Integrin $\alpha_3\beta_1$ directs the stabilization of a polarized lamellipodium in epithelial cells through activation of Rac1

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Summary

Epithelial cell migration is a crucial event in wound healing, yet little is known about mechanisms whereby integrins regulate epithelial cell polarization and migration. In the present work, we demonstrate the importance of adhesion through the $\alpha_3\beta_1$ integrin in promoting the stabilization of leading lamellipodia in migrating keratinocytes. We demonstrate that this integrin is found at the leading edge of migrating keratinocytes and that inhibition of $\alpha_3\beta_1$ binding to laminin-5 prevents the formation of stable leading lamellipodia. Consistent with this observation, keratinocytes derived from $\alpha_3\beta_1$-deficient mice fail to form stable leading lamellipodia but retain the ability to form actin-containing protrusions that rapidly extend and retract from the cell membrane. Formation of a leading lamellipodium also requires $\alpha_3\beta_1$-dependent activation of Rac1, because $\alpha_3\beta_1$-deficient keratinocytes show decreased activation of Rac1 compared with $\alpha_3\beta_1$-expressing cells, and formation of stable leading lamellipodia can be inhibited in the latter cells by expression of the dominant negative Rac1 mutant Rac1N17. Furthermore, $\alpha_3\beta_1$-deficient keratinocytes expressing constitutively active Rac1L61 failed to form stable lamellipodia when plated onto laminin-5, demonstrating that $\alpha_3\beta_1$ is required for Rac1-mediated formation of a stable lamellipodium. These observations identify a crucial role for integrin-mediated adhesion and signaling in the formation of large, polarized, stable lamellipodia by migrating epithelial cells. To our knowledge, this study is the first to demonstrate that signal transduction through a specific integrin is required to direct the development of a lamellipodium from an initial protrusion and promote persistent epithelial cell migration.

Movies available online

Key words: $\alpha_3\beta_1$ Integrin, Keratinocyte, Laminin-5, Migration, Lamellipodia, Rac1

Introduction

Cell migration is a crucial event that occurs during many processes including wound healing, angiogenesis, development and metastasis. Although migratory phenotypes and factors that promote migration vary greatly among cell types, the basic mechanism that drives cell migration in all cells is polarized actin polymerization (Pollard and Borisy, 2003). This process is initiated by chemotactic factors that stimulate localized actin polymerization and the formation of an initial protrusion. Adhesion to an extracellular matrix (ECM) is not required for formation of these initial protrusions, as demonstrated by the fact that cells in suspension extend protrusions in response to growth factor stimulation. However, these initial protrusions are never as large as the lamellipodia that form when cells are plated onto adhesive substrates, suggesting an important role for adhesion in promoting the formation of stable leading-edge lamellipodia (Bailly et al., 1998).

Integrins are the major cell surface receptors for cell adhesion to the ECM and they are known to play an important role in cell migration (Hynes, 2002; Ridley et al., 2003). Several integrins have been shown to localize to leading-edge lamellipodia in migrating cells, including $\alpha_5\beta_1$ in fibroblasts (Laukaitis et al., 2001), $\alpha_v\beta_3$ in endothelial cells (Kiosses et al., 2001) and $\alpha_4\beta_1$ in CHO cells (Goldfinger et al., 2003; Pinco et al., 2002). At the leading edge of a migrating cell, integrin-mediated adhesion is important in generating the traction required to pull the cell body forwards. More recently, integrin signaling has been suggested to play a role in the formation of a leading-edge lamellipodium by a positive-feedback mechanism involving convergence of integrin- and growth-factor-mediated pathways (Cho and Klemke, 2002). Integrins nucleate the formation of focal adhesions and focal complexes, which serve as platforms for the propagation of signal transduction (Giancotti and Ruoslahti, 1999), and some of these signal transduction events regulate cell migration (Ridley et al., 2003). However, the exact functions of integrins at the leading edge and the roles they serve in the establishment of a polarized leading-edge lamellipodium remain unclear.

Keratinocyte migration is crucial for re-epithelialization of cutaneous wounds. However, little is known about the mechanisms that regulate keratinocyte migration, in part because primary keratinocytes do not demonstrate a strong spontaneous migratory potential in culture (Decline et al., 2003). Recently, it was established that individually migrating keratinocytes assume a phenotype similar to that of migrating...
fish keratocytes, consisting of a large leading lamellipodium with no retraction fibers (Frank and Carter, 2004). One family of proteins known to be important in regulating keratinocyte functions is the integrins (Watt, 2002). A recent study in mice with an epidermis-specific knockout of β1 integrins showed that cutaneous wounds fail to heal properly owing to a defect in the initiation of cell migration (Grose et al., 2002). Keratinocytes express several β1 and non-β1 integrins, and many potential integrin ligands are present in the cutaneous wound bed (Watt, 2002). However, the interaction between integrin α3β1 and its principal epidermal ligand, laminin-5 (LN-5), seems to be of particular importance in keratinocyte function. In migrating keratinocytes, α3β1 dramatically changes its localization from primarily cell-cell contacts to the basal surface, suggesting that its interaction with the ECM is enhanced during cutaneous wound healing. Indeed, function-blocking antibodies that inhibit α3β1 adhesion to the ECM inhibit closure of scrape-wounded monolayers of primary human keratinocytes. In addition, expression of LN-5 by leading-edge cells of cutaneous wounds precedes both expression of other ECM proteins and initiation of cell migration (Nguyen et al., 2000). Furthermore, keratinocytes deficient in LN-5 expression show defects in proliferation and migration on collagen, suggesting a crucial role for cell-derived LN-5 in keratinocyte function (Ryan et al., 1999). Finally, mice deficient in expression of the α3-integrin subunit (e.g. α3-null mice) display defects in the assembly of the epidermal basement membrane, and α3-null keratinocytes show reduced spreading on LN-5, re-enforcing the importance of this integrin in maintaining normal epidermal function (DiPersio et al., 1997; Hodivala-Dilke et al., 1998).

