Laminin 5 deposition regulates keratinocyte polarization and persistent migration

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Summary

Repair of wounded epidermis requires both keratinocyte migration and deposition of laminin 5 over exposed dermal collagen. To understand the coupling between leading cell migration and laminin 5 deposition, we developed a novel migration assay using time-lapse microscopy. We demonstrate that in migrating, human keratinocytes the deposition of laminin 5 promoted 'processive migration', characterized by stable cell polarization that was tightly coupled to persistent, linear migration in the absence of a chemotactic gradient. Processive migration required deposition of laminin 5, which was restricted to the rear of the polar cell. Integrin $\alpha 3\beta 1$ interacted with these laminin 5 deposits at contact sites that did not require actin-dependent cross-linking. Further, we show that the migrating cells switched adhesion by integrin $\alpha 2\beta 1$ on collagen at the front of the cell to integrin $\alpha 3\beta 1$ on exogenous laminin 5 at the rear of the cell. Along with this switch of integrin usage was the removal of collagen from sites under the cell that precisely correlated with deposition of laminin 5. Processive migration was blocked with suppressors of microtubule dynamics (nocodazole and taxol) or rottlerin, a PKC-δ inhibitor. These drugs were also shown to block deposition of laminin 5 but, surprisingly, constitutive secretion was unimpaired, suggesting deposition was a regulated event. Thus, at the front of the cell, the leading lamellipodium was stabilized through integrin interactions in focal complexes with the exogenous substratum. However, at the rear of the cell, stable cell polarization and linear migration was promoted by laminin 5 deposits and integrin $\alpha 3\beta 1$.

Movies available on-line

Key words: Integrins, Keratinocytes, Cell migration, Cell adhesion, Extracellular matrix

Introduction

The major adhesive component of epithelial basement membranes is laminin 5 (Carter et al., 1991; Rousselle et al., 1991; Vailly et al., 1994). Laminin 5 mediates stable adhesion of basal keratinocytes through hemidesmosomes (Borradori and Sonnenberg, 1999; Burgeson and Christiano, 1997; Fuchs et al., 1997; Green and Jones, 1996). However, wounding of quiescent epidermis activates keratinocytes at the leading edge of the wound, forming a population of cells that are distinct from the following cells. We have previously shown that leading cells in an outgrowth are distinguished from following keratinocytes by their deposition of laminin 5, failure to communicate via gap junctions and sensitivity to toxin B, an inhibitor of Rho GTPase (Lampe et al., 1998; Nguyen et al., 2000a; Nguyen et al., 2001). Recently, it has been shown that these leading cells also express p16, a marker that correlates with increased linear migration (Natarajan et al., 2003). The leading cells therefore form a migratory population responsible for both advancing the edge of the epidermal outgrowth onto dermal collagen and providing laminin 5 for the stable adhesion of the following cell sheet and repair of the basement membrane. Leading keratinocytes express integrins $\alpha 6\beta 4$, $\alpha 3\beta 1$, $\alpha 2\beta 1$ and $\alpha 5\beta 1$ and can adhere to laminin 5, collagen or fibronectin (Nguyen et al., 2001). However, migration in closure of wounded monolayers requires only interactions of integrin $\alpha 3\beta 1$ with laminin 5 (Goldfinger et al., 1999; Lampe et al., 1998; Nguyen et al., 2000b; Santoro et al., 2003). Further, targeted disruption of laminin 5 in mouse keratinocytes blocks epidermal adhesion in the animal and inhibits migration of keratinocytes in response to epidermal growth factor (Nguyen et al., 2000b; Ryan et al., 1999). While the role of laminin 5 deposits in initial adhesion and spreading on exogenous collagen has been described, the role of laminin 5 deposition in the migration of the leading cells is not clear.

Keratinocytes express a second receptor for laminin 5, integrin $\alpha 6\beta 4$, that is a component of hemidesmosomes, cell junctions responsible for stable adhesion. Several reports have demonstrated regulatory interplay between migration by integrin $\alpha 3\beta 1$ in focal adhesions and stable adhesion by $\alpha 6\beta 4$ in hemidesmosomes (Goldfinger et al., 1999; Hintermann et al., 2001; Santoro et al., 2003; Xia et al., 1996). The binding of integrin $\alpha 6\beta 4$ to laminin 5 leads to Rho-independent activation of PI 3-kinase and cell spreading by integrin $\alpha 3\beta 1$ (Gu et al., 2001; Mainiero et al., 1997; Nguyen et al., 2000a). Understanding of migration of keratinocytes in culture is further complicated by the ability of laminin 5 to promote gap junctional communication and cell-cell interactions (Lampe et al., 1998). Previous studies have shown that laminin 5 promotes (Carter et al., 1991; Shang et al., 2001; Zhang and Kramer, 1996) or inhibits migration (Goldfinger et al., 1999; O'Toole et al., 1997; Yamada et al., 1996).

Cells at the leading edge of a wound form polarized cells with lamellipodia extended in the direction of the wound bed (Martin, 1997). It is still not clear what regulates the direction of migration in a wound outgrowth, whether it is in response to the free edge or to chemotactic factors released during wounding (Wells, 2000). We hypothesized that laminin 5 deposition plays a central role in polarization and migration of leading cells. The studies presented here focus not on the initial activation of leading cells but rather on their migratory response to the expression and deposition of laminin 5 onto an exogenous collagen surface. The mechanisms coupling deposition of extracellular matrix proteins and cell migration are not well established so we developed a system to examine this key event in wound repair.

We demonstrate that the deposition of laminin 5 regulated a polarized, linear and persistent 'processive migration' in leading keratinocytes. After initial adhesion to exogenous ligand, cells transitioned to a highly polarized cell that coupled to the onset of processive migration. During this transition and throughout migration, we found that laminin 5 was deposited at contacts restricted to the rear of the cell. Integrin α3β1 interactions with laminin 5 in these deposition contacts was required for processive migration, however, this α3β1 activation was not dependent on the actin cytoskeleton. Furthermore, we show that laminin 5 deposition is regulated separately from laminin 5 found in the culture media. Thus, stable cell polarization and persistent migration as observed in leading cells of epidermal outgrowths were replicated in the single-cell population, and this was promoted by laminin 5 deposition.

