EGF-induced MAPK Signaling Inhibits Hemidesmosome Formation through Phosphorylation of the Integrin β4

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Migration of keratinocytes requires a regulated and dynamic turnover of hemidesmosomes (HDs). We and others have previously identified three serine residues on the integrin β4 cytoplasmic domain that play a critical role in the regulation of HD disassembly. In this study we show that only two of these residues (Ser-1356 and Ser-1364) are phosphorylated in keratinocytes after stimulation with either PMA or EGF. Furthermore, in direct contrast to previous studies performed in vitro, we found that the PMA- and EGF-stimulated phosphorylation of β4 is not mediated by PKC, but by ERK1/2 and its downstream effector kinase p90RSK1/2. EGF-stimulated phosphorylation of β4 increased keratinocyte migration, and reduced the number of stable HDs. Furthermore, mutation of the two serines in β4 to phospho-mimicking aspartic acid decreased its interaction with the cytoskeletal linker protein plectin, as well as the strength of α6β4-mediated adhesion to laminin-332. During mitotic cell rounding, when the overall cell-substrate area is decreased and the number of HDs is reduced, β4 was only phosphorylated on Ser-1356 by a distinct, yet unidentified, kinase. Collectively, these data demonstrate an important role of β4 phosphorylation on residues Ser-1356 and Ser-1364 in the formation and/or stability of HDs.

Hemidesmosomes (HDs) are specialized junctional complexes that mediate firm adhesion of epithelial cells to the underlying basement membrane. Two types of HDs have been characterized: type I and II (1). Type I (classical) HDs are present in squamous and complex epithelia, such as the skin and the bladder. They contain integrin α6β4, plectin, the bivalent pempophilid antigens 180 (BP180) and 230 (BP230), and the tetraspanin CD151 (2). Type II HDs lack BP180 or BP230 and are present in simple epithelia, such as the intestine (3). As the integrin α6β4 binds to Ln-332 in the extracellular matrix (ECM) and associates intracellularly with plectin, which in turn interacts with the keratin filament system, a protein complex is formed that protects the cell against mechanical stress. The importance of this linkage for epidermal-dermal cohesion is substantiated by the finding that in both humans and genetically modified mice, mutations in the genes for these proteins that either prevent their expression or function, result in a skin blistering disorder known as epidermolysis bullosa (2, 4).

The primary interaction between plectin and β4 occurs through the first pair of fibronectin type III (FnIII) domains and a small part of the connecting segment (CS) of β4 and the actin binding domain of plectin (plectin-ABD) (5–7). Indeed, mice carrying a specific deletion of the C-terminal portion of the β4 cytoplasmic domain, which still contains the plectin-ABD binding site, can still form normal HDs (8). However, binding of β4 to the plectin-ABD is stabilized by adjacent binding sites in the CS and the C-tail of the β4 subunit that interact with the plakin domain of plectin (9, 10). In type I HDs, the interaction of β4 with plectin is further reinforced through additional interactions with BP180 and BP230 (11). As a result, type I HDs are believed to be less dynamic and more stable than type II HDs. Whereas type I HDs mediate firm adhesion of the epidermis to the underlying basement membrane, the presence of type II HDs in migrating intestinal epithelial cells suggests that these structures are dynamically regulated. One factor implicated in the regulation of type II HD stability is the epidermal growth factor (EGF) (12). EGF is one of many cytokines produced during wound healing, stimulating both keratinocyte proliferation and migration (13). Whether EGF also regulates type I HDs has not been investigated.

Previous studies have shown that activation of pathways downstream of the EGF receptor (EGFR) or protein kinase C (PKC) result in phosphorylation of three serines (Ser-1356, Ser-1360, and Ser-1364) located within the CS of β4 (12, 14). Substitution of the serines by phospho-mimicking aspartic acid residues destabilized the interaction between β4 and plectin and partially prevented the assembly of HDs (14). On the contrary, substitution of the serines by phosphorylation-resistant alanines resulted in a more stable association between β4 and plectin. PKC-dependent phosphorylation of the β4 cytoplasmic tail was also observed in keratinocytes stimulated with macrophage stimulating protein (MSP), and was suggested to create a binding site for 14-3-3 proteins (15).

Although it has been suggested that at least two of the aforementioned serines are substrates for PKCα phosphorylation downstream of EGFR, bioinformatic analysis showed that only Ser-1360 is part of a consensus sequence for PKC (pSXK/R). Furthermore, this consensus sequence is not evolutionarily conserved, unlike the three serine residues (16). This raised the question of whether phosphorylation of these residues down-
stream of EGFR is directly dependent on phosphorylation by PKCa. Therefore, we decided to reinvestigate the phosphorylation of residues downstream of the EGFR and PKC and determine their role in HD regulation in more detail.

