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β4 Integrin and Epidermal Growth Factor Coordinately Regulate Electric Field-mediated Directional Migration via Rac1

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Endogenous DC electric fields (EF) are present during embryogenesis and are generated in vivo upon wounding, providing guidance cues for directional cell migration (galvanotaxis) required in these processes. To understand the role of beta (β)4 integrin in directional migration, the migratory paths of either primary human keratinocytes (NHK), β4 integrin-null human keratinocytes (β4−), or those in which β4 integrin was reexpressed (β4+), were tracked during exposure to EFs of physiological magnitude (100 mV/mm). Although the expression of β4 integrin had no effect on the rate of cell movement, it was essential for directional (cathodal) migration in the absence of epidermal growth factor (EGF). The addition of EGF potentiated the directional response, suggesting that at least two distinct but synergistic signaling pathways coordinate galvanotaxis. Expression of either a ligand binding–defective β4 (β4+AD) or β4 with a truncated cytoplasmic tail (β4+CT) resulted in loss of directionality in the absence of EGF, whereas inhibition of Rac1 blinded the cells to the EF even in the presence of EGF. In summary, both the β4 integrin ligand–binding and cytoplasmic domains together with EGF were required for the synergistic activation of a Rac-dependent signaling pathway that was essential for keratinocyte directional migration in response to a galvanotactic stimulus.

INTRODUCTION

Directional migration is a fundamental process essential for numerous cellular responses including embryonic development, inflammation, and tumor metastasis as well as skin wound repair. When skin is wounded, keratinocytes migrate directionally into the wound bed to initiate reepithelialization, necessary for wound closure and restoration of barrier function. Many factors are involved in orchestrating the complex process of wound healing (Martin, 1997), and recent studies have led to the belief that a lateral electric field (EF) generated immediately upon wounding is one of the earliest signals to initiate directed cell migration (Ojingwa and Isseroff, 2003; McCaig *et al.*, 2005). The lateral EF is driven by the transepithelial potential through the low-resistance wound pathway, creating a negative pole (cathode) at the center of the wound (Borgens *et al.*, 1977). Endogenous DC EFs of 40–200 mV/mm have been measured near wounds in mammalian epithelia (Barker *et al.*, 1982; McGinnis and Vanable, 1986; Chiang *et al.*, 1992; Sta Iglesia *et al.*, 1996; Sta Iglesia and Vanable, 1998). Skin-derived keratinocytes can sense an EF of this magnitude within minutes, reform their lamellipodia facing the cathode, and migrate directionally in vitro, a process known as galvanotaxis (Nishimura *et al.*, 1996). The mechanism for keratinocyte sensing of the EF is largely unknown, but a role for Ca^{2+} influx (Trollinger *et al.*, 2002; Fang *et al.*, 1998), epidermal growth factor receptor (EGFR) phosphorylation (Fang *et al.*, 1999), and cAMP-dependent PKA (Pullar *et al.*, 2001; Pullar and Isseroff, 2005) have been implicated in previous work from this laboratory. Thus, in addition to providing a physiological mechanism for rapid orientation of directional migration of keratinocytes in wound healing, the galvanotaxis response of cells in vitro provides an experimental paradigm for dissecting the mechanisms that underlie directional migratory responses of cells.

α6β4 integrin, a receptor for laminin 332, plays a critical structural role in keratinocytes, localizing to the hemidesmosome (HD) and providing tight anchorage of basal keratinocytes to the basement membrane (Sonnenberg *et al.*, 1991). Indeed, in patients expressing a null mutation of the β4 integrin, epidermal blistering occurs because of the poor adhesion of the epidermis to the basement membrane (Niessen *et al.*, 1996; van der Neut *et al.*, 1996). However, the α6β4 integrin can switch from a structural adhesive role to a signaling component involved in cell migration. On wounding, growth factors secreted in the wound can transform

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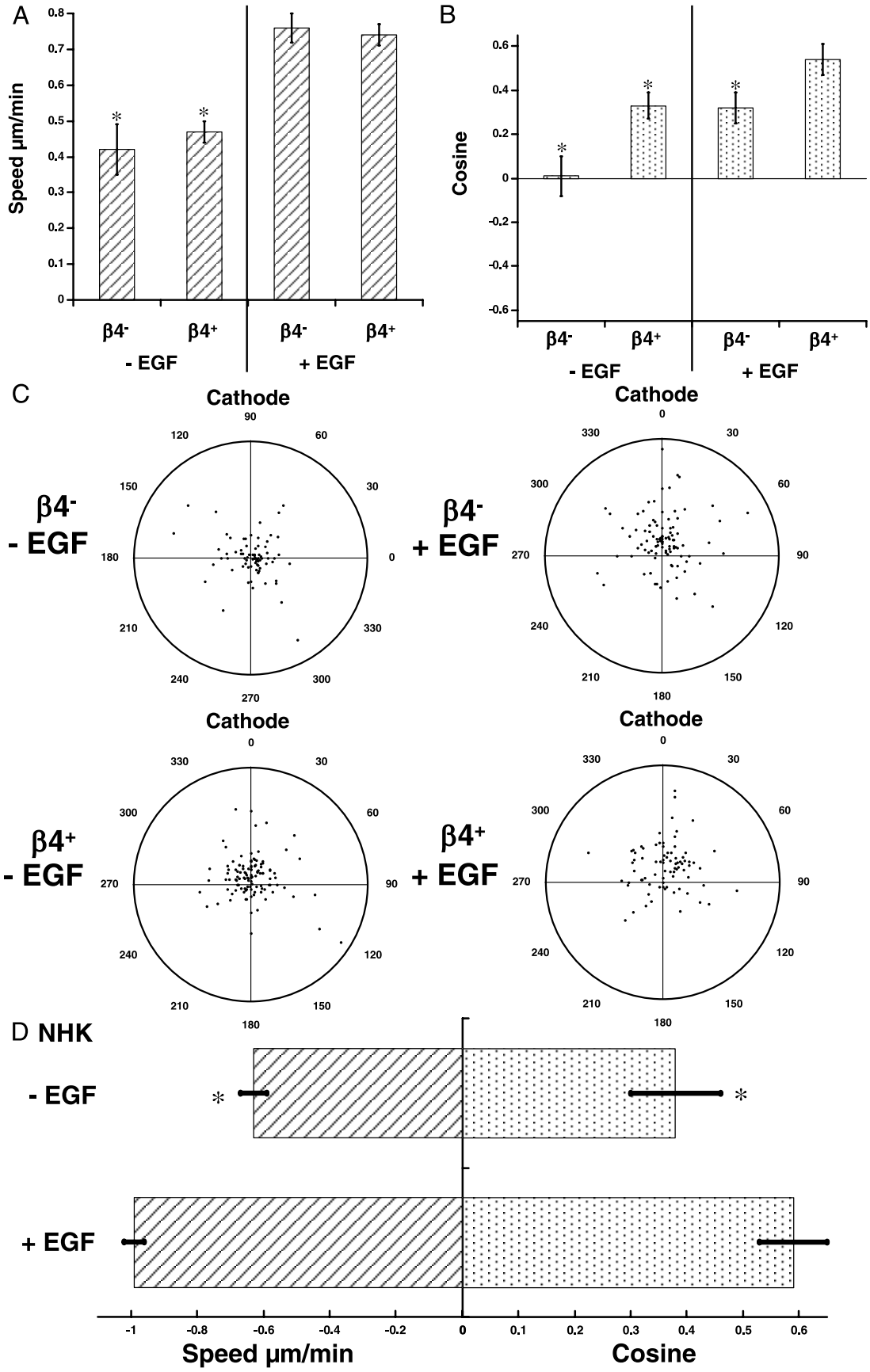
A
Speed µm/min
0.8
0.7
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β4-
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+ EGF
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Cosine
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-1
-0.8
-0.6
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Speed µm/min
Cosine

