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The subcellular distribution of the high molecular mass protein, HD1, is determined by the cytoplasmic domain of the integrin β4 subunit

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SUMMARY

The high molecular mass protein, HD1, is a structural protein present in hemidesmosomes as well as in distinct adhesion structures termed type II hemidesmosomes. We have studied the distribution and expression of HD1 in the GD25 cells, derived from murine embryonal stem cells deficient for the β1 integrin subunit. We report here that these cells possess HD1 but not BP230 or BP180, two other hemidesmosomal constituents, and express only traces of the α6β4 integrin. By immunofluorescence and interference reflection microscopy HD1 was found together with vinculin at the end of actin filaments in focal contacts. In OVCAR-4 cells, derived from a human ovarian carcinoma which, like GD25 cells, only weakly express α6β4, HD1 was also localized in focal contacts. Upon transfection of both GD25 and OVCAR-4 cells with cDNA for the human β4 subunit the subcellular distribution of HD1 changed significantly. HD1 is then no longer present in focal contacts but in other structures at cell-substrate contacts, colocalized with α6β4. These junctional complexes are probably the equivalent of the type II hemidesmosomes. Transfection of GD25 cells with β1 cDNA did not affect the distribution of HD1, which indicates that the localization of HD1 in focal contacts was not due to the absence of β1. Moreover, in GD25 cells transfected with cDNA encoding a β4/β1 chimera, in which the cytoplasmic domain of β4 was replaced by that of β1, the distribution of HD1 was unaffected. Our findings indicate that the cytoplasmic domain of β4 determines the subcellular distribution of HD1 and emphasize the important role of α6β4 in the assembly of hemidesmosomes and other junctional adhesive complexes containing HD1.

Key words: HD1, Integrin, Focal contact, Hemidesmosome

INTRODUCTION

Integrins form a family of widely expressed cell surface receptors, which participate in cell-cell and cell-extracellular matrix interactions (Hynes, 1992). All integrins are transmembrane heterodimeric proteins consisting of non-covalently associated α and β subunits. Both α and β subunits have large extracellular domains, whereas their cytoplasmic domains are usually short (Hynes, 1992; Sonnenberg, 1993).

Many integrins are concentrated in distinct plasma membrane regions where they are associated with cytoskeletal elements and mediate cell attachment to the extracellular matrix (ECM). In focal contacts, specialized junctions at sites of close membrane-substrate contact, integrins of the β1 and β3 subfamilies are linked to the actin microfilament network by a protein complex. Several cytoplasmic plaque proteins have been implicated in this linkage, including α-actinin, vinculin and talin. α-Actinin and talin have been shown to interact with the actin-binding protein vinculin (Turner and Burridge, 1991), as well as with the cytoplasmic domain of the integrin β1 subunit (Tapley et al., 1989; Otey et al., 1990), and may serve as a direct link between the cytoskeleton and the plasma membrane. In turn, integrins connect these cytoskeletal proteins to the ECM by their association with ECM components, such as fibronectin, vitronectin and various laminin isoforms (Delwel and Sonnenberg, 1996).

Hemidesmosomes (HDs) represent another type of specialized junction in some complex epithelia, such as those of the skin, bladder, trachea and amnion. They mediate firm attachment to the underlying basement membrane (Green and Jones, 1996; Borradori and Sonnenberg, 1996). Ultrastructurally, they appear as membrane-associated electron dense plaques linked to the keratin intermediate filaments (IF). Several molecular components of HDs have recently been identified, including two transmembrane proteins, the α6β4 integrin (Stepp et al., 1990; Sonnenberg et al., 1991) and the bullous pemphigoid antigen BP180 (Giudice et al., 1992; Li et al., 1993; Nishizawa et al., 1993). They are receptors for ECM proteins. The α6β4 integrin has been shown to bind to laminin-5 (Sonnenberg et al., 1993; Niessen et al., 1994), which is a major laminin isoform in the epidermal basement membrane (Carter et al., 1991; Rousselle et al., 1991). Results of recent studies suggest that BP180 interacts with the integrin α6 subunit and that this contributes to the stabilization of the HD complex (Hopkinson et al., 1995).

An important role of α6β4 in the assembly of HDs was first suggested by the observation that antibodies against α6 prevent formation of HDs (Kurpakus et al., 1991). Furthermore, overexpression of a cytoplasmic deletion mutant of the β4 subunit has a dominant negative effect on the assembly of HDs (Spinardi et al., 1995). Finally, in α6 and β4 null mice HDs are absent (van der Neut et al., 1996; Dowling et al., 1996; Georges-Labouesse et al., 1996). In patients with junctional epidermolysis bullosa associated with pyloric atresia who are deficient for the β4 integrin subunit, HDs are still formed, but they appear rudimentary and their number is reduced (Vidal et al., 1995; Niessen et al., 1996).

The hemidesmosomal plaque proteins HD1 (Hieda et al., 1992), plectin (Wiche et al., 1984), BP230 (Tanaka et al., 1990, 1991; Sawamura et al., 1991) and IFAP300 (Skalli et al., 1994), may play a role in linking the IF to the plasma membrane. A direct interaction of IF with plectin and IFAP300 has been demonstrated in vitro (Yang et al., 1985; Foisner et al., 1988). In further studies, involving the expression of specific domains of plectin in cells, it was shown that the COOH terminal part is the site which interacts with IF. Since the molecular mass of HD1, plectin and IFAP300 and their tissue distribution are similar, these proteins may be identical. The absence of both plectin and HD1 in a subset of junctional epidermolysis bullosa patients associated with muscular dystrophy, supports this contention (McLean et al., 1996; Smith et al., 1996; Gache et al., 1996). Certain sequence motifs in the COOH terminal domain of plectin are also present in the COOH terminus of desmoplakin, another IF binding protein, and in BP230, which suggests that BP230 also interacts with IF. Indeed, in mice deficient for BP230, HDs lack the inner plaque and are not attached to the cytoskeleton (Guo et al., 1995).