Our results show that EGFR and PKC activation leads to phosphorylation of the β4 subunit on Ser-1356 and Ser-1364 in keratinocytes. Furthermore, we present evidence that ERK1/2 and p90RSK1 phosphorylate β4 at these sites, resulting in a destabilization of the binding of β4 to plectin, a reduction in the number of type I and type II HDs formed and in α6β4-mediated strength of adhesion, while it leads to an increased migration speed. Finally, we demonstrate that β4 is phosphorylated on Ser-1356 during mitosis by an as yet unidentified kinase.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal rabbit antibodies specific for the phosphorylated residues Ser-1356, Ser-1360, and Ser-1364 on β4 were raised against a synthetic peptide with the sequence SCDDVLRSPPGSQRPSVSDD containing phosphate group on one of the serine residues shown in italics. The three synthetic peptides were conjugated to maleimide-activated mckLH (Pierce) and injected into rabbits. The rabbits received a booster immunization every 4 weeks and antisera were collected 1 week after the third booster. To prevent non-phosphospecific recognition of β4 by the antibody in immunoblotting, the antibodies were used in combination with 10 μM of the synthetic peptide without phosphate groups. The following anti-integrin monoclonal antibodies (mAbs) were used: anti-α2 (10G11), anti-α3 (J143), anti-α6 (8H4), anti-β1 (TS2/16), anti-β4 (450–9D or 450–11A). Antibodies against phospho ERK1/2 (T202/Y220; clone E10), phospho-p38 MAPK (116p38 MAPK (T180/Y182; clone 12F8), phospho-p90RSK1 (T359/S363), Akt (9272), phospho-Akt (S473), phospho-VASP (S157), and phospho-pan PKC (γT514) were purchased from Cell Signaling (Beverly, MA), and ERK2 (clone 23) from BD Bioscience (San Jose, CA). Human mAb 1D8 against β4 was kindly provided by Dr. T. Hashimoto (Keio University, Tokyo, Japan). Polyclonal antibodies against β1 (U21E) were obtained from Dr. U. Mayer (University of East Anglia, Norwich, UK). Other antibodies were anti-plectin (clone 31), α-tubulin (clone B-5–1–2, from Sigma-Aldrich), and anti-cyclin A and -B (from Santa Cruz Biotechnology, Santa Cruz, CA). The rabbit polyclonal antibody against the first pair of FNIII repeats (residues 1115–1355) of the integrin β4 subunit was generated as described previously (14). HRP-conjugated secondary antibodies were purchased from GE Healthcare (UK), TexasRed-conjugated goat anti-rabbit and FITC-conjugated goat anti-human were from Invitrogen, Cy5-conjugated donkey anti-mouse was from Jackson IR or goat anti-mouse antibody (M1204) from Sanquin (Amsterdam, The Netherlands).

**Cell Culture**—β4-deficient PA-JEB keratinocytes were cultured in keratinocyte serum-free medium (SFM; Invitrogen, Rockville, MD) supplemented with 50 μg/ml bovine pituitary gland extract, 5 ng/ml EGF, 100 units/ml penicillin and 100 units/ml streptomycin, as previously described (6, 17). PA-JEB/β4 keratinocytes were obtained by retroviral infection, as described previously (18, 19). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

**cDNA Constructs**—The generation of full-length β4 cDNA has been described previously (5). Single and double mutants of β4 were created by site directed mutagenesis using the PCR-based overlap extension method and Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). Wild-type and mutant β4 cDNA was cloned into the pcDNA3 vector (Invitrogen) with the BssH2/NotI restriction sites and subsequently into the retroviral vector LZRS-MS-IRES-ZEO with the EcoRI restriction sites (6, 20). The plectin-1A ABD in the pcDNA3-HA vector has been described previously (10, 21).

**Flow Cytometry**—Expression of wild-type and mutant integrins in PA-JEB keratinocytes was analyzed by flow cytometry using specific monoclonal antibodies and FITC conjugated secondary antibodies. Cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**Western Blotting and Co-immunoprecipitation Assays**—PA-JEB/β4 keratinocytes were starved in growth factor-free keratinocyte-SFM. Following pretreatment with the kinase inhibitors Gö6983 (100 μM, Calbiochem, San Diego, CA), BI-D1870 (10 μM; University of Dundee, Dundee, UK), U0126 (10 μM), PD98059 (20 μM), or SB203580 (10 μM) for 1 h, cells were incubated with or without 50 ng/ml EGF (Sigma-Aldrich), 100 ng/ml PMA (Sigma-Aldrich), 200 μM Sorbitol, or 25 μM forskolin (FSK; Calbiochem) and 100 nM 3-isobutyl-1-methylxanthine (IBMX; Calbiochem). Cells were lysed in radio immunoprecipitation assay (RIPA) buffer and cleared by centrifugation at 20,000 × g for 60 min at 4 °C. Proteins were separated on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp., Billerica, MA) and incubated with antibodies.

For the co-immunoprecipitation assays, COS-7 cells were co-transfected with the indicated cDNAs by using the DEAE-dextran method (22). Cells were lysed in MPER (Mammalian Protein Extraction Reagent, Pierce) supplemented with 0.1%Nonidet P-40 and a mixture of protease inhibitors (Sigma-Aldrich). After clearing by centrifugation, the lysates were incubated with either 2.5 μg of purified mAb 450–11A to precipitate β4 or 2.5 μg TS2/16 to precipitate β1, followed by an incubation for 4 h with GammaBind G-Sepharose (Amersham Biosciences). The immunoblots were analyzed using polyclonal antibodies against HA, β4, or β1, and secondary antibodies linked to horseradish peroxidase (HRP) (GE Healthcare, UK). Signals were visualized by chemiluminescence (GE Healthcare, UK).

**Adhesion Strengthening Assay**—PA-JEB/β4 keratinocytes expressing either S1356A/S1364A or S1356D/S1364D were, respectively, labeled with 10 μM Cell Tracker (TM) Orange CMTMR and Green CMFDA from Invitrogen for 30 min at 37 °C, seeded on coverslips coated with Ln-332-rich Rac-11P matrix in a 1:1 ratio, and after culturing overnight in serum-free medium, spun in PBS containing 1 mM MgCl2, 2 mM CaCl2, and...
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2.5% dextran (average mol wt 425,000–575,000; Sigma-Aldrich) using a spinning disc device built after Boettiger (23). Cover glasses were imaged on an AxioObserver Z1 CCD microscope equipped with a 5x/0.15 Plan-Neofluar objective and a Hamamatsu ORCA-ER camera. Adherent fractions were calculated as a function of applied shear stress using ImageJ and SigmaPlot (Systat Software Inc.).