Materials and Methods

Cells and cell culture

Primary keratinocytes from normal human foreskins (HFK) were prepared as described by Boyce and Ham (Boyce and Ham, 1985). Keratinocytes from individual JF/VS9-3-96 with junctional epidermolysis bullosa-pyloric atresia (JEB-PA) have premature termination mutations in both alleles of the *ITGB4* gene encoding the β4 integrin subunit (JF/VS9-3-96 is proband from family two in Pulkkinen et al. (Pulkkinen et al., 1998). Keratinocytes, from an individual with gravis JEB, that did not secrete laminin 5 because of a defect in the laminin β3 chain, were a gift from M. R. Pittelkow (Lim et al., 1996). Cultures were maintained at 150 μm CaCl$_2$ in serum-free keratinocyte growth media (KGM, Clonetics Corp.). All cells were used at passage 2-4 while in logarithmic phase of growth.

Reagents and antibodies

The mouse monoclonal antibodies mAb D2-1 against precursor laminin 5 α3 chain, C2-5 and C2-9 against both precursor and processed laminin α3 chain and mAb B4-6 against laminin γ2 chain were prepared and characterized as previously described (Nguyen et al., 2001). The mAb P1F2 and P1B5 against integrin α3 and P1H5 against integrin α2 were previously described (Wayner and Carter, 1987). mAb P5D2 against integrin β1 was a generous gift from Dr E. Wayner. mAb 3D5 against β4 integrin was made by Alis Karabulut Thorup while in the Carter laboratory. Other antibodies used were rabbit anti-human collagen type I (Chemicon), GoH3 against integrin α6 (Coulter-Immunotech), mAb 9EG7 against integrin β1 (Pharmingen) and purified rabbit polyclonal antibody FAK(pY397) against focal adhesion kinase (FAK) that is phosphorylated at tyrosine 397, and FITC- or rhodamine-conjugated goat anti-mouse IgG (Biosource International). The nuclear dye DAPI, rhodamine-conjugated phalloidin, Alexa-conjugated goat anti-mouse secondary

antibodies and Zenon mouse IgG labeling kits were obtained from Molecular Probes. Rottlerin was obtained from Alexis Biochemicals, Bis[sulfosuccinimidyl] suberate (BS3) was from Pierce and LY294002 from Calbiochem. Unless specified, all other chemicals were obtained from Sigma-Aldrich.

Immobilized ECM surfaces

Cover glasses for immunofluorescent microscopy or tissue culture dishes were coated with laminin 5 using one of three methods which all gave equivalent results. (1) Human foreskin keratinocytes (HFK) were cultured to confluence at 37°C on surfaces to allow for the deposition of laminin 5 and then the cells were removed by trypsin digestion as previously described (Xia et al., 1996). (2) Recombinant laminin 5 (BioStratum, Inc., Durham, NC) at 7 μg/ml was adsorbed overnight at 4°C. (3) Laminin 5 present in the conditioned culture media from HFK cultures was trapped onto surfaces using immobilized mAb C2-5. We used the first method, except when stated otherwise, to efficiently prepare large numbers of surfaces. Collagen surfaces were prepared by adsorbing human placental type I collagen (Wayner and Carter, 1987) at 10 μg/ml for 2 hours at 24°C.

Immunofluorescent microscopy

HFKs for use in immunofluorescent studies were plated onto prepared cover glass surfaces, cultured at 37°C and then fixed with 2% formaldehyde in 0.1 M sodium cacodylate, 0.1 M sucrose, pH 7.2 for 10 minutes at 24°C. Cells were permeablized with 0.5% Triton X-100 for 5 minutes at 24°C and then blocked in 0.5% heat-denatured BSA. Stained cover glasses were mounted in ProLong antifade (Molecular Probes) or 70% glycerol, 125 mM Tris, pH 9 and 5% n-propyl gallate. Images were collected by epifluorescence on a Zeiss Axiophot with a Photometrics SenSys cooled CCD digital camera (Roper Industries, Trenton, NJ) using MetaMorph software (Universal Imaging, Downingtown, PA). Deltavision images, when indicated, were collected at the specified wavelengths using 0.2 μm optical sections and deconvoluted using Deltavision Software (Applied Precision). All images were prepared using Photoshop software (Adobe).

Determining percent polarized fan cells

Keratinocytes were plated at a density of 1×10^4 cells/cm^2 onto surfaces for 2 hours and fixed with formaldehyde. Phase-contrast images were taken of three separate fields containing approximately 100-200 cells for each culture plate. For a cell to be scored as polarized, it needed to possess all three defining properties of the fan cell morphology as seen in Fig. 1C: (1) a phase-bright retracted rear, which extends across the cell diameter; (2) the nucleus polarized to the rear of the cell; (3) a lamellipodium that extends around the remaining circumference of the cell. Cells that did not fulfill these three parameters but were spread were counted as nonpolarized. Cells that were not spread were not counted.

Immunoblots

Adherent cells on laminin 5 were treated for 7 hours with 6 μm rottlerin then extracted with 1% Triton X-100 in PBS containing protease inhibitors (2 mM PMSF, 1 mM NEM), followed by extraction in 0.5% SDS/PBS and protease inhibitors. Detergent-soluble and -insoluble extracts from equal numbers of cells for each experiment were separated on SDS-page gels (Laemmli, 1970). Proteins were blotted onto nitrocellulose, blocked in 0.5% BSA and incubated with primary mouse monoclonal antibody against β4 integrin (3D5). Primary antibodies were reacted with peroxidase-conjugated rabbit anti-mouse IgG (DAKO) for 1 hour. Blots were developed with ECL chemiluminescence (Amersham) and direct

exposure to Hyperfilm MP (Amersham) and individual bands were quantitated using ImageQuant software (Molecular Dynamics). Volume intensity of the bands was corrected for background and the ratio of insoluble/soluble were calculated as (SDS/Triton) for untreated and rottlerin-treated lanes.