We have studied the distribution of the high molecular mass protein, HD1, in two distinct cell lines: in GD25 cells from a recently established murine embryonal cell line completely deficient for the β1 subunit (Fässler et al., 1995), and in OVCAR-4 cells, derived from a human ovarian carcinoma. Both cell lines weakly express the integrin α6β4. We have examined whether the subcellular localization of HD1 is affected by an increased expression of α6β4. We show that HD1 does not only occur in HDs, as previously described, but that it is also present in focal contacts. After transfection of GD25 or OVCAR-4 cells with the cDNA for the integrin β4 subunit, but not for a β4/β1 chimeric molecule in which the cytoplasmic domain of β4 has been replaced by that of β1, the distribution pattern of HD1 changed dramatically.

MATERIALS AND METHODS

Cell lines and antibodies

The murine β1 knockout cells (GD25) and the β1-transfected GD25 cells have been described previously (Fässler et al., 1995; Wennerberg et al., 1996). All cells, including OVCAR-4 cells, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-BRL, Paisley, UK), penicillin and streptomycin. For GD25 cells transfected with murine β1 cDNA, puromycin was added to the complete medium (Clontech laboratories, Palo Alto, CA) and for those transfected with β4 and β4/β1 chimera, hygromycin B (Calbiochem, La Jolla, CA) was added for selection of cells. The following antibodies were used: MB1.2, anti-β1 (gift from Dr B. Chan, University of Western Ontario, London, Canada); 2C9.G, anti-β3 (Pharmingen, San Diego, CA); 346-11A, anti-murine β4 (Kennel et al., 1989); 4.3E1 and 439-9B, anti-human β4 (Hessle et al., 1984; Kennel et al., 1989); 5HK2 and 5HK1, rabbit polyclonal anti-β5 and anti-β6 (gift from Dr H. Kemperman, The Netherlands, Cancer Inst., Amsterdam, The Netherlands); rabbit polyclonal antibodies against α3, α5 and αv (Defilippi et al., 1992); GoH3, anti-α6 (Sonnenberg et al., 1987); anti-α-actinin mAb, clone BM-75.2 (Sigma Chemical Co., St Louis, MO); rabbit polyclonal anti-vinculin (Geiger, 1979) and anti-vinculin mAb, clone VIN-11-5 (Sigma Chemical Co.); anti-HD1 (Hieda et al., 1992). Anti-rat IgG was prepared in rabbits, affinity purified and adsorbed on a gluteraldehyde cross-linked mouse serum column to remove all antibodies cross-reacting with mouse IgG (Sonnenberg et al., 1986). Fluorescein (FITC)-conjugated antibodies to mouse IgG were from Zymed Laboratories Inc. (San Francisco, CA) and to rabbit and hamster IgG were from Nordic Immunochemicals Laboratory (Tilburg, The Netherlands). Texas red-conjugated antibodies to mouse IgG were from Molecular Probes (Eugene, OR) and to rabbit IgG were from Amersham International (Buckinghamshire, UK). Rhodamine-conjugated phalloidin was purchased from Sigma Chemical Co.

Construction of β4/β1 cDNA chimera

Full length β4 cDNA in pUC 18 was completely digested with *Sal*I and partially with *Hin*dIII and a 2.7 kb fragment was isolated and inserted into the *Sal*I/*Hin*dIII sites of the pBluescript vector (Stratagene Corp., La Jolla, CA). The plasmid was digested with *Hin*dIII and *Afl*II, and a fragment, containing nucleotides 1,950-2,770 was exchanged for a fragment covering nucleotides 1,950-2,265. This latter fragment was generated by PCR using the primers 5′-CACTGCCACCAGCAGTCGCTC-3′ (positions 1,813-1,833) and 5′-GGGAAGCTTACCTCGGTTGCAGCACGG-3′ (positions 2,248-2,265) with a *Hin*dIII restriction site (underlined) and β4 cDNA as a template, and subsequent digestion with *Hin*dIII and *Afl*II. The cDNA encoding the β1 cytoplasmic domain was obtained by digestion of full length β1 cDNA with *Hin*dIII and *Dra*I. The isolated 280 bp fragment was ligated to the *Hin*dIII site at position 2,265, and to *Eco*RV of the polylinker. The β4/β1 cDNA was digested with *Xba*I and the 2.5 kb fragment inserted into the *Xba*I site of the pcDNA-1Hyg expression vector (Niessen et al., 1994). Sequence analysis revealed that during this procedure Arg at position 722 was replaced by Leu in β4.

Transfections

The GD25 and OVCAR-4 cells were transfected with the human β4 full length cDNA (Niessen et al., 1994) and the former also with cDNA encoding the extracellular domain of human β4 and the cytoplasmic tail of β1 (β4/β1 chimera) using $CaCl_2$ and HBS buffer (1 mM Na_2HPO_4, 6.5 mM glucose, 140 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.1). Cells were selected using complete medium in the presence of 400 μg/ml of hygromycin B (Calbiochem, La Jolla, CA). Transfected cells were sorted at least three times on the fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA) to obtain cells that strongly express β4, β1 or the β4/β1 chimera. The human β4 specific mAb 4.3E1 and the murine β1 mAb MB1.2 were used for these sortings.

Immunofluorescence microscopy

Immunofluorescence analysis of cultured cells was done as follows: cells were detached with trypsin, plated on uncoated glass coverslips in culture medium containing 10% FCS and incubated overnight at 37°C. Cells were fixed with 1% formaldehyde in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature. After rinsing and blocking with 1% BSA in PBS for 30 minutes, the permeabilized cells were incubated with primary antibodies for 30 minutes at 37°C. The cells were stained with FITC or Texas red-labeled anti-mouse, anti-hamster or rabbit IgG for 30 minutes. Several steps were carried out for double labeling experiments. The coverslips were then washed again, mounted in Vectashield (Vector Laboratories, Inc.) and viewed under a Zeiss micro-

scope (Carl Zeiss, Inc., Thornwood, NY) equipped with a confocal scanning laser microscope (Bio-Rad, Richmond, CA). For interference reflection microscopy (IRM) the coverslips were mounted in 50% glycerol plus 100 mg/ml 1,4-diazobicyclo-(2-2-2)octane. The pictures correspond to the basal surface of the cells and ×60 magnification was used. In some experiments, coverslips coated with laminin-5 were used. Laminin-5 was obtained from RAC-11P/SD cells, by growing them on glass coverslips as previously described (Sonnenberg et al., 1993). After 1-2 days, cells were detached with 20 mM EDTA in PBS and the coverslips were washed with PBS and blocked for 1 hour with 1% of BSA.

