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Abstract
Growth factor-induced cell migration and proliferation are essential for epithelial wound repair. Cell migration during wound repair also depends upon expression of laminin-5, a ligand for α6ß4 integrin. We investigated the role of α6ß4 integrin in laminin-5-dependent keratinocyte migration by re-expressing normal or attachment-defective ß4 integrin in ß4 integrin null keratinocytes. We found that expression of ß4 integrin in either a ligand bound or ligand unbound state was necessary and sufficient for EGF-induced cell migration. In a ligand bound state, ß4 integrin supported EGF-induced cell migration though sustained activation of Rac1. In the absence of α6ß4 integrin ligation, Rac1 activation became tempered and EGF chemotaxis proceeded through an alternate mechanism that depended upon α3ß1 integrin and was characterized by cell scattering. α3ß1 integrin also relocalated from cell-cell contacts to sites of basal clustering where it displayed increased conformational activation. The aberrant distribution and activation of α3ß1 integrin in attachment-defective ß4 cells could be reversed by the activation of Rac1. Conversely, in WT ß4 cells the normal cell-cell localization of α3ß1 integrin became aberrant after the inhibition of Rac1. These studies indicate that the extracellular domain of ß4 integrin, through its ability to bind ligand, functions to integrate the divergent effects of growth factors on the cytoskeleton and adhesion receptors so that coordinated keratinocyte migration can be achieved.

Keywords
alpha6beta4 integrin, EGF, laminin-5, keratinocyte, chemotaxis

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$\alpha6\beta4$ integrin regulates keratinocyte chemotaxis through differential GTPase activation and antagonism of $\alpha3\beta1$ integrin

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Summary

Growth factor-induced cell migration and proliferation are essential for epithelial wound repair. Cell migration during wound repair also depends upon expression of laminin-5, a ligand for α6β4 integrin. We investigated the role of α6β4 integrin in laminin-5-dependent keratinocyte migration by re-expressing normal or attachment-defective β4 integrin in β4 integrin null keratinocytes. We found that expression of β4 integrin in either a ligand bound or ligand unbound state was necessary and sufficient for EGF-induced cell migration. In a ligand bound state, β4 integrin supported EGF-induced cell migration though sustained activation of Rac1. In the absence of α6β4 integrin ligation, Rac1 activation became tempered and EGF chemotaxis proceeded through an alternate mechanism that depended upon α3β1 integrin and was characterized by cell scattering. α3β1 integrin also relocalated from cell-cell contacts to sites of basal clustering where it displayed increased conformational activation. The aberrant distribution and activation of α3β1 integrin in attachment-defective β4 cells could be reversed by the activation of Rac1. Conversely, in WT β4 cells the normal cell-cell localization of α3β1 integrin became aberrant after the inhibition of Rac1. These studies indicate that the extracellular domain of β4 integrin, through its ability to bind ligand, functions to integrate the divergent effects of growth factors on the cytoskeleton and adhesion receptors so that coordinated keratinocyte migration can be achieved.

Key words: α6β4 Integrin, EGF, Laminin-5, Keratinocyte, Chemotaxis

Introduction

Hemidesmosomes (HD) are specialized attachment structures of the basement membrane zone (BMZ) which bind laminin-5 (Rousselle et al., 1991) through α3β1 integrin (Carter et al., 1991) and α6β4 integrin (Sonnenberg et al., 1991). During wound healing, basal keratinocytes along the wound edge undergo transition from static adherent structures to motile, regenerative sheets of cells (Martin, 1997). Growth factors such as epidermal growth factor (EGF), secreted into the wound site by macrophages and keratinocytes induce HD disassembly, keratinocyte proliferation and migration (Barrandon and Green, 1987; Mainiero et al., 1996; Marikovsky et al., 1993; Martin, 1997). Keratinocytes at the wound front can migrate on dermal collagen using α2β1 integrin and MMP-1 (Pilcher et al., 1997) or laminin-5 through α3β1 integrin (Goldfinger et al., 1999).

α6β4 integrin has generally been viewed as a mediator of attachment and HD formation at sites more distal from the wound edge (Kurpakus et al., 1991; Nguyen et al., 2000a) or even as an inhibitor of keratinocyte motility (Hintermann et al., 2001). However, several lines of evidence suggest α6β4 integrin may play a more direct and active role in keratinocyte migration. α6β4 integrin interacts with receptor tyrosine kinases such as EGFR, ErbB-2 and Met (Falcioni et al., 1997; Hintermann et al., 2001; Trusolino et al., 2001). Stimulation of keratinocytes with EGF induces tyrosine phosphorylation of the cytoplasmic domain of β4 which is implicated in both HD disassembly and epithelial motility (Mainiero et al., 1996). α6β4 may play an active role in chemotactic migration through lysophosphatidic acid (LPA) by activation of a cAMP-specific phosphodiesterase and RhoA GTPase (O'Connor et al., 2000; O'Connor et al., 1998). and α6β4 can localize with filamentous actin and stabilize lamellipodial membrane protrusions (Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999).

Since growth factor stimulation is required to induce keratinocyte migration during wound healing, we examined the role of laminin-5, α3β1 integrin and α6β4 integrin in this process. We re-expressed wild-type and attachment-defective β4 integrin in β4 null patient keratinocytes and studied the effects of α6β4 integrin ligation on EGF-mediated keratinocyte chemotaxis. We found that EGF-induced keratinocyte migration response depends upon the interaction between laminin-5 and the β4 integrin ectodomain. When bound to laminin-5, α6β4 integrin promoted EGF-dependent cell migration through Rac1 activation. Without laminin-5 ligation through α6β4 integrin, EGF induced keratinocyte chemotaxis

through α3β1 integrin. We show evidence that these two pathways are antagonistic and suggest a mechanism through which α6β4 may coordinate these two signals to regulate integrated epithelial movement during wound healing.

Materials and Methods

Cell lines

Primary keratinocytes were obtained from an patient with epidermolysis bullosa with pyloric atresia (EB-PA) resulting from a compound heterozygote mutation in the β4 integrin gene (C738X/4791delCA) (Pulkkinen et al., 1998). Cells were immortalized with HPV18 E6 and E7 genes (Kaur et al., 1989). Additional studies were carried out on primary keratinocytes from an EB-PA patient deficient in β4 as a result of a premature termination codon (C658X). Neonatal human foreskin keratinocytes (NHK) and immortalized patient cells were cultured in serum-free medium (SFM) (Gibco). Modified human 293 PHOENIX cells (a gift from Dr G. Nolan, Stanford University, Stanford, CA) were cultured in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Antibodies

Mouse mAb 3E1, ASC-8 and rat mAb GoH3 recognizing the extracellular domains of β4 and α6 respectively, and rabbit polyclonal antiserum to β4 wwere obtained from Chemicon (Temecula, CA). Mouse mAb ASC-8 is inhibitory to α6β4 attachment and was used in all inhibition assays at 10 μg/ml. The mouse mAb 121 raised against HD1/plectin and the mouse mAb 233 raised against BP180 were a gift from Dr K. Owaribe (Nagoya University, Nagoya, Japan). The rabbit laminin-5 antisera has been characterized (Marinkovich et al., 1992). Anti-laminin-5 mAb BM165 (Rousselle et al., 1991) was purified through protein G affinity chromatography. BM165 prevents attachment to the α3 subunit of laminin-5 and was used at 10 μg/ml. Mouse anti-α3 integrin mAb P1B5 and mouse mAb HUTS-4 against the active conformation of β1 integrins were obtained from Chemicon. P1B5 is inhibitory to α3β1 integrin attachment and was used at 10 μg/ml. Mouse mAb 349 and rat mAb 346-11A against human paxillin and integrin were obtained from Transduction Labs and Pharmingen respectively (Lexington, NY). Rabbit sera 119 and P1 raised against RhoA and Cdc42 respectively were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb 23A8 against Rac1 was obtained from Upstate Biotech (Lake Placid, NY). Mouse mAb 9E10 to the myc tag was obtained from Oncogene Research Products (Boston, MA). Phosphotyrosine western blots were carried out with mouse mAb 4G10 (Upstate Biotech, Lake Placid, NY). Rabbit antibodies to p44/42 MAP kinase and phospho-p44/42 MAP kinase were obtained from New England Biolabs Inc (Beverly, MA). FITC- and TRITC-conjugated phalloidin was purchased from Sigma Chemical Co. (St Louis, MO). TRITC-conjugated goat anti-rabbit, Cy5-conjugated goat anti-rat and FITC-conjugated donkey anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (Westgrove, PA). The sheep anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham (Arlington Heights, IL).

cDNA constructs and vectors

β4pRK-5 was a generous gift from Dr F. G. Giancotti (Sloan Kettering Cancer Institute, NY). Previous reports have shown that a β4 integrin cDNA from this lab contained an in frame deletion of 7 amino acids (880-886) in the membrane proximal region (Dans et al., 2001). Therefore, before use we sequenced this region to ensure no deletions were present. β4 cDNA was cloned as a 5.6 kb *Eco*RI fragment into the *Eco*RI site of retroviral expression vector LZRS (Kinsella and Nolan, 1996) containing the encephalomyocarditis virus (EMCV)-IRES and blasticidin-resistance sequences (Deng et al., 1998). An attachment-deficient (AD) β4 construct was produced through cloning the *Eco*RI β4 cDNA insert into the *Eco*RI site of pSK and performing mutagenesis using the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison WI). Primers used for the point mutation of β4 sequences were as follows: β4(AD) (D230A, P232A, E233A, incorporating a novel *Nae*I site) 5′ GGCAACCTGGCTGCTGCTGCCGGCGGCTTCG 3′. Positive clones were sequenced and ligated into the *Eco*RI site of LZRS-IRES-blasticidin. Dominant inhibitory Rho family GTPase constructs cloned into the GFP fusion vector EGFP-C1 (Clontech) were a generous gift from Dr Eugene Butcher (Stanford Medical Center, CA). GFP tagged GTPase inserts were cloned into LZRS-IRES-blasticidin by PCR using the *Eco*RI tailed primer GTPaseF, 5′ CCCCCCGAATTACAGATCCGCTAGCGCTACCGGTC 3′ and GTPaseR 5′ CGGTACCGTCGACTGCAGAATTC 3′. PCR products were digested with *Eco*RI and cloned into LZRS and verified by sequencing. Myc tagged V12Rac1 was a kind gift of Dr Alan Hall (University College London, UK) and was cloned as an *Eco*RI fragment into LZRS-IRES-blasticidin. The GTPase pull-down construct pGEX-2T-RBD against GTP-RhoA was a kind gift from Dr Martin A. Schwartz (Scripps Research Insitute, La Jolla, CA) while pGEX-2T-PAK against GTP-Rac1 and GTP-Cdc42 was a kind gift from Dr John Collard (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Retroviral transduction

