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Integrin α6β4 Forms a Complex with the Cytoskeletal Protein HD1 and Induces Its Redistribution in Transfected COS-7 Cells

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The integrin α6β4 is a major component of hemidesmosomes, in which it is linked to intermediate filaments. Its presence in these structures is dependent on the β4 cytoplasmic domain but it is not known whether β4 interacts directly with keratin filaments or by interaction with other proteins. In this study, we have investigated the interaction of GST-cytoβ4A fusion proteins with cellular proteins and demonstrate that a fragment of β4A, consisting of the two pairs of fibronectin type III repeats, separated by the connecting segment, forms a specific complex containing a 500-kDa protein that comigrates with HD1, a hemidesmosomal plaque protein. A similar protein was also bound by a glutathione *S*-transferase fusion protein containing the cytoplasmic domain of a variant β4 subunit (β4B), in which a stretch of 53 amino acids is inserted in the connecting segment. Subsequent immunoblot analysis revealed that the 500-kDa protein is in fact HD1. In COS-7 cells, which do not express α6β4 or the hemidesmosomal components BP230 and BP180, HD1 is associated with the cytoskeleton, but after transfecting the cells with cDNAs for human α6 and β4, it was, instead, colocalized with α6β4 at the basal side of the cells. The organization of the vimentin, keratin, actin, and tubulin cytoskeletal networks was not affected by the expression of α6β4 in COS-7 cells. The localization of HD1 at the basal side of the cells depends on the same region of β4 that forms a complex containing HD1 in vitro, since the expression of α6 with a mutant β4 subunit that lacks the four fibronectin type III repeats and the connecting segment did not alter the distribution of HD1. The results indicate that for association of α6β4 with HD1, the cytoplasmic domain of β4 is essential. We suggest that this association may be crucial for hemidesmosome assembly.

INTRODUCTION

Integrins mediate cell adhesion to the extracellular environment and are also important for the integrity of cells by connecting the cytoskeleton to the plasma membrane (Hynes, 1992). The integrin α6β4 is concentrated in hemidesmosomes (Stepp *et al.*, 1990; Jones *et al.*, 1991; Sonnenberg *et al.*, 1991), adhesion junctions in stratified epithelial cells, which serve as anchoring sites for the keratin filaments (Borradori and Sonnenberg, 1996; Green and Jones, 1996). The α6β4 integrin is also expressed on certain subsets of endothelial cells (Kennel *et al.*, 1992), on immature thymocytes (Wadsworth *et al.*, 1992), and in peripheral nerves on Schwann cells and perineural fibroblasts (Sonnenberg *et al.*, 1990; Niessen *et al.*, 1994a). The β4 subunit is unique because of its unusually large cytoplasmic domain of approximately 1000 amino acids instead of the approximately 50 amino acids in the other β subunits. The cytoplasmic domain of β4 shows no similarity to other proteins except for the presence of four fibronec-

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tin type III repeats (FNIII[1] repeats), which reside in two pairs separated from each other by a connecting segment (Hogervorst *et al.*, 1990; Suzuki *et al.*, 1990). The RNA for this region of β4 is subject to alternative splicing: the β4B[2] (Hogervorst *et al.*, 1990) and β4C (Tamura *et al.*, 1990) variants have insertions of 53 and 70 amino acids, respectively, in the connecting segment, and, in the β4D variant (Clarke *et al.*, 1994), 7 amino acids are deleted from the fourth FNIII repeat. The sequences essential for localization of α6β4 in hemidesmosomes were shown to reside in the first pair of FNIII repeats and the connecting segment of the β4 subunit (Spinardi *et al.*, 1993).

It has been suggested that the large cytoplasmic domain of β4 plays an important role in linking the keratin filaments to the hemidesmosome, either directly or indirectly, via one or more associated proteins. Proteins that might be involved in this linkage are BP230 (Stanley *et al.*, 1981), plectin (Wiche *et al.*, 1984), IFAP-300 (Skalli *et al.*, 1994), HD1 (Hieda *et al.*, 1992), and the 200-kDa 6A5 antigen (Kurpakus and Jones, 1991). Plectin and IFAP-300 bind IF in vitro (Foisner *et al.*, 1988; Skalli *et al.*, 1994). The structure of HD1 has not yet been determined but its apparent molecular mass and tissue distribution are similar to those of plectin (Gache *et al.*, 1996). In addition, mutations in the plectin gene correlate with a loss of immunoreactivity not only of plectin but also of HD1 (McLean *et al.*, 1996; Smith *et al.* 1996). Together, these data suggest that HD1 and plectin are identical or related molecules. On the basis of its structural homology with plectin (Wiche *et al.*, 1991) and another IF-associated protein, desmoplakin (Green *et al.*, 1992), it has been proposed that BP230 also interacts directly with IF (Tanaka *et al.*, 1991). Recent work has demonstrated that in mutant mice lacking BP230, IFs are apparently not attached to hemidesmosomes, which supports this assumption (Guo *et al.*, 1995). Finally, a less common protein, the 200-kDa 6A5 antigen, is only present in hemidesmosomes in stratified and pseudostratified epithelium (Kurpakus *et al.*, 1991).

In this report we show that, in vitro, the β4 cytoplasmic domain forms a complex containing the 500-kDa protein HD1, and that this association is not disturbed by the 53 amino acids inserted in the connecting segment of the β4B variant. We also show that expression of human α6β4 in COS-7 cells results in a different distribution of HD1, with which α6β4 is co-localized, and that this effect is dependent on the cytoplasmic domain of β4.

[1] Abbreviations used: FNIII repeat, fibronectin type III repeat, IF, intermediate filament.

[2] We suggest a new nomenclature for the different β4 variants: β4A for the most abundant β4 subunit, previously designated as (−53)β4 by us, β4B for the (+53)β4 variant, β4C for the (+70)β4 variant, and β4D for the (−7)β4 variant.