Cell labeling and immunoprecipitation

Cells were surface-labeled with ^{125}I using lactoperoxidase as previously described (Sonnenberg et al., 1987), washed, lysed in 1% Nonidet P40 in 25 mM Tris-HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor and the cell lysates were clarified by centrifugation. Samples of lysates were immunoprecipitated with antibodies previously bound to Protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) or to Protein A-Sepharose to which rabbit anti-rat IgG or rabbit anti-mouse IgG was bound. The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions.

RESULTS

HD1 is present in focal contacts

The GD25 cell line used in the present study was derived from embryonal stem cells deficient for the β1 integrin subunit (Fässler et al., 1995). Flow cytometry analysis using anti-β1 mAb confirmed the absence of the β1 subunit from GD25 cells, whereas there was little reactivity with anti-α6 mAb (Fig. 1). However, both integrin subunits were detected on β1-transfected GD25 cells. To further analyze integrin expression by GD25 cells, the cells were surface-labeled with ^{125}I, lysed with nonionic detergent and immunoprecipitations were carried out using a panel of antibodies directed against individual α or β subunits. As shown in Fig. 2, GD25 cells express substantial amounts of αvβ3 (lanes 4 and 6) and αvβ5 (lanes 4 and 8) and small amounts of α6β4 (lanes 3 and 7) and the α5 subunit (lane 2). There was no evidence for the presence of α3 (lane 1) or β1 (lane 5). Fig. 2 also shows the results obtained with β1-transfected GD25 cells in which, as expected, α3 (lane 1), α5 (lane 2) and α6 (lane 3) were found in association with β1 (lane 5). We also investigated the presence of several components of HDs. HD1 was precipitated but two other hemidesmosomal components, BP230 or BP180, were not detected by immunoprecipitation using metabolically labeled cells (not shown).

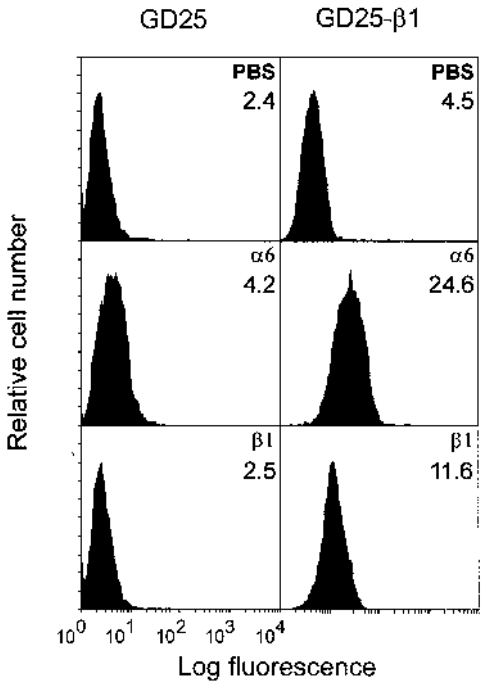

Fig. 1. Cell surface expression of integrin subunits of GD25 and β1-transfected GD25 cells. Flow cytometry of the cells was carried out using antibodies against α6, β1, or PBS as a negative control. FITC-labeled goat anti-rat was used as the secondary antibody. The median fluorescence in each case is shown in the upper right corner.

We next studied the distribution of HD1 in GD25 cells and compared it with that of αv. In double labeling experiments combined with IRM (Fig. 3), HD1 (B) and αv (C) were found to be colocalized and associated with the cell membrane at sites where the cells were in close contact with the underlying substrate (A). The integrin subunits β3 and β5 were also colocalized with HD1 (Fig. 4). The immunofluorescence pattern of discontinuous streaks observed for HD1 as well as for the αv integrins is clearly different from the hemidesmosome-like pattern in which HD1 is found in other cell lines such as RAC-

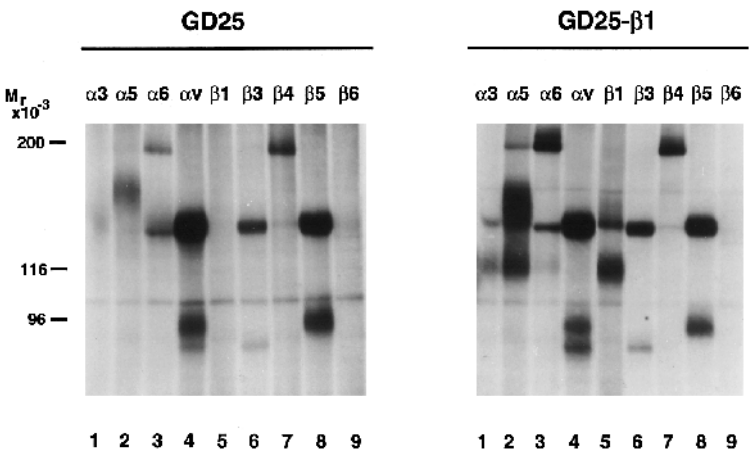

Fig. 2. Expression of integrins on GD25 and β1-transfected GD25 cells. Cells were labeled with ^{125}I, lysed and cell lysates were subjected to immunoprecipitation with antibodies against α3 (lane 1), α5 (lane 2), α6 (lane 3), αv (lane 4), β1 (lane 5), β3 (lane 6), β4 (lane 7), β5 (lane 8) or β6 (lane 9).