Amphotropic retrovirus was produced in modified 293 cells as previously described (Kinsella and Nolan, 1996). 1×10^5 keratinocytes were seeded into 6-well tissue culture plates and incubated for 24 hours 15 minutes prior to infection, 5 μg/ml polybrene (Sigma) was added to both viral supernatant and keratinocyte media. Media was removed and 4 ml retroviral supernatant added. Plates were centrifuged at 300 ***g*** for 1 hour at 32°C using a Beckman GS-6R centrifuge. Cells were incubated at 37°C for 24 hours followed by replacement with fresh SFM and selection with 5 μg/ml blasticidin (Calbiochem, La Jolla, CA).

Biochemical methods

Phosphorylation of α6β4 by EGF was assessed by immunoprecipitation of β4 from EGF-treated cell lysates. Briefly, keratinocytes in culture were starved of growth factors by incubating in keratinocyte SFM without additives (SFM/WA) for 16 hours. Cells were then treated with recombinant human EGF (100 ng/ml) before washing with ice cold PBS followed by the addition of lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM EDTA, 500 μM Na_3VO_4) for 20 minutes on ice. Equalized lysates (1 mg) were added to 3 μg mAb 3E1 and 100 μl protein A/G immobilized beads (Pierce, Rockford, IL) and incubated for 17 hours at 4°C. Beads were washed with lysis buffer twice then once with ice-cold water before being boiled for 10 minutes with 7 M urea sample buffer (125 mM Tris pH 6.95, 7 M urea, 1 mM EDTA, 2% SDS, 0.1% bromophenol blue, 10% β-ME). After SDS-PAGE of samples, the degree of phosphorylation was ascertained by western blot with mAb 4G10. GTPase activation assays were carried out using a modified GST pull-down protocol (Ren et al., 1999). Briefly, cells were growth starved as above, treated with 2 ng/ml EGF, harvested at intervals, washed once with ice cold PBS and extracted with lysis buffer (50 mM Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 50 mM NaCl, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM Na_3VO_4). Lysates were immediately incubated for 30 minutes with GST-PAK or GST-RBD beads at 4°C washed three times with lysis buffer, once with ice-cold water and eluted with 35 μl 7 M urea sample buffer with 20% β-mercaptoethanol before electrophoresis on a 12% SDS-PAGE gel.

Cell scattering and adhesion assays

Cell scattering was ascertained by examining clonal growth after 4 days. Briefly, cells were plated at low density (<5000 cells per 60 mm plate) and allowed to grow in each selected medium for 4 days. For studies with EGF-free medium, cells were plated in normal SFM for 16 hours then the medium was changed to SFM/WA. Cell scattering was quantified by counting colonies of less than eight cells, defining unscattered colonies as having at least 90% of the cells in contact with each other. Each count was performed with at least 50 colonies and repeated three times. Cell adhesion assays were performed using a crystal violet assay attachment assay (Wayner et al., 1991), coating 96-well plates with 10 μg/ml affinity purified laminin-5 and incubating cells for 60 minutes at 37°C. Laminin-5 secreted by cells was visualized by matrix extraction. Briefly, cells were allowed to adhere to 6-well plates as described then were removed with 2 ml 20 mM ammonium hydroxide for 5 minutes at room temperature. Plates were rinsed three times with PBS then 200 μl matrix extraction buffer added (8 M urea, 1% SDS, 10 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol) before removal by scraping. Western blotting was performed with 10 μg of each lysate.

Migration assays

Monolayer scratch assays were performed by plating 10^6 cells into 60 mm tissue culture plates and incubating cells in SFM for 24 hours. Medium was changed to SFM/WA for 16 hours. Fresh mitomycin-C (Sigma) was added at 10 μg/ml and cells incubated 3 hours on ice. Cells were washed twice with SFM/WA and scratched with a 1 mm cell scraper. Plates were washed three times with SFM/WA and marked areas photographed using a Zeiss Axiovert 25 microscope (50× magnification). Cells were incubated with or without 2 ng/ml EGF and photographed at defined time intervals. Migration was quantified by calculating percentage change in the area between migrating cell sheets using NIH image software and >3 repeats per data point. Chemotaxis assays were performed using a modified Boyden chamber assay (Leavesley et al., 1992). Briefly, 6.5 mm, 8.0 μm pore size transwell inserts (Costar, Corning, NY) were coated with extracellular matrix (ECM) diluted in 250 μl PBS for 3 hours at 37°C, rinsed twice with PBS and blocked with 5% BSA/PBS for 60 minutes at 37°C then placed in 750 μl medium in 24-well plates. 5×10^4 growth factor-starved keratinocytes were added to the upper chamber and incubated for 16 hours Chambers were washed twice with PBS, fixed with 3% paraformaldehyde/PBS for 15 minutes and stained with 0.1% crystal violet for 15 minutes. Non-migrating cells were removed by swabbing and cells quantified by counting three fields of view (100×) on a Zeiss Axioscope. Experiments were performed in triplicate and repeated at least twice.

Immunofluorescence microscopy

For HD components, cells were cultured in HAMF12:DMEM (1:3) containing 10% fetal calf serum, 0.4 μg/ml hydrocortisone and 10^{-6} M isoproterenol (both from Sigma Chemical Co., St Louis, MO). Cells were then fixed with 3% paraformaldehyde permeabilized with 0.5% Triton X-100 in PBS at room tempeerature (RT) for 30 minutes. For focal adhesion (FA) components, cells were fixed with 3% formaldehyde/0.5% Triton X-100 buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EGTA, 5 mM EDTA, 50 mM sodium pyrophosphate, 100 μM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 30 minutes at RT. Cells were blocked with 1% BSA for 60 minutes before staining with appropriate primary and secondary antibodies. Actin was labeled with FITC or TRITC-phalloidin diluted at 1 ng/ml. Labeled slides were viewed using an Applied Precision deltavision deconvolution system and a Bio-Rad confocal microscope.

Quantification of lamellipodial area

Cells were seeded into 8 chamber slides in SFM. After 6 hours, medium was changed to SFM/WA and cells were incubated at 37°C for 16 hours Cells were treated with recombinant EGF (2 ng/ml) and fixed with 3.4% formaldehyde at RT for 15 minutes. Cells were stained with TRITC-phalloidin and visualized on a Leitz Aristaplan microscope, capturing images with a digital spot camera (National Instruments, Austin TX). Lamellipodial area was calculated as described (Rabinovitz and Mercurio, 1997) using NIH image software. Each value is expressed as the mean of >50 cells.

Results

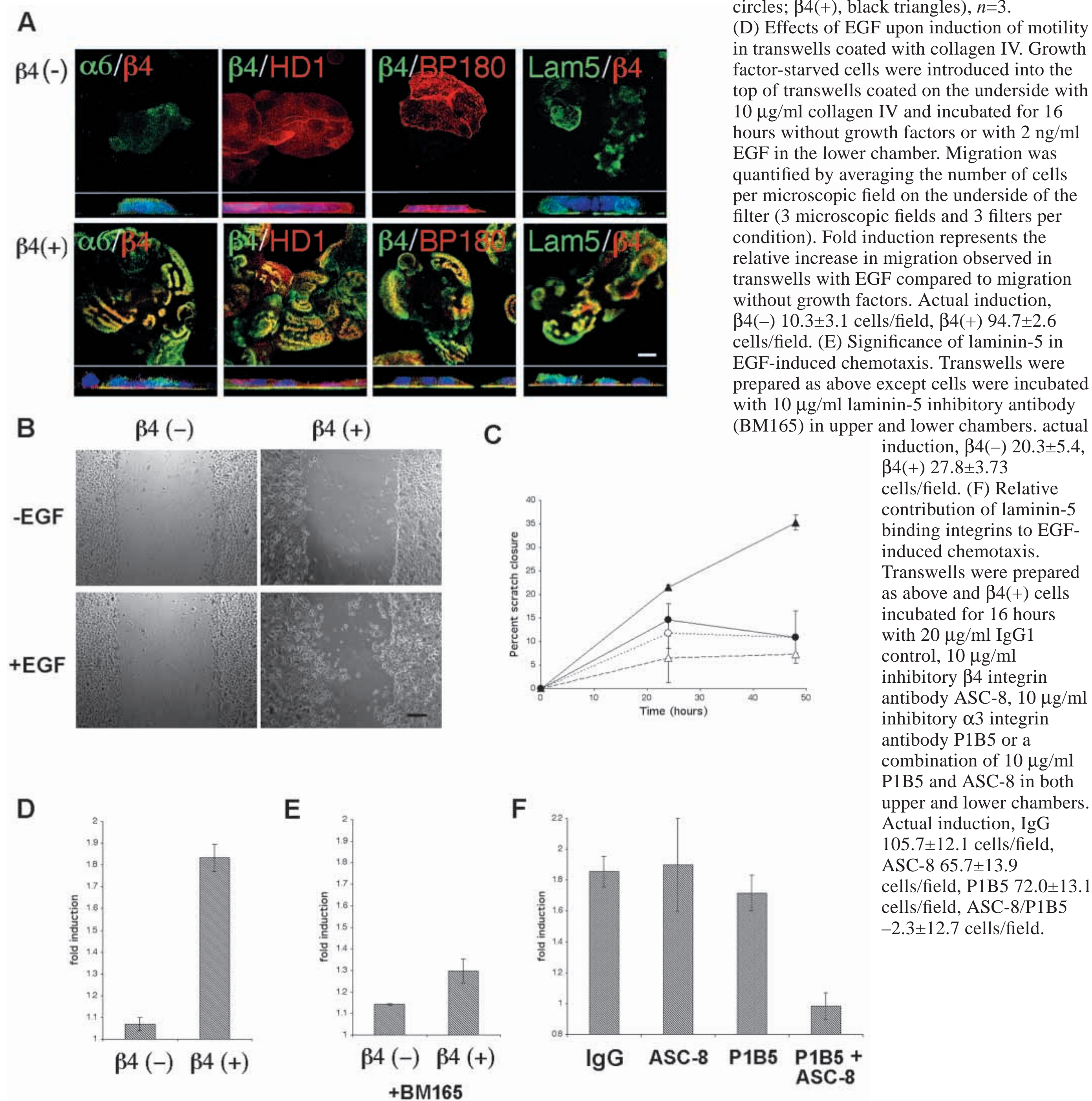
α6β4 integrin and laminin-5 are essential for EGF induced migration in keratinocytes

