The cytoplasmic domain of $\beta_4$ integrin contains two pairs of fibronectin-like repeats separated by a connecting segment. The connecting segment harbors a putative tyrosine activation motif in which tyrosines $1422$ and $1440$ are phosphorylated in response to $\alpha_6\beta_4$ binding to laminin-5. Primary $\beta_4$-null keratinocytes, obtained from a newborn suffering from lethal junctional epidermolysis bullosa, were stably transduced with retroviruses carrying a full-length $\beta_4$ cDNA or a $\beta_4$ cDNA with phenylalanine substitutions at either one of the tyrosines $1422$ and $1440$ are phosphorylated in response to the $\alpha_6\beta_4$ integrin and the $\alpha_6\beta_4$-null keratinocytes were devoid of sub-basal dense plates and of inner cytoplasmic plaques with keratin filament insertion. Collectively, our data suggest full genetic and functional correction of $\alpha_6\beta_4$-null keratinocytes. In cultures generated from $\beta_4$ integrin, $\beta_4$ mutants as well as $\alpha_6$ integrin, HD/plectin, and BP180 were not concentrated at the dermal-epidermal junction. Furthermore, the number of hemidesmosomes was strikingly reduced as compared with $\beta_4$-corrected keratinocytes. The rare hemidesmosomes detected in $\beta_4$-null cells were devoid of sub-basal dense plates and of inner cytoplasmic plaques with keratin filament insertion. Collectively, our data demonstrate that the $\beta_4$ tyrosine activation motif is not required for the localization of $\alpha_6\beta_4$ at the keratinocyte plasma membrane but is essential for optimal assembly of bona fide hemidesmosomes.

Human epidermis consists of a stratified squamous epithelium composed of keratinocytes organized in distinct cellular layers. Keratinocytes forming the basal layer firmly adhere to the basement membrane by means of hemidesmosomes (HDs), multiprotein complexes linking the epithelial intermediate filament network to the dermal anchoring fibrils (see Refs. 1 and 2 for review). HDs are formed by the clustering of several cytoplasmic and trans-membrane proteins (2). The cytoplasmic HD plaque components, which include HD/plectin (3) and the bullous pemphigoid antigen 1 (BP230) (4), act as linkers for elements of the cytoskeleton at the cytoplasmic surface of plasma membrane. The trans-membrane constituents of HDs, which include the $\alpha_6\beta_4$ integrin (5, 6) and the bullous pemphigoid antigen 2 (BP180) (7), serve as cell receptors connecting the cell interior to extracellular matrix proteins.

In particular, the $\alpha_6\beta_4$ integrin is a receptor for the basement membrane component laminin-5, a heterotrimeric protein composed of three distinct polypeptides, $\alpha_3$, $\beta_3$, and $\gamma_2$, which are encoded by three different genes known as LAMA3, LAMB3, and LAMC2, respectively (see Ref. 8 for review). Laminin-5 binds to the basal keratinocyte cell surface through the $\alpha_6\beta_4$ integrin and tightens the dermal-epidermal junction by binding also to the N-terminal NC-1 domain of type VII collagen (9). The crucial importance of the interaction between laminin-5 and its $\alpha_6\beta_4$ receptor in maintaining the integrity of the integument has been unambiguously proven by the generation of $\alpha_6^-$ and $\beta_4$-null mice (10–12) and by the identification of gene mutations in patients suffering from a devastating blistering disorder of the skin known as junctional epidermolysis bullosa (JEB). In most cases, JEB is due to mutations in LAMA3, LAMB3, and LAMC2 genes (13–15) and in ITGA6 and ITGB4 genes, which encode $\alpha_6$ and $\beta_4$ integrin subunits, respectively (16, 17). Mutations in ITGA6 and ITGB4 are usually associated to pyloric atresia (PA)-JEB (16, 17).