Figure 1.

basal wound edge keratinocytes from static cells to motile ones. This is accompanied by the disassembly of the HD and the relocalization of α6β4 integrin from the HD to the lamellipodia of migrating cells (Marikovsky *et al.*, 1993; Mainiero *et al.*, 1996; Martin, 1997; Santoro *et al.*, 2003). The ligand for this integrin, laminin 332 (also called laminin 5), is secreted by keratinocytes both in culture (Marinkovich *et al.*, 1992; Ryan *et al.*, 1996) and at the leading edge of an epidermal outgrowth into the wound bed (Sonnenberg *et al.*, 1991; Ryan *et al.*, 1999), providing a suitable binding partner for the integrin as the cell migrates.

Previously, the α6β4 integrin has been implicated in the chemotaxis response of keratinocytes, where it plays a role in EGF-induced cell migration through the sustained activation of Rac1 (Russell *et al.*, 2003). Mechanisms regulating directional migration in response to EF could be similar. Just as actin is polymerized preferentially at the leading edge of neutrophils during chemotaxis (Zigmond, 1977; Devreotes and Zigmond, 1988), it similarly accumulates at the cathodal-facing edge of corneal epithelial cells migrating directionally in an applied EF (Zhao *et al.*, 1999). Additionally, the EGFR is polarized in both corneal epithelial cell (Zhao *et al.*, 2002) and keratinocyte (Fang *et al.*, 1998, 1999) galvanotaxis and is required for fibroblast directional migration toward fibronectin or laminin (Li *et al.*, 1999). With this possible similarity in mind, we investigated the signaling roles of both the α6β4 integrin and EGFR in the galvanotaxis response in keratinocytes.

Here we describe the novel role for β4 integrin in keratinocyte galvanotaxis and delineate the corresponding signaling pathways. We report that although the expression of β4 integrin appears to have no effect on rate of migration on a collagen substrate, it is critical for EF-mediated directional migration. In the absence of exogenously supplied EGF, β4 integrin-null cells cannot respond with directional migration. The β4 integrin coordinates with EGF to initiate and maintain persistent, robust cathodal migration. The β4 integrin extracellular laminin-binding domain and its functional cytoplasmic domain are both required for β4 integrin–mediated galvanotaxis. We demonstrate that Rac1 is the common downstream target for both β4 integrin–mediated and EGF-mediated directional migration in an EF. Therefore, the molecular mechanisms underpinning both chemotaxis and galvanotaxis employ Rac1 as a critical mediator of directional responses.

Figure 1 (facing page). β4 integrin and EGF are both required for keratinocyte directional migration. β4+, β4−, and NHKs were starved for 16 h in basal medium containing no growth factors. Cells, 2×10^4,were plated in the presence or absence of 2 ng/ml EGF on the collagen-coated glass in the center of the galvanotaxis chambers as described in *Materials and Methods.* An electric field (EF) of 100 mV/mm was applied across the cells, and images were captured every 10 min over a 1-h period. The speed (μm/min; A) and directionality (cosine; B) of migration were calculated as described in *Materials and Methods.* (A) The speed of migration for β4− and β4+ cells in the presence and absence of EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+, no EGF, n = 113; β4−, EGF, n = 98 and β4+, EGF, n = 81. *$p < 0.01$ between all conditions and those in the presence of EGF. (B) The cosine of migration for β4− and β4+ cells in the presence and absence of EGF is shown. *$p < 0.01$ between all conditions and β4+ + EGF. (C) To clearly demonstrate the directionality and rate of migration for each condition the data were plotted using a circle graph. Each cell's position at time (t) = 0 is at the origin (0,0), and its final position is at the end of the 1-h exposure to the EF is plotted as a single point on the graph. The radius of each circle represents 100 μM of translocation distance. The cathode is at the top of each graph (0°) and the anode is at the bottom (180°). The data shown are combined from at least four independent experiments. (D) The speed and directionality for NHKs in the presence and absence of EGF is presented (NHK, no EGF, n = 59; NHK, EGF, n = 78). *$p < 0.01$ between all conditions and those in the presence of EGF. The data shown are combined from at least four independent experiments on three separate cell strains. Error bars, SEM.

MATERIALS AND METHODS

Primary Human Keratinocytes

Primary human keratinocytes (NHK) were isolated from neonatal foreskins as we have reported previously (Isseroff *et al.*, 1987; Pullar *et al.*, 2006), under an approved exemption granted by the Internal Review Board at the University of California, Davis and cultured using a modification of the method of Rheinwald and Green (1975). NHKs were grown in keratinocyte growth medium (KGM; Epilife, 0.06 mM Ca^{2+}; Cascade Biologics, Portland, OR), supplemented with human keratinocyte growth supplement (0.2 ng/ml EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 0.18 μg/ml hydrocortisone, and 0.2% bovine pituitary extract; Cascade Biologics) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin; Gemini Bio-Products, Calabasas, CA) at 37°C in a humidified atmosphere of 5% CO_2. Cell strains isolated from at least three different foreskins were used in all experiments, performed with subcultured cells between passages 3 and 7.

Cell Lines

Primary human keratinocytes lacking β4 integrin were obtained from a patient with epidermolysis bullosa with pyloric atresia (EB-PA) resulting from a premature termination codon (C658X; Russell *et al.*, 2003). Neonatal foreskin keratinocytes and EB-PA patient keratinocytes were immortalized with HPV18 E6 and E7 genes (Kaur *et al.*, 1989) and designated NIK and β4−, respectively. Cells were cultured in equal parts defined keratinocyte serum free medium (SFM; Invitrogen, Carlsbad, CA) and keratinocyte medium 154 (Cascade Biologics). Culture media contained 100 IU/ml penicillin and 100 IU/ml streptomycin, and cells were cultured at 37°C in a humidified 5% CO_2 incubator.