Time-lapse microscopy

Activated keratinocytes were plated at a density of 1×10^4 cells/cm^2 onto collagen or laminin 5 plates. For migration and polarization studies, mother plates of keratinocytes were treated with either 1 µM nocodazole, 1 µM taxol or 6 µM rottlerin for 3 hours or 5 µM LY294002 for 30 minutes, then trypsinized and replated in the presence of drugs. Cells were labeled prior to time-lapse recordings with a fluorescent nuclear dye (Hoescht 33372, Molecular Probes). Recordings of migration started 1-3 hours after plating. Migration was monitored in an environmental chamber at 37°C using a Nikon Eclipse TE300 inverted microscope equipped with an epifluorescence illumination source with EX 365/10 nm and EM 460/50 nm filters. Phase contrast or fluorescent images were collected every 2 minutes for 2 hours using a cooled interline CCD camera (Princeton Instruments) and stored as image stacks using MetaMorph software (Universal Imaging). There were 50-70 cells in each image field that were tracked. Trajectories of the centroid from fluorescent nuclei were measured using MetaMorph. We manually chose to not track cells that did not spread, that divided or that migrated off the field of view during 2-hour recordings. This represented less than 10% of the total cells. All other keratinocytes in the image were included in the analysis. Calculated from this data was the linear distance (l), defined as the distance between the start and end position of the nucleus centroid and the total distance (t) traveled, which yielded the Processive Index (P.I.)=l/d and the velocity of migration from the total distance as µm/minute.

BSA adhesion assay

Untreated Petri plates were blocked with 0.5% w/v heat-denatured BSA overnight at 4°C. HFKs were labeled with 0.5 µM calcein-AM (Molecular Probes), suspended in trypsin and plated at 5×10^6 cells/cm^2 for 4 hours at 37°C (Gil et al., 2002). The percentage of cells that adhered was determined as fluorescence of post-wash cells adhered/total cells on a CytoFluorII fluorescence plate reader (PerSeptive Biosystems). Three wells were averaged for each experiment and the averages and standard deviations from three independent experiments are presented as values of percentage adhered relative to untreated KGM=1.0.

ELISA and immunoprecipitation assay for secretion of laminin 5

Laminin 5 deposited into the extracellular matrix was quantitated by ELISA. HFKs were pretreated for 3 hours with 1 µM nocodazole, 1 µM taxol or 6 µM rottlerin in KGM, trypsinized and replated onto laminin 5 surfaces in the presence of drugs for 4 hours to allow for deposition of laminin 5 into the extracellular matrix. Cells were removed with 5 mM EDTA in PBS for 30 minutes at 37°C. The remaining extracellular matrix was fixed in 2% formaldehyde and blocked overnight with 0.5% BSA. Deposits were detected with either non-immune hybridoma medium SP2 or mAb D2-1 against newly deposited, precursor laminin 5 followed by rabbit anti-mouse horseradish peroxidase (Jackson Laboratories). After extensive washing, peroxidase substrate ABTS (KPL) was added, the reaction was allowed to develop for 20 minutes and absorbance at 405 nm was determined.

Laminin 5 secreted into the culture media was quantitated by radio-immunoprecipitation. HFKs were pretreated for 3 hours with 1 µM nocodazole or taxol in KGM, trypsinized and replated onto laminin 5 surfaces for 1 hour. The medium was then changed from KGM to cysteine/methionine-free KGM (Clonetics Corp., San Diego, CA) with 75 µCi/ml [^{35}S]methionine (ICN Biochemicals, Irvine, CA) and cells were labeled for 1 hour, followed by a 4-hour chase with unlabeled KGM to allow laminin 5 secretion into the medium. Labeled cell culture medium was collected and protease inhibitors were added. Immunoprecipitation with mAb C2-5 against laminin α3 chain or mAb B4-6 against laminin γ2 chain immobilized to Protein A-agarose with Rabbit anti-mouse IgG (ICN/Cappel) was carried out at 4°C in RIPA buffer (1% Triton X-100, 0.5% BSA, 1 mM EDTA, 50 mM Tris pH 7.5). Precipitates were washed extensively in 1% empigen BB, 50 mM Tris, 400 mM NaCl and analyzed by 8% SDS-PAGE. Gels were treated with Fluoro-Hance (Research Products International Corp.) before visualization by autoradiography. Each experiment was performed at least three times with equivalent results.

Results

Precursor laminin 5 interacted with integrin α3β1 to induce polarization and linear migration

To examine the role of laminin 5 deposition in the migration of keratinocytes, we first developed a system to generate robust migration in single cells. Deposits were visible by immunofluorescence at the site of initial adhesion (arrow, Fig. 1A) and were left behind in a linear trail that marked the path of migration, similar to a recent description of keratinocyte
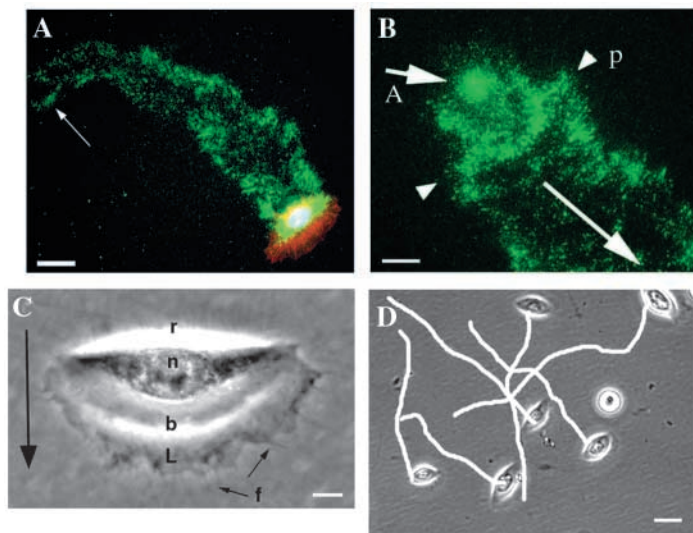