In the current study, we demonstrate the importance of a distinct integrin-mediated event that is required for establishment of a polarized migratory phenotype in epithelial cells. Mouse keratinocytes (MK cells) derived from integrin α3-null mice failed to form stable leading lamellipodia and become polarized, either as individually migrating cells or as leading-edge cells in an in vitro scrape wound. However, these α3β1-deficient cells retained the ability to form initial actin-containing protrusions that extended and retracted rapidly along the circumference of the cell. Interestingly, although α3β1-deficient cells remained motile on LN-5, they lost cell-cell adhesions and migrated randomly as individual cells into the wound area. This was in contrast to MK cells derived from wild-type mice and α3-null MK cells transfected with human α3, which formed stable leading lamellipodia and migrated as a contiguous sheet into the wound area. Consistent with a role for α3β1 in regulating cell polarization, α3β1 was detected by immunofluorescence at leading edges of lamellipodia, and blocking the interaction of α3β1 with LN-5 with a function-blocking antibody prevented formation of stable leading lamellipodia by wound-edge keratinocytes. We also identify a novel role for α3β1-mediated signal transduction in the establishment of cell polarity. α3β1 was required for the activation of Rac1, a small GTPase known for its ability to stimulate the formation of lamellipodia, and inhibition of Rac1 activity abrogated the ability of keratinocytes to form stable leading lamellipodia. However, constitutive activation of Rac1 in α3β1-deficient keratinocytes was not sufficient to initiate the formation of stable leading lamellipodia, demonstrating that α3β1 was also required for Rac-mediated lamellipodium formation. This work illustrates the importance of integrin-mediated adhesion and signaling functions in promoting formation of stable leading-edge lamellipodia in migrating epithelial cells.

Materials and Methods

MK cell culture

The MK+/+ cell line (MK-1.16) and MK−/− cell line (MK-5.4.6) were derived from keratinocytes isolated from wild-type mice or α3-integrin-knockout mice, respectively (DiPersio et al., 2000). MK−/− cells were stably transfected with a full-length human α3-encoding cDNA; such transfectants express high levels of α3β1 on the cell surface, as described previously (DiPersio et al., 2000). MK growth medium consisted of Eagle’s minimum essential medium (EMEM; BioWhittaker, Walkersville, MD) supplemented with 4% fetal bovine serum (FBS) (BioWhittaker) from which Ca2+ had been chelated, 0.05 mM CaCl2, 0.4 µg ml−1 hydrocortisone (Sigma, St Louis, MO), 5 µg ml−1 insulin (Sigma), 10 ng ml−1 epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA), 2 × 10−5 M 3,3′,5-triiodo-L-thyronine (Sigma), 10 units ml−1 interferon γ (IFNγ; Sigma), 100 units ml−1 penicillin, 100 µg ml−1 streptomycin (Invitrogen) and 100 µg ml−1 L-glutamine (Invitrogen). Stocks of MK cell lines were maintained at 33°C, 8% CO2, on tissue culture plates coated with 30 µg ml−1 denatured rat tail collagen (Cohesion, Palo Alto, CA). For experiments, MK cells were subcultured on LN-5 ECM prepared from the human squamous cell carcinoma cell line SCC-25, as described previously (Xia et al., 1996).

Immunofluorescence analysis of wound edges and sparse cultures

Cells were grown to confluence on collagen-coated glass coverslips, serum deprived for 8 hours and then scrape wounded with the narrow end of a 200 µl pipette tip. For antibody-blocking experiments, scrape wounds were treated immediately after wounding with function-blocking antibodies against α3β1 (10 µg ml−1 P1B5; Chemicon, Temecula, CA) or α6β4 (5 µg ml−1 GoH3; BD Biosciences), or a control IgG (10 µg ml−1). For establishment of sparse cultures, MK cells were serum deprived for 8 hours and then trypsinized from stock plates, resuspended in either serum-containing (4% chelated FBS) or serum-free EMEM, 0.05 mM CaCl2 and pre-incubated at 33°C for 30 minutes with rotation before plating onto LN-5 ECM at a density of approximately 2.5 × 105 cells per coverslip. For antibody blocking experiments, cells were treated with P1B5, GoH3 or a control IgG as indicated above during the 30 minute preincubation period. After 30 minutes of adhesion, cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes and blocked in 1% bovine serum albumin (BSA) in PBS for 1 hour. Staining for α3β1 was performed with the monoclonal antibody P1B5. Staining for β-actin was performed with a mouse anti-human monoclonal antibody (clone AC-15; Sigma). The secondary antibody used was FITC-conjugated goat anti-mouse antibody (Pierce). Staining for F-actin was performed with TRITC-conjugated phalloidin (Sigma). Samples were viewed on an Olympus BX60 upright microscope. Images were generated using SlideBook (Spectra Services) and Adobe Photoshop.