cDNA Constructs and Vectors

The cloning of the β4 integrin gene (β4+) and the adhesion defective β4 integrin gene (β4+AD) have been described previously (Russell *et al.*, 2003). The cDNAs for the constitutively active and inactive Rac1 mutants, V12Rac1 and N17Rac1, were a kind gift of Dr. Alan Hall (University College London, United Kingdom) and were cloned as EcoRI fragments into the retroviral expression vector LZRS (Kinsella and Nolan, 1996) containing the encephalomyocarditis virus (EMCV)-IRES and blasticidin-resistance sequences (Deng *et al.*, 1998). To generate the β4 integrin construct with a truncated cytoplasmic domain, (β4+CT) the WT β4 integrin cDNA was removed from the LZRS retroviral expression vector by EcoRI digestion and subsequently cloned into the EcoRI site of the pENTR1A vector (Invitrogen). The COOH-terminal region of the β4 cytoplasmic deletion mutant $\beta4^{1-1217}$ was obtained by PCR using β4-del (sac)-5′ (AGTACTGGATTCAGGGTGACTCCG, sense) and β4-1217+EcoRV-3′ (TAGATATCAGTGGGTGCGGCAGGACACCA, antisense) primers and subsequently exchanged with WT β4 in pENTR1A. The reverse primer included a stop codon followed by the EcoRV site. PCR was performed using Takara Ex Taq (Takara, Tokyo, Japan) with the β4 WT cDNA in LZRS as a template. The PCR fragment was cloned into a T-Easy vector (Promega, Madison, WI; $\beta4^{1-1217}$ in T-Easy), and its sequence was verified. The SacI/EcoRV fragment from $\beta4^{1-1217}$ in T-Easy was ligated into the SacI/EcoRV site within the WT β4 in pENTR1A. Finally, $\beta4^{1-1217}$ cDNA was recombined from the pENTR1A to the LZRS retroviral expression vector, including a Gateway cassette, using the LR Clonase II Enzyme mix (Invitrogen) by a recombination reaction.

Retroviral Transduction

Keratinocytes were transduced with retroviral vectors for the stable expression of WT β4 integrin (β4+), β4+AD, V12Rac1, N17Rac1, and β4+CT constructs. Amphotropic retrovirus was produced in modified 293 cells as previously described (Kinsella and Nolan, 1996). Keratinocytes, 2×10^5, were incubated in serum-free medium (SFM) containing 5 μg/ml poybrene for 15 min before infection. Medium was removed and 10 ml of retroviral supernatant containing 5 μg/ml polybrene (Sigma, St. Louis, MO) was added to both viral supernatant and keratinocyte media. Medium was removed, and 4 ml of retroviral supernatant was added. Plates were centrifuged at $300 \times g$ for 1 h at 32°C using a Beckman GS-6R centrifuge (Beckman Coulter, Fullerton, CA). Cells were incubated at 37°C for 24 h followed by replacement with fresh SFM and selection with 5 μg/ml blasticidin (Calbiochem, La Jolla, CA). Selection was omitted for primary cell cultures.

Antibodies and Inhibitors

Mouse mAb 3E1 against β4 integrin (immunostaining) and ASC-8 were 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. Mouse monoclonal anti-vinculin antibody (h-vin-1) was purchased from Sigma. The specific Rac1 inhibitor 553502 was purchased from Calbiochem (San Diego, CA) in solution and used at a concentration of 100 μM.

Cell Treatments

Cells were starved of growth factors in basal medium (Epilife containing antibiotics [100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin]) for 16 h, before plating for galvanotaxis studies. Cells were plated in the presence or absence of epidermal growth factor (EGF; Invitrogen), 2 ng/ml for 3 h before EF application. ASC-8 was added to the cells for 10 min before plating. Cells were preincubated with the Rac1 inhibitor for 30 min before EF application. Galvanotaxis was performed in the presence or absence of EGF, ASC-8, or 553502.

Galvanotaxis

The galvanotaxis chamber construction and DC application were performed as described previously (Pullar and Isseroff, 2005). Briefly, the galvanotaxis chamber is composed of a rectangular Plexiglas frame with two medium reservoirs on opposite sides to which a 45 × 50-mm piece of No. 1.5 glass coverslip is attached to form the chamber bottom. This allows for continuous observation of the plated cells on an inverted microscope. Keratinocytes, 2×10^4, are plated onto the collagen-coated center of the chamber between two 25 × 10-mm coverslip spacers and allowed to attach for 3 h at 37°C. A third 25-mm^2 coverslip is placed on top, straddling the two spacer coverslips and covering the cells plated on the collagen-coated center panel. This third coverslip rests ~100–105 μm above the center panel and is sealed on top of the spacer coverslips with silicone high-vacuum grease (Dow Corning, Midland, MI). This small height is chosen to minimize the cross-sectional area through which current flows. A small cross-section creates a high-resistance pathway, resulting in a higher voltage gradient for a fixed current. The aqueduct allows for medium and current flow across the cells. The voltage across the coverslip is measured using a voltmeter via silver-sliver chloride (Ag-AgCl) wire electrodes inserted into both medium reservoirs on either side of the center panel. Six-centimeter-long 2% agar/phosphate-buffered saline–filled pieces of polypropylene tubing connect each end of the chamber to a medium-filled well in which the Ag-AgCl electrodes are placed, to separate possible electrode byproducts from the cells themselves. A 100-mV/mm current is applied across the chamber. The current is measured with an ammeter in series, and we only use chambers for which the current flow is kept below 0.6 mA, to minimize joule heating. Furthermore, temperature of the medium in the chambers is maintained at 36°C by placing the chamber on a metal plate heated to and maintained at 39°C. The temperature is continuously monitored during the experiment using a YSI 400 analog temperature probe (Yellow Springs Instrument Co., Yellow Springs, OH) directly attached to the metal plate and does not vary by more than 1°C over the course of the experiment.

Time-Course Observation and Data Analysis

The galvanotaxis chambers rest on inverted Nikon microscopes (Melville, NY), and control and test assays were run simultaneously on duplicate setups side by side. Time-lapse images of the cell migratory response are digitally captured every 10 min over a 1-h period by a QImaging Retiga-EX cameras (Burnaby, BC, Canada) controlled by a custom automation written in Improvision Openlab software (Lexington, MA) on a Macintosh G4 (Apple Computer, Cupertino, CA). After each cell's center of mass is tracked using the Openlab software, directionality of migration is calculated and imported to Excel (Microsoft, Redmond, WA). Cosine θ describes the direction of migration and is a measure of the persistence of cathodal directedness, where θ is the angle between the field axis and the vector drawn by the cell migration path. The average cosine $\theta = \Sigma_i \cos \theta_i / N$, where Σ_i is the summation of 50–150 individual cells from at least three different cell strains. Angle zero ($\theta = 0°$) is assigned to the negative pole (cathode) and increasing angles assigned in a clockwise manner, with $q = 180°$ aligned with the positive pole (anode). Therefore, the cosine θ will provide a number between −1 and +1, and the average of all of the separate cell events yields an index of directional migration. For example, if a cell were to move directly to the negative pole, the angle $\theta = 0°$ and the cosine $\theta = 1$. "Cosine," therefore, refers to the average directional migration index of separate cell migration events at the end of a 1-h period. Results are given as average cosine θ ± the SEM. Significance is taken as $p < 0.01$, using Student's *t* test (unpaired) to compare the means of two cell populations.