Fig. 1. Activated keratinocytes migrated processively on laminin 5. (A) Immunofluorescence of precursor laminin 5 deposited by a keratinocyte migrating on exogenous laminin 5 for 3 hours. Arrow, site of initial adhesion. Anti-laminin 5 mAb D2-1 (green), phalloidin (red). Scale bar: 20 µm. (B) mAb D2-1 stains the initial site of adhesion (arrow, A) and the band of deposits that was under the symmetrically spread cell. The cell is no longer in this field. The deposit pattern reflects the change in cell morphology as it polarized and elongated perpendicular to the direction of migration (arrowheads, P) and began linear migration (large arrow). Scale bar: 10 µm. (C) Phase image of a keratinocyte with polarized fan cell morphology (see also Movie 1, http://dev.biologists.org.supplemental/). Large arrow denotes direction of migration. Cell characteristics r, retracted rear, n, nucleus, b, cell body-lamellipodium transition region, L, lamellipodium, f, filopodia. Scale bar: 5 µm. (D) Tracks from 2-hour migration on exogenous laminin 5, overlay of phase image of cells at the end (see also Movie 2, http://dev.biologists.org.supplemental/). Scale bar: 20 µm.

migration (Natarajan et al., 2003). mAb D2-1 is a useful tool for labeling newly deposited precursor laminin 5. mAb D2-1 recognizes an epitope in the precursor laminin $\alpha3_{200}$ LG4/5 domain and is absent from the extracellular matrix of confluent keratinocytes cultures (Goldfinger et al., 1998; Nguyen et al., 2000b). Activated, log-phase keratinocytes passaged onto exogenous laminin 5 at sparse cell densities to minimize cell-cell interactions showed a striking form of migration that has not been previously described for keratinocytes. Highly persistent migration directed along linear tracks was coupled to the deposition of precursor laminin 5 onto the surface (Fig. 1A). Robust migration began immediately after adhesion and spreading and continued for approximately 12 hours, after which cell-cell interactions dominated and migration declined. We therefore analyzed the migration of cells at low density during the first 6 hours after initial adhesion and spreading. This transition from adhesion and spreading was evident in the deposition history shown in Fig. 1B. Laminin 5 was deposited at the site of adhesion and the pattern of deposits reflected the change in morphology from a symmetrically spread cell to the elongated polarized cell shown in Fig. 1C as the cell began to migrate.

During migration, these cells retained the phenotype of a highly polarized cell (Fig. 1C; see also Movie 1, http://dev.biologists.org.supplemental/). The combination of persistent migration with retention of polarized morphology defines 'processive migration'. We observed that polarization always coincided with the immediate onset of processive migration. Polarized keratinocytes possessed a large, thin lamellipodium (L) extending at the leading edge that encompassed half the perimeter of the cell (Fig. 1C), giving the cell a fan-shape appearance as described in fish keratocytes (Euteneuer, 1984). However, while fish keratocytes migrate at rates up to 20 μm/minute, processive migration of human keratinocytes proceeded at a velocity of about 0.7 μm/minute, comparable to migration recently described for NBT-II cells (Huang et al., 2003). Unlike fibroblasts, there is no significant tail extending at the trailing edge of these fan cells (Fig. 1C, r). When plated onto laminin 5, more than 50% of the total activated cells polarized and migrated as processive fan cells, a remarkable synchrony for a primary cell population (Fig. 1D, see also Movie 2, http://dev.biologists.org.supplemental/). Thus, our approach generated a synchronized population of keratinocytes with the phenotype of leading cells in an epidermal outgrowth. Specifically, they possessed a highly polarized, migratory phenotype and deposited a precursor laminin 5 during migration. Utilizing this system, we were able to examine the integrin and extracellular matrix ligands responsible for mediating this migration.

Processive migration was first quantified by measuring the percentage of polarized cells in a population (Fig. 2). We later verified that the percentage of polarized cells reflected the percentage of cells undergoing processive migration over a 2-hour time-lapse recording. For a cell to be scored as polarized, it needed to possess all three defining properties of the fan cell morphology evident in Fig. 1C: (1) a phase-bright retracted rear, which extends across the cell diameter; (2) the nucleus polarized to the rear of the cell; (3) a lamellipodium, which extends around the remaining circumference of the cell. Activated keratinocytes were grown to log phase and plated onto surfaces for 2 hours before they were fixed with
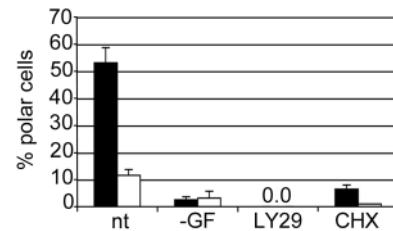

Fig. 2. Polarization is affected by exogenous ligand and requires growth factors, PI 3-kinase and protein translation. Keratinocytes were plated for 2 hours on laminin 5 (black bars) or collagen surfaces (white bars) with either no treatment (nt), absence of growth factors (–GF), presence of 5 μM LY294002 (LY29) or presence of 10 μg/ml cycloheximide (CHX). Values represent the average and standard deviation of at least three independent experiments.

formaldehyde and scored for polarization (Materials and Methods). Greater than 50% of activated keratinocytes formed polarized cells on laminin 5 (Fig. 2). The exogenous extracellular ligand influenced polarization. Only 11% of cells polarized when plated onto a surface coated with type I collagen. Cells grown in the presence of growth factors but plated in basal medium, which lacks EGF, insulin and pituitary extract, adhered and spread on exogenous laminin 5 but fewer than 3% were able to polarize on laminin 5 or collagen. Polarization could be recovered when the basal medium was supplemented with TGF-β1, EGF or insulin (data not shown). The direction of polarization and persistent migration could not be affected by applying concentration gradients of soluble TGFβ1, EGF or serum (data not shown). Thus, processive migration was not chemotactic but resembled chemokinetic migration in that directionality was defined by an intrinsic mechanism rather than an extrinsic source (Wells, 2000). PI 3-kinase has been associated with stimulating migration and cell polarization (Curnock and Ward, 2003). Consistently, when PI 3-kinase was inhibited with LY294002, polarization of cells on laminin 5 and collagen was blocked while adhesion and spreading were normal.

Protein synthesis was not required for initial adhesion and spreading exogenous ligands (DiPersio et al., 1995). However, cycloheximide-treatment blocked polarization and migration on laminin 5 and collagen, demonstrating protein translation was required. Thus, adhesion and spreading did not require protein translation but polarization and migration were distinct events requiring new protein synthesis.