MATERIALS AND METHODS

Cells and Antibodies

The mouse mammary tumor cell line RAC-11P (Sonnenberg *et al.*, 1993), the human squamous cell carcinoma cell line UMSCC-22B and the African Green monkey kidney cell line COS-7 were grown in DMEM supplemented with 7.5% fetal calf serum, streptomycin, and penicillin. The mouse monoclonal antibody (mAb) 4.3E1 (Hessle *et al.*, 1984) and the rat mAb 439–9B (Kennel *et al.*, 1989) are both directed against the extracellular domain of human β4. The mouse mAbs 450–10D and 450–11A are directed against the cytoplasmic domain of β4 (Kennel *et al.*, 1992). The rat mAb GoH3 is directed against human α6. Dr. Owaribe (Nagoya University, Nagoya, Japan) provided us with the mouse mAb 121 directed against the HD1 molecule (Owaribe *et al.*, 1991). The human autoimmune serum 81F94, recognizing both BP230 and BP180, was obtained from Dr. D. Boorsma (Free University, Amsterdam, the Netherlands). The mouse mAb 43A recognizing CK14 was a generous gift from Dr. D. Yvanyi (The Netherlands Cancer Institute, Amsterdam, the Netherlands). A mouse mAb PanCK recognizing different cytokeratin polypeptides was purchased from Immunotech (Marseille, France). The mouse mAbs RV202 directed against vimentin and E7 against tubulin were kindly provided by Dr. F. Ramaekers (University of Limburg, Maastricht, the Netherlands). Actin was visualized by using phalloidin conjugated to rhodamine (Sigma Chemical Co., St. Louis, MO).

cDNA Constructs and Preparation of Glutathione S-Transferase (GST) Fusion Proteins

To obtain cDNA fragments encoding the two pairs of FNIII repeats and the connecting segment, with or without the 53 amino acids insert, a polymerase chain reaction (PCR) was performed using primer 1: AATGGATCCAGATGTTGTCATCACAG, positions 3352–2269 of β4 cDNA (Hogervorst *et al.*, 1990), and primer 2: GACTCTATGTGATGATGCCCTCG, positions 5142–5165, and plasmids encoding either β4A or β4B as templates. In the PCR, a fragment was produced of 1637 bp for β4A and one of 1796 bp for β4B, respectively. These fragments were digested with *Bam*HI and subsequently ligated into the *Bam*HI-*Sma*I linearized vector pRP270, a derivative of the pGEX vector (K. Vink, The Netherlands Cancer Institute) for expression as GST fusion proteins. PCR was also performed to obtain the cDNA encoding the cytoplasmic domain of α6 using primer 1: GCGGTACCCAATAAGAAAGATCATTATGATGCC, positions 3279–3301 (Tamura *et al.*, 1990) of α6 cDNA, and primer 2: AGCTCTAGAAGTTAAAACTGTAGGTTCG, positions 3677–3695, and α6 cDNA plasmid. After digestion of the PCR product with *Kpn*I and *Bam*HI, the 190-bp fragment was ligated into plasmid pUC19. This plasmid was digested with *Kpn*I and *Hin*dIII and the 190-bp fragment was subsequently cloned into the bacterial expression vector pRP270. GST fusion proteins were expressed in JM109 bacteria as described (Smith and Johnson, 1988) and immobilized on glutathione-agarose beads (Sigma Chemical Co.).

For the generation of full-length β4A cDNA, a cDNA clone isolated from a λgt11 human keratinocyte library was used that encodes the β4A subunit from position 1880 to the 3′ terminus. The λ DNA was digested with *Sfi*I, and the resulting 2.2-kb fragment was exchanged for the 2.35-kb *Sfi*I fragment of the β4B cDNA (Niessen *et al.*, 1994b), resulting in full-length cDNA encoding the β4A subunit. The cDNA was then ligated into the *Xba*I site of the pRC-CMV vector. For the $\beta4^{1182}$ (amino acid numbering according to Suzuki *et al.*, 1990) cDNA, full-length β4 cDNA in the *Eco*RI site of pUC18 was digested with *Sac*I. A fragment of 3.5 kb was isolated and filled in by T4 polymerase. After ligation of a nonsense *Eco*RI linker, the fragment was digested with *Eco*RI and inserted into the *Eco*RI site of pRC-CMV. The correctness of all constructs was verified by sequencing.

Immunoprecipitation and Binding Assays

Cells were grown to near confluency, after which the cells were metabolically labeled overnight using a [^{35}S]methionine-cysteine mixture (50 μCi/ml) in methionine- and cysteine-free minimal essential medium (ICN Biomedicals Inc., Costa Mesa, CA) containing 10% DMEM. Cells were lysed in lysis buffer containing 0.5% Nonidet P-40 (NP-40) 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 for 1 h at 4°C. The cell lysates were clarified by centrifugation at 14,000 × *g* for 10 min and precleared with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 2 h at 4°C. Immunoprecipitations were carried out by incubating the precleared lysates for 1 h at 4°C with specific mouse or rat mAbs coupled to protein A-Sepharose via rabbit anti-mouse or rabbit anti-rat IgG (Sonnenberg *et al.*, 1993). The beads were washed three times with lysis buffer and twice with phosphate-buffered saline (PBS).

For binding assays with GST fusion proteins, cells were lysed and clarified as described above, diluted to 0.1% NP-40, and precleared with glutathione-agarose beads for 2 h at 4°C. Then equivalent amounts of the GST fusion proteins (approximately 5 μg) or GST alone, immobilized on glutathione-agarose beads, were incubated with the precleared lysate for 4 h at 4°C. In competition assays, a 100-fold (500 μg) excess of free GST or GST-cytoβ4A was added during the incubation of the lysate with the fusion proteins coupled to glutathione-agarose beads. The beads were washed three times with lysis buffer containing 0.1% NP-40 and twice with PBS. Associated proteins were eluted by boiling in SDS sample buffer containing 5% β-mercaptoethanol. Samples of the associated proteins, the supernatant after the first wash step and the cell lysate after incubation with the beads, were analyzed on a 5% SDS-polyacrylamide gel under reducing conditions.

Western Blot Analysis

Protein samples were separated on polyacrylamide gels and transferred to nitrocellulose for 5 h at 250 mA or overnight at 100 mA. The nitrocellulose filters were blocked in TBST (20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 1% bovine serum albumin (BSA), washed, and incubated with the primary antibody for 1 h at room temperature. After washing, filters were incubated with the appropriate biotin-labeled secondary antibody in TBST/1% BSA. The third step involved an incubation with a biotin/streptavidin alkaline phosphatase complex, which was made according to the manufacturer's recommendation (Boehringer Mannheim GmbH, Mannheim, Germany) and diluted 1:20 in TBST/1% BSA. Antibody binding was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$.

DNA Transfections and Immunofluorescence Microscopy

COS-7 cells were plated on glass coverslips and transfected using the DEAE-dextran method (Seed and Aruffo, 1987). After 1 or 2 d, cells were fixed with 1% formaldehyde for 10 min and then permeabilized with 0.5% Triton X-100 for 5 min and blocked with 1% BSA for 20 min. All incubations with antibodies were for 30 min at 37°C, after which the coverslips were washed three times with PBS. Cells were first incubated with the mouse mAbs 121 (anti-HD1), PanCK (antikeratin), E7 (antitubulin), or RV202 (antivimentin) and then with fluorescein isothiocyanate-conjugated goat anti-mouse Igb-fluorescein isothiocyanate (Zymed Laboratories, San Fransisco, CA). Subsequently, cells were incubated with either the rat mAb 439–9B or the rat mAb GoH3 followed by rabbit anti-rat-specific IgG and then with Texas Red-conjugated donkey anti-rabbit Igb (Amersham International, Buckinghamshire, United Kingdom). For double labeling of actin and β4, cells were first incubated with mAb 439–9B followed by rabbit anti-rat-specific IgG and then with fluorescein isothiocyanate-conjugated goat anti-rabbit Igb-fluorescein isothiocyanate (Zymed Laboratories), after which actin was detected with rhodamine-conjugated phalloidin. The coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed under a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Richmond, CA).