The rate and directionality of migration can be clearly represented graphically using a polar plot. Each cell is placed at the origin of the circle at time 0, and its final position is plotted as a single point on the graph. The distance of the points from the origin provides an indication of cell migration rate and the distribution of points between the four quadrants indicates the direction of migration. The radius of each graph is 100 μM.

Immunofluorescence Staining and Microscopy

Sterile glass cover slips (Fisher Scientific, Pittsburgh, PA) were transferred into 12-well dishes and collagen-coated with 60 μg/ml collagen I (Vitrogen 100; Collagen, Palo Alto, CA) in KGM for 1 h at 37°C. Coverslips were washed three times with KGM, and 3×10^4 cells were added per well and allowed to attach for 3 h. Coverslips were processed at room temperature unless otherwise noted. Coverslips were washed twice in PBS and fixed for 10 min in 4% paraformaldehyde. Coverslips were washed twice in PBS between each step. Cells were permeabilized for 5 min with 0.1% Triton X-100/PBS and blocked with 5% goat serum/PBS for 20 min. Primary monoclonal anti-β4 integrin antibody (3E1; 1:50) or primary monoclonal anti-vinculin antibody (h-vin-1; 1:100) was added dropwise in 1% goat serum/PBS (1:100) and incubated for 1 h at 37°C. A goat anti-mouse cy3 antibody (Jackson ImmunoResearch Laboratory, West Grove, PA; 1:100) was added in 1% goat serum/PBS for 1 h at 37°C. Standard controls were performed. Coverslips were incubated with the primary antibody alone or the secondary antibody alone to ensure specificity. Finally, Prolong gold anti-fade reagent (Molecular Probes, Eugene, OR) was used according to manufacturer's instructions to mount the coverslips onto glass microscope slides. Slides were viewed on an inverted fluorescent Nikon Diaphot microscope using a 40× pan fluor objective. Images were captured using QImaging Retiga-EX cameras and are presented in gray scale (β4 integrin) or pseudocolored red (vinculin).

RESULTS

Both β4 Integrin and EGF Are Required for Keratinocyte Directional Migration

Keratinocytes will quickly attach to collagen-coated culture plates, and within 3 h the majority of the cells will develop a motile phenotype, with a characteristic crescent shape and a broad lamellipodium along the convex side of the cell. They will begin to secrete laminin 332 during this time frame (Marinkovich *et al.*, 1992), switching from collagen- to laminin-dependent adhesion and signaling (Nguyen *et al.*, 2000a, 2000b; Frank and Carter, 2004).

To determine the role that β4 integrin plays in both keratinocyte motility and directional migration within a DC EF, human keratinocyte cell lines lacking the β4 integrin (β4−), those in which the β4 integrin has been reexpressed (β4+), or primary human keratinocytes (NHK) were examined for their migratory response in an EF. We have previously demonstrated that the application of a DC EF does not alter the keratinocyte migration rate (Nishimura *et al.*, 1996; Pullar and Isseroff, 2005). The absence (β4−) or presence (β4+) of β4 integrin appears to have no effect on the rate of cell migration in this system, whereas the application of EGF significantly increases their speed by 81 and 57%, respectively (Figure 1A).

In contrast, directional migration is markedly affected by the absence of the β4 integrin. The β4− cells, in the absence of EGF, are blinded to the directional stimulus (Figure 1B) and migrate randomly. Reexpression of β4 integrin (β4+) partially (61%) restores directional migration in the absence of EGF, whereas the directional responses of β4− and β4+ cells are both potentiated in the presence of EGF (Figure 1B). β4+ cells are 69% more directional than β4− cells in the presence of EGF. To clearly demonstrate these differences, the rate and directionality of migration for the β4− and β4+ cells are represented graphically using a polar plot as described. Although the distance from the origin of β4− and β4+ cells is similar in the absence of EGF, more cells are present in the top two quadrants in the presence of β4 integrin, demonstrating that reexpression of the β4 integrin partially restores directional migration in β4− cells and fully restores directional migration in β4+ cells (as visualized by the majority of cells plotted in the top two quadrants in polar plot Figure 1C). In primary human keratinocytes (NHK; Figure 1D), EGF also potentiates both motility and directional migration in response to the applied EF. It appears, therefore, that there are at least two distinct but synergistic