The cytoplasmic domain of $\alpha_6\beta_4$ contains two pairs of type III fibronectin (FN)-like repeats separated by a 142-amino acid connecting segment (CS). This CS is the target of multiple regulatory mechanisms, including tyrosine phosphorylation (18) and proteolytic processing (19). In particular, CS harbors tandem tyrosine phosphorylation sites (Ty-1422 and Tyr-1440), which resemble the tyrosine activation motif (TAM) of immune receptors and are phosphorylated in response to the binding of $\alpha_6\beta_4$ to laminin-5 (18). The potential TAM resides within a 303-amino acid segment of the $\beta_4$ cytoplasmic domain that includes the first pair of type III FN-like repeats and the CS. Mutational studies have indicated that this segment of $\beta_4$ is sufficient to mediate the incorporation of recombinant $\beta_4$ into the existing HD-like adhesion of 804G cells (20). We initially observed that phenylalanine substitutions at either one of the two tyrosines in the potential TAM decreased the incorporation of recombinant $\beta_4$ in HD-like adhesions (18). Although subsequent studies have yielded a contrasting result, they have...
provided evidence that the integrity of the TAM is required for efficient recruitment of BP180 by recombinant $\beta_4$ in HD-like adhesions of PA-JEB keratinocytes (21, 22). We have recently obtained evidence that the original TAM mutant used by Mainiero et al. (18) was generated starting from a version of $\beta_4$ that differs from the canonical form A because it lacks amino acids 941–948 (QDHTIVDT) in the membrane proximal portion of the cytoplasmic domain. The origin and nature of this variant form remain to be established. We have observed that this variant form of $\beta_4$ and a canonical form carrying phenylalanine substitutions at Tyr-1422 or Tyr-1440 are both normally incorporated in the HD-like adhesions of 804G cells (23). However, a mutant $\beta_4$ carrying both modifications is not, as shown previously (18).

In addition to resolving the prior controversies, these results reveal a functional synergy between amino acid stretches located relatively far apart in the linear sequence of the $\beta_4$ cytoplasmic domain and highlight the necessity to further examine the potential role of the $\beta_4$ TAM in HD assembly. Moreover, the potential role of specific portions of the $\beta_4$ cytoplasmic domain, and in particular of the TAM, in interaction with other HD components and in HD assembly is based solely on the results obtained using immortalized cell lines cultured on plastic. Under these conditions, both keratinocytes and 804G cells do not form HDs. Instead, HD components (such as $\alpha_2\beta_1$, BP180, and HD1/plectin) are organized in typical patches in

\[ \text{in vivo} \]

skin ("leopard skin" pattern, as described in Ref. 24), often referred to as HD-like adhesions (22, 25).

This said, the functional role of HD components in the proper assembly of mature HDs can in fact be studied in vitro because normal human primary keratinocytes can be cultivated in conditions that allow full epithelial differentiation (26–28) and proper assembly of mature HDs (29, 30). Keratinocytes can generate cohesive sheets of stratified epithelium that maintains virtually the same differentiation features and gene expression pattern of its in vivo counterpart so that it can be routinely transplanted in patients suffering from large skin or mucosal defects (31–33). When primary keratinocytes are seeded onto dead-de-epidermized dermis in organotypic cultures (29), mature HDs are formed in vitro (30). Therefore, the availability of human $\beta_4$-deficient primary keratinocytes (see "Results"), the possibility of stably transducing primary keratinocytes with high efficiency (34), and the possibility of subsaturating stably transduced cells in conditions in which HDs are formed (30) provide a unique opportunity to clarify the above uncertainties and to investigate the role of $\beta_4$ and its potential TAM in the formation of mature HDs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, cDNA Constructs, and Amphotropic Producer Cell Lines**—Swiss mouse 3T3-J2 cells (a gift from Howard Green, Harvard Medical School, Boston), GP+E-86 ecotropic packaging cells, and GP+ene Am12 amphotropic packaging cells were grown as described previously (34). Normal human epidermal keratinocytes were obtained from skin biopsies of healthy volunteers. Primary $\beta_4$-null keratinocytes were obtained from a 1-cm² biopsy taken from a newborn patient suffering from PA-JEB (see "Results"). Informed consent was obtained from the parents.