Cell Cycle Analysis—To synchronize PA-JEB/β4 keratinocytes in the G0/G1 phase of the cell cycle, they were starved overnight in growth factor-free medium, and then cultured in complete medium. After 15 h, the cells were treated with 250 ng/ml nocodazole for 4.5 h to arrest them at the G2/M transition. Mitotic (M) cells were collected by mechanical shake off. G2-enriched cells were obtained from the cells that remained attached to the flask. After washing, a portion of the mitotically selected cells were plated in fresh medium for 2.5 h to progress into the G1 phase. Cell lysates were prepared at the different time points after the addition of complete medium and nocodazole and analyzed by immunoblotting. Cell synchronization was evaluated by monitoring the expression of cyclin A and B1, whose expression peaks in the S/G2 phase and at the G2/M transition of the cell cycle.

Immunofluorescence—PA-JEB/β4 keratinocytes were seeded on glass coverslips and starved for 18 h before treatment with or without EGF (50 ng/ml) for 1 h. The cells were fixed in 1% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100 for 5 min. Cells were blocked with PBS containing 2% BSA for 1 h and incubated with the primary antibodies for 45 min. Cells were washed three times before incubation with the secondary antibody. After three wash-steps with PBS, the coverslips were mounted onto glass slides in Mowiol-DAPCO and studied by using a confocal microscope Sp2/AOBS (Leica, Mannheim, Germany). The sequentially acquired images were analyzed with the image processing program ImageJ. The colocalization of β4, plectin and BP230 in HDs was calculated from two 8-bit images in which the overlapping pixels, with an intensity of 50% and a ratio of 50%-<, were highlighted. The percentage of HD1 represents the ratio of co-localization of β4 and BP230 (type I HDs) and of β4 and plectin (type I and II HDs). To exclude pixel overlap by unspecific events generated by background noise, the ratio of co-localization of plectin and BP230 (type HD) and of β4 and BP230 (type I HD) was determined.

Fluorescence Recovery after Photobleaching—Fluorescence recovery after photobleaching (FRAP) experiments were performed with a Leica TCS SP2 confocal microscope (Leica, Mannheim, Germany). Clusters of HDs of PA-JEB/β4-EGFP keratinocytes were bleached using an Argon/Krypton laser for 2 s at maximal laser power. Recovery of fluorescence in the bleached region was analyzed from images collected every 15 s for 10 min with a low laser power (20%). The fluorescence intensity was corrected for the background intensity outside the cell and normalized to the fluorescence intensity of a non-bleached region containing HDs.

Cell Migration Assays—For the wound-scratch assays, PA-JEB/β4 keratinocytes were grown to confluency in 24-well plates coated with 10 μg/ml collagen-I (PureCol, Inamed Bio-materials, CA). After starvation in keratinocyte-SFM, a wound was introduced by scraping the monolayer with a 200 μl pipette tip, followed by two washes with PBS to remove cell debris. PA-JEB/β4 keratinocytes were treated with EGF (50 ng/ml) and cell migration was observed at three positions along the scratch by live cell imaging. Images were acquired every 5 min for 24 h using an AxioCam MRm Rev.3 camera equipped with a Zeiss Axiovert 200 m inverted microscope. The images were analyzed by using the image processing program ImageJ and Matlab (Mathworks). In the scratch assays, wound closure is defined as the area closed per second. The data shown represent the mean ± S.E. of three independent experiments performed in triplicate.

For the single cell migration assay, keratinocytes were sparsely seeded on laminin-332 rich Rac-1P matrices blocked with 0.5% BSA, serum-starved over night and transferred on a Zeiss Axiovert 200 m microscope at 37°C and 5%CO2. Images were captured every 6–8 min using a 10 × 0.5 NA Plan objective with a Zeiss Axiocam camera. G06983 (100 nM), U0126 (100 nM) and EGF (50 ng/ml) were added at the indicated time points to inhibit PKC and MEK1/2 or stimulate migration, respectively. Cell tracks were automatically determined and quantified using polytrack (24) on Matlab (Mathworks). The graphs depict the average velocity over time (sliding average = 9) of 200–300 cells ± S.E.

Statistics—Data were analyzed using a non-parametric t test (Mann-Whitney) in which p < 0.05 was considered statistically significant. Calculations were performed using Prism 3.0 GraphPad software (San Diego, CA).

RESULTS

PMA and EGF Induce the Phosphorylation of β4 on Ser-1356 and Ser-1364 in Keratinocytes—To obtain further insight into the role of β4 phosphorylation in the regulation of HDs, we produced polyclonal antibodies specific for the individual phosphorylated Ser-1356, Ser-1360, and Ser-1364 residues. However, only those antibodies that were specific for the phosphorylated residues Ser-1356 and Ser-1364 reacted with β4 in lysates of EGF- and PMA-stimulated PA-JEB cells that lack integrin α6β4 expression. As expected, p38 MAPK and ERK1/2 phosphorylation increased after EGF or PMA stimulation of PA-JEB/β4 keratinocytes (Fig. 1A), suggesting that Ser-1360 is not phosphorylated after treatment with these agents. The polyclonal antibodies were specific for phosphorylated Ser-1356 and Ser-1364, as they did not react with Ser-1356 phosphorylated residues when Ser-1356 and Ser-1364 reacted with β4 in lysates of EGF- and PMA-stimulated PA-JEB/β4 keratinocytes (Fig. 1A), suggesting that Ser-1360 is not phosphorylated after treatment with these agents. The polyclonal antibodies were specific for phosphorylated Ser-1356 and Ser-1364, as they did not react with these residues were substituted to alanine (supplemental Fig. S1). Furthermore, besides a reaction of the phosphospecific antibody against Ser-1364 with another unidentified protein, no reactivity was observed with PA-JEB cells that lack integrin α6β4 expression. As expected, p38 MAPK and ERK1/2 phosphorylation increased after EGF or PMA stimulation of PA-JEB/β4 keratinocytes. In contrast, only PMA-treatment led to a reduction in Akt phosphorylation.