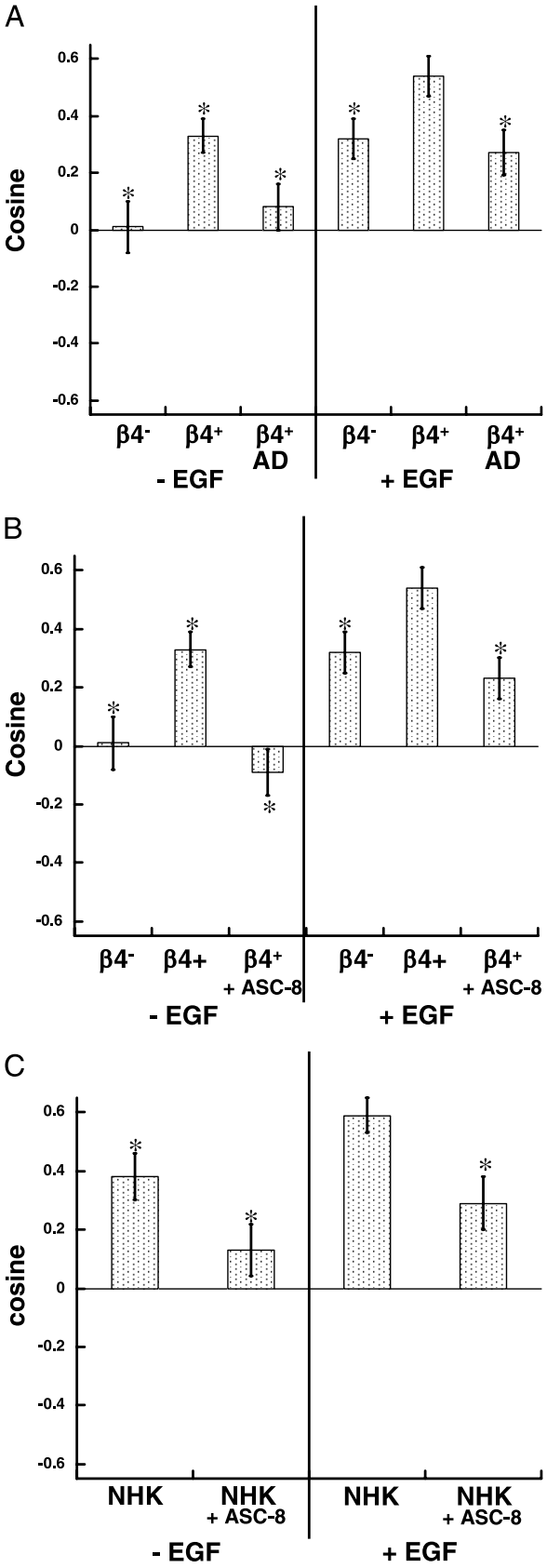

Figure 2. Laminin 332–mediated ligation of β4 integrin is required for keratinocyte directional migration in the absence of EGF. β4+, β4+ADs were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. (A) The cosine of migration for β4+ and β4+AD cells in the presence and absence of EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+, no EGF, n = 113; β4+AD, no EGF, n = 73; β4−, EGF, n = 98; β4+, EGF n = 81; and β4+AD EGF, n = 82. The data shown are combined from at least four independent experiments. Error bars, SEM. *$p < 0.01$ between all conditions and β4+ cells in the presence of EGF. (B) β4+ and NHKs were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. The cosine of migration for β4+ cells in the presence and absence of ASC-8 and EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+, no EGF, n = 113; β4+, no EGF + ASC-8, n = 75; β4−, EGF, n = 98; β4+, EGF, n = 81; and β4+, EGF + ASC-8, n = 114). The data shown are combined from at least four independent experiments. Error bars, SEM. *$p < 0.01$ between all conditions and β4+ in the presence of EGF. (C) The cosine of migration for NHKs in the presence and absence of ASC-8 and EGF is shown. The number of cells is as follows: NHK, no EGF, n = 59; NHK, no EGF + ASC-8, n = 70; NHK, EGF, n = 78; and NHK, EGF + ASC-8, n = 62. The data shown are combined from at least four independent experiments on three separate cell strains. Error bars, SEM. *$p < 0.01$ between all conditions and NHK in the presence of EGF.

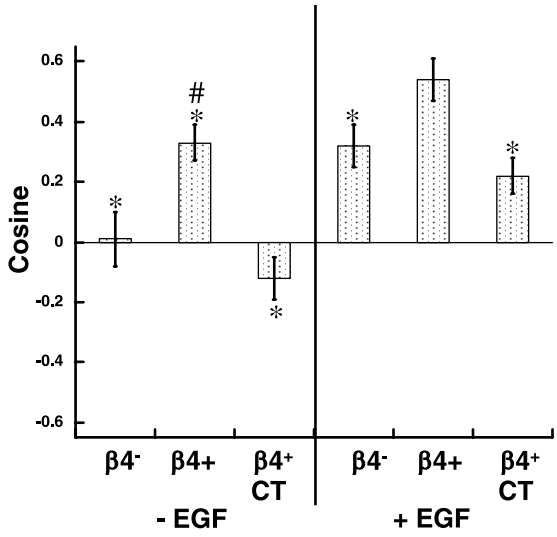

Figure 3. The cytoplasmic tail of β4 integrin is essential for β4 integrin–mediated keratinocyte directional migration in the absence of EGF. β4+, β4+CTs were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. The cosine of migration for β4+ and β4+CT cells in the presence and absence of EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+, no EGF, n = 113; β4+CT, no EGF, n = 116; β4−, EGF, n = 98; β4+, EGF, n = 81; and β4+CT, EGF, n = 141. The data shown are combined from at least four independent experiments. Error bars, SEM. *$p < 0.01$ between all conditions and β4+ in the presence of EGF. #$p < 0.01$ between β4+ in the absence of EGF and both β4− and β4+CT in the absence of EGF.

signaling pathways that can coordinate EF-mediated keratinocyte directional migration, a β4 integrin– and an EGF-dependent pathway.

β4 Integrin Is Localized to Focal Adhesions within the Keratinocyte Lamellipodium

Actin remodeling plays an important role in cell polarization (Ridley *et al.*, 2003), essential for chemoattractant-driven

(Merlot and Firtel, 2003) and EF-driven (Zhao *et al.*, 2002) directional migration and motility (Pantaloni *et al.*, 2001). Actin filaments terminate in focal adhesions (FAs), signaling centers that mediate the mechanical attachment of cells to the extra cellular matrix (Gilmore and Burridge, 1996), and regulate gene expression, cell growth, and survival (Sastry and Burridge, 2000). We reasoned that if the β4 integrin is localized within FAs, then binding to its ligand, laminin 332, could initiate the signaling required for β4 integrin–mediated directional migration. We found that after plating on collagen for 3 h, the keratinocytes exhibit β4 integrin staining within linear focal adhesions (identified as vinculin immunopositive structures) localized at the leading edge of the migrating cells (unpublished data), supporting this hypothesis.

Laminin 332-mediated Ligation of β4 Integrin Is Required for Keratinocyte Directional Migration in the Absence of EGF

To determine if ligand binding to laminin 332 was required for the directional response, we expressed an adhesion-defective mutant of β4 integrin that fails to bind laminin 332 (β4+AD) in the β4− cells and observed their migratory response in an applied EF. Loss of adhesion by laminin 332