Keratinocytes were cultivated on a feeder layer of lethally irradiated 3T3-J2 cells as described (28, 30, 33) and passaged at the stage of subconfluence. pRC/CMV-$p\beta_4^{1142EF}$, pRC/CMV-$p\beta_4^{1154OF}$, and pRC/CMV-$p\beta_4^{1142EF/Y1154OF}$, encoding $\beta_4$ with phenylalanine substitutions in the TAM sequence, were constructed from partial cDNA clones cDNA-unique, by nucleotide sequence of the canonical form A of $\beta_4$, including the amino acid sequence QDHTIVDT (941–948) (35). A PCR fragment from pRC/CMV-$p\beta_4$ restricted with EcoRV and XhoI, containing the 3’ end of $\beta_4$ (0.443 kilobase pairs), was inserted in pBBS/SK to obtain pBBS/SK3'endY. A 4.968-kilobase pair fragment from pRC/CMV-$p\beta_4$, restricted with EcoRI and EcoRV was inserted in pBBS/SK3'endY to obtain full-length pBBS-SK-$p\beta_4$. pL84SN was constructed by cloning the 5.4-kilobase pair fragment from full-length pBBS/SK-$p\beta_4$ into the EcoRI/Xhol sites of pLXSN retroviral vector (36) as described previously (34). The other constructs were inserted into the EcoRI/Xhol sites of pLXSN retroviral vector as described above. All constructs were sequenced before the generation of producer cell lines.

Amphotropic producer cell lines carrying each of the above constructs were generated by the transfection protocol as described (30, 34). A control amphotropic packaging cell line was generated as above using the pLXSN retroviral vector. For each producer cell line, the viral titre was higher than $1 \times 10^6$ colony-forming units/ml.

**Transduction Gene Transfer, in Situ Hybridization, and Southern and Northern Analysis**—Infection of primary keratinocytes was performed as described previously (30, 34). Briefly, subconfluent primary $\beta_4$-null keratinocytes were trypsinized and seeded (5 × $10^3$ cells/cm²) onto a feeder layer (2.3 × $10^5$ cells/cm²) composed of lethally irradiated 3T3-J2 cells and producer GP+ene Am12 cells (a 1:2 mixture). After 3 days of cultivation, cells were collected and plated onto a regular 3T3-J2 feeder layer. Subconfluent cultures were used for further analysis.

Analysis of integrated proviral genomes was performed by Southern analysis as described (34). In situ hybridization was performed using the DIG Nucleic Acid Detection kit (Roche Molecular Biochemicals) following the manufacturer's instructions. Sections of cultured epidermis were hybridized with a $\beta_4$ integrin antisense probe. For Northern analysis, cellular RNA was extracted with RNAfast (Sagam). 10 μg of total RNA was size-fractionated through 1% agarose-formaldehyde gels and transferred to nylon membrane (Hybond N°, Amersham Pharmacia Biotech). Blots were prehybridized at 68 °C for 2 h in 50% formamide, 5× SSC, 0.02% SDS, 2% blocking reagent, and 0.1% N-laurylsarcosine. Hybridization was performed overnight in the same conditions with the addition of 32P-labeled $\beta_4$ integrin fragment SacII (335/HindIII (1129) probes (2 × 10^6 cpm/ml). Filters were washed at high stringency in standard conditions.

**Immunological Analysis**—The following antibodies were used: mouse 3E1 mAb, raised against the extracellular domain of $\beta_4$ (Life Technologies, Inc.); goat (N20, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal T20 (Santa Cruz Biotechnology), recognizing the $\alpha_4$ integrin; and HD121 and 1D1 mAbs (gift from Dr. K. Owaribe, Nagoya University, Nagoya, Japan) recognizing HD1/plectin and BP180, respectively.