We utilized α6β4 integrin null EB-PA keratinocytes to study α6β4 integrin in keratinocyte migration. Control vector (LacZ) or full-length β4 integrin were retrovirally expressed in EB-PA cells to create β4(–) and β4(+) cells respectively. β4(–) cells showed normal laminin-5 secretion but no detectable β4 integrin and the HD proteins BP180, BP230 and HD1/plectin were diffusely localized (Fig. 1A top panels; BP230 not shown). In contrast, β4(+) cells basally accumulated β4 integrin which co-localized with α6 integrin, HD1/plectin, BP180, BP230 and laminin-5 (Fig. 1A bottom panels; BP230 not shown). Flow cytometry showed that β4(+) cells expressed cell surface β4 integrin at a level comparable to normal keratinocytes (73.2±12.2% of control, *n*=3).

Without EGF, neither β4(–) nor β4(+) cells migrated in a monolayer scratch assay (Fig. 1B,C; <10.9±5.6% scratch closure over 48 hours), however upon addition of EGF only β4(+) cells migrated significantly into the wound scratch (β4(–) 10.9±5.6% closure vs. β4(+), 35.3±1.6% scratch closure over 48 hours, $P<0.05$). Similar observations were obtained when migration was assayed using ECM-coated transwell chambers. EGF also induced the migration of β4(+) cells but not β4(–) cells across transwells coated with collagen IV, collagen I, fibronectin or laminin-1 (Fig. 1D, collagen IV shown, overall fold induction: β4(+) 2.04±0.18 versus β4(–) 1.08±0.03). These ECM substrates are not ligands for α6β4 integrin (with the exception of laminin-1), therefore we investigated whether interactions between α6β4 integrin and autocrine laminin-5 were responsible for mediating cell migration. In the presence of BM165, an antibody to laminin-5 that inhibits cell adhesion, EGF-induced migration of β4(+) cells was significantly reduced, suggesting ligation of β4 integrin by laminin-5 was necessary for EGF-induced keratinocyte chemotaxis (Fig. 1E, collagen IV shown). Similar results were also obtained with collagen I, fibronectin and, laminin-1 (data not shown).

To identify the cellular receptors for laminin-5 responsible for mediating EGF-induced chemotaxis in keratinocytes, the transwell assays were repeated using β4(+) cells in the presence of α6β4 integrin (mAb ASC-8) and α3β1 integrin (mAb P1B5) inhibitory antibodies or an IgG control (Fig. 1F). Collagen IV was selected as a migration substrate as it is neither a ligand for α6β4 nor for α3β1 integrin. These studies showed that although β4 integrin expression is necessary for attachment, either α6β4 or α3β1 integrin is sufficient for EGF-induced chemotaxis in keratinocytes. Thus, both α6β4 integrin and laminin-5 expression are essential for EGF-induced

Fig. 1. α6β4 integrin and laminin-5 are required for EGF induction of motility in human keratinocytes. (A) Distribution of HD components in β4(–) and β4(+) cells. Cells were cultured for 24 hours on glass coverslips fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunofluorescence microscopy was performed to identify α6 integrin subunit (with rat mAb GoH3) in combination with the β4 subunit (mouse mAb 3E1); β4 integrin (rat mAb 346-11A) in combination with plectin/HD1 (mouse mAb 121) or BP180 (mouse mAb 233), and r β4 integrin (with mouse mAb 3E1) in combination with laminin-5 (rabbit polyclonal anti-laminin-5 antisera). Mouse antibodies are colored red while rat and rabbit antibodies are colored green, colocalization is therefore represented by a yellow color. Narrow images under each figure represent z-sections of the image above (nuclei are stained blue with Hoechst dye). Scale bar: 10 μm. (B) Effects of α6β4 expression on keratinocyte monolayer scratch migration. Integrin β4-deficient EB-PA keratinocytes expressing either LacZ, [β4(–)] or β4 cDNA, [β4(+)] were starved of growth factors for 16 hours before treatment with 10 μg/ml mitomycin C for 3 hours on ice to prevent subsequent proliferation. Cell monolayers were wounded by scraping and migration of the cells into the scrape wound was photographed 48 hours later after incubation in either supplement free medium (top panels) or in medium supplemented with 2 ng/ml EGF (lower panels; Scale bar: 200 μm). (C) Quantification of monolayer scratch assays. Marked areas were photographed at 24 and 48 hours periods and areas between scratch fronts calculated to generate percentage scratch closure in conditions of no EGF (β4(–), white circles; β4(+), white triangles) or 2 ng/ml EGF (β4(–), black circles; β4(+), black triangles), *n*=3. (D) Effects of EGF upon induction of motility in transwells coated with collagen IV. Growth factor-starved cells were introduced into the top of transwells coated on the underside with 10 μg/ml collagen IV and incubated for 16 hours without growth factors or with 2 ng/ml EGF in the lower chamber. Migration was quantified by averaging the number of cells per microscopic field on the underside of the filter (3 microscopic fields and 3 filters per condition). Fold induction represents the relative increase in migration observed in transwells with EGF compared to migration without growth factors. Actual induction, β4(–) 10.3±3.1 cells/field, β4(+) 94.7±2.6 cells/field. (E) Significance of laminin-5 in EGF-induced chemotaxis. Transwells were prepared as above except cells were incubated with 10 μg/ml laminin-5 inhibitory antibody (BM165) in upper and lower chambers. actual induction, β4(–) 20.3±5.4, β4(+) 27.8±3.73 cells/field. (F) Relative contribution of laminin-5 binding integrins to EGF-induced chemotaxis. Transwells were prepared as above and β4(+) cells incubated for 16 hours with 20 μg/ml IgG1 control, 10 μg/ml inhibitory β4 integrin antibody ASC-8, 10 μg/ml inhibitory α3 integrin antibody P1B5 or a combination of 10 μg/ml P1B5 and ASC-8 in both upper and lower chambers. Actual induction, IgG 105.7±12.1 cells/field, ASC-8 65.7±13.9 cells/field, P1B5 72.0±13.1 cells/field, ASC-8/P1B5 –2.3±12.7 cells/field.



chemotaxis in keratinocytes but this process can be mediated by laminin-5 induced ligation of either α6β4 or α3β1 integrin.

Mutation of β4 integrin extracellular domain permits recruitment of HD components but prevents laminin-5 attachment and EGF induced β4 integrin phosphorylation

To further examine the contribution of α6β4 integrin ligation to EGF induced chemotaxis we designed and expressed an attachment-defective β4 integrin mutant in the EB-PA cells. An extracellular ligand-binding mutant of β4 integrin was designed according to published data that identified two regions in the β3 integrin extracellular domain that were essential for attachment in the platelet integrin αIIbβ3 (asterisks, Fig. 2A) (Baker et al., 1997). Homology analysis of the exodomains of β3 and β4 integrins revealed a high level of conservation between these two ligand attachment regions. Accordingly, a mutant β4 cDNA construct was engineered that incorporated three substitutions within the second homology domain at D230A, P232A and E233A and termed adhesion defective β4, or β4(AD).

EB-PA cells expressing β4(AD) had strong basal expression of the β4 integrin that co-localized in type I HD clusters with α6 integrin, plectin, BP180 and secreted laminin-5, in a pattern that was similar to that exhibited by the β4(+) cells (Fig. 2B). Recruitment of HD components by β4(AD) is in agreement with previous reports that β4 integrin recruitment to HDs is driven by cytoplasmic interactions with BP180 and plectin and not by α6β4 attachment to laminin-5 (Homan et al., 1998; Nievers et al., 2000; Nievers et al., 1998).

β4(AD) and β4(+) cells were studied by attachment assays using affinity purified laminin-5 (Fig. 2C). Contributions of α6β4 and α3β1 integrin to adhesion were analyzed using inhibitory antibodies (ASC8; α6β4 integrin, and P1B5; α3β1 integrin, respectively). Both cell types attached at comparable levels to laminin-5, however inhibition of α3β1 integrin completely prevented β4(AD) attachment while not affecting β4(+) cells. Note that these assays measure substrate attachment and not the strength of substrate attachment, which could be enhanced in β4(+) cells. α6β4 integrin can uniquely mediate attachment to laminin-5 even at 4°C (Xia et al., 1996). While β4(+) cells could attach effectively at 4°C, β4 (AD) cells could not (Fig. 4D). However, pre-incubating β4(+) cells with β4 integrin inhibitory antibody (ASC-8) reduced the adhesion level of the β4 (+) cells at 4°C to the same level exhibited by β4(AD) cells. These studies demonstrate that although β4(AD) cells fail to adhere through α6β4 integrins they retain normal function of α3β1 integrin. We conclude that the adhesion defect of β4(AD) cells is a consequence of direction mutation of the extracellular domain of the β4 integrin subunit rather than of a non-specific effect upon α3β1 integrin expression or function.