RESULTS

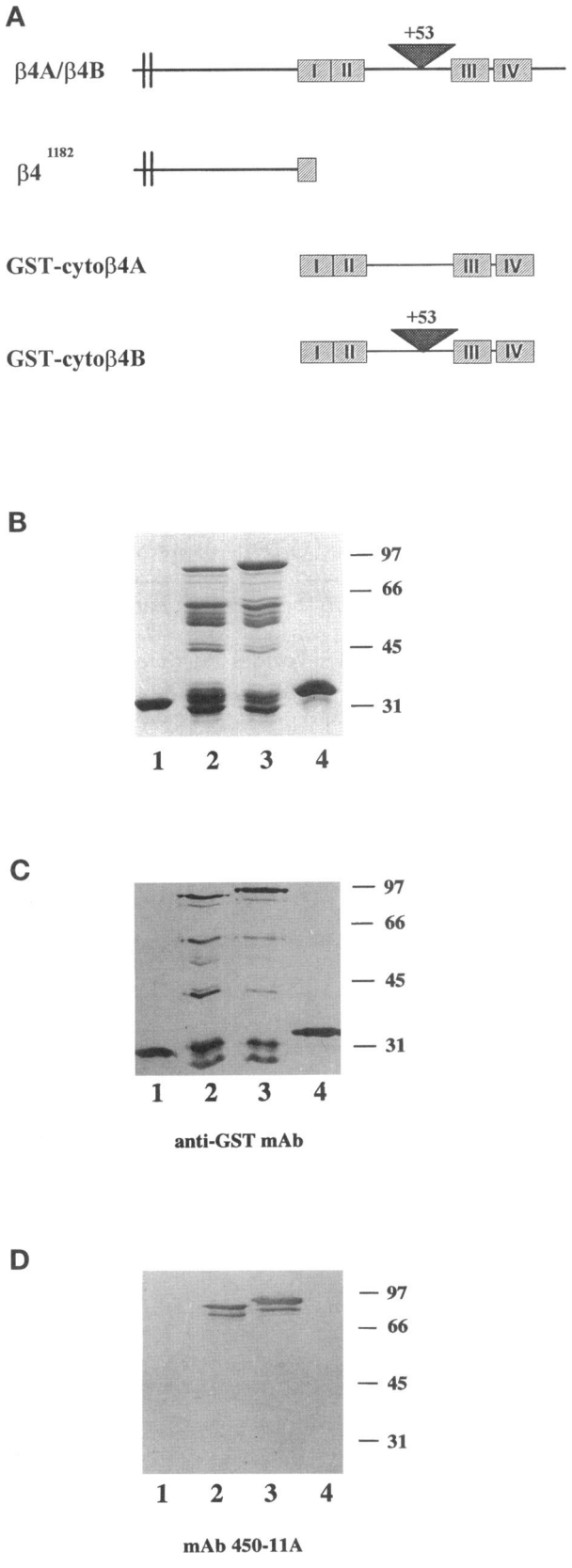
Generation of GST-β4 Fusion Proteins

We used an in vitro binding assay to study the interaction of the integrin β4 subunit with other cytoplasmic proteins. A fragment of the β4 cytoplasmic domain consisting of the two pairs of FNIII repeats and the connecting segment (GST-cytoβ4A; Figure 1A) was expressed in *Escherichia coli* and then isolated by binding to glutathione-agarose beads. Similarly, GST-cytoβ4B, which is identical to GST-cytoβ4A, except for the 53 amino acids inserted in the connecting segment as in the β4B variant (Figure 1A), was also isolated. Analysis of the purified GST-β4 proteins on SDS-polyacrylamide gels followed by Coomassie blue staining revealed, in addition to the expected bands of 87.5 kDa for GST-cytoβ4A and 93 kDa for GST-β4B, the presence of several smaller bands. Purified GST-cytoα6A and GST alone migrated as single protein bands of 31 kDa or 27 kDa, respectively (Figure 1B).

In several eukaryotic cells, it has been found that β4 is sensitive to proteolytic degradation (Giancotti *et al.*, 1992; Potts *et al.*, 1994), especially at its COOH-terminus. To demonstrate that the smaller bands in the purified GST-β4 protein preparation represent degradation products of the GST fusion proteins and not bacterial proteins, replicate samples were analyzed by immunoblotting with a mAb to GST and mAb 450–11A, which recognizes an epitope in the third FNIII repeat of the cytoplasmic domain of β4. As shown in Figure 1C, immunoblotting with the mAb to GST produced the same set of multiple bands that were detectable in the Coomassie blue-stained gel. The mAb 450–11A reacted with the intact GST-β4 fusion proteins and with a slightly faster migrating component (Figure 1D). These results indicate that the smaller bands in the GST-β4 preparations were derived from the larger GST-β4 fusion proteins by proteolysis at their COOH-terminal ends.

Association of β4 and a High Molecular Mass Protein In Vitro

In a first series of experiments, we explored the possible interaction of the integrin β4 subunit with cellular proteins using immunoprecipitation. Different procedures for labeling, cell lysis, including lysis with digitonin, and immunoprecipitation were used. However, in none of the experiments did any protein other than α6 coprecipitate with β4. Perhaps the binding between β4 and cellular proteins is weak and a large



amount of recombinant β4 might then shift the equilibrium of the reaction in a favorable direction and allow binding to be detected. Therefore, we produced large amounts of the GST-cytoβ4A fusion protein, which was immobilized on glutathione-agarose beads and further used to precipitate associated proteins from lysates of metabolically labeled RAC-11P cells. These were chosen because they express the components necessary for assembling hemidesmosomes (Sonnenberg *et al.*, 1993). As shown in Figure 2, a high molecular mass protein of 500-kDa was bound to GST-cytoβ4A but not to GST alone or GST-cytoα6A. The amount of protein associated with this immobilized GST-cytoβ4A fusion protein could be reduced by adding a 100-fold excess of free GST-cytoβ4A as competitor. No such competition was observed with the GST protein alone (Figure 3). The specificity of this interaction is further ascertained by the fact that, of the two closely spaced high molecular mass proteins which are present in approximately equal amounts in the lysates of RAC-11P cells, only one, i.e., the one that migrated more slowly, was precipitated by the GST-cytoβ4 fusion protein. Moreover, β4 and the 500-kDa protein were found to be associated when instead of GST maltose binding protein fusion proteins of β4 were used in the in vitro binding assay (our unpublished observations). We conclude that the formation of the complex containing the 500-kDa protein and β4 is specifically mediated by the cytoplasmic domain of β4.

