site being identified in the tail domain, Thr 4542, for the $p34^{cdc2}$ kinase (*37*). Thirdly, the antibodies might cross-react with other protein(s) weakly. All the reported antigenic sites for mAbs on plectin, including those identified here, have been primarily mapped to the rod domain (*38*). The coiled-coil domain is rather simple and generally conservative in structure, with polypeptides containing similar or identical amino acid sequences. For example, human plectin (U53204) and the human cardiac alpha-myosin heavy chain (D00943), both containing coiled-coil domains, have QAEEAE and AEEAEE as common amino acid sequences. Considering that all isolated cDNA clones included the coiled-coil rod domain, the possibility of cross-reactivity of antibodies cannot be excluded. Further analyses on plectin at the protein level will clarify the source of the heterogeneity.

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