Kinetic studies show that after 10 min of stimulation with PMA or EGF, the β4 subunit was readily phosphorylated on both Ser-1356 and Ser-1364 and that this phosphorylation was sustained for 1–2 h after EGF and for up to 4 h after PMA-treatment (Fig. 1B). The difference in phosphorylation times likely reflects different kinetics of EGF receptor and PKC down-regulation. We conclude that of the three serines located within the CS of β4 only Ser-1356 and Ser-1364 are phosphorylated in PA-JEB/β4 keratinocytes.
EGF-induced Phosphorylation of β4 Is PKC Independent—Previous studies suggested that EGF stimulates a PKCα-dependent pathway that results in the phosphorylation of β4 on serine residues, and in its redistribution to actin-rich structures (12, 25). To investigate whether the EGF-induced phosphorylation of β4 in keratinocytes depends on PKC, we treated PA-JEB/β4 cells with the PKC inhibitor G66983 prior to and during their stimulation with EGF. Pretreatment with the inhibitor completely prevented the PMA-induced phosphorylation of β4 on both Ser-1356 and Ser-1364, while it has only a minor effect on the phosphorylation induced by EGF (Fig. 2). Moreover, down-regulation of PKC by prolonged treatment with PMA prevented the phosphorylation of β4 by PMA, but not that by EGF. Both PMA and EGF stimulated the phosphorylation of ERK1/2 in PA-JEB/β4 keratinocytes. However, as was observed with the phosphorylation of β4, PKC activity was not required for EGF to stimulate phosphorylation of ERK1/2, consistent with previously published reports (26). These data indicate that PKC and EGF independently induce MAPK signaling and phosphorylation of β4 in PA-JEB/β4 keratinocytes.

PMA- and EGF-stimulated Phosphorylation of β4 Is Not Mediated by PKA and p38 MAPK—We have previously shown that Ser-1364 on the integrin β4 subunit is a PKA phosphorylation site in vivo (14). To investigate whether PKA activation can result in the phosphorylation of Ser-1364 downstream of PMA and EGF treatment, we compared the phosphorylation of VASP, a known substrate of PKA with that of β4 (27). Consistent with previous findings, stimulation of PA-JEB/β4 keratinocytes with forskolin/IBMX resulted in the phosphorylation of VASP, as well as that of Ser-1364, but not of Ser-1356 (Fig. 3). However, as judged by the absence of VASP phosphorylation after EGF or PMA treatment, PKA is not activated downstream of the EGFR or PKC.

Because PKC and EGFR activation result in the up-regulation of p38MAPK activity and Ser-1356 is localized in a consensus site for this proline-directed kinase, we treated the cells with sorbitol, a known p38MAPK activator (28). Although a strong activation of p38MAPK occurred, it did not cause an increased phosphorylation of either Ser-1356 or Ser-1364. In fact, phosphorylation of Ser-1364 was below the basal levels usually observed in the absence of exogenous growth factor (Fig. 3). This may point to the activation of a phosphatase downstream of p38MAPK that specifically de-phosphorylates Ser-1364. We conclude that neither PKA nor p38MAPK are involved in the phosphorylation of Ser-1356 and Ser-1364 downstream of the EGFR or PKC.

PMA- and EGF-induced Phosphorylation of β4 Depends on ERK1/2 Activation—Next we investigated whether ERK1/2 kinases are involved in the phosphorylation of β4 downstream of EGFR and PKC activation. These kinases are activated in EGFR and PKC signaling pathways and are proline-directed kinases similar to p38MAPK. PA-JEB/β4 keratinocytes were treated with two inhibitors (U0126 and PD98059) of the upstream kinases MEK1/2 which are known to phosphorylate and activate ERK1/2. As a control, and to further substantiate our finding that p38MAPK (α and β) does not mediate the phosphorylation of Ser-1356, we also treated the cells with the p38MAPK inhibitor SB203580. Incubation with SB203580
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PMA- and EGF-induced Phosphorylation of β4 on Ser-1364 Depends on p90RSK Activation—Although the data thus far indicated that β4 could be directly phosphorylated by ERK1/2, a downstream effector kinase of ERK1/2 might also be involved in the phosphorylation of Ser-1356 or Ser-1364. P90RSK is a multifunctional ERK effector that participates in the regulation of diverse cellular processes and recognizes a consensus sequence (RRXpS or RXRXpS), which closely resembles the sequence in which Ser-1364 resides (30, 31). To investigate if p90RSK is involved in the phosphorylation of Ser-1364, PA-JEB/β4 keratinocytes were stimulated with PMA or EGF in the presence of the p90RSK inhibitor BI-D1870 (32). Inhibition of p90RSK activity prevented the phosphorylation of Ser-1364, and had little effect on the phosphorylation of Ser-1356 (Fig. 5A).

To substantiate this notion, we overexpressed β4 with either wild-type or dominant-negative p90RSK1/2 in COS-7 cells. In contrast to our findings with ERK1/2, overexpression of p90RSK1/2 by itself induced phosphorylation of Ser-1364 (Fig. 5B). Stimulation of the cells with either PMA or EGF increased Ser-1364 phosphorylation, while expression of a dominant-negative p90RSK1/2 prevented PMA- and EGF-induced phosphorylation of Ser-1364 by endogenously activated p90RSK (Fig. 5B and data not shown). Interestingly, expression of both p90RSK1/2 and dominant-negative p90RSK1/2 decreased PMA- and EGF-induced phosphorylation of Ser-1356, suggesting that they may compete with β4 for phosphorylation by ERK1/2. In conclusion, we identified ERK1/2 and p90RSK1/2 as kinases that mediate phosphorylation of Ser-1356 and Ser-1364, respectively, downstream of PKC and EGFR.