We next characterized the extracellular matrix-integrin interactions involved in polarization and processive migration. Activated keratinocytes express integrin α2, a receptor for collagen, and integrin α3, a receptor for laminin 5. As we have previously shown (Carter et al., 1990a), adhesion to exogenous collagen or laminin 5 was blocked with anti-α2 or anti-α3 antibodies, respectively (data not shown) indicating that cells used exogenous ligand for initial adhesion. Cells plated onto laminin 5 in the presence of inhibitory anti-α2 integrin antibodies were able to polarize normally (Fig. 3). Cells that were adhered and migrating on laminin 5 lost polarization when anti-α3 antibodies were added and there was considerable rounding of the cells. These keratinocytes express active integrin α6β4, another receptor for laminin 5. Integrin α6β4 was not required for polarization as anti-α6 antibodies
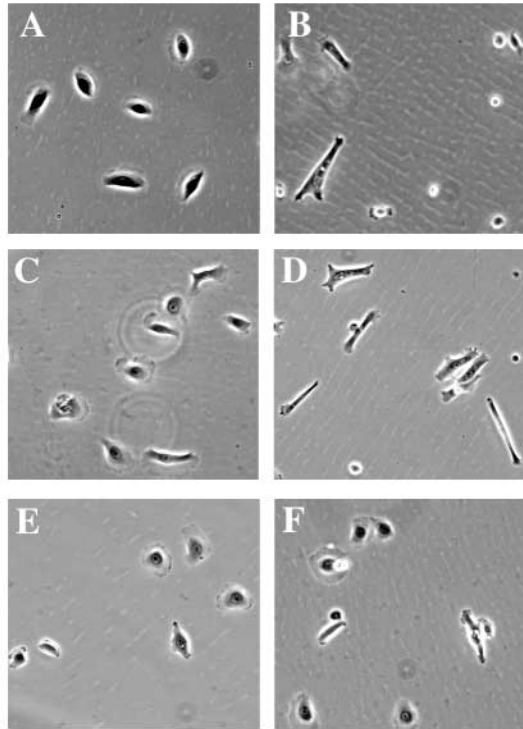
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Surface	Antibody	% polar cells	
Lm5	nt	53 ± 5.6	*
	anti- α3	0	**
	anti- α2	64 ± 10	*
	anti- α6	43 ± 19	*
	JEB-PA	74 ± 10	*
Col	nt	11 ± 2.1	
	anti- α3	0	**
	anti- α2	3.8 ± 3.8	**
	anti- α6	14 ± 12	*
	anti- lm5	5.7 ± 5.2	**

* Not significantly different values, P>0.05; Student's
t-test. ** Significantly different from nt value,
P<0.05; Student's t-test.

Fig. 3. Polarization on collagen requires interactions of integrin
α3β1 with precursor laminin 5 deposits. Cells were plated onto
exogenous laminin 5 (A,B) or collagen (C-F) in the absence of
antibody (C) or the presence of anti-α2 mAb P1H5 (A), anti-α3 mAb
P1B5 (D), anti-α6 goH3 (G) or anti-laminin 5 mAb C2-9 (E) for 5
hours then the percentage of polarized cells were determined (see
Materials and Methods). Alternatively, for those antibodies that
would block initial adhesion, cells were instead plated for 1 hour to
allow for adhesion and spreading prior to the addition of anti-α3
mAb P1B5 (B) or anti-α2 P1H5 (F) for 3 hours before cells were
quantified. (G) Summary of data obtained from polarization
experiments. Values represent the average and standard deviation of
at least three independent experiments.

had no effect on polarization. Furthermore, keratinocytes
derived from patients with junctional epidermolysis bullosa-
pyloric atresia (JEB-PA), which lack β4 integrin, were able to
polarize (Fig. 3G). As expected, cells that were adhered and
migrating on collagen lost polarization when anti-α2
antibodies were added. Anti-α6 antibody did not affect

polarization or migration of cells on collagen. Anti-α3 blocked
polarization on collagen and random migration resulted from
the loss of stable rear retraction and the formation of elongated
cells. Cells migrating on collagen surfaces deposited laminin
5 in deposition trails similar to those observed on exogenous
laminin 5, thus providing a ligand for integrin α3. That
inhibiting integrin α3 blocks polarization suggested these new
deposits of laminin 5 contributed to processive migration.
Indeed, anti-laminin 5 antibodies also blocked polarization on
collagen (Fig. 3E).

Processive migration is characterized by small, punctate focal complexes

Optical sections from deconvoluted micrographs revealed
adhesion components restricted to the basal surface of
polarized keratinocytes (Fig. 4). Anti-phospho FAK(pTyr397),
a marker for active focal adhesions, localized to small, punctate
clusters under lamellipodia but rarely under the cell body or at
the rear of the cell, characteristic of focal complexes. Focal
complexes are newly formed contacts that are readily
disassembled allowing for rapid migration (Huang et al., 2003;
Kaverina et al., 2002). Paxillin and vinculin colocalized with
FAK in focal complexes (data not shown). Integrin β1 (Fig.
4B,C) and integrin α3 (Fig. 4E,F) colocalized with FAK.
Consistent with our prior observations (Carter et al., 1990b),
integrin β1 was not found in retractions fibers of keratinocytes
migrating on exogenous laminin 5 or collagen. Cell surface
staining of integrin α6β4 in keratinocytes was uniformly
distributed over the basal and apical cell surface (data not
shown). However, in processively migrating cells, all of this
staining was extractable with detergent, as shown in Fig. 6D,E,
demonstrating that it is not present in hemidesmosome-like cell
junctions. Consistent with the polarization data in Fig. 3, α2
integrin was not found in focal complexes of keratinocytes on
exogenous laminin 5 (data not shown). In our assay α2β1 did
not contribute to processive migration on laminin 5, in contrast
to previous studies (Decline et al., 2003; Decline and
Rousselle, 2001). When the cells were plated onto collagen
surfaces, integrin α2 colocalized with FAK in more discrete
structures resembling focal adhesions both under the
lamellipodium and under the cell body (Fig. 4G-I). Integrin
α3β1 colocalized with diffuse FAK distribution at the leading
edge (Fig. 4J-L), in agreement with the ability of anti-α3
antibodies to block polarization on collagen. However, α3 was
not found in the more discrete focal contacts under the cell
body where α2 was found. Thus, polarization and processive
migration was characterized by: (1) deposition of laminin 5;
(2) absence of focal adhesions; (3) presence of minimal focal
complexes containing integrin α3; (4) stable polarization
during persistent migration.