Five Hundred-Kilodalton Protein Associated with β4 Fusion Proteins Comigrates with HD1

To investigate whether the molecular mass of the 500-kDa protein associated with GST-cytoβ4 corresponds to that of the hemidesmosomal plaque protein HD1 (Hieda *et al.*, 1992), we compared the mobilities of these proteins in SDS-polyacrylamide gels. As shown in Figure 4A, the 500-kDa protein comigrated with the band obtained by immunoprecipitation of HD1 with mAb 121. The GST-cytoβ4B fusion protein also bound to a protein that comigrated with HD1 but in smaller quantities, suggesting that the affinity of β4B either for this protein or for another protein participating pro-

Figure 1. Characterization of β4 deletion mutants and expression of GST fusion proteins in *E. coli*. (A) Schematic representation of the cytoplasmic domain of full-length β4, $\beta4^{1182}$, and the GST-cytoβ4A and GST-cytoβ4B fusion proteins. The four FNIII repeats (I–IV) are shown as boxes with oblique lines. In the β4 splice variant, 53 amino acids (inverted triangles) have been inserted in the region connecting the two pairs of FNIII repeats. GST fusion proteins were expressed as described in MATERIALS AND METHODS and immobilized on glutathione-agarose beads. Samples of fusion proteins were resolved by SDS-PAGE on a 10% gel and either visualized by staining with Coomassie blue (B) or immunoblotted with a mAb to GST (C) or antiβ4 mAb 450–11A (D). Lane 1, GST alone; lane 2, GST-cytoβ4A; lane 3, GST-cytoβ4B; and lane 4, GST-cytoα6A.

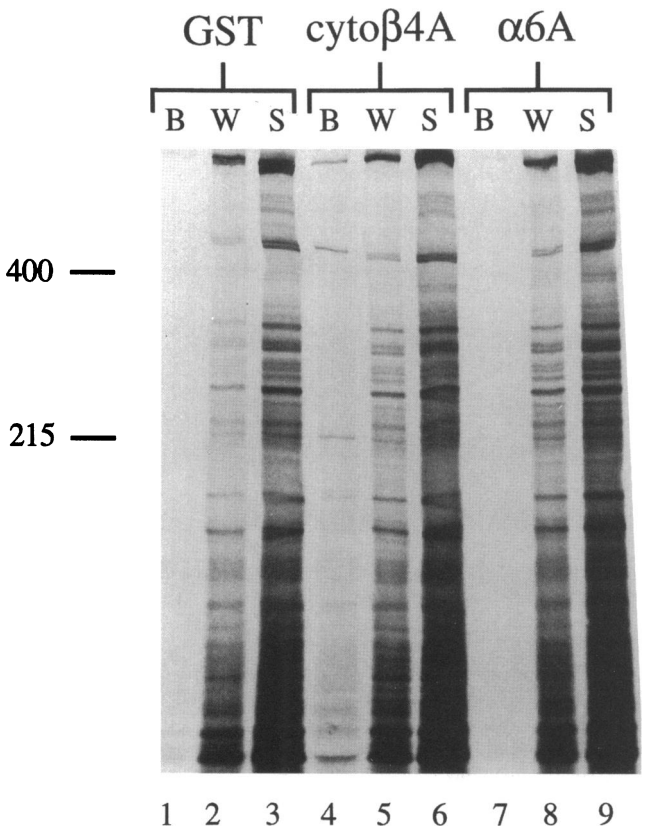


Figure 2. A GST-cytoβ4A fusion protein forms a complex with a 500-kDa high molecular mass protein from RAC-11P cells. Lysates prepared from metabolically labeled RAC-11P cells were incubated with 5 μg of immobilized GST (lanes 1–3), GST-cytoβ4A (lanes 4–6), and GST-α6A (lanes 7–9). Following binding of the proteins, the beads were pelleted and washed three times. Parts of the bound fraction, 40% (B); of the wash, 5% (W); and of the unbound, 1% (S) fractions were analyzed by SDS-PAGE under reducing conditions.

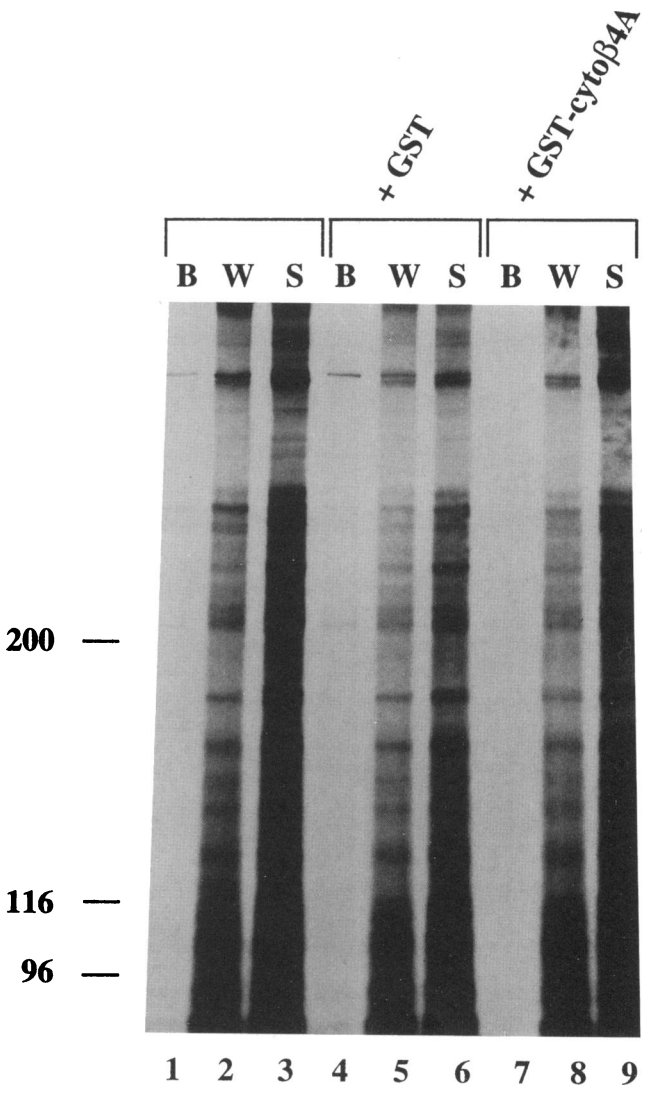


Figure 3. Binding of the 500-kDa high molecular mass protein is inhibited by free GST-cytoβA. Lysates prepared from metabolically labeled RAC-11P cells were incubated with 5 μg of immobilized GST-cytoβ4A in the absence (lanes 1–3) of added proteins or in the presence of a 100-fold excess of free GST (lanes 4–6) or free GST-cytoβ4A. Similar amounts of the bound (B), wash (W), and supernatant (S) fractions as in the experiment described in Figure 2 were tested.

tein is weaker than that of β4A. We also tested the ability of the GST-β4 fusion proteins to associate with proteins from another cell line, the human UMSCC-22B line, which contains hemidesmosome-like structures (Hogervorst *et al.*, 1993). Both GST-cytoβ4A and GST-cytoβ4B precipitated a protein of 500-kDa that comigrated with HD1 from these cells as well (Figure 4B), and again the affinity of cytoβ4B for this protein appeared to be lower than that of cytoβ4A. Neither GST-cytoβ4A nor GST-cytoβ4B specifically bound a protein that comigrated with BP230 from lysates of either of the cell lines used.