α6β4 integrin becomes tyrosine phosphorylated following stimulation with high concentrations of EGF (Mainiero et al., 1996). To further evaluate the ligand binding characteristics of our β4 integrin mutant (AD) cells, we tested the ability of EGF to induce tyrosine phosphorylation of β4 integrin. Although EGF induced phosphorylation of β4 integrin in β4(+) cells, no phosphorylation of β4 integrin was observed in β4(AD) cells (Fig. 2E, compare top panel to the lower panel). All three cell types secreted and processed laminin-5 to a similar degree regardless of EGF treatment (Fig. 2F). Finally, we examined the possibility that expression of the β4(AD) mutant induced non-specific effects on EGFR expression or signaling. Flow cytometry of surface EGFR of β4(+) compared with β4(AD) cells revealed similar levels of expression (mean fluorescence 162.1±10.6 vs. 199.4±11.6). Signaling by the EGFR was tested by examination of the effects of EGF on MAP kinase activation (Fig. 2G). Treatment of all cell types resulted in strong activation of p44/42 MAP kinase. Separate studies also confirmed that the kinetics of this activation was unchanged (data not shown). Taken together, these experiments show that mutation of conserved extracellular residues within the β4 subunit prevents attachment to laminin-5 and inhibits ligation-dependent tyrosine phosphorylation of β4 integrin. However, loss of attachment function does not prevent β4 integrin from mediating its other cellular functions including the recruitment of HD components.

Ligation of α6β4 integrin is required for sustained activation of Rac1, lamellipodia formation and RhoA-independent chemotaxis

Members of the Rho GTPase family drive chemotaxis by EGF in many cell types (Nobes and Hall, 1995). Therefore, we examined the effects of α6β4 expression and ligation upon EGF-dependent Rho GTPase activity (Fig. 3A,B). β4(–) cells showed transient EGF induced stimulation of Rac1 activity and modest activation of Cdc42. Expression of wild-type α6β4 integrin resulted in rapid and sustained activation of both Rac1 and Cdc42 (for at least 2 hours). In contrast, expression of β4(AD) resulted in a truncated Rac1 activation profile similar to β4(–) cells. Interestingly, while β4(–) and β4(+) cells exhibited similar EGF-dependent RhoA activity β4(AD) cells showed higher RhoA activation both before and after EGF treatment (Fig. 3B). Thus we concluded that α6β4 expression and ligation are essential for sustained EGF-dependent Rac1 and Cdc42 activation. Interestingly, in the absence of α6β4 integrin laminin-5 interactions, Rac1/Cdc42 activation is truncated whereas RhoA activity appears to be amplified.

Since Rac1 induces membrane ruffling and lamellipodia extension, structures of known importance to cell migration (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996), we next investigated the relevance of α6β4 integrin-dependent Rac1 activation to lamellipodia formation after EGF exposure. Cells were treated with EGF and fixed at time intervals following EGF stimulation and lamellipodial area was quantified (Fig. 3C). EGF induced membrane extension in all cells tested, and this effect peaked after 5 minutes. However, β4(–) and β4(AD) cells failed to sustain lamellipodia induction beyond 20 minutes. In contrast, β4(+) cells maintained lamellipodia for at least 2 hours following EGF exposure. Therefore, sustained lamellipodia formation in β4(+) cells mirrors the activation of Rac1 in that it requires both α6β4 integrin expression and ligation to laminin-5. To more directly test this observation, NHK were incubated with β4 inhibitory antibody (ASC-8) and lamellipodial induction was measured in response to EGF (Fig. 3D). Consistently, inhibition of α6β4 integrin ligation markedly truncates sustained lamellipodia formation similar to that observed in β4(–) and β4(AD) cells.

To further explore the significance of Rac1 activation in

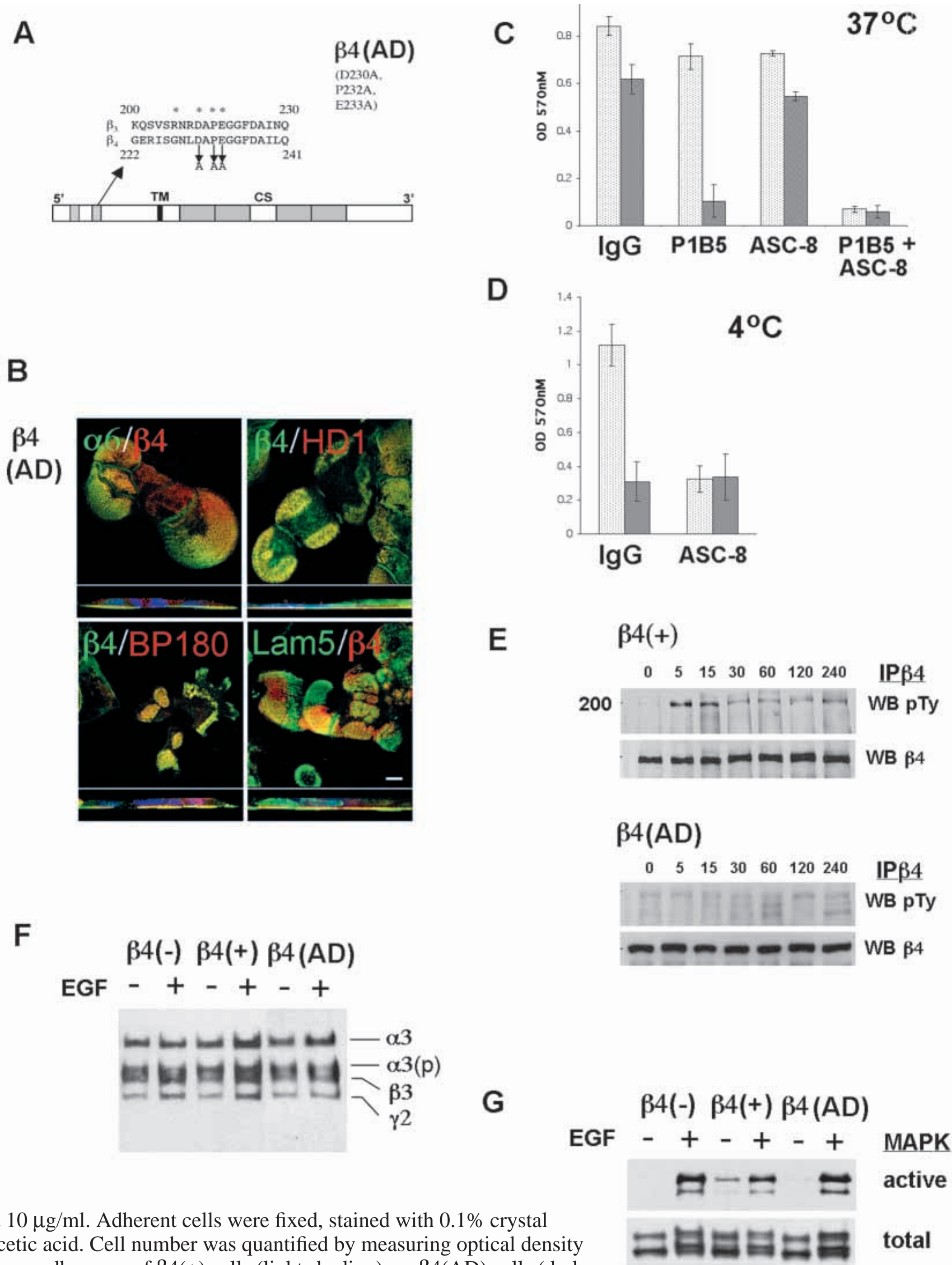


Fig. 2. Mutation of homologous polar residues within the extracellular domain of β4 prevents attachment of α6β4 to laminin-5 and tyrosine phosphorylation after EGF treatment but not recruitment of HD components. (A) Amino acid substitutions to generate an attachment defective β4 subunit, β4(AD). Homology analysis was carried out with the extracellular domain of the β3 integrin subunit. Asterisks indicate residues essential for ligand binding in integrin αIIbβ3 (Baker et al., 1997). Arrows represent sites of point mutation and alanine substitution. (B) Distribution of HD components in β4(AD) cells. Cells were cultured for 24 hours on glass coverslips fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunofluorescence microscopy was performed as described for β4(–) and β4(+) cells (see Fig. 1A). Mouse and rabbit antibodies are colored red while rat antibodies are colored green, colocalization is therefore represented by a yellow color. Narrow images under each figure represent z-sections of the image above (nuclei are stained blue with Hoescht dye) Scale bar: 10 μm. (C) Attachment of keratinocytes to laminin-5 in the presence of inhibitory antibodies to α3 integrin (P1B5) and/or cells in suspension were applied to 96-well plates coated with 10 μg/ml laminin-5 and incubated for 60 minutes at 37°C before unattached cells were washed off. Inhibitory antibodies and control IgG were supplemented at 10 μg/ml. Adherent cells were fixed, stained with 0.1% crystal violet and solubilized with 10% acetic acid. Cell number was quantified by measuring optical density at 570 nM (n=4). The bar chart shows adherence of β4(+) cells (light shading) vs. β4(AD) cells (dark shading). (D) Attachment of keratinocytes to laminin-5 at 4°C in the presence of β4 inhibitory antibody (ASC-8). Cells in suspension were applied to 96-well plates coated with 10 μg/ml laminin-5 and incubated for 60 minutes at 4°C before washing off unattached cells. Inhibitory antibodies and control IgG were supplemented at 10 μg/ml. Adherent cells were fixed, stained with 0.1% crystal violet and solubilized with 10% acetic acid. Cell number was quantified by measuring optical density at 570 nM (n=4). The bar chart shows adherence of β4(+) cells (light shading) compared with β4(AD) cells (dark shading). (E) Tyrosine phosphorylation of the β4 subunit after stimulation of cells with EGF. β4(+) and β4(AD) keratinocytes were growth factor starved for 16 hours then stimulated with 100 ng/ml EGF. At time intervals indicated (in minutes), cells were lysed and immunoprecipitated with β4 mAb, 3E1. Western blots show tyrosine phosphorylation (mAb 4G10, upper panels) and total β4 in immunoprecipitates (with rabbit polyclonal antiserum 1922, lower panels). (F) Laminin-5 secretion and processing by transduced cells. Cells were grown on plastic culture dishes with or without 2 ng/ml EGF. After 24 hours, cells were removed with 20 mM ammonium hydroxide and matrix was extracted with 8 M urea buffer before western blotting with a polyclonal laminin-5 antibody. Symbols to the right of the blot indicate the separate laminin-5 subunits with a (p) indicating a processed subunit. (G) Activation of p44/42 MAP kinase by EGF. Cells were growth factor starved and treated for 5′ with 2 ng/ml EGF before lysis and western blotting with phospho-p44/42 MAP kinase antibody (upper panel). Blots were then stripped and reblotted with total p44/42 MAP kinase antibody (lower panel).