Substitution of Ser-1356 and Ser-1364 by Phospho-mimicking Aspartic Acid Prevents the Binding of β4 to the Plectin-ABD Resulting in Defective Adhesion to Ln-332—To investigate whether phosphorylation of Ser-1356 and Ser-1364 modulates the binding of β4 to plectin, these residues were mutated to either phosphorylation-resistant alanines or phospho-mimicking aspartic acids. Interaction between mutant β4 subunits and plectin-ABD was assessed by co-immunoprecipitation assay from lysates of COS-7 cells co-transfected with the respective expression constructs. In agreement with previously reported findings, mutation of Ser-1356 and Ser-1364 to aspartic acids reduced binding to β4 (Fig. 6A) (14). In fact, binding of plectin-
we studied their phosphorylation levels at different times during the cell cycle. Keratinocytes were synchronized at the G0 phase by growth factor deprivation for 18 h and then cultured for 15 h in complete medium (G1 phase) before they were treated with nocodazole for 3.5 h to enrich the content of cells at the G2/M phase. Additionally, mitotic cells were collected by a shake-off. A proportion of these cells were directly lysed, while the rest were re-plated in complete medium to allow them to re-enter the G1 phase. Addition of complete medium transiently increased phosphorylation levels of Ser-1356 and Ser-1364 before returning to basal levels after 15 h (Fig. 7). However, phosphorylation of Ser-1356 specifically increased in both nocodazole mediated G2/M phase arrested cells and the mitotic cell population obtained by shake-off. After replating mitotic cells, levels of Ser-1356 phosphorylation decreased to basal levels (Fig. 7). Because ERK1/2 is not activated during mitosis, phosphorylation of Ser-1356 must have been mediated by another kinase, most likely the proline-directed cyclin-dependent kinase 1 (CDK1), which

**FIGURE 4. PMA- and EGF-stimulated phosphorylation of β4 is dependent on ERK1/2 activation.** A, PA-JEB/β4 keratinocytes, starved overnight in growth factor-free medium and pretreated for 1 h with U0126, PD98059 or SB203580, were left unstimulated or stimulated with PMA or EGF for 10 min. Cells lysates were analyzed by immunoblotting with antibodies specific to phosphorylated β4 (Ser-1356 and Ser-1364), total β4, phospho-ERK1/2, and total ERK1/2 (8 COS-7 cells, transiently expressing β4 alone or together with ERK1 or a dominant-negative version of ERK1, were starved overnight in growth factor-free medium, and then left unstimulated or stimulated with EGF for 10 min. Phosphorylated β4 (Ser-1356 and Ser-1364), total β4, phospho-ERK1/2 and total ERK1/2 were detected by immunoblotting. B, PA-JEB keratinocytes, stably expressing wild-type β4 (FAPP) or β4 with a mutated ERK docking site (AAAP), were starved overnight in growth factor-free medium, and then left unstimulated or stimulated with EGF or PMA for 10 min. Phosphorylated β4 (Ser-1356 and Ser-1364), total β4, phospho-ERK1/2, and total ERK1/2 were detected by immunoblotting. The graphs show the fold increase in phosphorylation of Ser-1356 and Ser-1364 after EGF or PMA stimulation. The intensity of the bands corresponding to phosphorylated β4 were related to that of total β4 using ImageJ.

**ABD to β4** was reduced comparably to that of β4, a mutant that is unable to recruit plectin into HDs (6, 20). On the contrary, substitution of the two serines by alanine seemed to slightly increase the binding of β4 to the plectin-ABD. No significant interaction was seen between plectin-ABD and β1 integrin, indicating that plectin-ABD binding to β4 was specific.

Because cellular adhesion to the ECM is influenced by the interaction of adhesion receptors with cytoskeletal elements, we measured the effect of β4 phosphorylation on the adhesion strength of keratinocytes to Ln-332. Using a spinning disc device (23) we subjected PA-JEB/β4, and PA-JEB/β4 keratinocytes to a variety of shear stress and measured the total amount of cellular detachment. We found that PA-JEB/β4 keratinocytes adhered significantly more strongly to Ln-332 than cells expressing the phosphomimics (Fig. 6B). The results shown indicate that phosphorylation of Ser-1356 and Ser-1364 can potentially modulate the interaction between β4 and plectin and weaken cellular strength of adhesion.

**β4 Is Phosphorylated on Ser-1356 during Mitosis**—During mitosis, when keratinocytes undergo cellular rounding and adherence to their substrate is reduced, HDs are partially disassembled (19). To investigate whether HD disassembly is accompanied by the phosphorylation of Ser-1356 and Ser-1364, at this phase of the cell cycle becomes highly active. We conclude that during mitosis β4 is phosphorylated on Ser-1356 but not on Ser-1364.

**Phosphorylation of Ser-1356 and Ser-1364 Partially Prevents the Assembly of Type I and II HDs**—Previous studies have shown that substitution with aspartic acid of Ser-1356, Ser-1360, and Ser-1364 in β4 compromise the formation of HDs in PA-JEB/β4 keratinocytes (14). The findings here indicate that only two of these serines (Ser-1356 and Ser-1364) are phosphorylated upon EGF and PMA stimulation. This prompted us to investigate whether substitutions by alanines or aspartic acids affected the formation of HDs. First, we studied the effects of these mutations on the formation of all HDs (i.e. type I and II) by examining and comparing the subcellular distribution of both β4 and plectin in PA-JEB keratinocytes that stably express β4 or β4 using double immunofluorescence microscopy. For comparison, we included PA-JEB keratinocytes expressing wild-type β4 and deprived of growth factors to minimize β4-phosphorylation. As shown in Fig. 8A, all three keratinocyte cell lines were able to form HDs. Scatter plot analysis revealed that less β4 co-localized with plectin in the PA-JEB keratinocytes expressing β4 compared with those expressing wild-type β4 or β4. These results suggest that phosphorylation of Ser-1356 and
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Ser-1364 leads to a partial reduction in the formation of HDs in keratinocytes.