Time-lapse video microscopy

For time-lapse video microscopy of scrape-wound migration, MK cells were grown to confluence on either collagen-coated surfaces or LN-5 ECM, serum deprived for 8 hours and then scrape wounded as described above. The morphology of the wound edge was observed for 8 hours on an Olympus IX70 inverted microscope. Images were recorded and movies analysed using ImagePro software.
used model for the coordinated cell migration that occurs during re-epithelialization of a cutaneous wound. In order to determine the effect of integrin α3β1 ablation on the behavior of leading-edge keratinocytes in a wound, we used time-lapse video microscopy to monitor wound-edge morphology and migration of mouse keratinocytes (MK cells) into a scrape wound. Three different immortalized MK cell lines were used in this work; MK+/+ cells were derived from a wild-type mouse; MK−/− cells were derived from an α3-integrin-null mouse; and MKα3 cells are MK−/− cells transfected stably with human α3 that express similar levels of α3β1 to MK+/+ cells, as described previously (DiPersio et al., 2000). Confluent monolayers of MK cells on collagen-coated surfaces were scrape wounded and migration of wound-edge keratinocytes was observed for 8 hours. As expected, MK+/+ cells migrated into the wound area as a contiguous sheet and cells at the wound edge maintained cell-cell adhesions as they migrated into the wound area (Fig. 1A,D). In stark contrast to this, scrape wounds of MK−/− cells lost integrity of the wound edge and cells scattered randomly as individual cells into the wound area (Fig. 1B,E). Importantly, MKα3 cells migrated into the scrape wound as an intact sheet, demonstrating that restoring expression of α3β1 in MK−/− cells rescued the ability of wound-edge cells to migrate as an intact monolayer (Fig. 1C,F). These results demonstrate that α3β1 is required to maintain cohesiveness of the epidermal sheet at the wound edge. In order to determine whether the loss of wound-edge integrity and scattering seen in MK−/− cells was a collagen-dependent phenomenon, wound-edge cell migration was also observed on LN-5 ECM. It has been established that keratinocyte adhesion to this LN-5 ECM depends entirely on LN-5-binding integrins (DiPersio et al., 1997; Xia et al., 1996). Wound-edge morphology and migration phenotypes observed on LN-5 were identical to those on collagen-coated surfaces for each cell line (Fig. 1G-L). The changes in migration phenotype seen in MK−/− cells (i.e. loss of cell-cell cohesion and increased scattering) are further illustrated in online movies (see Movies 1 and 2, http://jcs.biologists.org/supplemental). We believe that the similarity in migration phenotype on LN-5 ECM and collagen is due to the fact that keratinocytes on collagen migrate on newly deposited LN-5, as described previously (Frank and Carter, 2004; Ryan et al., 1999).

In order to examine cellular morphologies further and to gain more insight into events at the wound edge, cells were grown to confluence on collagen-coated glass coverslips, wounded and then fixed either immediately after wounding (0 hours) or 2 hours after wounding. Lamellipodium formation is a crucial event in epithelial wound closure and is indicative of a cell that has become polarized to migrate (Ridley et al., 2003). MK+/+ and MKα3 cells at the wound edge displayed lamellipodia as early as 2 hours after wounding, as seen in both bright-field panels and by phalloidin staining (Fig. 2A, arrowheads; Movie 1, http://jcs.biologists.org/supplemental). Phalloidin staining for F-actin was consistent with that normally seen in lamellipodia, with staining evident in the ruffles that define the entire border of the lamellipodium (Small et al., 2002). α3β1-expressing cells also displayed structures consistent with actin purse strings as visualized by phalloidin staining (Fig. 2A, arrows), as described previously in epithelial cell lines and indicating coordinated behavior.

**Results**

**Genetic ablation of α3β1 causes disorganized keratinocyte migration and loss of an organized leading edge in scrape wounds**

Scrape-wounded monolayers of keratinocytes are a widely
among wound-edge cells (Bement et al., 1993; Martin and Lewis, 1992). In addition, leading-edge lamellipodia of MKα3 cells stained positive for β-actin (Fig. 2B), indicating that these lamellipodia are true motility-promoting structures (Kislauskis et al., 1997). In contrast to α3β1-expressing cells, MK–/– cells showed neither lamellipodia nor structures reminiscent of actin purse strings. Instead, cells that had lost contact with the monolayer scattered into the wound as early as 2 hours after wounding (Fig. 2A; Movie 2, http://jcs.biologists.org/supplemental). Scattering cells displayed actin-containing pseudopodia and filopodia that were not oriented in any particular direction. These results are in agreement with a recent study of epidermal explants derived from β1-integrin-deficient mice, in which migrating keratinocytes displayed actin cytoskeletal abnormalities, including lack of polarized actin filaments and purse strings (Raghavan et al., 2003).