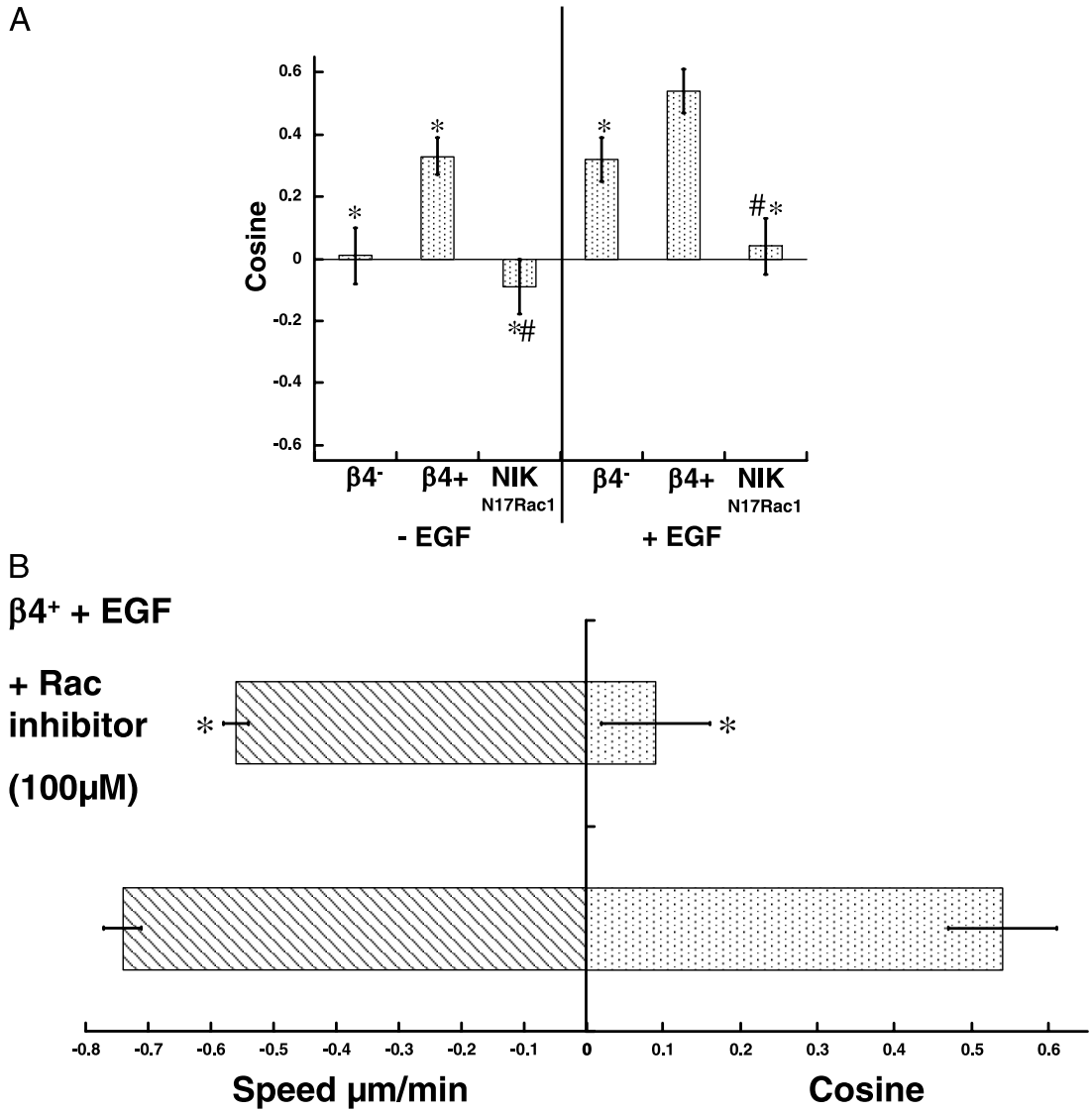

Figure 4. Rac1 is the common downstream target for both β4 integrin– and EGF-mediated keratinocyte directional migration in an EF. β4+, β4−, and NIK-N17Rac1s were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. (A) The cosine of migration for β4−, β4+, and NIK-N17Rac1 cells in the presence and absence of EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+, no EGF, n = 113; NIK-N17Rac1, no EGF, n= 58; β4−, EGF, n = 98; β4+, EGF, n = 81; and NIK-N17Rac1, EGF, n = 54. *$p < 0.01$ between all conditions and β4+ + EGF. #$p < 0.01$ between NIK-N17Rac1 and β4−/+ cells in both the presence and absence of EGF. The data shown are combined from at least four independent experiments. Error bars, SEM. β4+ cells, 2×10^4, were plated, in the presence of 2 ng/ml EGF, on the collagen-coated glass in the center of the galvanotaxis chambers as described in *Materials and Methods.* Thirty minutes before EF application 100 μM 553502 (Rac1 inhibitor) was added to the medium of half the prepared chambers. Galvanotaxis was performed in the presence or absence of 553502, as described in *Materials and Methods.* (B) The speed and directionality of migration for β4+ in the presence and absence of 553502 are shown. The number of cells is as follows: β4+, EGF, n = 81 and β4+ + 553502, EGF, n = 117. *$p < 0.01$. The data shown are combined from at least four independent experiments. Error bars, SEM.

has no effect on rate of migration in the presence or absence of EGF (unpublished data). In stark contrast, however, β4+AD cells are blinded to the EF in the absence of EGF and migrate randomly (Figure 2A). The inclusion of EGF in the medium recovers some of the directional deficit, as it does in the β4− cells. Indeed, their responses to the EF are very similar to β4− cells (Figure 2A).

To confirm the dependence of the results on laminin 332 binding, we preincubated β4+ cells and NHKs with an antibody known to inhibit the attachment of β4 integrin to laminin 332 (ASC-8). The effect of ASC-8 on EF-mediated directional migration of β4+ cells and NHKs is similar. The antibody blinds both β4+ cells and NHKs to the EF in the absence of EGF and they migrate randomly, whereas the ability to sense and respond to the EF is partially restored by EGF to 43 and 54% of the directionality observed for β4+ cells, respectively (Figure 2, B and C).

The Cytoplasmic Tail of β4 Integrin Is Essential for β4 Integrin–mediated Keratinocyte Directional Migration in the Absence of EGF

Integrins transmit signals from the extracellular matrix by the association of adapter proteins with their cytoplasmic tails (CT; Giancotti and Ruoslahti, 1999). Previously, it has been demonstrated that the cytoplasmic tail of β4 integrin is essential for the α6β4-mediated activation of signaling pathways required for the anchorage-independent survival of mammary tumors (Zahir *et al.*, 2003) and for epidermal growth and wound healing (Nikolopoulos *et al.*, 2005). To further examine the role of the β4 integrin in EF-mediated directional migration, we expressed a β4 integrin construct lacking a cytoplasmic tail in β4− cells (β4+CT). There was no decrease in the rate of migration between β4+ and β4+CT cells in the absence or presence of EGF (unpublished data), suggesting that the B4 cytoplasmic tail and its downstream signaling are not critical components for motility per se. In contrast, however, the β4+CT cells are unable to sense and respond with directional migration to the EF in the absence of EGF, indicating the requirement of the β4 cytoplasmic tail for the response (Figure 3). Addition of EGF partially rescues the directional response in the β4+CT cells, again suggesting that a second, β4 integrin–independent, EGF-mediated signaling pathway is required for a directional migratory response to an applied EF (Figure 3).

Rac1 Is the Common Downstream Target for Both β4 Integrin- and EGF-mediated Directional Migration in an EF

The small Rho guanosine triphosphatase Rac1 has been shown to play a role in chemotaxis, and its absence prevents neutrophils from responding to a chemoattractant with directional migration (Merlot and Firtel, 2003; Sun *et al.*, 2004). Because Rac1 is required for β4 integrin–mediated EGF-dependent chemotaxis (Russell *et al.*, 2003), we speculated that Rac1 could likewise mediate either the β4 integrin–mediated or the EGFR-mediated galvanotaxis response, or both. To test this hypothesis, a dominant inhibitory form of Rac1, N17Rac1, was expressed in an immortalized human keratinocyte line (NIK) and N17Rac1 cells were monitored for migration rate and directionality in an applied EF. Expression of N17Rac1 decreases the rate of migration by 26 and 46% in the absence and presence of EGF, respectively (unpublished data). However, NIK-N17Rac1 cells were unable to sense and respond to the applied EF, even in the presence of EGF (Figure 4A). To confirm that Rac1 is essential for both β4 integrin– and EGF-mediated directional migration in an EF, we preincubated β4+ cells in a Rac inhibitor (553502) before monitoring motility and directionality. In the presence of the Rac inhibitor, the speed of β4+ cell migration is reduced by 24%, whereas the cells are completely blinded to the EF and migrated randomly (Figure 4B).