Laminin 5 deposits mediate processive migration on collagen

We next quantified the persistence of processive migration
using time-lapse microscopy. Analysis of 2-hour migration
tracks of keratinocytes on laminin 5 revealed highly persistent
migration, evident in the relatively straight migration paths
in Fig. 1A,D and Fig. 5A,a. Migration proceeded at
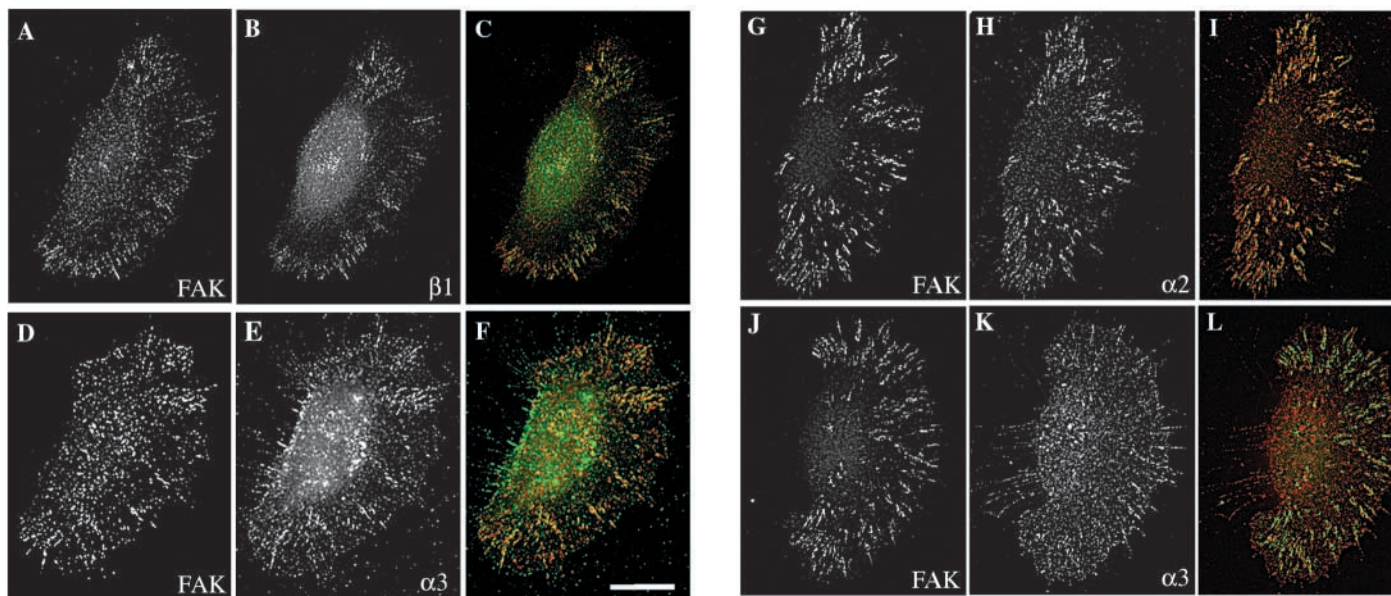approximately 0.7 μm/minute. Cells migrating on collagen,

Fig. 4. Activated keratinocytes on laminin 5 assembled small, punctate focal complexes during processive migration. Cells were plated onto exogenous laminin 5 (A-F) or collagen (G-L) for 2 hours, then fixed and stained for immunofluorescence. Deconvoluted optical sections of the basal cell surface are shown. Phospho-FAK-Y397 (red) (A,D,G,J), β1 integrin mAb 9EG7 (B) and α3 integrin mAb P1F2 (E,K) and α2 integrin mAb P1H5 (H) (green). Scale bar: 10 μm.

however, traveled shorter distances and were less linear (Fig. 5A,b). These differences in persistence were quantified by calculating the processive index, PI, of migration. We define PI=l/t, where l is the linear distance between start and end and t is the total distance traversed by the cell. Keratinocytes migrating on exogenous laminin 5 had an average PI of 0.8, approaching the ideal PI=1.0 for linear migration (Fig. 5B,a). Also apparent in this analysis was that the majority of the cells were in the category of processively migrating cells (shaded area in Fig. 5B,a), defined as moving greater than 40 μm, more than the average width of the cell, with a PI>0.7. In contrast to

robust processive migration on exogenous laminin 5, cells on collagen moved shorter distances and fewer cells migrated processively (Fig. 5B,b).