The lack of cell cohesiveness at the wound edge and scattering of MK–/– cells into the wound area is likely to reflect a defect in formation and/or maintenance of cell-cell contacts, consistent with a recently described role for α3β1 in the regulation of E-cadherin-dependent cell-cell contacts in kidney epithelial cells (Wang et al., 1999). Presumably, in the absence of mature cell-cell contacts, MK–/– cells are free to migrate away from the wound edge. Importantly, because lamellipodium formation is a prerequisite for the formation of epithelial cell-cell contacts, the inability of MK–/– cells to form lamellipodia might be the cause of cell-cell adhesion defects seen in these cells (Ehrlich et al., 2002).

Integrin α3β1 is a component of lamellipodia on wound-edge cells and its adhesion to LN-5 is required to form stable leading-edge lamellipodia

Integrins are present in leading lamellipodia of certain cell types, where they are thought to stabilize lamellipodia and to promote signal transduction that stimulates motility (Cho and Klemke, 2002). In order to determine whether integrin α3β1 localizes to the leading-edge lamellipodia of keratinocytes, MKα3 cells were grown to confluence on collagen-coated surfaces, wounded and fixed 2 hours after wounding. The localization of α3β1 in leading-edge lamellipodia of wound-edge cells was demonstrated by indirect immunofluorescence using a monoclonal antibody specific for human α3, P1B5 (Fig. 3A,B, arrowhead). This observation is consistent with a previous report that α3β1 localizes to the leading edge of scrape-wounded breast-epithelial cells (Goldfinger et al., 1999). In order to confirm the specificity of P1B5 for α3β1, confluent monolayers of MKα3 cells were shown to demonstrate the expected cell-cell contact localization of α3β1 (Fig. 3C), and MK–/– cells were shown to lack P1B5 staining (Fig. 3D).

In order to determine whether α3β1 ligation to LN-5 is required for lamellipodium extension into the wound area, scrape-wounded monolayers of MKα3 cells were treated with P1B5, which blocks α3β1 adhesion to LN-5 (Symington and Carter, 1995). Cells fixed immediately after wounding exhibited no lamellipodia; however, by 2 hours after wounding, about 50% of leading edge keratinocytes treated with control IgG exhibited lamellipodia (Fig. 3E,F). By contrast, treatment
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Integrin α3β1 regulates lamellipodium formation with P1B5 dramatically decreased the number of wound-edge cells with lamellipodia to about 10%. Treatment with GoH3, which blocks keratinocyte adhesion to LN-5 via the α6β4 integrin (Niessen et al., 1994), did not affect the ability of leading-edge cells to form lamellipodia (Fig. 3E,F). This finding was expected because α6β4 promotes stable adhesion of keratinocytes but is not required for migration (Nguyen et al., 2000). The ability to abrogate lamellipodium formation in leading-edge keratinocytes by blocking α3β1 adhesion to LN-5 is consistent with the lack of lamellipodium formation in MK−/− cells. α3β1 has been reported to have other functions in addition to cell-ECM adhesion (Kreidberg, 2000), including the formation of adherens junctions (Chattopadhyay et al., 2003), binding to uPAR (Zhang et al., 2003) and binding to the tetraspanin CD151 (Yauch et al., 2000), and these functions are also absent from MK−/− cells. However, results from the P1B5 blocking experiment strongly suggest that lamellipodium formation by keratinocytes is dependent on the ability of α3β1 to bind LN-5 and perhaps not on other α3β1 functions.

Cultures of α3β1-deficient mouse keratinocytes show fewer fan-shaped cells when plated sparsely onto LN-5. The scrape-wound system is a good model in which to study coordinated cell behavior. However, it is limited in that only a small proportion of cells undergo phenotypic changes associated with cell migration (i.e. only leading-edge cells display large, stable lamellipodia). Therefore, sparsely plated cultures were used in an attempt to increase the number of cells that assume the characteristics of wound-edge keratinocytes, especially a large, stable leading lamellipodium or fan. Indeed, plating α3β1-expressing cells sparsely onto LN-5 induced the formation of many fan-shaped cells reminiscent of scrape-wound-edge cells. This polarized fan shape was recently described to be the characteristic of keratinocytes migrating on LN-5 (Frank and Carter, 2004; Nguyen et al., 2000). In the presence of serum, about 28% of MK+/+ cells assumed a fan shape after 30 minutes on LN-5 (Fig. 4D). In serum-free conditions, the number of fan-shaped MK+/+ cells was reduced to about 15%. By contrast, only 8% and 1% of MK−/− cells assumed a fan shape in serum and serum-free conditions, respectively (Fig. 4D), and the vast majority of MK−/− cells remained without stable lamellipodia (Fig. 4B). Furthermore, MK−/− cells that did form fans appeared to be significantly smaller than the MK+/+ cells (data not shown). The number of MKα3 cells that assumed a fan shape was similar to that seen for MK+/+ cells under both serum and serum-free conditions (Fig. 4D), indicating that formation of fan-shaped cells is dependent on integrin α3β1. The increased number of fan-shaped cells seen in the presence of serum demonstrates that growth factors enhance the formation of lamellipodia, as expected. However, serum stimulation alone was not sufficient

Fig. 2. Cells that lack α3β1 do not extend lamellipodia at a wound edge. (A) MK+/+, MK−/− or MKα3 cells were grown to confluence on collagen-coated glass coverslips, scrape wounded and fixed either immediately after wounding (0 hours) or 2 hours after wounding. Cells were stained for F-actin with TRITC-conjugated phalloidin. (B) MKα3 cells were stained for β-actin 2 hours after wounding. The corresponding phase image is shown for each field. Arrowheads point to edges of lamellipodia; arrows point to actin purse strings. Bar, 10 μm.
to fully stimulate cells to assume a fan shape, because cultures of MK+/+ cells in the presence of serum displayed fewer fan-shaped cells than α3β1-expressing MK cells did even in the absence of serum (Fig. 4D). The ability of MK+/+ and MKα3 cells to form fans in the absence of serum demonstrates that exogenous growth factors are not required for α3β1-mediated lamellipodium formation.