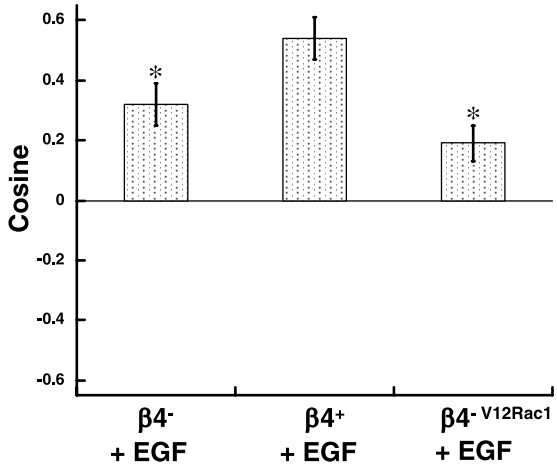

Figure 5. Activated Rac1 cannot rescue keratinocyte directional migration in β4 integrin-null cells. β4−, β4+, and β4-V12Rac1 cells were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. The cosine of migration for β4− and β4-V12Rac1 cells in the presence of EGF is shown. The number of cells is as follows: β4−, EGF, n = 98; β4+, EGF, n = 81; and β4-V12Rac1, EGF, n = 129. *$p < 0.01$ between all conditions and β4+ + EGF. The data shown are combined from at least four independent experiments. Error bars, SEM.

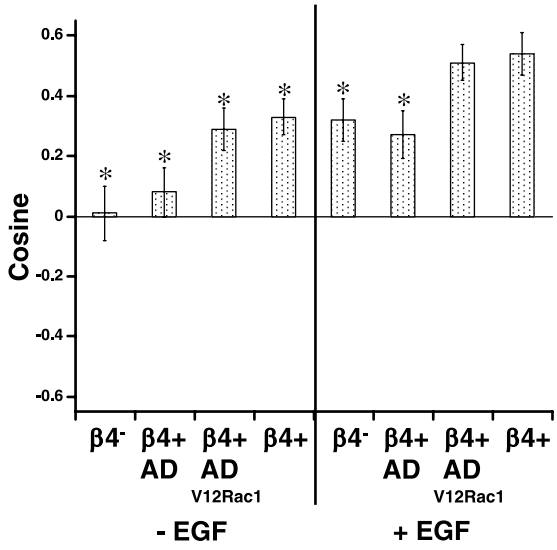

Figure 6. Activated Rac1 fully restores β4 integrin–mediated keratinocyte directional migration in the absence of laminin 332 ligation. β4−, β4+AD, β4+AD-V12Rac1, and β4+ cells were starved for 16 h in basal medium containing no growth factors, and experiments were performed as described in the legend for Figure 1. The cosine of migration for β4−, β4+AD, β4+AD-V12Rac1 cells in the presence or absence of EGF is shown. The number of cells is as follows: β4−, no EGF, n = 64; β4+AD, no EGF, n = 73; β4+AD-V12Rac1, no EGF, n = 75; β4+, no EGF, n = 113; β4−, EGF, n = 98; β4+AD, EGF, n = 82; β4+AD-V12Rac1, EGF, n = 102; and β4+, EGF, n = 81. *$p < 0.01$ between all conditions and β4+AD-V12Rac1 + EGF. The data shown are combined from at least four independent experiments. Error bars, SEM.

Activated Rac1 Cannot Rescue Keratinocyte Directional Migration in β4 Integrin-Null Cells

Rac activation appears to be critical for keratinocyte EF-mediated directional migration, and in some instances can rescue the cellular deficits induced by the absence of the β4 integrin. For example, expression of a constitutively active Rac mutant (V12Rac1) can sustain the viability of mammary tumors lacking β4 integrin (Zahir *et al.*, 2003). We wondered, therefore, if expression of V12Rac1 could rescue the directional migratory defect of the β4− cells. There is no significant difference in the rate of migration of β4− and β4-V12Rac1 cells (unpublished data). Although the V12Rac1 cells exhibit partial EF-mediated directional migration in the presence of EGF (35%), the constitutively active Rac mutant is not sufficient to compensate for absence of the β4 integrin (Figure 5).

Activated Rac1 Fully Restores β4 Integrin–mediated Keratinocyte Directional Migration in the Absence of Laminin 332 Ligation

Although the activate Rac1 mutant was unable to restore directional migration in cells lacking β4 integrin, we wondered if it could compensate for the lack of laminin 332–mediated ligation in cells expressing a functional cytoplasmic tail (β4+AD). β4+AD cells have a truncated Rac1 activation profile, similar to β4− cells and fail to sustain lamellipodial induction beyond 20 min (Russell *et al.*, 2003).

The expression of the active Rac1 mutant has no effect on migration rate in both the presence and absence of EGF (unpublished data); however, β4+AD cells are now able to fully respond to an applied EF. Indeed, they exhibit a robust directional response in the presence of EGF, and in its absence they are indistinguishable from β4+ cells (Figure 6).

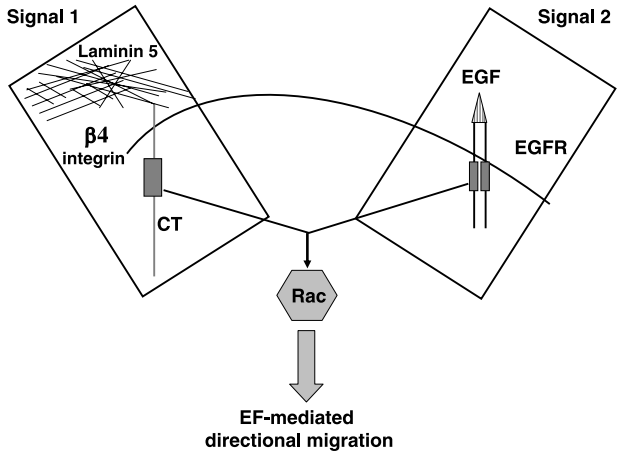

Figure 7. A diagrammatic illustration is shown outlining the synergistic pathways required for keratinocyte EF-mediated directional migration.

DISCUSSION

The signaling pathways that initiate and maintain cell polarity are of fundamental importance in directional cell migration. In this study, we establish a novel role for the α6β4 integrin for regulating cell polarity and maintaining directional migration in response to the cueing signal provided by an applied DC electric field. We demonstrate that although the expression of β4 integrin appears to have no effect on the rate of keratinocyte migration, it is essential for EF-mediated directional migration in the absence of EGF. In the presence of EGF, the signaling pathway initiated by β4 integrin ligand–binding converges with that of EGF receptor activation to initiate and maintain robust, persistent directional (cathodal) migration. Adhesion to laminin 332 and a functional cytoplasmic domain are both required for the β4 integrin–mediated directional migratory response, and Rac1 activation is a required downstream element for both β4 integrin– and EGF-mediated cathodal directional migration (Figure 7). We believe that this is the first description of a role for β4 integrin in sensing and responding to an EF-mediated directional signal.

Keratinocytes express two integrin receptors for laminin 332, α6β4 (Sonnenberg *et al.*, 1991) and α3β1 (Carter *et al.*, 1991). Previously, there have been reports of both laminin 332–mediated inhibition (Miyamoto *et al.*, 1996; O'Toole *et al.*, 1997; Goldfinger *et al.*, 1999) and promotion (Carter *et al.*, 1991; Zhang and Kramer, 1996; Shang *et al.*, 2001; Raymond *et al.*, 2005) of keratinocyte migration. Wound edge keratinocytes secrete abundant laminin 332 as they migrate into the wound, which is thought to facilitate and promote α3β1-mediated migration and form the foundation of a new basement membrane during reepithelialization (Goldfinger *et al.*, 1999; Nguyen *et al.*, 2000a, 2000b). Indeed the closure of wounded keratinocyte monolayers requires only α3β1 interaction with laminin 332 (Lampe *et al.*, 1998; Goldfinger *et al.*, 1999; Nguyen *et al.*, 2000a, 2000b). We observe no alteration in the rate of keratinocyte migration in the absence of β4 integrin, suggesting that laminin-5–dependent keratinocyte migration is occurring exclusively via α3β1 in our system.