We measured the percentage of cells in the region of processive migration. Processive migration was observed in greater than 60% of the cells on laminin 5 but in less than 20% of the cells on collagen (Fig. 6). These values agree with the percentage polarized cells in Figs 2, 3 demonstrating that the fan cell phenotype of Fig. 1C is a hallmark of processive migration. Keratinocyte cell lines isolated from patients with junctional epidermolysis bullosa-gravis (JEB-G), which fail to
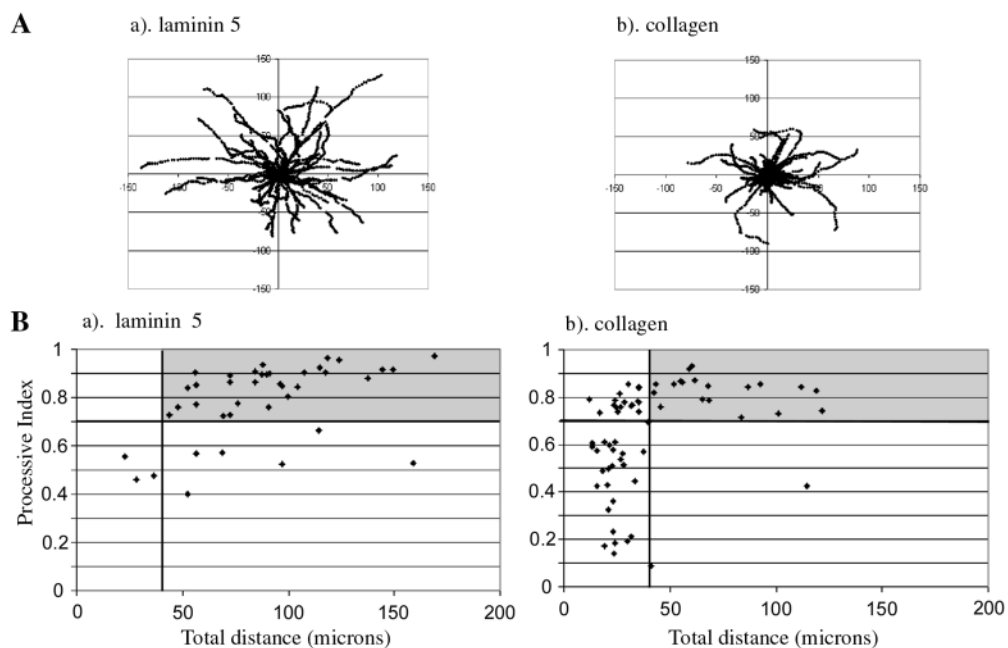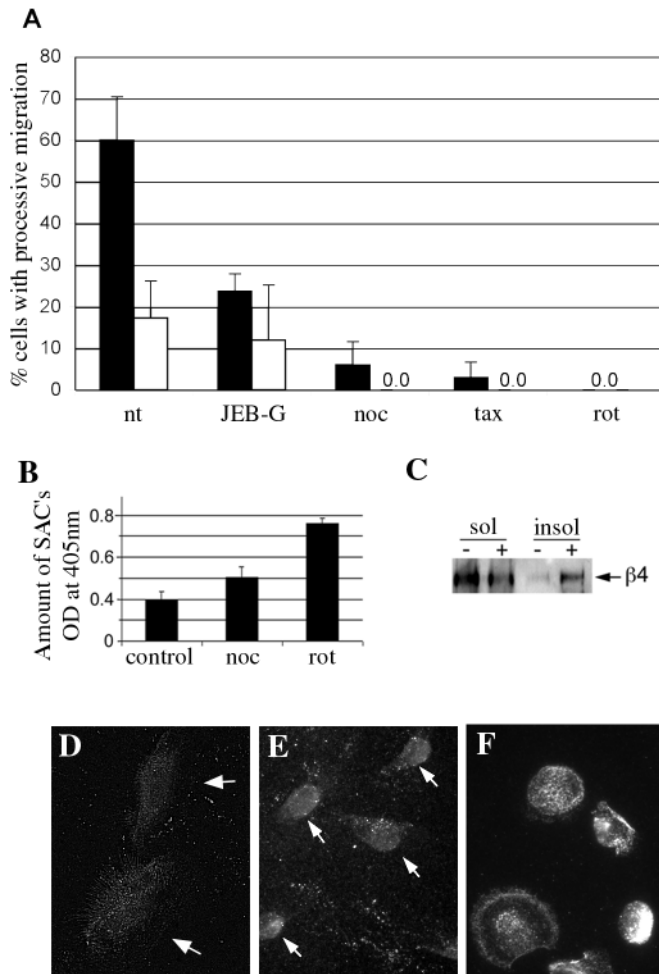

Fig. 5. Laminin 5 provided a better substratum for processive migration than did collagen. Cells were plated onto exogenous laminin 5 or collagen for 1-2 hours before migration was recorded at 37°C by time-lapse imaging every 2 minutes for a total of 2 hours. (A) Plots of cells on exogenous laminin 5 (a) or collagen (b) showing 2 minute interval positions for all cells in one 10× field oriented so that cell origins are at x(0), y(0). Distance migrated in microns is indicated on the plots. (B) Processive index for each cell in A is shown as a function of the total distance traveled for cells on laminin 5 (a) or collagen (b). Shaded regions indicate processively migrating cells with a total migration >40 μm and PI>0.7.

A

B   **C**

D   **E**   **F**

Fig. 6. Processive migration required deposition of precursor laminin 5. (A) Time-lapse analysis from 2-hour recordings as described in Fig. 5 were analyzed to determine the percentage of cells that fell into the region defining processive migration (shaded region, Fig. 5) on laminin 5 (black bars) or collagen (white bars). Cells that do not synthesize laminin 5 (JEB-G) showed decreased processive migration. Treatment of cells with 1 μM nocodazole (noc), 1 μM taxol (tax) or 6 μM rottlerin (rot) blocked processive migration. Values are averages and standard deviations from at least three independent experiments. (B) Rottlerin and nocodazole caused an increase in detergent-insoluble integrin α6β4. Triton-insoluble integrin α6 was quantified by ELISA using anti-α6 antibody goH3. Cells pretreated for 3 hours with 1 μM nocodazole or 6 μM rottlerin were plated on recombinant laminin 5 adsorbed to Petri plates for 5 hours then extracted and processed for ELISA. Representative data from one of three experiments is shown. (C) Triton-insoluble integrin β4 was quantified by western blot analysis using mAb 3D5. Log phase cells plated on recombinant laminin 5 adsorbed to Petri plates were either not treated (–) or treated (+) with 6 μM rottlerin for 7 hours, extracted with 1% Triton (sol) followed by 0.5% SDS (insol) and immunoblotted with anti-β4 antibodies. Equal amounts of protein from Triton X-100 extracts and equal amounts for SDS samples were loaded. Gels were quantified using ImageQuant software (Materials and Methods). (D) Integrin α6 stained with goH3 antibody is absent from basal contacts in polarized cells (indicated by arrows) migrating on laminin 5. Cells were fixed and processed for immunofluroescence as described in Fig. 4 using deconvoluted microscopy. (E,F) Cells were plated on laminin 5 for 5 hours then extracted with 1% Triton X-100, fixed and processed for standard epifluorescence. (E) Processively migrating cells on laminin 5, indicated by arrows, lack detergent-insoluble integrin α6β4. (F) Cells pretreated with 6 μM rottlerin for 3 hours then plated on laminin 5 had increased α6β4 that was resistant to Triton X-100 extraction.

make laminin 5, did not migrate as processively on laminin 5. While the JEB-G cells did migrate on collagen, we were not able to statistically distinguish them from normal keratinocytes because of the error associated with the low percentage of cells that migrate on collagen. We showed that inhibition of integrin α3 or laminin 5 blocked polarization on collagen (Fig. 3), therefore, the deposition of laminin 5 promoted processive migration on collagen.