Five Hundred-Kilodalton Protein Is Identical to HD1

To demonstrate that the 500-kDa protein is identical to HD1, bound GST fusion proteins and associated proteins were analyzed by immunoblotting with antibodies to HD1. As shown in Figure 5, the 500-kDa protein associated with either GST-cytoβ4A or GST-cytoβ4B was recognized by the anti-HD1 mAb 121 (lanes 4 and 7). The anti-HD1 mAb did not react with the proteins bound to GST alone (lane 1). The 500-kDa protein was also detected in the supernatant of the first wash step and in the lysate after incubation with the different GST fusion proteins. Obviously, the GST-β4 fusion proteins did not bind all of the HD1 molecules present in the cell lysate. Thus, the 500-kDa protein bound by GST-cytoβ4A and GST-cytoβ4B was identified as HD1.

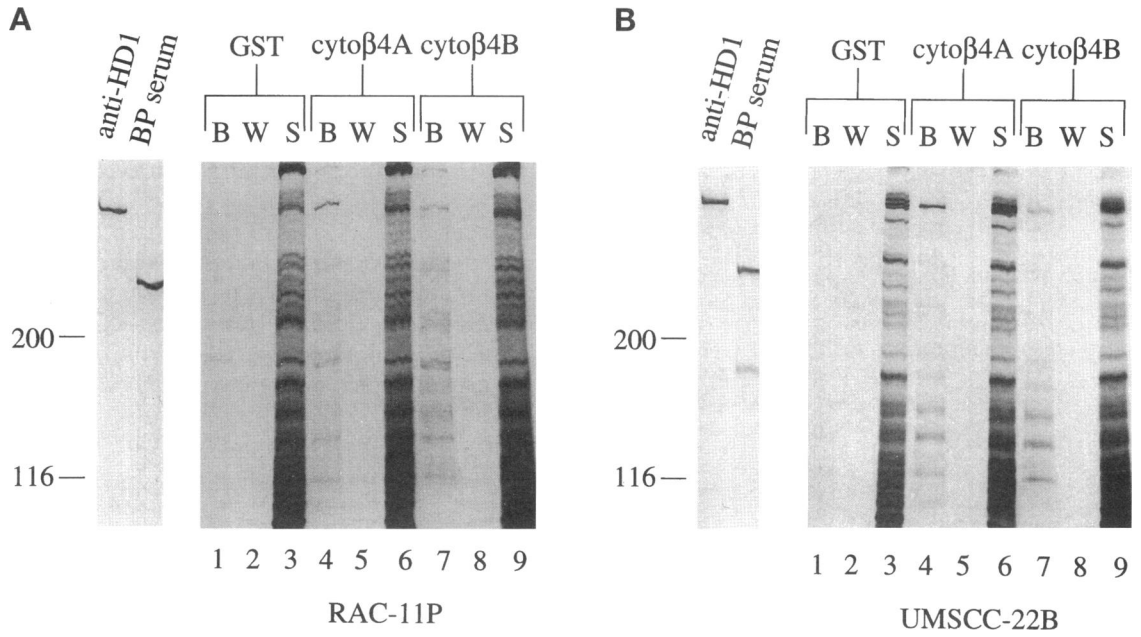


Figure 4. The 500-kDa protein forms a complex with cytoβ4A and cytoβ4B and comigrates with the hemidesmosomal plaque protein HD1. Lysates prepared from metabolically labeled RAC-11P (A) or UMSCC-22B (B) cells were incubated with immobilized GST (lanes 1–3), GST-cytoβ4A (lanes 4–6), or GST-cytoβ4B (lanes 7–9). Similar amounts of the bound (B), wash (W), and supernatant (S) fractions as in the experiment described in Figure 2 were tested. BP230 and HD1 were immunoprecipitated from the same lysates and examined on the same gel to compare their mobilities with those of the proteins bound by the GST fusion proteins.

Since we used total cell lysates, the possibility had to be excluded that HD1 is precipitated as part of a complex also containing keratins. Immunoblots of samples obtained by in vitro binding experiments were incubated with a keratin 14-specific antiserum (CK14). As shown in Figure 5, the same amount of CK14 protein was bound to GST-cytoβ4A, GST-cytoβ4B, and GST alone, which suggests that HD1 does not bind to β4 via keratins.

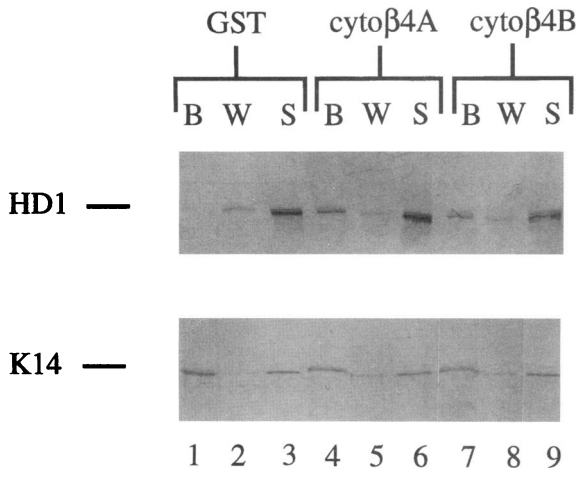


Figure 5. The GST-cytoβ4A- and GST-cytoβ4B-associated 500-kDa protein is HD1. Lysates from unlabeled RAC-11P cells were used in the in vitro binding assay with GST (lanes 1–3), GST-cytoβ4A (lanes 4–6), and GST-cytoβ4B (lanes 6–9) proteins. Similar amounts of the bound (B), wash (W), and supernatant (S) fractions as in the experiment described in Figure 2 were analyzed by SDS-PAGE and immunoblotted with either the anti-HD1 mAb 121 or the antikeratin mAb 43A.

HD1 and α6β4 Are Colocalized at Sites of Cell Substratum in COS-7 Cells Transfected with α6 and β4 cDNAs

The subcellular distribution of HD1 varied among different cell lines. Whereas in some cells HD1 was found to be associated with the cytoskeleton, in others it was present in adhesion structures, along with α6β4, at the basal surface of the cell. To investigate whether this different basal localization was dependent on the presence of α6β4, we performed transfection assays using COS-7 cells.