Previously, we have demonstrated that EGF enhances both keratinocyte motility and EF-mediated directional migration (Fang *et al.*, 1998), as we also demonstrate here. However, the current studies reveal a novel and absolute requirement for the coordinated action of both EGF and β4 integrin to initiate and maintain robust EF-mediated directional migration. It is known that EGFR and α6β4 integrin cross-talk. α6β4 integrin can physically interact with a number of receptor tyrosine kinases including the EGFR, ErbB-2, Ron, and Met (Falcioni *et al.*, 1997; Hintermann *et al.*, 2001; Mariotti *et al.*, 2001; Trusolino *et al.*, 2001), and EGF induces tyrosine phosphorylation of the β4 integrin cytoplasmic tail, which has been implicated in hemidesmosome disassembly and epithelial motility (Mainiero *et al.*, 1996). Additionally, integrin adhesion can result in the phosphorylation of the EGFR on numerous tyrosine residues (Moro *et al.*, 2002) and can potentiate signaling pathways in response to EGF, PDGF, FGF, VEGF, and insulin (reviewed by (Schwartz and Baron, 1999). A considerable amount of evidence supports a role for integrin-mediated adhesion in coordinating growth-factor–mediated responses (reviewed by Cabodi *et al.*, 2004). Integrin-mediated signaling is required for EGF-dependent proliferation, survival, and migration, demonstrating that a cooperative interaction is necessary to achieve full biological responses (Cabodi *et al.*, 2004). It appears that α6β4 integrin and EGF also cooperate to support robust keratinocyte directional migration in response to an applied EF cue.

β4 integrin has been demonstrated to localize to lamellipodia and filopodia of migrating carcinoma cells and associate with F-actin (Rabinovitz and Mercurio, 1997). Indeed, inhibition of α6β4 function resulted in the collapse of lamellipodia and filopodia (Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999). α6β4 has also been localized to actin-containing structures in keratinocytes (Geuijen and Sonnenberg, 2002; Santoro *et al.*, 2003; Spinardi *et al.*, 2004), but we observe its presence in fine linear structures at the leading edge, suggesting that in migrating keratino-

cytes, β4 integrin becomes incorporated within focal adhesion sites, centers for traction control (Ridley *et al.*, 2003).

The cytoplasmic tail of β4 integrin is unique with no known homology to other β subunits. It is unusually long (~1000 amino acids), associates with the hemidesmosome cytoskeleton (Sonnenberg *et al.*, 1991) via HD 1/plectin (Nievers *et al.*, 2000) and contains a tyrosine activation motif (TAM) that can facilitate the docking of src homology 2 (SH2) domains while phosphorylated. In primary keratinocytes both Shc and Grb2 are recruited to the β4 integrin–phosphorylated TAM sequentially activating MAP kinase pathways and promoting proliferation (Mainiero *et al.*, 1995, 1997). Furthermore, α6β4 can activate phosphoinositide 3-kinase (PI3-K), increasing the invasive capacity of breast and colon carcinoma cells (Shaw *et al.*, 1997).

The loss of the β4 integrin cytoplasmic tail has a negative effect on the ability of the cells to sense and respond to the applied EF in the absence of EGF. β4+CT-expressing cells are blinded to the applied EF and migrate randomly, reminiscent of β4+AD cells. It appears that a fully functional β4 integrin ligand-binding domain and a fully functional β4 integrin cytoplasmic tail are both required in order for keratinocytes to respond to an applied EF in the absence of EGF and respond robustly in the presence of EGF. This suggests that in the absence of EGF, ligation of β4 integrin by laminin 332 transmits a signal via its cytoplasmic tail to initiate and respond to an applied EF, allowing the cell to form a new lamellipodium facing the cathode, turn, and migrate directionally.

The ability of a cell to respond to an applied EF requires the formation of a new lamellipodium facing the cathode. Rac1 plays a role in lamellipodial formation (Hall, 1998) and expression of a constitutively active Rac1 mutant accelerates cutaneous wound repair (Hassanain *et al.*, 2005). Previously, it has been demonstrated that β4 integrin can activate the PI3 kinase signaling pathway (Shaw *et al.*, 1997; Gambaletta *et al.*, 2000; Shaw, 2001) resulting in Rac1 activation (Shaw *et al.*, 1997). Our studies highlight Rac1 as a critical convergence point and pivotal regulator of both β4 integrin– and EGF-mediated keratinocyte galvanotaxis, coordinating the signals to allow cells to migrate directionally in response to the applied EF. Although the expression of a dominant negative Rac1 mutant or the application of a specific Rac inhibitor slows keratinocyte migration, it prevents galvanotaxis in both the presence and absence of EGF. Importantly, we demonstrate that β4 integrin expression is essential for keratinocytes to sense and respond to an EF with robust directional migration, because a constitutively active Rac1 mutant cannot restore directional migration in cells lacking β4 integrin expression. Meanwhile, expression of the constitutively active Rac1 mutant in cells expressing adhesion-defective β4 integrin has no effect on migration rate but fully restores keratinocyte galvanotaxis. It has been suggested that while growth factors can activate Rac globally within the cell, integrins determine the specific locality where Rac will bind to its effectors, thus determining where to extend lamellipodia, facilitating directional migration (Del Pozo *et al.*, 2002; Grande-Garcia *et al.*, 2005). Although lamellipodial formation is absent in Rac1 genetic null cells (Vidali *et al.*, 2006), a partial, rather than total, decrease in levels can actually increase persistent directional motility by suppressing peripheral and sustaining dominant lamellipodial formation (Pankov *et al.*, 2005). Indeed, here it appears that β4 integrin coordinates with EGF to localize Rac1 activation to the dominant lamellipodium, initiate and maintain Rac1-dependent persistent directional migration in response to EF cueing.

In conclusion, we describe a role for α6β4 integrin in sustaining directional migration in keratinocytes in response to an applied EF and demonstrate that the cooperative interaction of both EGF and β4 integrin is necessary to achieve the full biological response of EF-mediated directional migration. Rac1 is critical for both EGF-mediated and β4 integrin–mediated keratinocyte directional migration in an applied EF as well as in EGF-driven chemotaxis (Russell *et al.*, 2003). Perhaps underpinning the role of β4 integrin in directional migration is its ability to regulate cell turning or mediate stabilization of the site at which the new lamellipodium will form to allow migration in the direction of the applied gradient. Studying EF-induced directional migration will improve our understanding of directional sensing and the signaling mechanisms required for these biological processes that play important roles in embryonic development, inflammation, tumor metastasis, and wound repair.

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