Immunofluorescence and immunohistochemistry were performed as described (28, 30, 38). For immunoblotting, subconfluent keratinocytes were trypsinized and rinsed on ice with PBS and lysed on ice with 50 mm Tris/HHCl, pH 8.5, 150 mm NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.2% sodium azide). Protein content was determined by the BCA assay (Pierce). Equal amounts of total proteins were immunoprecipitated with an excess of antibody, separated by SDS-PAGE, and transferred to a nitrocellulose filter. The blot was incubated in TBST (10 mm Tris-HCl, pH 8.0, 150 mm NaCl, and 0.05% Tween 20) containing 1% BSA and washed, and probed with specific antibodies for 1 h at room temperature. Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Pharmacia Biotech).

Immunoprecipitations were carried out on surface-labelled keratinocytes as described (39). Briefly, subconfluent keratinocytes were detached with 10 mm EDTA in phosphate-buffered saline (PBS), pH 7.4, and then washed and resuspended in PBS (2 × 10^6 cells/ml). Iodination was carried out for 15 min at room temperature in the presence of 1 mCi/ml of [125I]iodine (Amersham Pharmacia Biotech), 0.6 mg of lactoperoxidase, and 0.003% H₂O₂. After washing with 5 mm KI in PBS, cells were lysed in radioimmune precipitation buffer, pH 8.5, containing protease inhibitors (Complete™, Roche Molecular Biochemicals). Immunoprecipitations were carried out by overnight incubation at +4°C of the immuneadsorbs (antibodies adsorbed on Protein A-Sepha-rose, Amersham Pharmacia Biotech) with samples of cell lysates followed by extensive washing and elution by boiling in Laemmli sample buffer. Samples were then analyzed by SDS-PAGE under nonreducing conditions on 6% polyacrylamide gels followed by autoradiography. Protein-bound radioactivity in cell lysates was counted, and equivalent amounts of radioactivity were loaded for each sample.

**Organotypic Cultures and Transmission Electron Microscopy**—Keratinocytes (5 × 10^4 cells/cm²) were seeded onto dead-de-epidermized dermis and cultivated as described (30). 7 days later, cultures were lifted at the air-liquid interface, cultured for 1 additional week, and then processed for electron microscopy. Briefly, specimens were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in...
graded alcohols, embedded in Epon resin, and sectioned on an ultramicrotome (Reichert Ultratome E, Leica, Wien, Austria). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (CM100, Philips, Eindhoven, The Netherlands). Organotypic cultures were also sectioned on a cryostat and then analyzed by immunofluorescence as described (30).

Morphometry—Electron micrographs of overlapping fields of the dermal-epidermal junction, taken at a magnification of ×−15,500, were printed and assembled into a montage with a final magnification of ×40,000. The prints were digitalized, using a scanner (HP ScanJet 4c), in bitmap format, and the files were analyzed using a semi-automatic image analysis system (Kontron Elektronic Imaging System KS 300). The length of dermal-epidermal junction was measured for each point, commencing at one end of the montage, and the number of HDs was counted. For each HD, the area was measured, and the percentage of HDs associated with tonofilaments was calculated. A total of 1,291 μm of cell membrane was examined, divided into: 162 μm for control keratinocytes, 116 μm for β4-null keratinocytes, 116 μm for β4-corrected keratinocytes, and 244, 441, and 212 μm for β4αY1422F, β4αY1440F, and β4αY1422F/Y1440F keratinocytes, respectively. A total of 443 HDs were examined, divided into: 150 for control keratinocytes, 0 for β4-null keratinocytes, 106 for β4-corrected keratinocytes, and 27, 130, and 30 for β4αY1422F, β4αY1440F, and β4αY1422F/Y1440F keratinocytes, respectively.