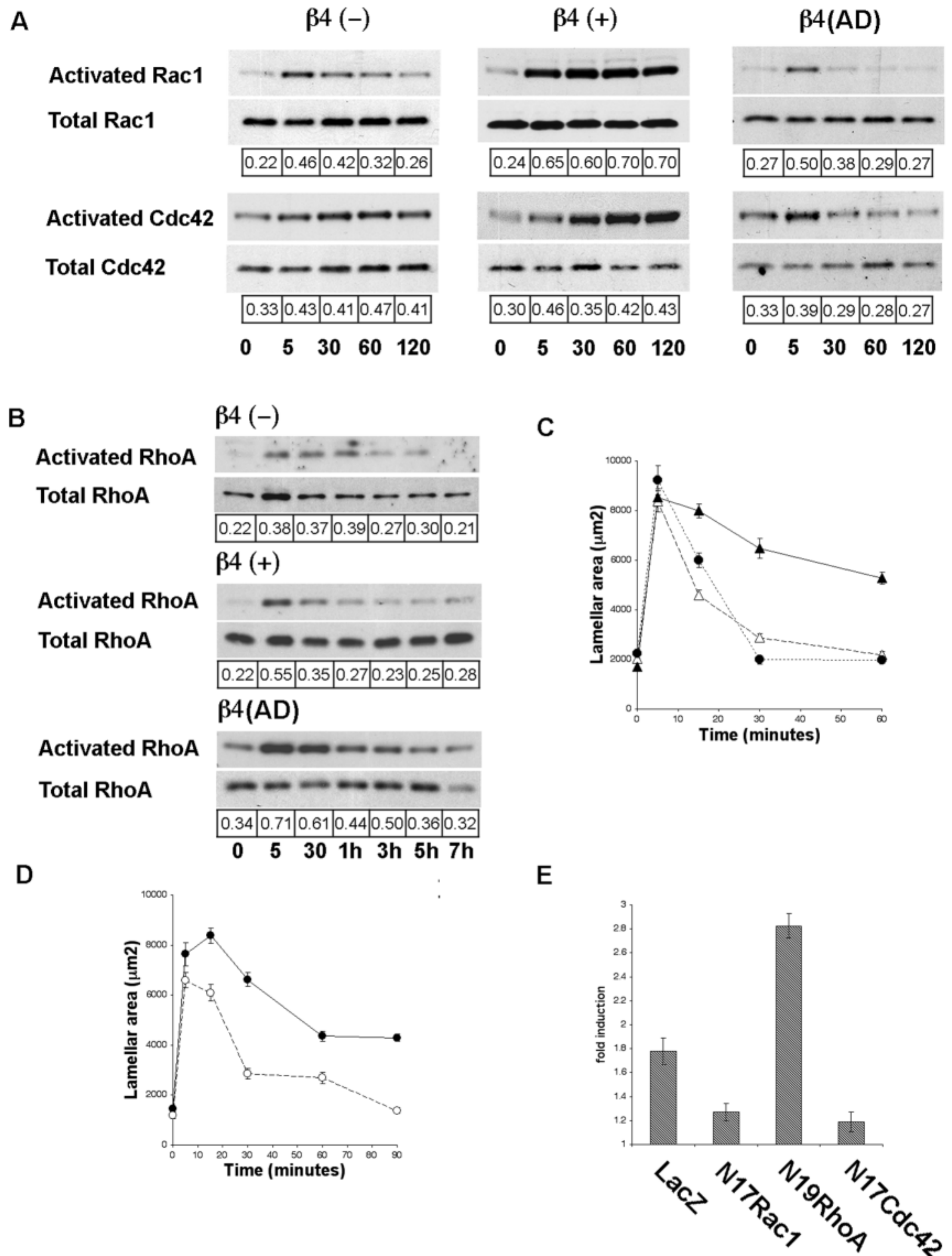


Fig. 3. α6β4 ligation is required for sustained activation of Rac1, lamellipodia formation and RhoA independent chemotaxis. (A) Effect of α6β4 expression and ligation upon Rac1 and Cdc42 GTPase activation by EGF. Growth factor-starved cells were stimulated with 2 ng/ml EGF for the indicated times. Cells were lysed, and incubated with GST-PAK and glutathione-sepharose beads. Beads were washed and bound proteins separated on a 12% SDS-polyacrylamide gel. GST-PAK pull down blots were initially probed for Rac1 before stripping and reblotting for Cdc42. Control gels show relative quantities of GTPases present in total lysates. Numbers under each profile represent average optical density of pull down lanes from at least 2 separate blots. (B) Effect of α6β4 expression and ligation upon RhoA GTPase activation by EGF. Growth factor starved cells were stimulated with 2 ng/ml EGF for the indicated times. Cells were lysed, and incubated with GST-RBD and glutathione-sepharose beads. Beads were washed and bound proteins separated on a 12% SDS-polyacrylamide gel. Control gels show relative quantities of GTPases present in total lysates. Numbers under each profile represent average optical density of pull down lanes from at least 2 separate blots. (C) Effect of EGF treatment upon lamellipodia formation. β4(–), β4(+) and β4(AD) cells were starved of growth factors for 16 hours and stimulated with 2 ng/ml EGF for the indicated times before fixing with 3.4% formaldehyde in PBS and stained with TRITC-phalloidin to identify filamentous actin. The area of lamellipodial projections was measured by tracing around membrane extensions on digital images. The lamellar area was then calculated using NIH image software. The graph shows total lamellar area for β4(–) cells (open triangles), β4(+) cells (black triangles) and β4(AD) cells (black circles). (D) Effect of β4 inhibitory antibody ASC-8 upon lamellipodia formation. Growth factor-starved NHKs incubated with 10 μg/ml IgG or ASC-8 were stimulated with 2 ng/ml EGF and lamellipodial area calculated as described. The graph shows lamellar area for NHK plus IgG (black circles) and NHK plus ASC-8 (open circles). (E) Effect of GTPase inhibition upon α6β4-dependent chemotaxis. β4(+) cells were retrovirally transduced with control LacZ or inhibitory GTPase constructs, N17Cdc42, N17Rac1 or N19RhoA. Transwell chemotaxis experiments were performed with collagen IV-coated transwells and 2 ng/ml EGF stimulation. Actual induction, β4(+)LacZ 106.4±9.0 cells/field, β4(+)N17Rac1 23.8±3.7 cells/field, β4(+)N19RhoA 121.1±3.0 cells/field, β4(+)N17Cdc42 11.8±9.1 cells/field.

keratinocyte chemotaxis, we retrovirally expressed dominant inhibitory forms of GFP-tagged N17Rac1, N17Cdc42 or N19RhoA in β4(+) cells and tested for effects on chemotaxis using the transwell assay (expression verified by western blot and immunofluorescence microscopy, data not shown). Transwell assays conducted with collagen IV revealed significant inhibition of EGF-induced chemotaxis in β4(+) cells after N17Rac1 or N17Cdc42 were expressed (Fig. 3D). However, expression of N19RhoA did not inhibit induction. These results suggest that expression and ligation of α6β4 integrin is required for sustained stimulation of Rac1, lamellipodia formation and chemotaxis in a process that appears to be independent of activated RhoA.

Attachment defective α6β4 integrin undergoes chemotaxis through an alternate pathway involving RhoA and integrin α3β1

We observed that expression and ligation of α6β4 integrin is required for sustained activation of Rac1 and lamellipodia formation. However, we previously observed that blocking α6β4 ligation with the inhibitory antibody ASC-8 does not block chemotaxis of β4(+) cells (Fig. 1F). We therefore used β4(AD) cells and asked whether the absence of α6β4 integrin ligation results in chemotaxis through an alternative EGF-dependent mechanism. In both monolayer scratch (Fig. 4A, 24-hour time point shown, full closure in 48 hours; *n*=3) and transwell migration assays (using collagen I, collagen IV, fibronectin or laminin-1; Fig. 4B, average fold induction 6.16±1.51, collagen IV shown), EGF-induced chemotaxis was enhanced in β4(AD) cells. To ascertain the contribution of laminin-5 and integrin α3β1 to this process we repeated the transwell assay in the presence of inhibitory antibodies. Treatment of β4(AD) cells with either laminin-5 or α3β1 integrin antibodies inhibited the chemotactic response to EGF (Fig. 4B), indicating that laminin-5-α3β1 integrin interactions are required for chemotaxis in β4(AD) cells.