Immunofluorescent staining for F-actin in fan-shaped MKα3 cells was consistent with actin staining in lamellipodia (Fig. 4A, arrowhead). By contrast, F-actin staining in MK−/− cells revealed circumferential pseudopodia and filopodia (Fig. 4B, arrows). Immunofluorescent staining showed that α3β1 in MKα3 cells localized to the leading-edge lamellipodia (Fig. 4A, arrowhead), similar to the localization of α3β1 in wound-edge keratinocytes (Fig. 3A). Staining for α3β1 was also detected under the cell body, consistent with the previously described location of α3β1 in migrating keratinocytes (Nguyen et al., 2001). In addition, a ‘trail’ of α3β1 was visible behind the trailing edge of the keratinocyte, presumably representing integrin that had been ripped out of the plasma membrane as the cell migrated, as previously described (Kirfel et al., 2003). Finally, the requirement for α3β1-mediated adhesion to LN-5 for assumption of a fan shape was confirmed by the fact that blocking this interaction with P1B5 inhibited fan formation in sparsely plated MKα3 cells (Fig. 4C).

In order to confirm that α3β1-dependent establishment of a stable leading lamellipodium is a precursor to persistent migration, the migratory behavior of fan-shaped MK+/+ and MKα3 cells was compared with that of MK−/− cells. Time-lapse video microscopy (Fig. 5; Movies 4 and 6, http://jcs.biologists.org/supplemental) revealed that fan-shaped MK+/+ and MKα3 cells migrated persistently in the direction of the fan. Upon initial adhesion, a small unpolarized lamellipodium was extended within 4 minutes. This initial unpolarized, circumferential lamellipodium rapidly polarized by 12 minutes, and grew in size as the cell began to migrate. We routinely observed cells that maintained a stable, polarized lamellipodium while migrating for up to 2 hours. By contrast, MK−/− cells extended and retracted pseudopodia rapidly, never establishing a stable lamellipodium, and did not exhibit persistent migration (Fig. 5, arrow; Movie 5, http://jcs.biologists.org/supplemental). Furthermore, whereas the leading lamellipodia seen in α3β1-expressing MK cells frequently persisted for up to 2 hours, the protrusions displayed by MK−/− cells lasted only several minutes before retracting. To our knowledge, this is the first demonstration that a specific integrin regulates stabilization of protrusions leading to the formation of a lamellipodium. Importantly, MKα3 cells treated with P1B5 did not display stable lamellipodia but extended

Fig. 3. Integrin α3β1 is localized at leading lamellipodia and its adhesion to LN-5 is required for lamellipodia formation by wound-edge cells. (A,B) MKα3 cells were grown to confluence on collagen-coated glass coverslips, wounded, fixed 2 hours after wounding and then stained with P1B5 for α3β1 integrin (B); the corresponding phase-contrast image is shown in (A). Arrowheads point to lamellipodium edge. Bar, 10 μm. (C) P1B5 staining of the MKα3 monolayer away from the scrape wound shows the expected cell-cell localization of α3β1. (D) Confluent MK−/− cells show a lack of staining with P1B5. (E) MKα3 cells on collagen-coated surfaces were scrape wounded and photographed immediately after wounding (0 hours) or 2 hours after wounding in the presence of a control IgG, a function-blocking antibody against integrin α3β1 (P1B5) or a function-blocking antibody against integrin α6β4 (GoH3), as indicated. Bar, 100 μm. (E¢) Panels show close-up images of wound-edge cells from IgG-treated or P1B5-treated scrape wounds, as indicated. (F) The proportions of leading-edge cells displaying lamellipodia were determined for three separate wound edges, 60 cells for each wound edge. Error bars represent s.e.m.
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 transient, circumferential protrusions similar to MK+/− cells (Fig. 4C; Movie 3, http://jcs.biologists.org/supplemental). In addition, P1B5-treated cells migrated in the same random, non-persistent manner as did MK−/− cells (see Movie 3). These results indicate that α3β1-mediated adhesion promotes a type of cell migration that is characterized by cell polarization, stable lamellipodium formation and persistent migration. Taken together, data in Figs 1-5 demonstrate that α3β1 ligation to LN-5 stabilizes initial protrusions from the cell membrane and promotes the formation of stable leading lamellipodia, and that this α3β1-dependent lamellipodia formation is a prerequisite for persistent keratinocyte migration.