RESULTS

Keratinocytes were cultivated from a 1-cm² skin biopsy taken from a newborn patient presenting with the clinical hallmarks of PA-JEB. The proband was a compound heterozygote for a 3-base pair deletion (ΔN318) in exon 8 of the maternal allele of the ITGB4 gene and an as yet unidentified paternal genetic defect. Allele-specific amplification of transcripts did not show any mRNA deriving from the paternal mutant allele, even in cycloheximide-treated cells.2 Immunohistochemical analysis showed absence of the β4 integrin in the skin of the proband (not shown). The keratinocytes of the proband are hereafter referred to as β4-null keratinocytes.

Northern blot analysis (Fig. 1) and in situ hybridization performed on cultured epidermal sheets (Fig. 2) showed similar levels of β4 transcripts in β4-null keratinocytes (Fig. 1A, lane 2, arrowhead, and Fig. 2C) as compared with normal control cells (Fig. 1A, lane 1, arrowhead, and Fig. 2A). Absence of the β4 polypeptide in β4-null cells was confirmed by immunoprecipitation followed by Western blot analysis (Fig. 1, B and C, lanes 2), immunofluorescence performed on β4-null colonies (not shown), and immunohistochemistry performed on cultured epi-

2 Zambruno, G., unpublished data.
levels comparable with those detected in normal control cells. Northern analysis showed abundant levels of exogenous \( \beta_4 \) transcripts (Fig. 1, lane 3, arrow). In situ hybridization (performed on epithelial sheets generated by \( \beta_4 \)-transduced keratinocytes) showed abundant levels of exogenous \( \beta_4 \) transcripts both in basal and suprabasal \( \beta_4 \)-corrected cells (Fig. 2E). The suprabasal expression of exogenous \( \beta_4 \) transcripts is expected because expression of the transgene is driven by the retroviral long terminal repeat. However, immunohistochemical analysis revealed that both in normal control cells (Fig. 2B) and in \( \beta_4 \)-corrected keratinocytes (Fig. 2F), the expression of the \( \beta_4 \) polypeptide was restricted to the basal layer of cultured epidermal sheets. It is possible that, in the absence of its natural \( \alpha_6 \) partner, exogenous \( \beta_4 \) is rapidly degraded in the ER of suprabasal layers.

The localization of \( \alpha_6\beta_4 \) and of other HD components was then investigated in organotypic cultures, namely in conditions allowing the formation of mature HDs. In normal control cultures, \( \alpha_6\beta_4 \) was clearly concentrated at the basal pole of basal keratinocytes (Fig. 3A). As described previously (40), in wound healing and in organotypic cultures, a faint labeling of the lateral and apical surfaces of the basal and first suprabasal cell layer was observed (Fig. 3A). The dermal-epidermal junction was also blotted by anti-HD1/plectin (Fig. 3B) and anti-BP180 (not shown) mAbs. In \( \beta_4 \)-null organotypic cultures, \( \beta_4 \) was virtually undetectable (Fig. 3C), whereas the \( \alpha_6 \) subunit was not polarized and was diffusely distributed in the basal keratinocyte cytoplasm (not shown). Similarly, HD1/plectin (Fig. 3D) and BP180 (not shown) were not concentrated at the dermal-epidermal junction but were diffusely distributed in the cytoplasm of basal keratinocytes. Gene correction of \( \beta_4 \)-null keratinocytes restored the normal expression pattern of \( \beta_4 \) (Fig. 3E), HD1/plectin (Fig. 3F), and BP180 (not shown). Indeed, the level of expression and the localization at the dermal-epidermal junction of the polypeptides were very similar to those observed in normal control cells (Fig. 3, A and B).