The GTPase activation profile of β4(AD) cells showed elevated levels of RhoA activation before and after treatment with EGF. We expressed inhibitory Rac1, RhoA and Cdc42 constructs in β4(AD) cells to determine whether changes in GTPase activation profiles reflected altered Rho family GTPase requirements for β4(AD) chemotaxis. Chemotaxis was uniformly inhibited by N17Rac1, N17Cdc42 and N19Rho expression (Fig. 4C) suggesting cell motility in response to EGF was now also dependent upon RhoA. Activation of RhoA in epithelial cells is often associated with cell scattering (Sander et al., 1999). In support of a role for RhoA in β4(AD) migration, β4(AD) cells appeared to migrate predominantly as individual cells following stimulation with EGF as opposed to migrating as an intact sheet of cells (Fig. 4A). The scattering phenotype of β4(AD) cells was quantified by examining colony formation following growth in normal EGF-supplemented medium (Fig. 4D). Four days after plating (5000 cells per 60 mm plate) β4(AD) cells showed extensive colony scattering (65.3±1.6% of total colonies), while β4(–) and β4(+) cells predominantly formed epithelial colonies with intact cell-cell interactions (18.2±4.2% and 23.1±4.2% scattered respectively).

To verify that the scattering effects that we observed were indeed due to loss of β4 integrin adhesion, colony scattering experiments were carried out using β4(+) cells treated with the β4 integrin inhibitory antibody (ASC-8) following 16 hours of EGF treatment (Fig. 4E). β4 (+) cells incubated with the β4 integrin adhesion blocking antibody (ASC-8) exhibited increased cell scattering ($P<0.05$; Fig. 4E, right), similar to that exhibited by β4(AD) cells, although the scattering was maintained for a shorter duration, possibly due to antibody internalization and turnover.

These data illustrated that while ligation of α6β4 mediates EGF induced chemotaxis through Rac1, in the absence of α6β4 ligation (but not expression) chemotaxis appears to be mediated through an alternative pathway that depends upon α3β1 integrin and utilizes RhoA. If true then in the absence of β4 integrin ligand binding, loss of RhoA activity should inhibit EGF-induced keratinocyte migration. We tested this hypothesis by conducting transwell assays in the presence of β4 integrin ligand blocking antibody (ASC-8) using β4(+) cells that expressed a dominant-negative RhoA (Fig. 4F). As anticipated, when α6β4 integrin ligation is prevented, EGF-induced chemotaxis is decreased, suggesting an increased dependency on RhoA for migration upon inhibition of α6β4 ligation.

Expression and ligation of α6β4 integrin change the distribution and conformational activation of α3β1 integrin

Our studies thus far suggested that EGF-induced keratinocyte chemotaxis through α3β1 integrin becomes altered following expression and ligation of α6β4 integrin. Additional studies were therefore conducted to further examine the effects of α6β4 integrin on α3β1 integrin function in our keratinocyte model. Since reports have suggested that keratinocyte immortalization with HPV18 E6 and E7 genes might alter adhesion and cytoskeletal organization (Nguyen et al., 2000a), we also conducted studies using retrovirally transduced primary cells isolated from a second EB-PA patient. Because α3β1 integrin regulates the actin cytoskeleton, we first analyzed the effect of expression and ligation of α6β4 integrin on the distribution of actin and the FA component paxillin using immunofluorescence. β4(–) cells had diffusely organized actin and paxillin (Fig. 5A) with basally distributed α3β1 integrin (Fig. 5D). In contrast, β4(+) cells displayed organized, cortical stress fibers and FAs (Fig. 5B) with basal laterally distributed α3β1 integrin (Fig. 5E), comparable to normal keratinocytes (Symington et al., 1993). Importantly, when compared with β4(+) cells, β4(AD) cells had reduced α3β1 integrin at sites of cell-cell contact and exhibited prominent stress fibers, increased focal adhesions (which is a phenotype that is consistent with enhanced RhoA activity; Fig. 5C) and clustered basal α3β1 integrin (Fig. 5F). Furthermore, parallel cultures immunostained with the β1 integrin activation specific antibody, HUTS-4, suggested that these cells had a notable increase in β1 integrin activity (Fig. 5I). In contrast, β4(–) cells had more punctate basal β1 integrin activation (Fig. 5G) and β4(+) cells had only limited peripheral staining e(Fig. 5H), similar to normal keratinocytes (Penas et al., 1998). Using immunofluorescence, we compared the distribution of β1 integrin binding partners (α1, α2, α3, α4, α5) to determine the specific contribution of different heterodimer partners to this activation (data not shown). Data showed that only α3 integrin relocalized from sites of cell-cell to cell-matrix adhesions in

association with activated β1 integrin in β4(AD) cells. These data suggested that the β4(AD) cell phenotype is potentially linked to enhanced α3β1 integrin activation at the basal membrane of cells, which is consistent with focal adhesions.

α6β4 integrin controls relocalization and inactivation of α3β1 integrin through Rac1 activation and suppression of RhoA