Rac1 activation in MK cells is dependent on integrin α3β1

In addition to physically stabilizing lamellipodia, integrin-mediated adhesion might activate signal-transduction pathways that promote the growth and stabilization of leading lamellipodia (Cho and Klemke, 2002). The small GTPase Rac1 stimulates the formation of lamellipodia in many cell types (Nobes and Hall, 1999). Because β1 integrins have been shown to stimulate Rac1 activity (Berrier et al., 2002; Hirsch et al., 2002; Price et al., 1998), we sought to determine whether Rac1 activation is α3β1-dependent in MK cells cultured on LN-5. Immunoblot analysis revealed that levels of total Rac1 protein were equivalent between MK+/+, MK−/− and MKα3 cells (Fig. 6A). The amount of active Rac1 in each of the MK cell lines was determined by GST-PAK pull-down. The amount of active Rac1 in MK−/− cells was reduced to about 50% of levels seen in α3β1-expressing MK cells (Fig. 6B,C). The basal level of active Rac1 in MK−/− cells might be a result of α6β4 binding to LN-5, which has also been reported to stimulate Rac1 activity in keratinocytes (Russell et al., 2003). Our results demonstrate that full activation of Rac1 in keratinocytes requires α3β1, and suggest that reduced Rac1 activity might be a contributing factor to the absence of lamellipodium formation in MK−/− cells.

Rac1 activity is required to form leading lamellipodia in α3β1-expressing MK cells

We determined that both activation of Rac1 and stable lamellipodium formation in MK cells are α3β1-dependent, and so we sought to determine whether Rac1 activity is required for assumption of a fan shape by keratinocytes. Rac1 activity was inhibited in MK cells by introducing a dominant-negative
mutant of Rac1 (Rac1N17) into MK+/+ and MKα3 cells by adenoviral transduction. Infected cells were plated sparsely onto LN-5 coated glass coverslips for 30 minutes in the presence of serum. Because the adenovirus used to deliver RacN17 also directs the expression of green fluorescent protein (GFP) from a separate promoter, cells expressing Rac1N17 were identified easily by fluorescence microscopy. As a control, MK cells were infected with an adenovirus that expresses GFP only. MK+/+ cells that expressed Rac1N17 failed to assume a fan shape (Fig. 7A-C, arrowhead), in contrast to uninfected cells in the same field, which retained the ability to form a leading lamellipodium (Fig. 7A-C, arrow). Identical results were obtained with MKα3 cells (Fig. 7G-I). However, the Rac1N17-expressing MK+/+ and MKα3 cells retained the ability to extend pseudopodia and filopodia, consistent with a role for Rac1 in specifically promoting the formation of lamellipodia (Nobes and Hall, 1999). GFP-control infected cells assumed a fan shape similar to uninfected cells in the same field, indicating that inhibitory effects are specific to Rac1N17 (Fig. 7D-F,J-L). These results demonstrate that Rac1 activity is required for α3β1-dependent formation of a stable leading lamellipodium by keratinocytes. Finally, in order to determine whether Rac1N17 inhibits lamellipodium formation by inhibiting α3β1 binding to LN-5, we treated Rac1N17-expressing MK+/+ and MKα3 cells with GoH3. This treatment completely eliminates α6β4-mediated adhesion of MK cells to LN-5 (DiPersio et al., 1997), leaving α3β1 as the only receptor for LN-5. GoH3-treated cells bound LN-5 to the same degree as control IgG-treated cells (data not shown), indicating that α3β1 binding to LN-5 does not require Rac1 activity and that the lack of lamellipodia formation by Rac1N17-expressing cells is not due to decreased binding of α3β1 to LN-5.

Stimulation of Rac1 activity in the absence of α3β1-mediated adhesion is not sufficient to induce formation of a stable leading lamellipodium

Because α3β1-dependent lamellipodium formation required Rac1 activity, we sought to determine whether increased Rac1
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Integrin $\alpha_3\beta_1$ regulates the phenotype of migrating keratinocytes

In this study, we established a novel role for integrin $\alpha_3\beta_1$ in regulating cell polarization during migration of epithelial cells.
Integrins are thought to be involved at several distinct steps in the migratory cycle, including stabilizing initial actin-containing protrusions, stimulating signal transduction and generating tractional forces required to pull the cell body forward (Pollard and Borisy, 2003). Previously, α3β1 was identified as an important factor in migration based on changes in localization during keratinocyte migration and the fact that treatment of scrape-wounded keratinocyte monolayers with a function-blocking antibody against α3β1 inhibits wound closure (Goldfinger et al., 1999; Nguyen et al., 2000). Here, we have identified a role for α3β1 at a distinct step in the migratory cycle. Using integrin α3β1-deficient keratinocytes, we determined that α3β1-mediated adhesion to LN-5 is required for establishment of a polarized phenotype by migrating cells. α3β1-deficient keratinocytes were unable to maintain a cohesive wound edge in a scrape wound. Further, the wound-edge cells failed to form lamellipodia and to polarize in the direction of the wound. Instead, these cells scattered into the wound randomly as individual cells rather than as a cohesive monolayer. Furthermore, whereas α3β1-expressing keratinocytes at the wound edge extended broad lamellipodia shortly after wounding, α3β1-deficient keratinocytes extended circumferential pseudopodia as the cells scattered into the wound area. The ability of α3β1-deficient cells to migrate into the wound area was unexpected because function-blocking antibodies against α3β1 have been shown previously to inhibit keratinocyte migration into scrape wounds (Nguyen et al., 2000). The inability of α3β1-deficient keratinocytes to maintain a cohesive unit as they migrate over a scrape wound is probably due to a defect in formation and/or maintenance of cell-cell adhesions. Consistent with this hypothesis, formation of both gap junctions in human keratinocytes (Lampe et al., 1998) and E-cadherin-dependent cell-cell contacts in murine kidney epithelial cells requires α3β1 function (Wang et al., 1999).