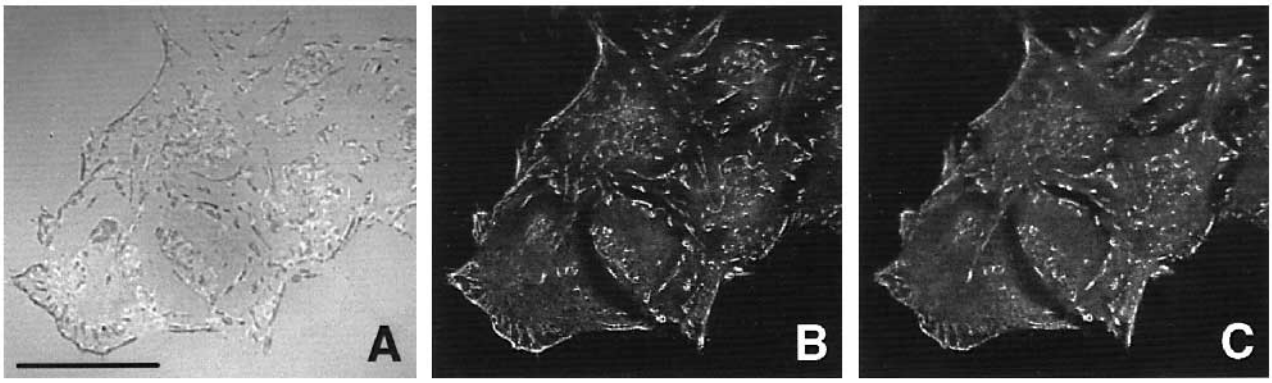

Fig. 3. Interference reflection microscopy and double labeling immunofluorescence of GD25 cells showing the distribution of cell contacts (A) with respect to the localization of HD1 (B) and αv (C). The cells were fixed and incubated with murine mAb 121 specific for HD1 and rabbit polyclonal antiserum against αv, followed by species specific FITC- and Texas red-conjugated secondary antibodies. Bar, 25 μm.

11P or 804G (Sonnenberg et al., 1993; C. M. Niessen et al., unpublished observations). The same distribution of HD1 and αv integrins was found when experiments were carried out in the presence of 1% BSA instead of FCS (not shown) which indicates that when the cells adhere to an ECM produced by themselves the discontinuous streaks were also formed.

Since αv is a constituent of some integrins that can be located in focal contacts, and since both HD1 and αv were detected in cell-substrate contacts, we further characterized the relation of these proteins to the actin filament network. As shown in Fig. 5, GD25 cells grown on glass coverslips contained F-actin filament bundles with the αv subunit (A) at their ends. Interestingly, HD1 was also localized at the end of the actin filaments (B). Furthermore, double labeling experiments showed that αv and HD1 were colocalized with vinculin (Fig. 6), as well as with α-actinin, another component of focal contacts (not shown). Taken together, our results provide the first evidence that HD1 can be localized in focal contacts.

To investigate whether the presence of HD1 in focal contacts is unique for GD25 cells, we determined the distribution of HD1 and αv in OVCAR-4 cells. In these cells, HD1 and αv occurred together in discrete accumulations at the end of actin filaments as well as in the cytoskeleton (Fig. 7). Thus, also in OVCAR-4 cells, HD1 is present in focal contacts.

The pattern of discontinuous streaks observed for HD1 in GD25 cells was more conspicuous at low cell density;

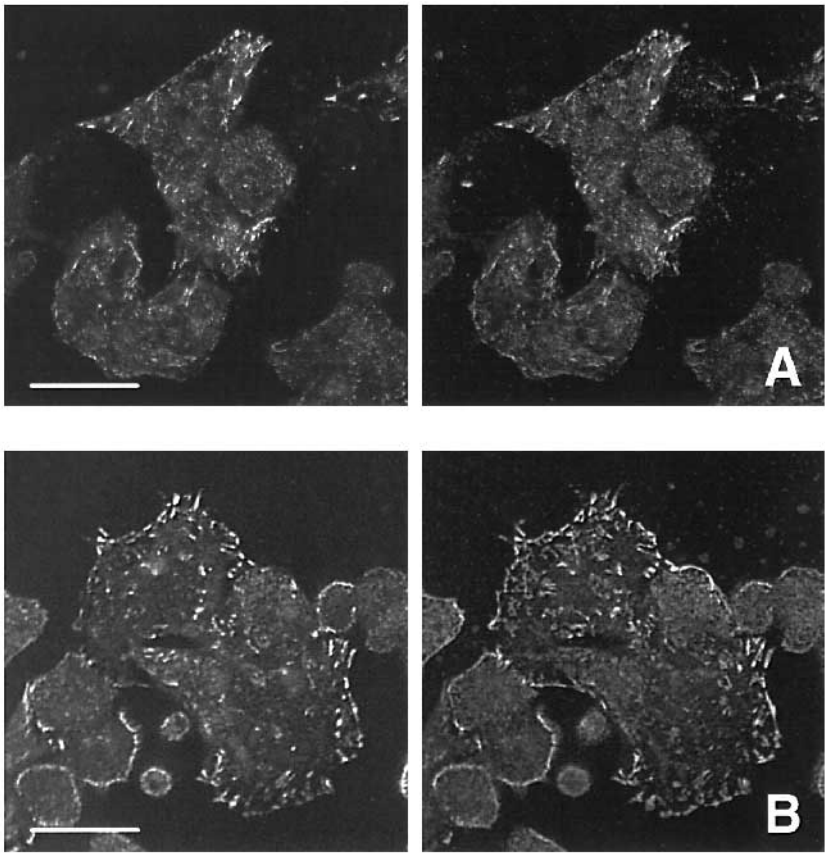

Fig. 4. Double labeling immunofluorescence of GD25 cells for HD1 and β3 (A) or β5 (B). Cells were fixed and incubated with an anti-HD1 murine mAb (left panels), followed by an anti-β3 hamster mAb (right, A) or polyclonal rabbit antiserum against β5 (right, B). In A bound antibodies were detected with species specific Texas red-conjugated goat anti-mouse IgG (left) and FITC-conjugated rabbit anti-hamster IgG (right), and in B with FITC-conjugated goat anti-mouse IgG (left) and Texas red-conjugated donkey anti-rabbit IgG (right). Bars, 25 μm.

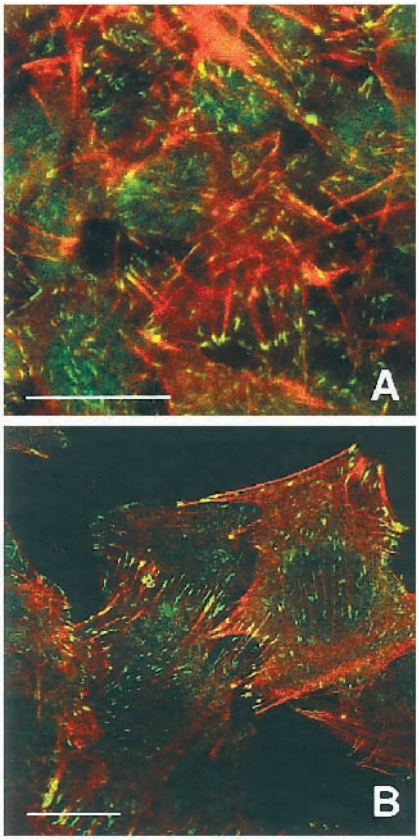

Fig. 5. Double labeling immunofluorescence of GD25 cells for actin filaments and αv (A) or HD1 (B). Cells were incubated with rhodamine-conjugated phalloidin (red in A and B) and anti-αv (green in A) or anti-HD1 (green in B), respectively. FITC-conjugated antibodies were used to detect bound anti-αv and anti-HD1 antibodies. Bars, 25 μm.