The formation of mature HDs was investigated by transmission electron microscopy performed on ultrathin sections of organotypic skin cultures. As shown in Fig. 4, normal control keratinocytes (A) and \( \beta_4 \)-corrected keratinocytes (B) assembled mature HDs (stars), displaying clearly recognizable sub-basal dense plates and cytoplasmic outer and inner plaques associated with keratin intermediate filaments (arrows) distributed along their basal plasma membrane. In contrast, very few rudimentary HDs, which appeared as small, moderately electron-dense spots almost completely lacking a tripartite structure and association with keratin filaments, could be identified in \( \beta_4 \)-null keratinocytes (F). Thus, \( \beta_4 \)-corrected keratinocytes were almost indistinguishable from normal control cells in terms of \( \alpha_6\beta_4 \) expression, the localization of HD components, and HD structure and density, suggesting that the adhesive properties of \( \beta_4 \)-null keratinocytes were fully restored.

Expression of \( \beta_4 \) Mutants in Primary \( \beta_4 \)-null Keratinocytes—To investigate the role of \( \beta_4 \) TAM in HD formation and maturation, subconfluent primary cultures of \( \beta_4 \)-null keratinocytes were stably transduced with replication-defective retroviruses carrying cDNA(s) encoding: (i) \( \beta_4 \) with a phenylalanine substitution at Tyr-1422 (\( \beta_4^{Y1422F} \)), (ii) \( \beta_4 \) with a phenylalanine substitution at Tyr-1440 (\( \beta_4^{Y1440F} \)), and (iii) \( \beta_4 \) with a combined replacement of Tyr-1422 and Tyr-1440 (\( \beta_4^{Y1422F/Y1440F} \)) (Fig. 5A). Proviral integration was demonstrated by Southern hybridization (not shown). As shown in Fig. 5B, variable levels of the different \( \beta_4 \) transcripts were detected in transduced \( \beta_4 \)-null keratinocytes (arrow).

The assembly of the \( \alpha_6\beta_4 \) heterodimer in cells transduced with different mutants was investigated by immunoprecipitation of cell lysates using anti-\( \beta_4 \) mAbs (3E1) followed by immunoblot using antisera raised against either \( \alpha_6 \) or \( \beta_4 \) (T20 and N20, respectively). As shown in Fig. 5C, all mutants were able to associate to the \( \alpha_6 \) subunit (lanes 2–4). It is worth noting that comparable amounts of the \( \alpha_6\beta_4 \) heterodimer were expressed in all transduced keratinocytes (Fig. 5C).

The exposure of \( \beta_4 \) on the keratinocyte plasma membrane was evaluated by immunoprecipitation of cell lysates prepared from surface-derivatized cells, using the anti-\( \beta_4 \) 3E1 mAb. As shown in Fig. 5D, equal amounts of \( \beta_4 \) were exposed on the cell surface in normal control cells (lane 1), \( \beta_4 \)-corrected cells (lane 2), and \( \beta_4 \)-null cells transduced with different TAM mutants (\( \beta_4^{Y1422F} \), \( \beta_4^{Y1440F} \), \( \beta_4^{Y1422F/Y1440F} \), lanes 3, 4, and 5, respectively). These data suggest that the \( \beta_4 \) TAM is not essential for the localization of the \( \alpha_6\beta_4 \) integrin at the keratinocyte plasma membrane.

The Role of \( \beta_4 \) TAM Domains in HD Formation and Maturation—The localization of \( \beta_4 \) mutants and of other HD components as well as the formation of mature HDs were investi-
showed that, similarly to normal control keratinocytes (A), \( \beta_4 \)-corrected keratinocytes (B) assemble mature HDs displaying sub-basal dense plates and outer and inner cytoplasmic plaques associated with bundles of keratin intermediate filaments (arrows). (Anchoring filaments transversing the lamina lucida are also visible, more frequently below the HD.) \( \beta_4^{1422F} \) (C) and \( \beta_4^{1440F} \) (D) keratinocytes also display HDs (stars), which appear, however, less numerous and smaller with reduction in keratin filament association. More severe HD alterations typify \( \beta_4^{Y1422F/Y1440F} \) keratinocytes (E, at arrow), in which sub-basal dense plates appear greatly attenuated and cytoplasmic inner plaques and keratin filament insertion are almost undetectable. A marked decrease in anchoring filament density is also evident in \( \beta_4 \)-null and \( \beta_4^{Y1422F/Y1440F} \) keratinocytes. Bar, 200 nm.