Our data indicate that chemotaxis of β4(AD) cells requires

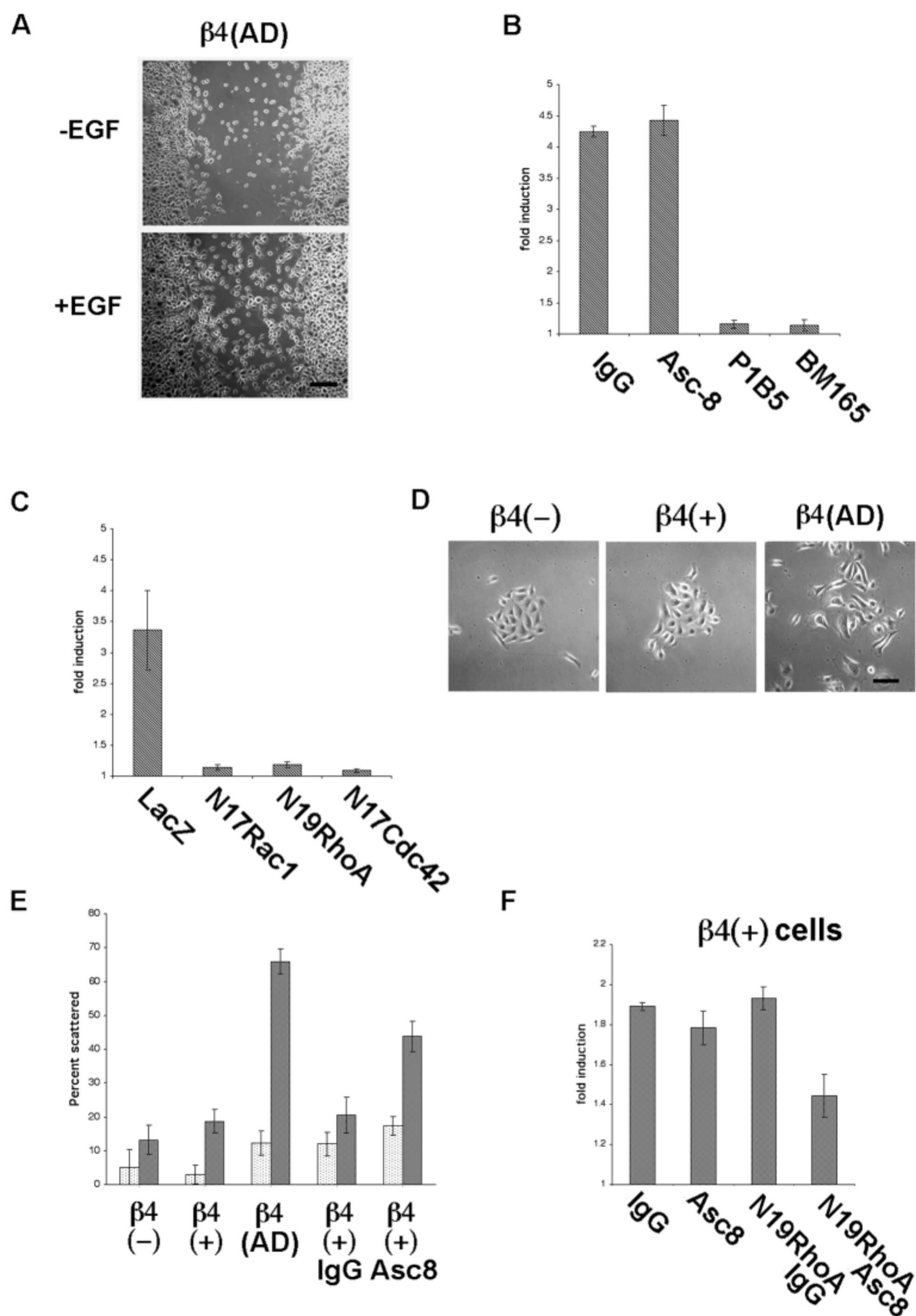


Fig. 4. EGF stimulates enhanced chemotaxis and reduced epithelial integrity in β4(AD) cells dependent upon an alternate pathway utilizing RhoA and integrin α3β1. (A) Effect of β4(AD) expression upon monolayer scratch migration. Cells were prepared as in Fig. 1A and incubated without growth factors (top panel) or with 2 ng/ml EGF (lower panel) for 24 hours at 37°C. Scale bar: 300 μm. (B) Effect of β4(AD) expression upon transwell chemotaxis. Transwell experiments with β4(AD) keratinocytes were performed with collagen IV-coated transwells and 2 ng/ml EGF. Cells were incubated for 16 hours with media supplemented in upper and lower chambers with IgG1, inhibitory β4 integrin antibody ASC-8, inhibitory α3 integrin antibody P1B5 or inhibitory laminin-5 antibody BM165. Actual induction IgG 60.0±4.2 cells/field, ASC-8 60.0±4.4 cells/field, P1B5 10.0±1.2 cells/field, BM165 16.1±1.1 cells/field. (C) Effect of GTPase inhibition upon β4(AD)-dependent chemotaxis. β4(AD) cells were retrovirally transduced with control LacZ or inhibitory GTPase constructs, N17Cdc42, N17Rac1 or N19RhoA. Chemotaxis experiments were performed with collagen IV-coated transwells and 2 ng/ml EGF. Actual induction, β4(AD)LacZ 136±23.1 cells/field, β4(AD)N17Rac1 15.0±3.7 cells/field, β4(AD)N19RhoA 21.0±3.6 cells/field, β4(AD)N17Cdc42 8.4±2.7 cells/field. (D) Effect of β4(AD) expression upon cell scattering. Cells were incubated at low density for 4 days in SFM before being photographed under phase contrast illumination. Scale bar: 40 μm. (E) Effect of EGF upon cell scattering. Cells were grown in growth factor free SFM for 4 days and scattered colonies counted using criteria described in materials and methods (light shaded columns). Cells were then incubated for 16 hours with 2 ng/ml EGF and colony scatter counts repeated (dark shaded columns). For antibody treatments, β4(+) cells were incubated for 3 days in normal SFM then 1 day in growth factor free SFM with 10 μg/ml mouse IgG or β4 integrin inhibitor ASC-8, before colony counts (light shaded columns). Colony scattering was measured after 6 hours with 2 ng/ml EGF (dark shaded columns). Each point represents the data from at least 50 colonies (n=3). (F) Effect of N19RhoA expression upon β4(+) transwell chemotaxis. Transwell experiments with β4(+)LacZ and β4(+)N19RhoA keratinocytes were performed with fibronectin-coated transwells and 2 ng/ml EGF. Cells were incubated for 16 hours with media supplemented in upper and lower chambers with IgG1 or inhibitory β4 integrin antibody ASC-8. Actual induction, β4(+)LacZ IgG 116.5±18.5 cells/field, β4(+)LacZ ASC-8 113.5±16.9 cells/field, β4(+)N19RhoA IgG 77.7±7.5 cells/field, β4(+)N19RhoA ASC-8 34±21.3 cells/field.

RhoA. RhoA is essential for the formation of actin stress fibers and focal adhesions and its functions can be antagonized by Rac1 (Nimnual et al., 2003; Sander et al., 1999). We therefore asked whether RhoA could play a role in the basal clustering and activation of α3β1 integrin and if this could be antagonized by Rac1 activation through α6β4 integrin. We expressed inhibitory N19RhoA or activated V12Rac1 in β4(AD) cells and recorded their impact upon the α3β1 integrin activation and actin cytoskeletal organization. Both inhibitory RhoA (Fig. 6A, middle row) and activated Rac1 (Fig. 6A, bottom row) prevented stress fiber formation, basal clustering of α3β1 integrin and α3β1 integrin activation. In addition, expression of N19RhoA induced relocalization of α3β1 integrin to sites of cell-cell contact while activated Rac1 enhanced cortical actin staining, reminiscent of its status in β4(+) cells. (Interestingly, expression of N19RhoA in β4(–) cells was not sufficient to alter the basal distribution of α3β1 integrin, data not shown.) These studies lead us to conclude that the cytoskeletal phenotype of β4 (AD) cells is dependent upon RhoA and can be inhibited by activating Rac1. Our previous experiments indicated that α6β4 ligation permits a sustained EGF-dependent activation of Rac1. We therefore asked whether inhibition of Rac1 in β4(+) cells could recapitulate any of the cytoskeletal characteristics exhibited by β4 (AD) cells (Fig. 6B). Expression of inhibitory N17Rac1 in β4(+) cells enhanced FA formation, basal clustering of α3β1 and β1 integrin activation (Fig. 6B, bottom panels).

In conclusion, these studies showed that α6β4 integrin ligation regulates the cellular localization of a3b1 integrin and tempers its activity by potentiating Rac1 activity. The coordination of this process is essential for facilitating EGF-induced chemotaxis via regulation of Rho GTPase cross-talk.

Discussion

Soluble EGFR ligands increase during wound healing (Marikovsky et al., 1993) and this is essential for re-epithelialization (Tokumaru et al., 2000). Our studies implicate α6β4 integrin as a primary control point for translation of EGF stimulation into keratinocyte migration. By rescuing the β4 integrin subunit in β4-null EB-PA cells we were able to show that α6β4 integrin and laminin-5 expression are essential for EGF-induced chemotaxis. However, the relationship between α6β4 expression and chemotactic signal transduction is complex and appears to be controlled by the ligand bound status of the β4 integrin subunit.

We found that ligation of α6β4 integrin drove chemotaxis by sustained activation of the Rho family GTPase Rac1. EGF stimulation may result in Rac1 activation through Fyn-mediated tyrosine phosphorylation of the β4 integrin cytoplasmic domain (Mainiero et al., 1996; Mariotti et al., 2001). However, at the physiological levels of EGF used in our chemotaxis assays we did not observe tyrosine phosphorylation of the β4 subunit. Alternately, Rac1 activation may be potentiated through increased phosphoinositide 3 kinase (PI3 kinase) activity following α6β4 ligation (Nobes and Hall, 1995; Shaw et al., 1997). Sustained Rac1 activation appears to redirect α3β1 integrin away from basal focal contacts and towards sites of cell-cell contact thereby functioning to temper its activation state. This implies that α6β4 integrin antagonizes α3β1 integrin through Rac1 to ultimately modulate signal transduction and thereby regulating cell motility (summarized in Fig. 7).

In the absence of β4 integrin ligation, Rac1 activation is no longer sustained and keratinocytes migrate through an alternative chemotactic pathway that depends upon α3β1 integrin and RhoA. The nature of this migration is also less directed and cells exhibit scattering. Interestingly, recent data have shown that introduction of an attachment-defective EGFP/β4 fusion into EB-PA cells also increased keratinocyte migration (Geuijen and Sonnenberg, 2002). This increase was apparently associated with a destabilization of the link between laminin-5 and the cytoskeleton through plectin. Although a similar situation may also occur in β4(AD) cells, this does not explain how EGF chemotaxis is restored by β4(AD) expression. In this regard, our data show that while β4(–) cells exhibit basal clustering of α3β1 integrin, α3β1 activity is reduced and they fail to undergo chemotaxis in response to EGF stimulation. Thus, expression of β4(AD) must facilitate

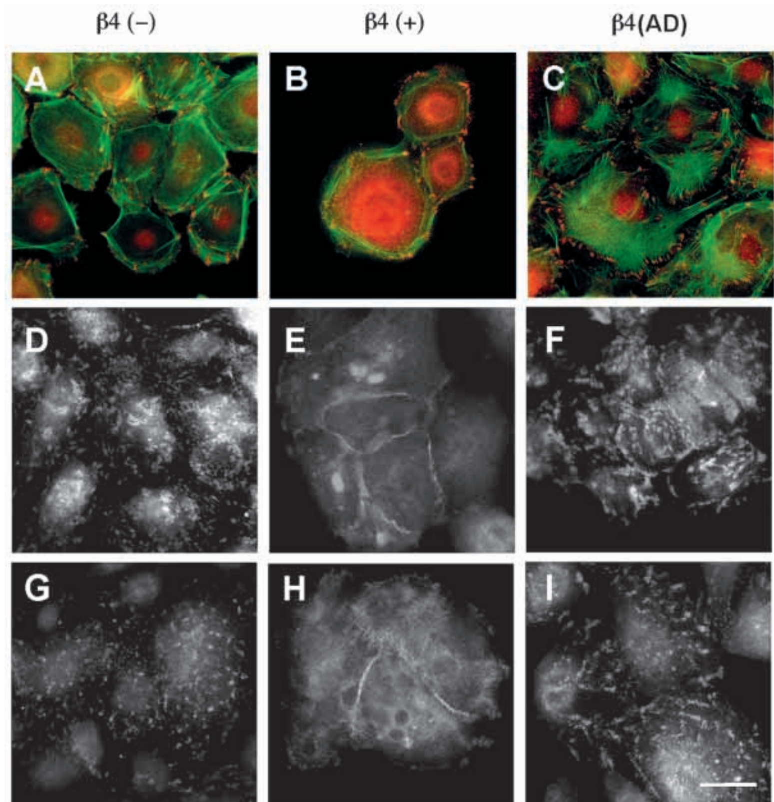


Fig. 5. Expression and ligation of α6β4 integrin changes the localization and activation state of α3β1 integrin. Cells were cultured on glass coverslips for 4 days prior to immunofluorescence staining for FA components. Cells were fixed with 3% formaldehyde and solubilized with 0.5% Triton X-100 buffer for 30 minutes at RT. Fixed cells were washed with PBS and blocked with 1% BSA for 1 hour. Cells were stained with FITC-phalloidin for filamentous actin (stained in green A-C) and anti-paxillin mAb (stained in red A-C). Further samples were stained for α3 integrin (D-F) and the conformationally active form of the β1 integrin subunit with mAb HUTS-4 (G-I). Scale bar; 20 μm.

the activation of alternative chemotactic pathways through effects on EGFR signaling. This activity could arise through destabilization of hemidesmosome-associated proteins, conformational alterations secondary to introduction of β4(AD) or more direct effects on β4 integrin activity mediated via loss of β4 ligation.

Indirect activation of α3β1 integrin though β4(AD)

So how might expression of non-ligated β4 integrin modulate α3β1 integrin function? One attractive possibility is that α6β4 integrin might compete for a regulatory molecule that modifies α3β1 integrin activation state. For example, members of the tetraspanin family of cell surface molecules have been shown to modify integrin function, particularly those of the β1 integrin family (Berditchevski and Odintsova, 1999). At least one member of this family, CD151 also associates preferentially with α6β4 integrin (Sterk et al., 2000), and expression of integrin β4 subunit in EB-PA cells relocates CD151 from α3β1 to α6β4 receptor clusters. It is possible that in our experiments introduction of β4(AD) results in a competitive relocalization of tetraspanins away from α3β1 integrin, which then alters the conformational activation of α3β1 integrin.