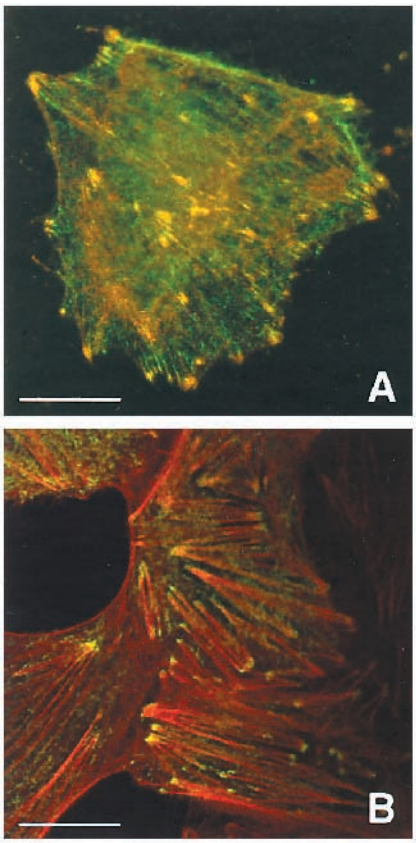

Fig. 7. Double immunofluorescence of OVCAR-4 cells showing the distribution of HD1 and αv (A) and of HD1 and F-actin (B). In A cells were incubated with anti-HD1 (green) and anti-αv (red) antibodies, followed by FITC and Texas red-labeled secondary antibodies, respectively. In B cells were incubated with rhodamine-conjugated phalloidin and anti-HD1 antibodies, followed by FITC-conjugated antibody to detect bound antibodies to HD1 (green). Bars, 25 μm.

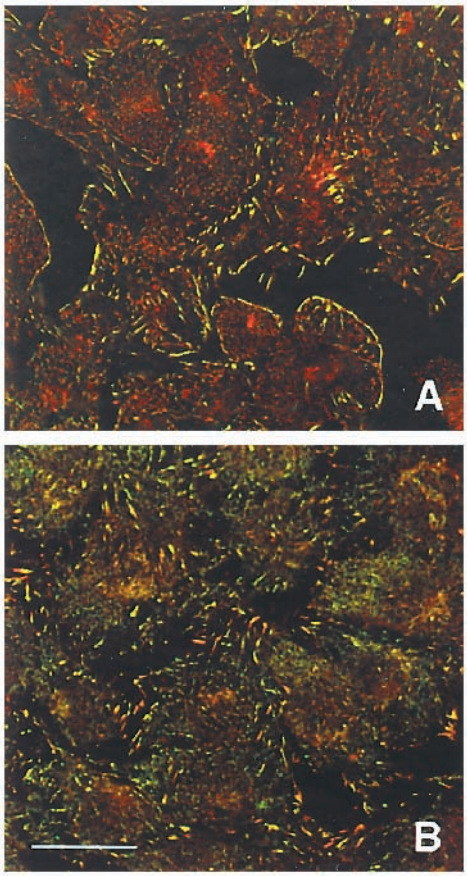

Fig. 6. Double labeling immunofluorescence of GD25 cells showing the distribution of vinculin and αv (A), and of vinculin and HD1 (B). Cells were incubated with murine mAb VIN-11-5 specific for vinculin (green, A) and a rabbit polyclonal antiserum against αv (red, A) or anti-HD1 murine mAb (green, B) and a polyclonal rabbit antiserum against vinculin (red, B), followed by species specific FITC- and Texas red-conjugated secondary antibodies. Bar, 25 μm.

therefore, the following experiment was carried out: part of a confluent layer of cells, obtained by extended incubation, was scraped off. During further incubation for 7 hours, cells migrated from the rest of the confluent layer into the denuded area. The streaky pattern was obvious in the migrated cells, in contrast to the more diffuse distribution pattern of HD1 in the confluent cells (Fig. 8).

Increased expression of α6β4 results in a different distribution of HD1

Although both GD25 and OVCAR-4 cells expressed α6β4 on their cell surface, the amount proved to be too small to define its subcellular localization by immunofluorescence. Therefore, to increase the expression of α6β4, the cells were transfected with full length human β4 cDNA. The expression of human β4 on transfected GD25 cells was evaluated by flow cytometry. As shown in Fig. 9, the level of human β4 expressed on GD25 cells was clearly greater than on the negative controls. Immunoprecipitation from ^{125}I-labeled β4-tranfected GD25 cells confirmed the presence of the human β4 subunit (Fig. 10A, lane 1) and showed that it was associated with endogenous α6 (Fig. 10A, lane 4). The α6β4 integrin was also detected on β4-tranfected OVCAR-4 cells by immunoprecipitation with anti-α6 or anti-β4 mAbs (Fig. 10B, lanes 1 and 2). No differences in the expression of other integrin subunits were observed.