To quantify the number of HDs, the level of their maturation, and the extent of their association to intermediate filaments, a morphometric analysis of the dermal-epidermal junction was undertaken on electron micrographs of overlapping fields (41) (see “Experimental Procedures”). We have analyzed 1,291 \( \mu m \) of basal membrane and 443 HDs (see Table I and “Experimental Procedures”). All measurements were made by the same observer at least three times on randomly selected montages. Measurements were made on montages obtained from two different experiments, and average values are indicated.

As shown in Table I, we did not detect mature HDs in \( \beta_4 \)-null keratinocytes, whereas the mean values for HD counts in control cells (9.3 HDs/10 \( \mu m \)) and \( \beta_4 \)-corrected keratinocytes (9.1 HDs/10 \( \mu m \)) were similar. In contrast, the number of detectable HDs was strikingly reduced (up to 8-fold) in \( \beta_4^{1422F} \) (1.1 HDs/10 \( \mu m \)), \( \beta_4^{1440F} \) (2.9 HDs/10 \( \mu m \)), and \( \beta_4^{Y1422F/Y1440F} \) (1.6 HDs/10 \( \mu m \)) keratinocytes. Statistical analysis of the size of HDs was calculated using KS 300, a semiautomatic image analysis system, and data fell into a Gaussian distribution. The average size of HDs of control (3,897 nm\(^2\)) and \( \beta_4 \)-corrected (3,366 nm\(^2\)) keratinocytes was similar. Phenylalanine substitution at tyrosine 1422 and 1440 determined a reduction of HD size (2692 nm\(^2\) and 2181 nm\(^2\), respectively). Analysis of keratin filament association showed a marked reduction of the ability of \( \beta_4^{1440F} \) and \( \beta_4^{Y1422F/Y1440F} \) HDs to associate to intermediate filaments as compared with control and \( \beta_4 \)-corrected cells (Table I). It is worth noting, however, that even if the number of HDs formed by \( \beta_4^{1422F} \) keratinocytes was dramatically reduced, their ability to associate to intermediate filaments was only slightly altered. Taken together, these data indicate that \( \beta_4 \) TAMS are essential for the formation of a correct number of mature HDs in basal keratinocytes.

**DISCUSSION**

The requirement for the cytoplasmic domain of \( \beta_4 \) integrin in HD assembly has been clearly documented (42), and the results of this study indicate that the integrity of both tyrosine 1422 and 1440 of the \( \beta_4 \) cytoplasmic TAM is demanded for the optimal assembly of bona fide HDs in human epidermis. This conclusion stands in clear contrast to prior studies indicating that TAM-mutant \( \beta_4 \) localizes efficiently to endogenous HD-like adhesions of 804G cells and that it promotes assembly of HD-like adhesions in (immortalized) PA-JEB keratinocytes (22, 23), thus indicated that \( \beta_4 \) TAM is dispensable for HD formation (22).

It is likely that the assembly of mature HDs has more complex molecular requirements than the formation of HD-like adhesions, which reflect the co-localization of HD components at the basal pole of cells cultivated on plastic (24). For instance, it has been suggested that the first pair of type III FN-like modules and the initial segment of the CS of \( \beta_4 \) interact directly with the actin binding domain of HD1/plecnicin (21, 43, 44), whereas the cytoplasmic N terminus of BP180 associates with BP230 (45). In turn, HD1/plecnicin and BP230 associate with keratin filaments (46). Thus, both \( \alpha_6 \beta_4 \) and BP180 can interact.
interact as a functional unit with the two plakins and thereby with the keratin cytoskeleton.