We next determined the effect of phosphorylation on the formation of type I HDs. Cells were stained for β4 and plectin, as well as for BP230, which served as a marker for type I HDs. Clusters of HDs that contain all three proteins were analyzed and the percentage of type I HDs was determined by dividing the colocalized signals of β4 and BP230 (type I HDs) by those of β4 and plectin (type I and II HDs). Growth factor starved PA-JEB/β4 keratinocytes contained ~80% type I HDs (Fig. 8, B and C). This percentage was significantly lower in cells expressing β4S1356A/S1364A, and slightly higher in the β4S1356D/S1364D-expressing PA-JEB keratinocytes. As a control, we determined the ratio of co-localization between that of β4 and BP230 and of plectin and BP230. Ideally, this value should be 1, which was indeed the case (Fig. 8C). Thus, under conditions in which the phosphorylation of β4 is minimized, the number of type I HDs in keratinocytes containing wild-type β4 is comparable to those expressing β4S1356A/S1364A. When treated with EGF, the percentage of type I HDs in the wild-type β4 expressing PA-JEB keratinocytes, but not in those expressing the mutant β4 subunits decreased from 80 to 65%. Therefore, the percentage of type I HDs in the keratinocytes with wild-type β4 became significantly lower than that in the β4S1356A/S1364A-expressing keratinocytes and approached the percentage found in the β4S1356D/S1364D-expressing cells (Fig. 8, B and C). Similar to what was observed in the untreated cells, the ratio of β4 and BP230 co-localization to that of plectin and BP230 co-localization was close to 1 (Fig. 8C).

To further substantiate the role of EGF-induced phosphorylation in the regulation of type I and II HDs, we generated PA-JEB cells expressing GFP-tagged wild-type β4 and compared the β4 dynamics before and after EGF stimulation using fluorescence recovery after photobleaching (FRAP) (Fig. 8, D and E). Under starved conditions β4 showed two distinct dynamic behaviors in HD clusters: it either recovers relatively slowly or much faster. These different recovery rates were likely represented by differences in the dynamic behavior of β4 within type I and II HDs, since in type I HDs, β4 is associated with BP180 and BP230 in addition to plectin. Indeed, staining of the clusters of HDs for both plectin and BP230 after FRAP analysis, revealed that the two recovery populations of GFP-tagged β4 represent those in type I and II HDs (supplemental Fig. S4). After EGF stimulation, the recovery rates for β4 ranged from slow to very fast. The β4 molecules with the fastest mobility may represent those molecules that were clustered by laminin-332 (Ln-332) but were not associated with plectin. The recovery rate of this latter population is similar to that observed for GFP-tagged β4R1281W (19). FRAP analysis of GFP-tagged β4S1356A/S1364A and β4S1356D/S1364D showed comparable dynamic behaviors as wild-type β4 before and after EGF stimulation, respectively. These data suggest that phosphorylation of β4 does not change the dynamics of HDs already containing β4, but likely interferes with the formation of new HDs.

Phosphorylation of Ser-1356 and Ser-1364 Regulates Keratinocyte Migration—We have previously shown that binding of β4 to plectin reduced migration of PA-JEB/β4 keratinocytes (19). Because phosphorylation of Ser-1356 and Ser-1364 diminished the number of HDs and decreased the strength of α6β4-mediated adhesion to Ln-332, we wanted to determine if there was a functional consequence on cell migration using a wound-scratch assay. PA-JEB/β4 keratinocytes grown to confluency on collagen-I and deprived of growth factors for 1 day, were scrape-wounded and then stimulated with EGF. In the presence of EGF, PA-JEB/β4 cells expressing wild-type β4 migrated nearly as fast as those expressing the β4R1281W (plectin-binding deficient) mutant. Interestingly, substitution of Ser-1356 and Ser-1364 by alanine reduced cell migration by 30%, whereas the substitution by aspartic acid had an intermediate effect (Fig. 9, A and B). As a control, integrin-subunit expression profiles and cell proliferating capacity were similar for each of the PA-JEB/β4 cell lines used (supplemental Fig. S2).
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To directly assess the influence of Ser-1356/1364 phosphorylation of β4 on the velocity of migrating keratinocytes, we carried out single cell migration assays on Ln-332-rich matrices. The velocity of PA-JEB/β4S1356A/S1364A keratinocytes under starved conditions was highest, compared with cells expressing either β4WT or β4S1356A/S1364A. After the addition of EGF, however, the migration rate of PA-JEB/β4WT keratinocytes increased considerably more strongly than that of PA-JEB/β4S1356A/S1364A. In fact, PA-JEB keratinocytes expressing β4WT migrated at a similar velocity as those expressing β4S1356A/S1364A with PA-JEB/β4S1356A/S1364A keratinocytes migrating ~15% more slowly (Fig. 9C). The described increase in velocity after stimulation with EGF can at least in part be attributed to the activity of MEK1/2, since pretreatment with 10 μM U0126 slowed down basal cell migration and completely abrogated the characteristic EGF response. Inhibiting PKC directly phosphorylated by PKCα by *in vitro* analysis. As we previously mentioned, the only serine of these three residues present in a PKC phosphorylation consensus site is Ser-1360; however, in this study we were unable to obtain evidence that β4 is phosphorylated on Ser-1360 downstream of the EGFR. Furthermore, we provide evidence that the phosphorylation of Ser-1356 and Ser-1364 leads to a reduced number of type I and II HDs in keratinocytes by hindering the association between β4 and the plectin-ABD. Furthermore, we present data that suggest that phosphorylation of these two serine residues downstream of the EGFR decreased α6β4-Ln332-mediated adhesion strength, whereas it increased cell migration. Finally, β4 phosphorylation at Ser-1356 was shown to take place during mitosis.