When plated sparsely onto LN-5, α3β1-expressing keratinocytes assumed a fan shape, typical for migrating keratinocytes and very similar to the highly motile fish keratocyte. The cells that assumed this fan shape maintained the leading lamellipodia for long periods and underwent persistent migration (i.e. they maintained movement in the direction of the fan). By contrast, α3β1-deficient cells extended circumferential pseudopodia and filopodia that rapidly extended and retracted from the cell surface. These cells were still able to migrate, but not in the persistent manner displayed by α3β1-expressing cells. The ability of α3β1-deficient cells to migrate on LN-5 ECM indicates an alternate migratory mechanism. It is tempting to speculate that the α3-null cells use the α6β4 integrin to migrate on LN-5. Indeed, a promigratory role for α6β4 has been described in keratinocytes and carcinoma cells (Mercurio et al., 2001; Russell et al., 2003). However, in normal epidermis,
α6β4 is thought to promote stable adhesion through hemidesmosomes (Jones et al., 1991; Sonnenberg et al., 1991; Stepp et al., 1990). It is possible that, in the absence of α3β1, α6β4 assumes promigratory functions that are not normally evident in keratinocytes. In support of this notion, we have detected α3β1-dependent differences in α6β4 phosphorylation and subcellular distribution between MK+/+ cells and MK+/− or MKα3 cells (D.P.C. and C.M.D., unpublished).

Recently, the existence of two distinct migratory pathways in keratinocytes was proposed, each mediated by one of the two LN-5 binding integrins expressed by keratinocytes (Russell et al., 2003). Russell et al. showed that the α3β1-mediated pathway resulted in cell scattering from a wound edge, whereas the α6β4-mediated pathway promoted coordinated cell migration. These results contrast with our findings, in which α3β1 was required for coordinated migration and α3β1-deficiency led to cell scattering. These seemingly conflicting results might be resolved by the recognition that coordinated keratinocyte migration requires independent contributions from both α3β1 and α6β4, such that a loss of expression or function of either integrin might lead to aberrant migratory patterns.

Role of integrins in promoting cell polarization
The determination and organization of a leading-edge lamellipodium is a complex process. Initially, a cell responds to an extracellular stimulus by extending an actin-containing protrusion in the direction of the stimulus. If a stimulus is sensed in more than one area then more than one process is extended. Positive-feedback mechanisms stimulate the growth of a protrusion into a lamellipodium while inhibiting the growth of other processes, which eventually results in cellular polarization and formation of a defined leading edge. Extracellular stimuli that trigger this process are diverse and include growth factors, cytokines and ECM proteins (Ridley et al., 2003). Although growth factors and integrins can stimulate similar signal-transduction pathways known to regulate cell migration, a recently proposed model of cell polarization incorporates distinct contributions from both growth factors and integrins in the feedback systems that generate a leading edge (Cho and Klemke, 2002). The best-studied functions for integrins in cell migration are their roles in promoting adhesion and providing the traction that is required to pull the cell body forward. Integrin-mediated adhesion has also been recognized as a potentially important step in stabilizing the initial protrusions generated by growth-factor-receptor activation; without adhesion, these initial protrusions are unstable and short-lived (Bailly and Condeelis, 2002). The ability of the α3β1-deficient keratinocytes to extend actin-containing pseudopods indicate that α3β1-mediated adhesion is not required for this initial step in formation of a leading lamellipodium. However, α3β1-mediated adhesion is required for the subsequent step of stabilizing a leading-edge lamellipodium. These conclusions are supported by the fact that treatment of α3β1-expressing cells with a function blocking antibody against α3β1 prevented assumption of a fan shape but cells still rapidly extended and retracted pseudopodia.

In the current study, we have demonstrated that α3β1 is localized to the leading-edge lamellipodia of migrating wound-edge keratinocytes. Previously, α3β1 has been localized either underneath the cell body in migrating keratinocytes or to cell-contacts in monolayers of quiescent keratinocytes. We propose a model (Fig. 9) wherein, upon wounding, actin-containing protrusions push the plasma membrane in the direction of the wound, so that α3β1 that was once at a cell-cell contact is pushed out and down with the membrane into contact with the ECM. This event would occur coincident with redistribution of α6β4 and disassembly of hemidesmosomes which is thought to be a crucial event if cell migration is to proceed (Nguyen et al., 2000). α3β1-mediated adhesion of the protrusion might be facilitated by deposition of LN-5 by keratinocytes at the wound edge (Fig. 9B). This model suggests a passive mechanism for integrin localization to a leading-edge lamellipodium that might occur in addition to active mechanisms that have been proposed for trafficking integrins to a leading edge (Webb et al., 2002). This passive mechanism of