We then studied the distribution of HD1 in β4-transfected GD25 cells. Cells were plated on glass coverslips previously coated with laminin-5, a ligand for α6β4, and fixed after 2 hours of incubation. As shown in Fig. 11A, in cells transfected with β4 cDNA, HD1 was observed in a honeycomb, spot-like

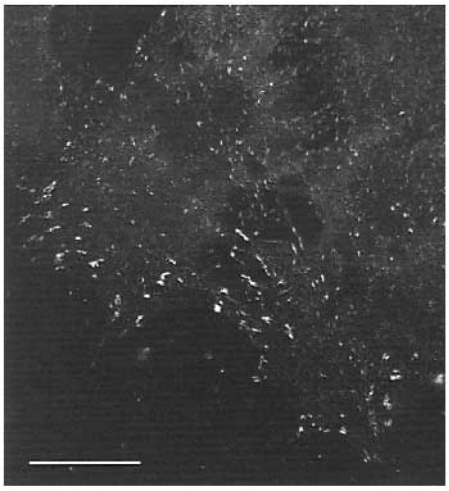

Fig. 8. Immunofluorescence microscopy of GD25 cells showing the distribution of HD1 in migrating cells. Cells were incubated sequentially with anti-HD1 mAb and FITC-conjugated secondary antibody. Bar, 25 μm.

distribution pattern similar to that of the β4 subunit, although staining for α6β4 was more extensive than that for HD1. Double staining for β4 and F-actin or α6 and vinculin demonstrated that these spot-like structures were clearly distinct from focal contacts, because β4 was not associated with actin filaments (Fig. 11C), nor was α6 codistributed with vinculin (Fig. 11D). Moreover, HD1 was not colocalized with vinculin in such spot-like structures (not shown). Similar to what has been observed in GD25 cells, in OVCAR-4 cells transfected with the human β4 full length cDNA, HD1 was colocalized with β4 (Fig. 11B). Again no β4 was observed at the end of the actin filaments and it was not codistributed with vinculin (data not shown).

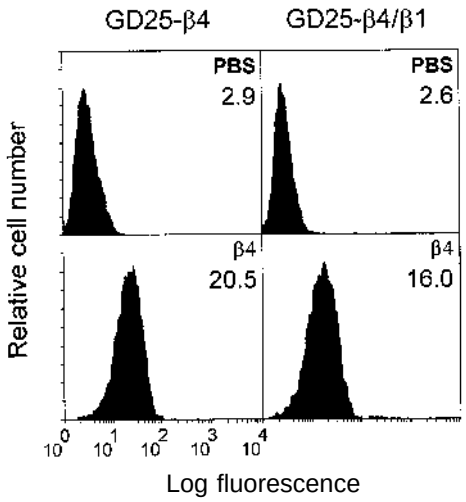

Fig. 9. Cell surface expression of integrin subunits of transfected GD25 cells. Flow cytometry of the transfectants was carried out using a mAb against the extracellular domain of human β4 (4.3E1) or PBS as a negative control. FITC-labeled goat anti-mouse was used as the secondary antibody. The median fluorescence in each case is shown in the upper right corner.

To further confirm the involvement of the β4 subunit in the distribution of HD1, GD25 cells were transfected either with cDNA for the β1 subunit (Fig. 1) or with cDNA for a β4/β1 chimeric construct encoding the extracellular domain of β4 fused to the intracellular domain of β1 (Fig. 9). Immunoprecipitation analysis performed on the transfected cells showed that β1 was expressed in association with different α subunits (Fig. 2, lanes 1 and 2), while the β4/β1 subunit was only associated with α6 (Fig. 10A, lane 9). As expected, the β4/β1 chimera was recognized by antibodies against both the extracellular domain of β4 (Fig. 10A, lane 6) and by antibodies against the cytoplasmic domain of β1 (Fig. 10A, lane 8). In GD25 cells transfected with the β1 subunit, HD1 was found together with β1 (Fig. 12), αvβ3 and αvβ5 at the end of actin filaments in focal contacts (not shown). A similar distribution of HD1 was found in cells transfected with the β4/β1 chimera and bound to laminin-5, in which HD1 was colocalized with β4/β1 as detected with a mAb against the extracellular domain of β4 (Fig. 13). These findings indicate, first, that the presence of HD1 in focal contacts is not due to the absence of β1 and, second, that the cytoplasmic domain of β4 determines the distribution of HD1 in spot-like structures instead of focal contacts.

DISCUSSION

In this study we have investigated the distribution of the hemidesmosomal plaque protein HD1 in GD25 cells, a cell line derived from embryonal stem cells deficient for the β1 subunit (Fässler et al., 1995). Due to this deficiency, most α subunits are not expressed on the cell surface with the exception of αv, which is associated with the β3 and β5 subunits. Although previous studies have claimed that α6β4 was absent in these

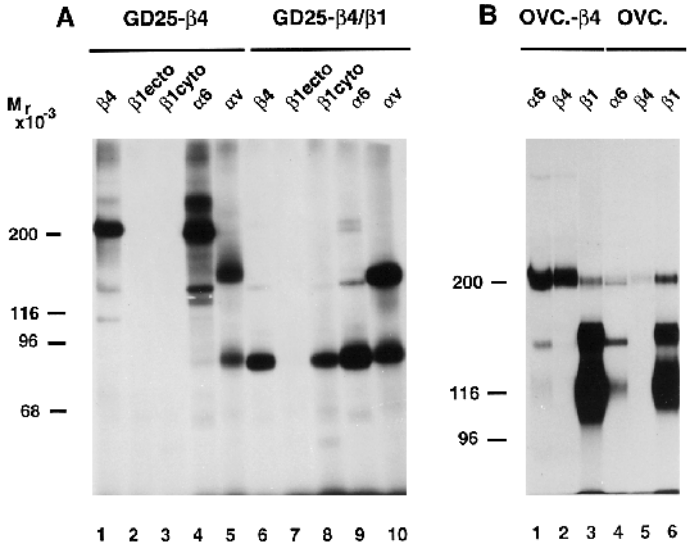

Fig. 10. Expression of integrins on GD25 cells transfected with β4 or β4/β1 chimera and OVCAR-4 cells transfected with β4. Cells were labeled with ^{125}I, lysed and subjected to immunoprecipitation with antibodies against the extracellular (MB1.2) and cytoplasmic domains of β1 (rabbit anti-β1), the extracellular domain of α6 (GoH3) and β4 (4.3E1), and against αv.

cells (Wennerberg et al., 1996), low levels of this integrin were detected. We also found that these cells possess HD1, but lack two other hemidesmosomal constituents, the bullous pemphigoid antigens BP230 and BP180. By immunofluorescence microscopy and IRM we have shown that in these cells HD1 is localized, together with αv and vinculin, at the end of actin filaments in focal contacts. The identical distribution observed for HD1 and focal contact proteins was unexpected. HD1 has previously been described as a structural protein present in typical HDs (Hieda et al., 1992), as well as in distinct adhesion plaques termed type II HDs which possess α6β4 but not the BP230 and BP180 antigens (Uematsu et al., 1994). Type II HDs have been suggested to represent the first step in the assembly of HDs in cells possessing the BP antigens (Uematsu et al., 1994). HD1 has also been found in glial cells and some endothelial cells that lack HDs (Hieda et al., 1992) in which its exact role remains unclear.