Immunofluorescence analysis revealed that these cells contain HD1, but not α6β4, and that HD1 is part of their cytoskeleton. This was confirmed by immunoprecipitation which furthermore showed that COS-7 cells express the integrin α3β1 and small amounts of α6β1 (Figure 6). The hemidesmosomal proteins BP230 and BP180 were not detected in these cells.

To obtain further information about the association of β4 with HD1, we have, in addition to a cDNA-construct encoding the full-length β4 sub-

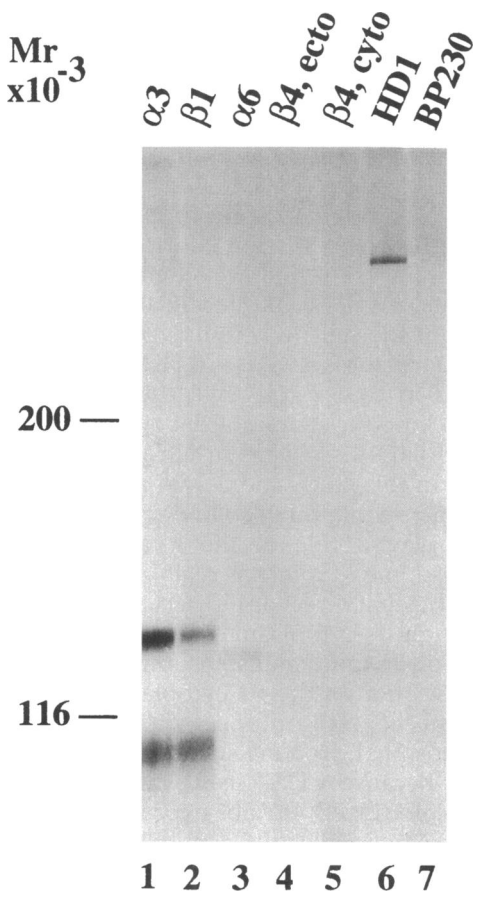


Figure 6. Expression of integrins and hemidesmosomal proteins in COS-7 cells. Lysates from metabolically labeled COS-7 cells were immunoprecipitated with the following antibodies: the anti-β1 mAb TS2/16 (lane 1), anti-α3 mAb J143 (lane 2), anti-α6 mAb GoH3 (lane 3), anti-β4 mAb 450–9D (lane 4), anti-β4 mAb 450–10D (lane 5), anti-HD1 mAb 121 (lane 6), or the 81F94 serum recognizing both BP230 and BP180 (lane 7).

unit, also generated one encoding a truncated form of β4 ($\beta4^{1182}$) that lacks the COOH-terminal half of the molecule (Figure 1A). This latter construct almost completely lacks the domain that has been identified to interact with HD1 in vitro. Together with α6 cDNA, these constructs were transiently transfected in COS-7 cells. By immunoprecipitation with an anti-α6 antibody, human and monkey α6 subunits in association with endogenous β1 were detected in COS-7 cells transfected with the human α6 cDNA (Figure 7). From COS-7 cells transfected with both the α6 and β4 cDNAs, α6 was precipitated in association with β4 by the anti-α6 mAb or by antibodies directed against either the extracellular or the cytoplasmic domain of β4 (Figure 7). The α6 subunit was also complexed to the truncated $\beta4^{1182}$ subunit in COS-7 cells transfected with the α6 and $\beta4^{1182}$ cDNAs (Figure 7).

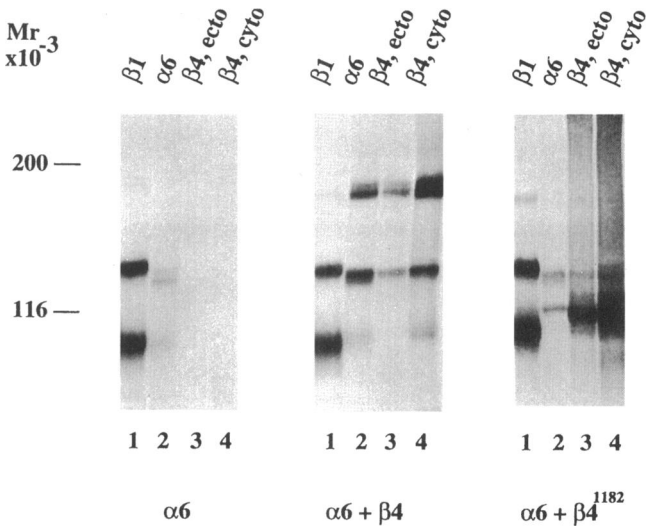


Figure 7. Immunoprecipitation of integrins from transfected COS-7 cells. COS-7 cells were transfected with α6 cDNA, α6 and β4 cDNAs, or α6 and β^{1182} cDNAs. After 2 d, cells were metabolically labeled, lysed, and precipitated with anti-β1 mAb TS2/16 (lane 1), anti-α6 mAb GoH3 (lane 2), anti-β4 extracellular mAb 450–9D (lane 3), and anti-β4 cytoplasmic mAb 450–10D (lane 4).

As shown in Figure 8, in COS-7 cells transfected with both human α6 and β4 cDNAs, the distribution of HD1 was strikingly different from that in cells transfected with the α6 cDNA alone. Although in the former HD1 was present at the basal side of the cell, along with α6β4, in the α6 transfected cells (and also in untransfected cells) HD1 was found to be part of the cytoskeleton and it was not colocalized with the α6 subunit. The dense staining pattern of α6β4 and HD1 at sites of cell substratum in COS cells resembled their distribution pattern in RAC-11P cells, in which it is typical for hemidesmosomes (Sonnenberg *et al.*, 1993). The distribution of HD1 was not different in COS-7 cells cotransfected with α6 and $\beta4^{1182}$ cDNAs in which HD1 was localized in the cytoskeleton, whereas $\alpha6\beta4^{1182}$ was distributed evenly over the entire surface of the cells. Thus, the colocalization of HD1 with α6β4 at the basal side of the COS-7 cells depends on the presence of the COOH-terminal part of the cytoplasmic domain of β4 which includes the sequences involved in the formation of a complex containing HD1 in vitro.