This said, mature HDs are formed in vitro only when keratinocytes are cultivated onto de-epidermized dermis (29, 30), as in the organotypic cultures shown here. This suggests that HD-like adhesions do not recapitulate the assembly of mature HDs and might explain discrepancies between our data and data reported previously (22).

What is the mechanism by which the two tyrosine residues of the potential \( \beta_4 \) TAM regulate HD assembly? The immune receptor TAMs interact in a phosphorylation-dependent manner with the tandem SH2 domains of downstream target effectors, such as the protein kinase Syk and ZAP70 (47). Based on this observation, we have previously hypothesized that phosphorylation of the potential \( \beta_4 \) TAM might activate a signaling pathway necessary for proper HD formation (18). Two lines of evidence suggest that this hypothesis has to be re-evaluated. First, phosphorylation of Tyr-1422 and Tyr-1440 in response to activation of the EGF receptor correlates with disassembly (not shown) of HDs (37). Second, we have recently observed that exposure to the tyrosine phosphatase inhibitor, pervanadate, promotes tyrosine phosphorylation of \( \beta_4 \) and disrupts HDs. Interestingly, this effect is largely suppressed by

Fig. 5. A, a schematic map of the different \( \beta_4 \) isoforms used to transduce \( \beta_4 \)-null keratinocytes. EC, TM, and IC indicate extracellular, transmembrane, and cytoplasmic domain, respectively. Amino acid substitutions in the CS segment are indicated. Black circles indicate the FN-like repeats. B, Northern analysis. 10 \( \mu \)g of total RNA obtained from control (1), \( \beta_4 \)-null (2), \( \beta_4 \)-corrected (3), \( \beta_4^{1422F} \) (4), \( \beta_4^{1440F} \) (5), and \( \beta_4^{1422F/1440F} \) (6) keratinocytes was separated by electrophoresis, transferred to nylon filters, and hybridized to a \(^{32}P\)-labeled \( \beta_4 \) probe or to a \(^{32}P\)-labeled \( \beta_4 \) probe and to a \(^{32}P\)-labeled \( \beta_4 \) probe and to a \(^{32}P\)-labeled \( \beta_4 \) probe and to a

Fig. 6. Immunofluorescence analysis of HD components in organotypic skin cultures. A and B, \( \beta_4^{1422F} \) cells. C and D, \( \beta_4^{1440F} \) and \( \beta_4^{1422F/1440F} \) cells. Sections of organotypic cultures were stained with an anti-\( \beta_4 \) mAb (A, C, and E) and with an anti-HD1/plectin mAb (B, D, and F). Note that \( \beta_4 \) and HD1/plectin staining was mostly pericellular in cells transduced with \( \beta_4 \) mutants. \( \beta_4 \) and HD1/plectin appeared occasionally polarized in some basal \( \beta_4^{1422F} \) and \( \beta_4^{1440F} \) cells but very rarely in \( \beta_4^{1422F/1440F} \) keratinocytes (arrows in panels A–F). Thus, the cellular distribution of HD/1/plectin was very similar in \( \beta_4 \)-null cells (Fig. 3, panel D) and in \( \beta_4^{1422F/1440F} \) keratinocytes (panel F).
phenylalanine substitutions at Tyr-1422 and Tyr-1440 (23). These more recent findings suggest the alternative hypothesis that the hydroxyl groups of Tyr-1422 and Tyr-1440 may be necessary for interaction with HD components such as, for instance, BP180 (22). In this model, phosphorylation of the two tyrosines may have a similar or even larger effect than their substitution to phenylalanine. Finally, there is evidence suggesting that the C-terminal tail of $\beta_4$ folds and binds intramolecularly to a 321 amino acid segment that includes the first pair of type III FN-like repeats and part of the CS (22, 43).

Since it has been speculated that this intramolecular bond may be disrupted to allow for association of $\beta_4$ with HD/plec汀 and/or BP180, it is possible that substitutions of the two tyrosines with phenylalanine interfere with this postulated conformational change. Future studies will distinguish among these possibilities.

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