The localization of HD1 in focal contacts in GD25 cells is not due to the absence of the β1 subunit, since transfection of these cells with β1 cDNA did not affect the subcellular distribution of this protein. Moreover, HD1 was not exclusively found in focal contacts in this cell line, but also in OVCAR-4 cells, which do express various β1 integrins (Sonnenberg et al., 1990). In both cases, HD1 was colocalized with β3 and β5. Taken together, our observations provide strong evidence that HD1 is not only localized in typical HDs and in type II HDs, together with α6β4, but can also be present in focal contacts together with other integrins.

Interestingly, the distribution of HD1 was different when the GD25 and OVCAR-4 cells were transfected with human β4 cDNA, and bound to laminin-5, a ligand for α6β4 (Niessen et al., 1994). In both cell lines, after transfection, HD1 was no longer distributed in focal contacts, but in other types of structures, together with α6β4. These structures may correspond to type II HDs. It is intriguing that the staining patterns of α6β4 and HD1 did not entirely overlap, the distribution of α6β4 being more extensive than that of HD1. Perhaps, the epitope of HD1 is less readily available for reacting with the antibody against it under these conditions. Alternatively, the binding of α6β4 to laminin-5 precedes the association of α6β4 with HD1 and represents an intermediate step in the formation of these type II HD-like structures.

Our results indicate that the cytoplasmic domain of β4 is required for the subcellular redistribution of HD1. This conclusion is based on the observation that a β4/β1 chimera, in which the cytoplasmic domain of β4 was replaced by that of β1, did not affect the distribution of HD1, which together with the β4/β1 chimeric protein remained in focal contacts. The distribution of the β4/β1 chimera in focal contacts is most likely due to the ability of the cytoplasmic domain of β1 to bind to certain cytoskeletal proteins associated with this type of cell junction, such as talin or α-actinin (Tapley et al., 1989; Otey et al., 1990).

It has been suggested that the assembly of HDs is regulated by α6β4-mediated signal transduction, subsequent to its binding to laminins (Mainiero et al., 1995). It is possible that

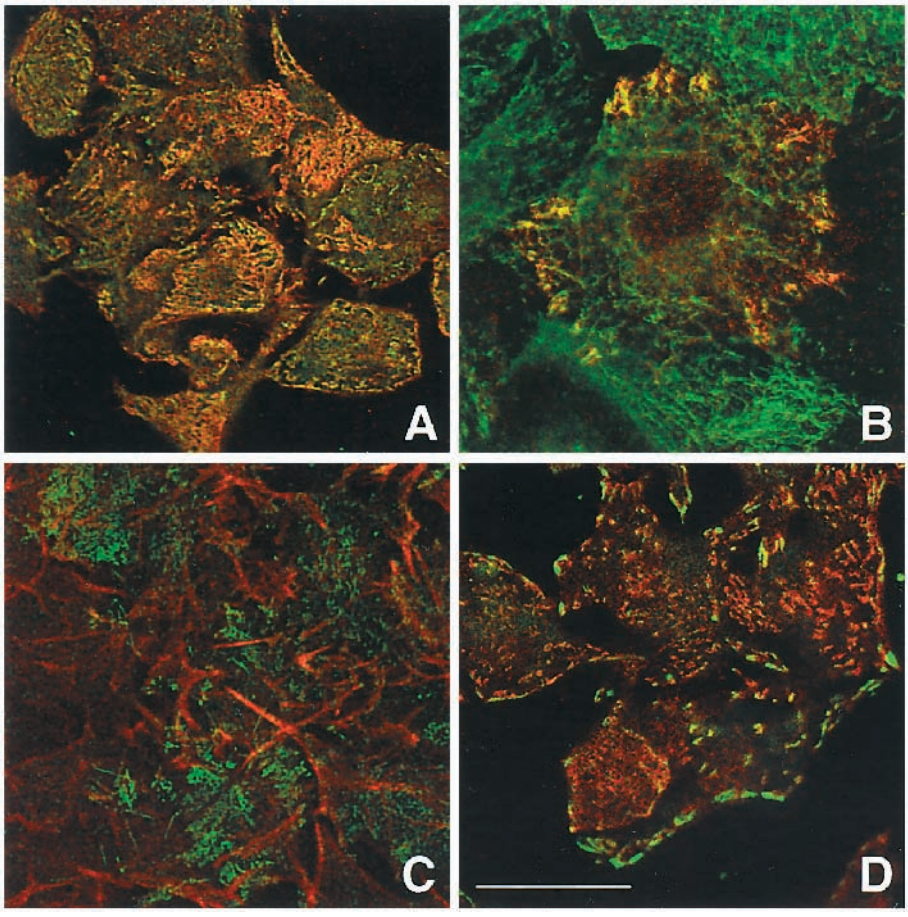

Fig. 11. Double labeling immunofluorescence microscopy of GD25 (A,C and D) and OVCAR-4 cells (B) transfected with β4 showing the distribution of HD1 and β4 (A and B), actin and β4 (C), and vinculin and α6 (D). Cells were grown on laminin-5-coated coverslips, fixed and permeabilized. Staining for HD1 (green, A and B) was performed using anti-HD1 murine mAb followed by FITC-conjugated goat anti-mouse IgG, and for actin by incubation with rhodamine-conjugated phalloidin (red, C). Vinculin (green, D) was detected with murine mAb VIN-11-5 followed by FITC-conjugated goat anti-mouse IgG. β4 (red, A and B; green, C) and α6 (red, D) were stained using an anti-human β4 rat mAb or anti-α6 rat mAb, respectively, rabbit anti-rat IgG (absorbed against mouse IgG) and Texas red-conjugated donkey anti-rabbit (A,B and D) or FITC-labeled goat anti-rabbit (C). Bar, 25 μm.