Expression of α6β4 Does Not Affect Other Cytoskeleton Networks

Double immunofluorescence experiments were performed to determine whether the endogenous cytoskeletal organization was affected by the expression of α6β4. The staining patterns for vimentin and actin were similar in transfected and untransfected COS-7 cells (Figure 9, A–D). Furthermore, both the keratin and tubulin networks were unaltered in cells express-

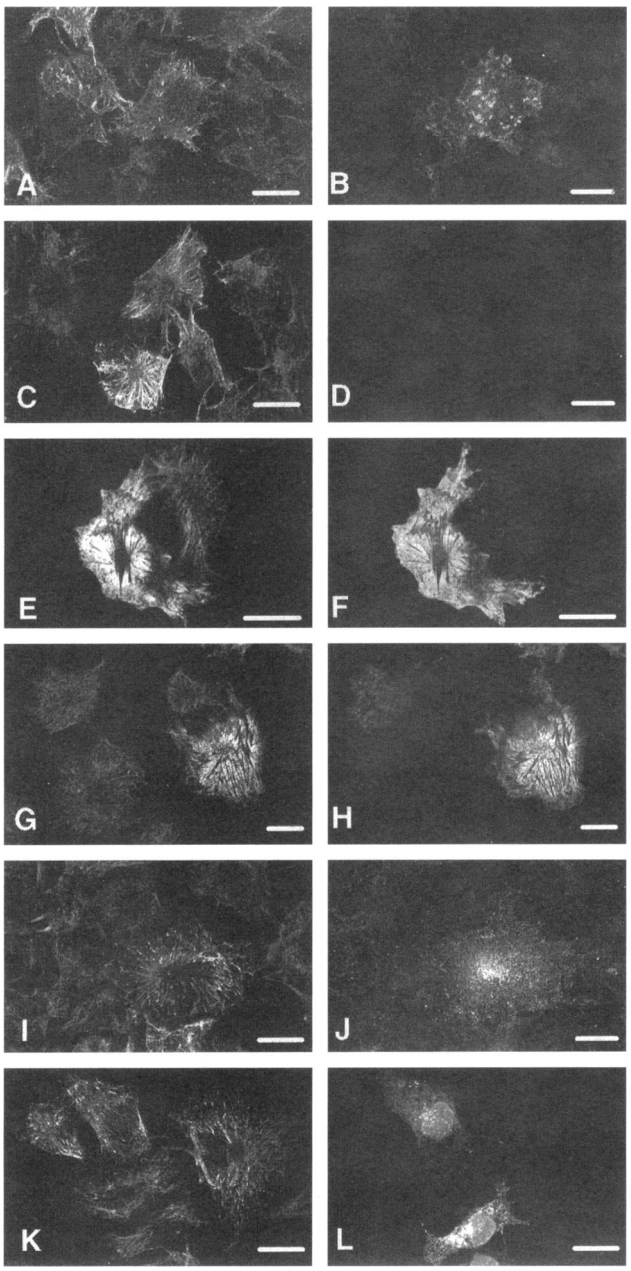


Figure 8. Double labeling immunofluorescence of transfected COS cells for HD1 and α6 or β4. COS-7 cells plated on glass coverslips were transfected with human α6 cDNA alone (A–D), human α6 and β4 cDNAs (E–H), or human α6 and $\beta4^{1182}$ cDNAs (I–L). After 2 d, cells were fixed and double stained for either HD1 (A, E, and I) and α6 (B, F, and J) or HD1 (C, G, and K) and β4 (D, H, and L). HD1 is colocalized with α6β4 in cells transfected with full-length α6 and β4 cDNAs (E–H), while it is associated with the cytoskeleton in cells transfected with either α6 cDNA alone (A–D) or α6 and $\beta4^{1182}$cDNAs (I–L).

ing α6β4 (Figure 9, E–H). Thus, when α6β4 is expressed, there is a change in the localization behavior of HD1 without an obvious reorganization of endogenous cytoskeletal networks.

DISCUSSION

In this article, evidence is presented that the integrin β4 subunit associates with the cytoskeletal-associated protein HD1. This conclusion is based on the following observations: 1) HD1 was bound by proteins expressed in bacteria consisting of GST fused to the FNIII repeats and the connecting segment of either β4A or β4B. 2) Expression of β4A in COS-7 cells, which contain HD1 but no other hemidesmosomal components, results in the localization of HD1 at the basal side of the cells, where it is present in the same junctional structures as α6β4. 3) HD1 and α6β4 are not colocalized in COS-7 cells that express a mutant β4 subunit lacking the region of the cytoplasmic domain with which HD1 associates in vitro. The sequences in β4 shown in this study to be important for formation of a complex containing HD1 have previously been found to play a role in the localization of β4 in hemidesmosomes (Spinardi *et al.*, 1993). It is, therefore, possible that direct or indirect association of β4 to HD1 through its cytoplasmic domain leads to the localization of β4 in hemidesmosomes.

The association of the β4 cytoplasmic domain with HD1 might be indirect, i.e., via binding to other hemidesmosomal proteins such as BP230 and BP180. However, because COS-7 cells, in which HD1 and α6β4 are colocalized, do not express either BP230 or BP180, these two molecules cannot be involved. Moreover, proteins of their size were not detected by SDS-PAGE in the samples of cellular proteins associated with GST-cytoβ4A from lysates of metabolically labeled RAC-11P or UMSCC-22B cells (Figure 4). It is unlikely that keratin 14 links β4 to HD1 because equal amounts of this protein were bound by the GST-β4 fusion proteins and by GST protein alone. Furthermore, GST-β4 fusion proteins also associate with HD1 in a lysate of RAC-5E cells, a fibroblast-like cell line, which does not contain keratins (Niessen, unpublished observations). However, attempts to demonstrate a direct interaction in gel or blot overlay assays have until now been unsuccessful. Thus, it remains possible that β4 does not bind directly to HD1 but via other components.

The structures found in COS-7 cells after transfection with α6 and β4 cDNAs that contain both HD1 and α6β4 may be identical to the type II hemidesmosomal structures described by Uematsu *et al.* (1994). These structures were linked to keratin filaments in the mammary gland cell line BMGE-H, which contains HD1 and α6β4 but no other hemidesmosomal components (Uematsu *et al.*, 1994). Similar structures were found in cells of the mouse TA3/Ha carcinoma cell line that normally grows in suspension because the large amount of the mucin epiglycanin on their surface prevents attachment of the cells to the substratum (Kemperman