Rabinovitz et al. (12) have previously reported that β4 is phosphorylated on three serine residues, Ser-1356, Ser-1360, and Ser-1364 after EGFR activation and suggest that at least two of these residues are phosphorylated by PKCα by *in vitro* analysis. As we previously mentioned, the only serine of these three residues present in a PKC phosphorylation consensus site is Ser-1360; however, in this study we were unable to obtain evidence that β4 is phosphorylated on Ser-1360 downstream of the EGFR. Furthermore, we provide evidence that the phosphorylation of Ser-1356 and Ser-1364 downstream of the EGFR is PKC-independent. Additionally, we were also unable to detect phosphorylation of Ser-1360 after PMA addition in these studies, contrary to our previously published results obtained from *in vivo* phosphopeptide mapping experiments (14). We are unable to definitively explain this discrepancy except that we now believe that the reduced phosphorylation of β4 in the phosphopeptide experiment was due to a low expression level of S1360A mutant subunit. Consistent with this revised conclusion, it is of note that in the original mapping experiments mutation of Ser-1360...
resulted in the loss of two phosphopeptides, and not just of one as would have been expected. These considerations, combined with the consistent non-reactivity of our phosphospecific antibodies against Ser-1360 with phosphorylated β4, led us to modify our initial conclusion that Ser-1360 is phosphorylated by PKC. We now conclude that this residue is not phosphorylated in keratinocytes stimulated with either PMA or EGF. Our data, however, do not rule out the possibility that Ser-1360 becomes phosphorylated after treatment with other stimuli (e.g. growth factors and cytokines that are released during wound healing).

Several reports document the activation of PKC downstream of EGFR activation. Rabinovitz et al. (12) argued that the activation of a PKCα-dependent pathway downstream of the EGFR is responsible for the phosphorylation of the β4 subunit. However, this conclusion was based on in vitro phoshopeptide mapping experiments. We have been unable to verify that any of the three aforementioned serine residues are phosphorylated by PKC in vitro, and in fact, we have even witnessed the non-specific phosphorylation of extracellular β4 peptides in these assays (data not shown). Moreover, a role of PKC in the phosphorylation of either Ser-1356 or Ser-1364 downstream of the EGFR could be excluded by the fact that in the presence of the PKC inhibitor G6983, as well as after depletion of PKC by overnight incubation with PMA, EGF was still able to induce the phosphorylation of β4 on these residues. It is interesting to note that the pan-pPKC (βII Ser-660) antibody we used in our previous studies to monitor PKC activation downstream of the EGFR is directed against the phosphorylated serine residue in the hydrophobic loop (bulky ring motif, FXXXSF) of PKCβII (14). This epitope also happens to be present in the C-terminal kinase of p90RSK1/2 and therefore, it most likely recognizes activated p90RSK1/2 kinase as well.

In line with previous work, we confirmed that PKA may phosphorylate β4 at Ser-1364 in vivo (14). However, we found no evidence that this kinase is activated downstream of PKC or the EGFR. Therefore, it is not likely to be responsible for the phosphorylation of Ser-1364 in cells that have been stimulated with either PMA or EGF. In fact, our data clearly show that two different kinase classes, ERK1/2 and p90RSK1/2, phosphorylate β4 on Ser-1356 and Ser-1364, respectively. There is precedent in the literature for the phosphorylation of proteins by both ERK1/2 and p90RSK1/2, including that of c-Fos and LKB1 (33, 34). It has been suggested that the cooperation between ERK1/2 and p90RSK1/2 in protein regulation serves to ensure that they are selectively activated by the ERK/RSK signaling pathway and not by another kinase that has a substrate specificity overlapping with that of either ERK1/2 or p90RSK1/2 (35).

CDK1 recognizes the same phosphorylation motif as ERK1/2 (36). This kinase is active at the transition of G2 to M during mitosis and therefore might well be the kinase that phosphorylates β4 at Ser-1356 at this stage of the cell cycle. We previously have shown that the phosphorylation of only one serine residue in the CS is not sufficient to prevent the interaction between β4 and plectin, and therefore, for the disassembly of HDs in mitotic cells other phosphorylation events might be necessary (14). In this respect, it is interesting to mention that Geramini et al. (37) have recently identified a novel phosphorylation site, Ser-1424, on the β4 subunit that plays a role in the disassembly of HDs. Although this site is constitutively phosphorylated and is modestly intensified after EGF stimulation, we do not believe that it is involved in the EGF- or PMA-mediated regulation of HDs since our previous phosphopeptide mapping experiments show that only the two phosphopeptides containing phospho-Ser-1356 and/or Ser-1364 increase in intensity after PMA- or EGF-stimulation of keratinocytes. However, a role of this site in the disassembly of HDs during mitosis cannot be excluded.

In a previous article, we reported that there was no difference in the migration rates of keratinocytes expressing the wild-type β4 subunit and those expressing the S1356A/S1360A/S1364A or S1356D/S1360D/S1364D triple mutants (14). Although double mutants were used in this report, we believe that the discrepancy in the results can be explained as follows. The surface expression levels of the β4 mutants were not assessed in the first report and we now have evidence suggesting they were not equivalent. Additionally, the new data were obtained using tissue culture plates pre-coated with collagen-1 and the cells were stimulated to migrate with ten times more EGF (i.e. 5 ng/ml versus 50 ng/ml). Therefore, the added effects of these differences may account for the observed differences in migration rates.