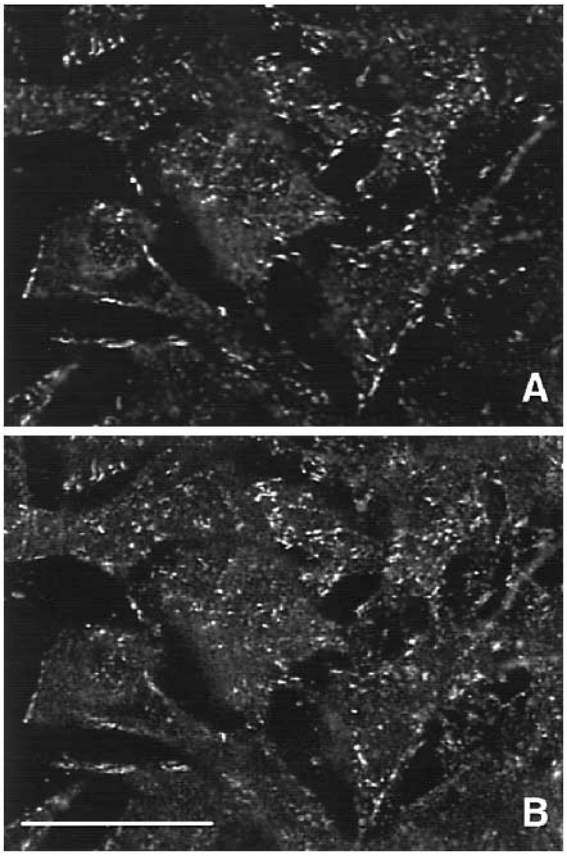

Fig. 12. Double immunofluorescence labeling of GD25 cells transfected with β1 for HD1 (A) and β1 (B). Staining for HD1 was performed using anti-HD1 murine mAb followed by FITC-conjugated anti-mouse IgG. The integrin β1 was then detected using an anti-murine β1 rat mAb, rabbit anti-rat IgG (adsorbed against mouse IgG) and Texas red-conjugated donkey anti-rabbit. Bar, 25 μm.

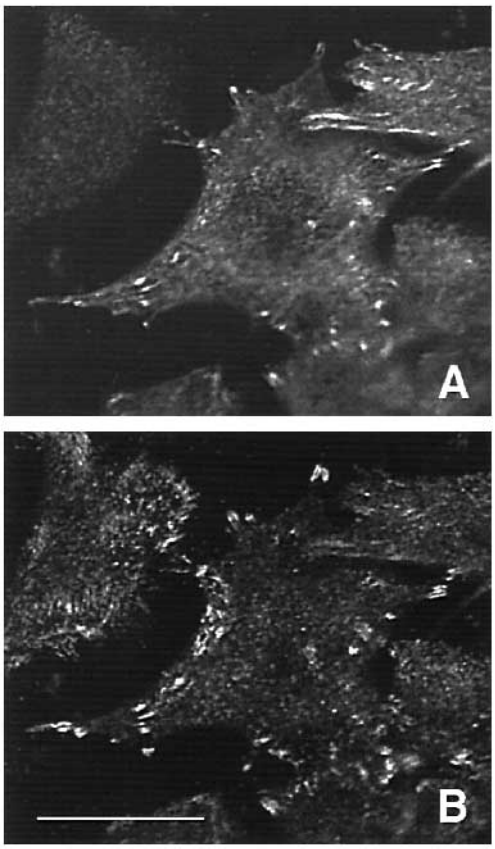

Fig. 13. Double immunofluorescence of GD25 cells transfected with β4/β1 chimera for HD1 (A) and β4/β1 (B). Cells were grown for 2 hours on laminin-5-coated coverslips, fixed and permeabilized, and incubated with antibodies against HD1 and human β4. FITC- and Texas red-conjugated antibodies were used to detect bound antibodies. Bar, 25 μm.

the interaction between HD1 and β4 is one of these triggered events. Although further investigations are needed to identify the nature of this possible interaction and to assess the potential requirement of additional molecules, our findings clearly indicate that the association between HD1 and β4 is strong enough to induce a change in the distribution and a preferential subcellular localization of HD1 with β4 when the level of expression of β4 is sufficiently high.

HD1 is not the only hemidesmosomal protein found in other cell-substrate junctions, such as focal contacts. For instance, plectin has previously been shown to be present in both focal contacts and HDs (Wiche et al., 1984; Seifert et al., 1992) while IFAP300 is present in both desmosomes and HDs (Skalli et al., 1994). Finally, plakoglobin is found in desmosomes and in the zonula adherens (Garrod, 1993). However, unlike other proteins present in different types of structures, HD1 is preferentially localized in HDs rather than in focal contacts. The HD1 molecule is only found in focal contacts in cells that do not possess HDs or do not express sufficient amounts of the integrin α6β4. Since focal contacts and HDs are cell junctions with different involvement in cellular migration, HD1 might be involved in the coordinated regulation of cell migration versus anchorage. It is noteworthy that the β4 cytoplasmic domain is subject to proteolytic cleavage (Giancotti et al., 1992) and tyrosine phosphorylation (Mainiero et al., 1995) and that its RNA is alternatively spliced (Tamura et al., 1990). It may be that by these mechanisms the integrin α6β4 regulates the cytoskeletal organization.

The exact relationship between HD1 and plectin is as yet not clear. A growing body of evidence, however, suggests that these two proteins are identical. First, patients with a distinct form of epidermolysis bullosa associated with muscular dystrophy were found to have mutations in the gene encoding plectin (McLean et al., 1996; Smith et al., 1996). In these patients neither plectin nor HD1 could be detected by immunofluorescence. Second, the two proteins have similar molecular masses and antibodies to plectin react with HD1 in immunoblots and vice versa (Gache et al., 1996; P. Sánchez-Aparicio, unpublished observations). Finally, as mentioned above, we have shown that HD1, like plectin, can be found not only in hemidesmosomes but also in focal contacts. If plectin and HD1 are indeed identical, our observation that HD1 can be localized into two adhesion structures with distinct cytoskeletal connections is consistent with the presence of an actin and intermediate filament binding domain within the plectin molecule. Thus, plectin, in analogy to a newly recognized neuronal isoform of BP230 (Brown et al., 1995), which has a structural organization similar to that of plectin, may

serve as a protein connecting actin with the intermediate filament cytoskeleton (Yang et al., 1996).

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