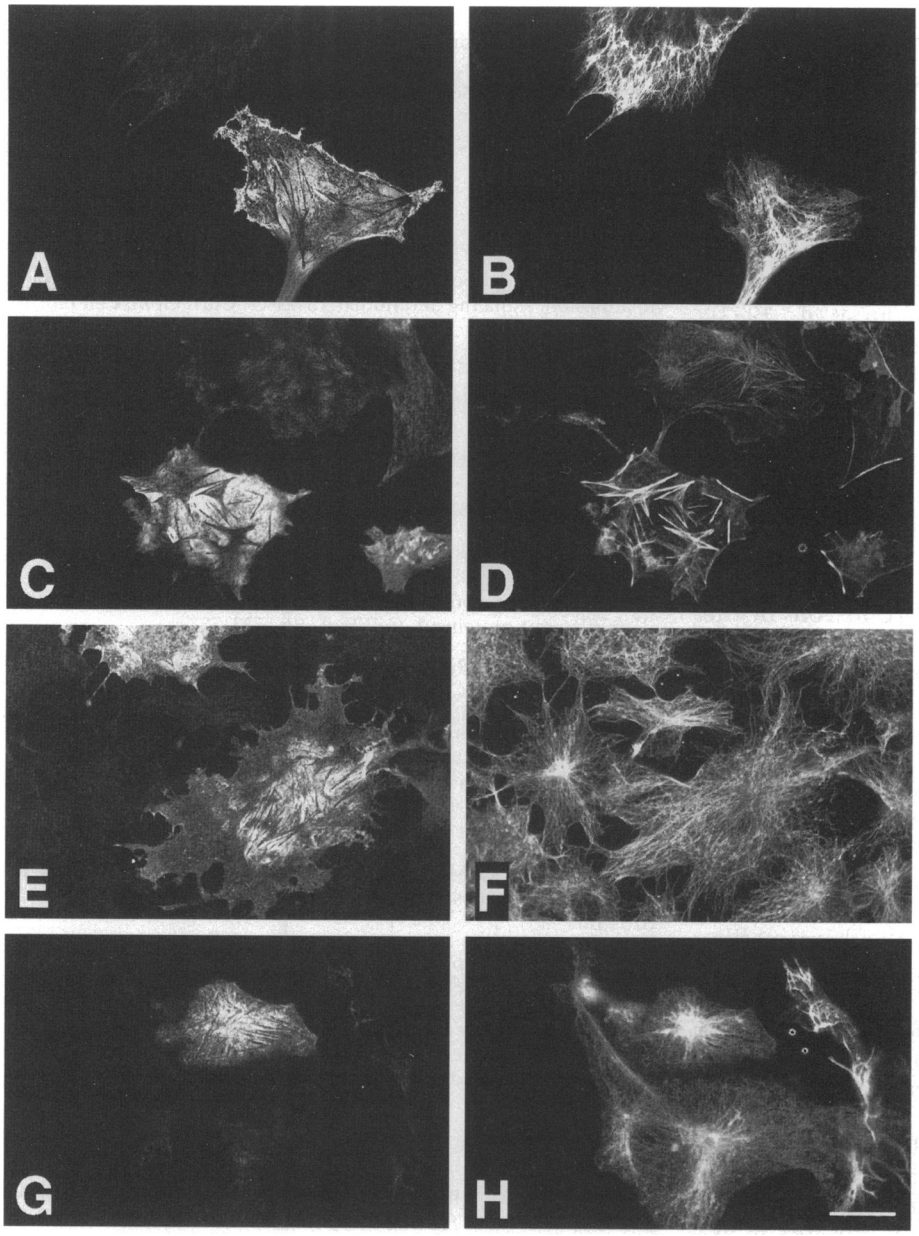


Figure 9. Double labeling immunofluorescence of transfected COS-7 cells for vimentin, actin, tubulin, or keratin and β4. COS-7 cells were grown on glass coverslips and transfected with α6 and β4 cDNAs. After 2 d, cells were fixed and double stained for β4 (A, C, E, and F) and vimentin (B), actin (D), tubulin (F), or keratin (H). The staining pattern of the different cytoskeleton components is similar in untransfected and transfected cells.

et al., 1994). After capping of the epiglycanin molecules, these cells adhere to laminin-1 and -5 by the α6β4 integrin, and this integrin in combination with HD1 but not BP230 was found in adhesion structures. The presence of α6β4 and HD1 in distinct structures at the basal side of the COS-7 cells suggests that α6β4 is involved in their adhesion to endogenously synthesized and deposited matrix, composed most likely of one or more laminin isoforms.

It is not clear why HD1 becomes colocalized with α6β4 after expression of this integrin in COS-7 cells. Perhaps, the affinity of HD1 for the cytoplasmic domain of β4 is stronger than for any other molecule and, therefore, HD1 will preferably interact with α6β4 when it is expressed. Alternatively, the association with HD1 could be induced by binding of α6β4 to its ligand, either by triggering a conformational change or signaling events (Maineiro *et al.*, 1995). Taken together, these findings indicate that an interaction between α6β4 and HD1 can occur in the absence of other hemidesmosomal components such as BP180 and BP230. This is interesting because the expression of HD1 along with α6β4 is not confined to cells that assemble hemidesmosomes (Sonnenberg *et al.*, 1990; Hieda *et al.*, 1992; Kennel *et al.*,

1992), and therefore these molecules may also associate in other cell types to form a complex for anchoring other cytoskeleton components to the plasma membrane.

Recent evidence indicates that HD1 and plectin are similar or identical proteins (Gache *et al.*, 1996; McLean *et al.*, 1996; Smith *et al.*, 1996). Plectin is considered to be a general cross-linker of cytoskeletal networks (Foisner and Wiche, 1991), and in COS-7 cells plectin has been shown to associate with the vimentin and keratin networks (Wiche *et al.*, 1993). The identification of an actin-binding domain in the NH_2-terminus of plectin suggests that it also associates with actin filaments (McLean *et al.*, 1996). If indeed HD1 is identical to plectin, then the altered localization of HD1 induced by $\alpha6\beta4$ in COS-7 cells might affect the structure of the different cytoskeletal networks. However, we found no obvious alterations in the vimentin, keratin, actin, and tubulin networks after expression of $\alpha6\beta4$.

The $\alpha6\beta4$ integrin plays an important role in the assembly of hemidesmosomes (Vidal *et al.* 1995; Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; Niessen *et al.*, 1996; Van der Neut *et al.*, 1996), and its association with HD1 may provide a link between the keratin filaments and the plasma membrane during early hemidesmosome formation. This role of HD1/plectin is supported by the observation that in patients deficient for plectin/HD1 no keratin filaments are attached to hemidesmosomes (Gache *et al.*, 1996; McLean *et al.*, 1996; Smith *et al.*, 1996). However, a stable attachment of the keratin filaments probably also requires the involvement of other components such as BP230. This is suggested by the fact that in BP230 knockout mice hemidesmosomes lack the inner plaque, and their association with the keratin filaments is reduced (Guo *et al.*, 1995). The exact role of BP180 in the formation of hemidesmosomes is not known. However, it seems to be required for their full maturation because the deficiency of BP180 found in patients with Generalized Atrophic Benign Epidermolysis Bullosa is associated with abnormal hemidesmosomes (Jonkman *et al.*, 1995; McGrath *et al.*, 1996). One function of BP180 could be to stabilize the hemidesmosomal complex by providing additional sites for protein interactions such as those with the integrin $\alpha6$ subunit (Hopkinson *et al.*, 1995).

We have shown that HD1 associates with the cytoplasmic domain of the integrin $\beta4$ subunit, which may be of relevance in the formation of adhesion structures in various types of cells.

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