Despite the fact that PMA induces the phosphorylation of β4 at Ser-1356 and Ser-1364, it did not stimulate migration of PA-
FIGURE 8. Phosphorylation of Ser-1356 and Ser-1364 prevents the assembly of both type I and II HDs. A, PA-JEB/β4WT, PA-JEB/β4S1356A/S1364A, and PA-JEB/β4S1356D/S1364D were starved overnight in growth factor-free medium and the cells were immunolabeled with antibodies against β4 (red) and plectin (green). Co-localization is visualized in the overlay images (yellow). The scatter plots display the intensity of distribution and the degree of co-localization in the corresponding cells. In the right panels, high intensity co-localizing pixels are shown in yellow, non-colocalizing β4 pixels in red and non-colocalizing plectin pixels in green. B, PA-JEB/β4WT, PA-JEB/β4S1356A/S1364A, and PA-JEB/β4S1356D/S1364D were starved overnight, with or without EGF-stimulation for 1 h. The cells were immunolabeled with antibodies against β4 (red), plectin (green), and BP230 (blue) and their co-localization is visualized in the overlay image (white). C, percentage of type I HDs is determined by the ratio of co-localization of β4 and BP230 (type I HDs), and that of β4 and plectin (type I and II HDs). As a control, the ratio between β4 and BP230, and that of plectin and BP230 was determined, which should be 1. D, PA-JEB cells stably expressing EGFP-tagged wild-type or mutant β4 subunits were starved overnight in growth factor-free medium and left untreated or treated with EGF for 60 min. The graphs show the kinetics of recovery of fluorescence intensity over time in bleached regions containing HDs of the indicated cell lines. The fluorescence intensity in the bleached region is expressed as relative recovery. Each line represents data obtained from a different region of a cell. Note the slow, intermediate, and fast recovery of fluorescence in the different cell lines, which represents the mobility of β4 in type I (red), type II (green), mixed clusters of type I and II HDs (yellow), and β4 molecules that are not bound to plectin but are clustered by having bound to Ln-332 deposits (purple). E, representative images of PA-JEB/GFP-β4WT cells left untreated or treated with EGF are shown pre-bleach, immediately after bleach (t = 0), and post-bleach (t = 10 min).
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**D**

**PA-JEB/β4\(^{WT}\) (-EGF)**

Fractional fluorescence recovery (%)

| Time (min) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------|---|---|---|---|---|---|---|---|---|---|----|
| EGF        | 10| 20| 30| 40| 50| 60| 70| 80| 90| 100| 110|

**PA-JEB/β4\(^{WT}\) (+EGF)**

Fractional fluorescence recovery (%)

| Time (min) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------|---|---|---|---|---|---|---|---|---|---|----|
| EGF        | 10| 20| 30| 40| 50| 60| 70| 80| 90| 100| 110|

**PA-JEB/β4\(^{S1356A/S1364A}\) (-EGF)**

Fractional fluorescence recovery (%)

| Time (min) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------|---|---|---|---|---|---|---|---|---|---|----|
| EGF        | 10| 20| 30| 40| 50| 60| 70| 80| 90| 100| 110|

**PA-JEB/β4\(^{S1356D/S1364D}\) (-EGF)**

Fractional fluorescence recovery (%)

| Time (min) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------|---|---|---|---|---|---|---|---|---|---|----|
| EGF        | 10| 20| 30| 40| 50| 60| 70| 80| 90| 100| 110|

**E**

**Pre-bleach**

**Bleach**

**Post-bleach (t=10 min)**

**Type I HD**

β4 (wild-type) -EGF

**Type II HD**

β4 (wild-type) -EGF

**β4 clusters**

β4 (wild-type) +EGF

FIGURE 8 — continued
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JEB/β4 keratinocytes. This suggests that phosphorylation of β4 by itself is not sufficient to induce migration and that other signaling events, e.g., those that are activated downstream of the EGFR but not of PKC, are essential. Alternatively, PKC activation may negatively regulate a signaling pathway that is critical for supporting cell migration. Indeed, we have observed that PMA stimulation of PA-JEB/β4 keratinocytes leads to the inactivation of basal PI-3 kinase signaling in these cells, as evidenced by a diminution of Akt phosphorylation. PI3-kinase signaling supports cell migration by Rac-dependent remodeling of the actin cytoskeleton and focal contacts (38).

Before epithelial cells can migrate during wound healing, type I HDs have to be completely disassembled. However, the disassembly of type II HDs is likely to be part of a more dynamic
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process. These structures are found in rapidly migrating intestinal epithelial cells and their dynamic regulation may allow for rapid adhesion and de-adhesion necessary for an efficient migration. The disassembly of HDs in wounds occurs under the influence of many cytokines and growth factors, including EGF, which are released by different cell types present in the wound milieu (13). However, because EGF only partially prevents the formation of HDs, it is anticipated that other cytokines also play a role in the prevention of their formation, and/or the disruption of these structures. These cytokines may induce the phosphorylation of β4 on other residues than those whose phosphorylation is induced by EGF, thereby augmenting the disruptive effects of EGF on the interaction between β4 and the plectin-ABD. For example, they may phosphorylate residues important for the binding of β4 to the plakin domain of plectin. Binding of β4 to this domain of plectin is not sufficient to mediate the recruitment of plectin by β4 into HDs, but it may nevertheless facilitate and stabilize the binding of the plectin-ABD to β4 (9, 10). Other phosphorylation events may interfere with the binding of β4 to either BP180 or BP230, and/or the binding between BP180, B230, and plectin, and the intermediate filament system (39, 40).

Several reports have suggested that β4 is also phosphorylated on tyrosine residues. Although most of these studies involved the use of carcinoma cells overexpressing the EGF receptor, data have also been presented that tyrosine phosphorylation may occur in normal keratinocytes (41). We have not observed tyrosine phosphorylation of β4 in PA-JEB/β4 keratinocytes after EGF stimulation and therefore have excluded a role of such kinases in the regulation of HD disassembly. However, the tyrosine phosphorylation of β4 in carcinoma cells has been well documented and is likely to contribute to a more efficient disruption of HDs in these cells (16, 42).

Acknowledgments—We thank John Blenis, Philippe Lenormand, Takashi Hashimoto, and Ulrike Mayer for providing reagents, and Jaco van Rheenen, Rob Woltluis, and Mike Ports for helpful discussions.

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