![Fig. 9. Model for role of α3β1 in the establishment of polarity by migrating wound-edge keratinocytes. (A) In quiescent epidermis, α6β4-containing hemidesmosomes mediate stable adhesion of basal keratinocytes to the basement membrane, whereas α3β1 is localized primarily to cell-cell borders. (B) Upon wounding, growth factors and cytokines in the wound bed stimulate actin polymerization and the formation of an initial protrusion. These initial protrusions contain α3β1 that interacts with newly deposited LN-5 (C), stabilizing the protrusion and subsequently stimulating Rac1 activity. Rac1 activation leads to growth of a stable leading lamellipodium.](image)
localizing \( \alpha3\beta1 \) to lamellipodia in wound-edge cells might ensure an efficient response of activated keratinocytes during cutaneous wound closure. This model is also consistent with the possibility that \( \alpha3\beta1 \) might, at cell-cell contacts and in addition to functions associated with cadherins (Chattopadhyay et al., 2003), represent a reserve that is ready at any time to respond rapidly to cutaneous wounding. After adhering to LN-5 deposited by keratinocytes at the wound edge and stabilizing the lamellipodium, \( \alpha3\beta1 \) could propagate signal transduction, causing continued growth and persistence of the lamellipodium (Fig. 9C, and see below). It is conceivable that other integrin-ligand interactions also contribute to this process. However, the fact that leading keratinocytes produce LN-5 at the wound edge and express high levels of \( \alpha3\beta1 \), combined with results from the current study, support a critical role for \( \alpha3\beta1/LN-5 \) interactions in promoting adhesion-dependent lamellipodium formation and initiating re-epithelialization.

Integrin-mediated activation of Rac1 in cell migration

A key signal-transduction molecule in generating lamellipodia is the small GTPase Rac1 (Nobes and Hall, 1999). We demonstrated that Rac1 activation is dependent on integrin \( \alpha3\beta1 \) in keratinocytes, consistent with other studies showing that adhesion is essential for Rac1 activation (Berrier et al., 2002; Hirsch et al., 2002; Price et al., 1998). A pathway from \( \alpha3\beta1 \) ECM ligands to Rac1 activation has been proposed previously, although a role for \( \alpha3\beta1 \) was not demonstrated directly (Gu et al., 2001). This pathway was shown to depend on FAK/Crk-mediated activation of the Rac1 guanine-nucleotide-exchange factor Dock180. We have determined that MK cells do display \( \alpha3\beta1 \)-dependent activation of FAK and phosphorylation of Cas (Manohar et al., 2004), and we are currently investigating whether Rac1 activation in our cells is dependent on this pathway. In addition to stimulating Rac activity, integrins have been shown to regulate the localization of Rac and its interaction with downstream effectors (Del Pozo et al., 2000; Del Pozo et al., 2002). Although we did not determine whether \( \alpha3\beta1 \) regulates Rac1 localization in keratinocytes, it is possible that mislocalization of Rac1 in \( \alpha3\beta1 \)-deficient keratinocytes contributes to the lack of cell polarization seen in these cells.

In the current study, we have shown that formation of a stable lamellipodium is dependent on Rac1 activity, because expression of dominant negative Rac1N17 in \( \alpha3\beta1 \)-expressing keratinocytes inhibited assumption of a fan shape. Conversely, increasing Rac1 activity in MK-2 cells by introducing a constitutively active Rac1L61 failed to induce stable lamellipodia, highlighting the importance of \( \alpha3\beta1 \)-mediated adhesion in Rac-dependent cell polarization. In our model, we propose that \( \alpha3\beta1 \) participates in growth of a lamellipodium by physically stabilizing initial protrusions and simultaneously promoting activation of Rac1, which promotes continued growth and maintenance of the lamellipodium (Fig. 9C).

Another small GTPase, cdc42, contributes to cell polarity and integrin-mediated adhesion has also been shown to regulate cdc42 activation (Nobes and Hall, 1999). Initial experiments suggest that \( \alpha3\beta1 \)-deficient MK cells also display reduced activation of cdc42 (D.P.C. and C.M.D., unpublished); however, it remains to be determined whether reduced cdc42 activity contributes to the failure of these cells to form polarized, stable leading lamellipodia.

In summary, our work demonstrates a requirement for \( \alpha3\beta1 \) in Rac-mediated cell polarization and persistent migration of keratinocytes on LN-5. Specifically, it defines a role for \( \alpha3\beta1 \)-mediated adhesion and signal transduction in establishment of a leading-edge lamellipodium, one of the earliest phenotypic changes that occurs during keratinocyte activation in a wound. In addition, our observations suggest that \( \alpha3\beta1 \)-deficient keratinocytes can adopt an alternate mechanism of migration, which has interesting implications in the analysis of cell migration. It is becoming increasingly apparent that many cell types exhibit a great deal of plasticity when it comes to modes of cell migration. Recently, an alternate migratory program, termed ‘ameboid migration’, was defined as migration that occurs independently of \( \beta1 \)-integrin-mediated adhesion and proteolysis of the extracellular environment (Webb and Horwitz, 2003). Whether or not the \( \alpha3\beta1 \)-deficient keratinocytes adopt such an ameboid migratory program is not yet known. Future studies of atypical migratory patterns in these cells might yield a more complete understanding of how a cell responds to extracellular cues and might lead to the discovery of novel therapeutic targets to either promote migration in cases of impaired cutaneous wound healing, as in the case of diabetic ulcers, or to inhibit migration in the case of tumor metastasis.

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