Direct activation of α3β1 integrin though β4(AD)

In a number of tumor systems, the ability of α6β4 to mediate invasion and activate PI3K does not require ligation of the β4 integrin subunit (Gambaletta et al., 2000; Shaw et al., 1997). Significantly, part of this process has been elucidated, since β4 integrin has an affinity for the hepatocyte growth factor receptor, Met, and amplifies invasive signals, including PI3 kinase through Met, irrespective of its ability to ligate with laminins (Trusolino et al., 2001). This signal transduction proceeds via tyrosine phosphorylation of the β4 integrin subunit. In our experiments, EGF did not mediate tyrosine phosphorylation of the β4 subunit in β4(AD) cells so an analogous ligand-independent function for EGF does not immediately appear likely. However, EGFR family member ErbB2, like Met, can associate with β4 integrin and amplify PI3 kinase activation and invasion independent of α6β4 ligation, (Gambaletta et al., 2000). Therefore, it is possible that loss of α6β4 ligation may trigger an alternative

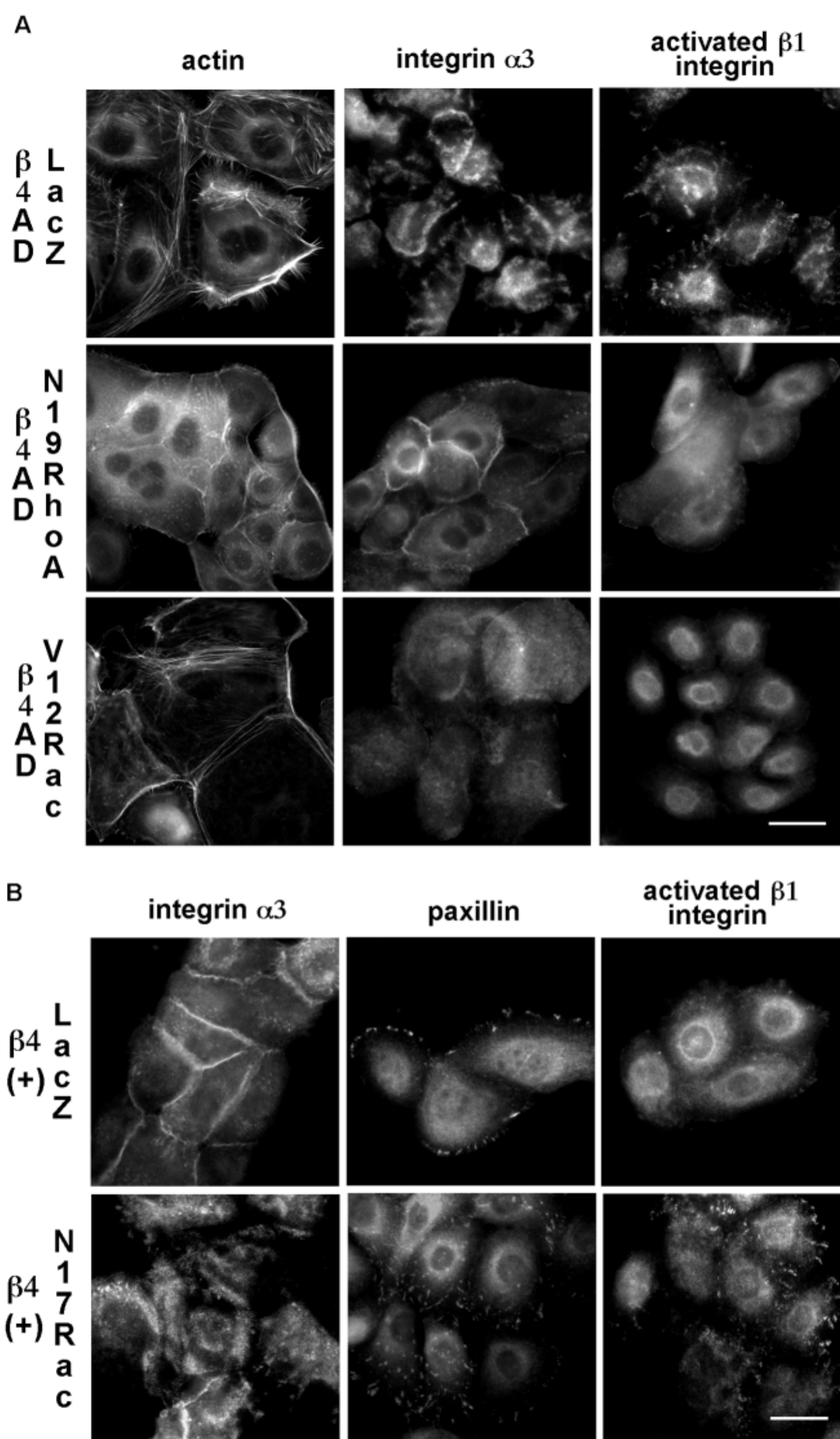


Fig. 6. Activation of Rac1 and suppression of RhoA through α6β4 integrin control the localization and activation of α3β1 integrin. (A) Effect of RhoA inhibition or Rac1 activation on α3β1 distribution. β4(AD) cells were transduced with control LacZ (top row), inhibitory N19RhoA (middle row) or activated V12Rac1 (bottom row) and examined by immunofluorescence microscopy. Left panel shows actin distribution with TRITC-phalloidin, center and right panels show α3 integrin and conformationally active β1 integrin. (B) Effect of Rac1 inhibition on distribution and activation of α3β1 integrin. β4(+) cells were retrovirally transduced with control LacZ (upper row) or inhibitory N17Rac1 (lower row) and studied by immunofluorescence microscopy. Left panel shows α3 integrin, center panel shows paxillin and right panel shows distribution of conformationally active β1 integrin. Scale bar: 20 μm.

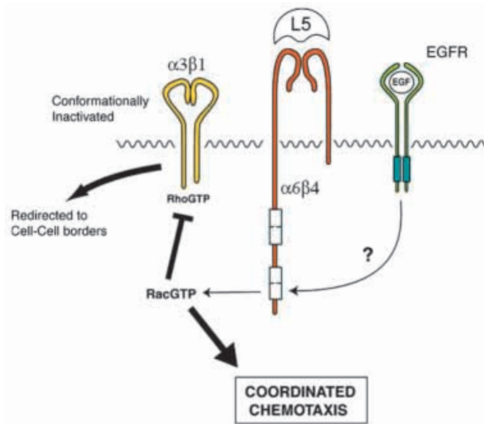


Fig. 7. Model explaining the role of α6β4 integrin in the coordination of migration through EGF. Cells without α6β4 integrin cannot sustain chemotactic EGFR signals, either from a loss of EGFR/β4 interactions and/or due to suppression of α3β1 integrin activity. Upon expression and ligation of α6β4 integrin, Rac1 activation by EGF is sustained, suppressing α3β1 integrin and RhoA. α3β1 integrin is redirected from sites of basal focal contact to sites of cell-cell contact and cells migrate as an integral epithelial sheet.

chemotactic pathway through EGFR that is independent of tyrosine phosphorylation.

Potential significance of EGFR signaling through α6β4 integrin during wound healing

Previous studies have ruled out a significant function for α6β4 integrin in epithelial migration because inhibitory antibodies to α6 or β4 fail to markedly impair chemotaxis or wound healing (Goldfinger et al., 1999; Hintermann et al., 2001; Nguyen et al., 2000b). We suggest that inhibition of α6β4 ligation results in the activation of a secondary chemotactic pathway that is dependent upon α3β1 integrin.

What would be the significance of this antagonstic relationship between α6β4 and α3β1 integrins to migrating keratinocytes during wound healing? Studies have shown that keratinocytes rely on two distinct pathways for attachment and spreading as cells move across the provisional dermal collagen matrix (Nguyen et al., 2000a). Cells at the wound front in vivo are dependent upon Rho family GTPases for attachment while cells distal from the wound edge mediate attachment and spreading via ligation of α6β4 to secreted laminin-5 and PI3 kinase activation. These differences in integrin activity and signaling have been attributed to changes in ECM composition from dermal collagen to laminin-5. In light of our current observations this model can now be integrated with the observed functions of α6β4 in the control of chemotactic migration. We believe that it is unlikely that keratinocytes make isolated contact between collagen I and α2β1 integrin at the front edge of a wound. Indeed, leading cells highly express unprocessed laminin-5, and upregulate expression of many integrins including α2β1, α3β1 and α6β4 (Kainulainen et al., 1998; Kurpakus et al., 1991; Larjava et al., 1993). Therefore, growth factor-induced chemotactic signals must be translated from several of these inputs into a functionally coordinated response.

We suggest that α6β4 integrin acts as a central control point for coordinated chemotactic responses during wound healing. At the wound front, changes in the kinetics of α6β4 attachment, ECM composition or its degree of processing, or even the density of laminin-5 deposition (Geuijen and Sonnenberg, 2002) may compromise ligation of α6β4 integrin resulting in increased dependence on chemotaxis through α3β1 integrin. As cells advance, α6β4 integrin ligates with secreted laminin-5, which enhances Rac1 activity and suppresses α3β1-dependent chemotaxis. This may be required to help maintain epithelial cohesion between leading cells and the epithelial sheet during reepithelialization. In support of this, spatial activation of Rac1 in epithelial cells plated on laminin has been implicated in the regulation of cellular cohesion through E-cadherin (Sander et al., 1998). Activated Cdc42 and Rac1 have also been implicated in the maintenance of both epithelial polarity and formation of tight junctions (Jou et al., 1998; Kroschewski et al., 1999; Nobes and Hall, 1999). Thus, we conclude, that activation of Rac1 through EGF stimulation of α6β4 integrin is important for EGF-mediated motility and possibly for the maintenance of cellular polarity and cohesion during wound healing.

In summary, we have elucidated a novel mechanism by which α6β4 integrin coordinates EGF signaling to keratinocytes to mediate chemotaxis. The divergent nature of this signal transduction may help to explain how keratinocytes coordinate EGF-stimulated migration and maintain epithelial integrity while migrating over matrix of changing composition. It may also help our understanding of the distinct roles of α6β4 during tumor progression.

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