Nocodazole and taxol, drugs that destabilize or stabilize microtubules, respectively, blocked migration on collagen (Fig. 6). While the cells adhered, they were not polarized and showed non-polarized ruffling of the lamellipodium around the circumference of the cell. In contrast, cells on exogenous laminin 5 were able to adhere, spread and migrate with an average total distance traveled of 32±19 μm and 26±4 μm over 2 hours in the presence of nocodazole and taxol, respectively. However, processive migration was reduced in comparison to untreated cells, which traveled an average of 91±29 μm. Rottlerin, an inhibitor of protein kinase C-δ (PKC-δ), blocked migration on collagen and laminin 5 but did not inhibit adhesion or spreading. Consistently, rottlerin and nocodazole increased the amount of Triton-insoluble integrin α6β4 relative to the low levels seen in untreated keratinocytes when on exogenous laminin 5 (Fig. 6B). Western blots of β4 (Fig. 6C) were quantified to determine the ratio of insoluble to soluble β4 of cells on exogenous laminin 5. Rottlerin increased the

ratio (insoluble/soluble) from 0.12 to 0.51. Studies have shown that PKC-δ and PKC-α are responsible for phosphorylation of α6 and β4 integrin that affects localization to soluble or triton-insoluble fractions (Alt et al., 2001; Mariotti et al., 2001; Rabinovitz et al., 1999; Santoro et al., 2003). Keratinocytes in culture localize integrin α6β4 to hemidesmosome-like stable anchoring contacts, or SACs (Carter et al., 1990b). SACs are Triton-insoluble cell junctions containing α6β4. In contrast to stationary cells, which make robust SACs, polarized cells on exogenous laminin 5 did not localize Triton-insoluble α6-integrin to SACs (Fig. 6D). In agreement with ELISA and western blot data (Fig. 6B,C), rottlerin increased the amount of Triton-insoluble α6β4 (Fig. 6E,F) in keratinocytes on exogenous laminin 5. An increase in SAC formation correlated with a loss of migration on both collagen and laminin 5. Conceivably, these drugs may have blocked laminin 5 deposition in addition to their other known effects on migration, a possibility we examined below.

Keratinocytes use integrin α3 for adhesion to their own laminin 5 deposits in the absence of exogenous ligand

Our data suggested that polarization and processive migration depended on interactions of integrin α3β1 with precursor laminin 5 deposits. To confirm that this interaction occurred, we used adhesion to BSA surfaces to examine interactions in

the absence of migration. Keratinocytes could adhere and spread when plated onto a BSA-blocked surface (Gil et al., 2002). This adhesion was blocked with anti-laminin 5 inhibitory antibody mAb C2-9 (Fig. 7). Keratinocytes lacking laminin 5 (JEB-gravis) were unable to adhere to BSA surfaces. Therefore, interaction with newly deposited precursor laminin 5 was required for adhesion. Nocodazole, taxol and rottlerin also completely blocked BSA adhesion suggesting that they altered the deposition or interactions with laminin 5. We later biochemically show that nocodazole, taxol and rottlerin blocked laminin 5 deposition (Fig. 8).

We used inhibitory antibodies to determine which integrins were involved in adhesion to the new deposits of laminin 5. Anti-β1 integrin blocked adhesion to BSA (Fig. 7). Anti-α3 integrin antibodies blocked adhesion but also caused cells in suspension to aggregate during the relatively long time course of the BSA adhesion assay (Symington et al., 1993). Anti-α2 integrin antibodies did not block adhesion. Anti-α6 integrin antibodies reduced but did not block adhesion and JEB-PA keratinocytes that lack integrin β4 were able to adhere to BSA-blocked surfaces. Cytochalasin D disrupts the actin cytoskeleton, blocks cell spreading, phosphorylation of FAK and migration. Cells treated with cytochalasin D could adhere but not spread by their own laminin 5 deposits on the BSA surface. Anti-laminin 5 and anti-α3 antibodies blocked this adhesion, as seen in the absence of cytochalasin D (data not shown). Anti-α6 antibodies could not block adhesion in the presence of cytochalasin D. JEB-PA cells could adhere in the presence of the drug. Thus, integrin α6 was not required for adhesion and integrin α3 was capable of mediating adhesion to laminin 5 deposits in these activated keratinocytes in the absence of actin-dependent cross-linking.

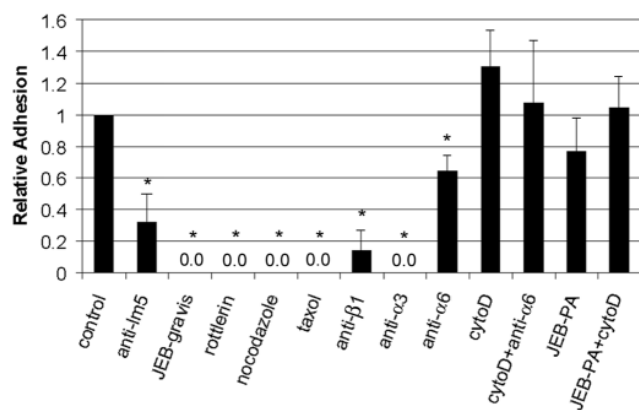**Fig. 7**. Laminin 5 deposits and integrin α3β1 mediate adhesion to BSA. Activated keratinocytes were plated onto a BSA blocked surface for 4 hours and the percentage adhered cells relative to nontreated control (nt=1.0) was determined. mAb C2-9 against laminin 5 (lm5) blocked adhesion, and JEB-gravis cells, which lack laminin 5, failed to adhere. Nocodazole and taxol at 1 µM and rotterlin at 6 µM completely blocked adhesion. Inhibitory antibodies against integrin β1 (P5D2) and integrin α3 (P1B5) inhibited adhesion while antibodies against integrin α6 (goH3) did not. JEB-PA cells lacking integrin β4 were able to adhere to BSA. Cytochalasin D at 5 µM did not block adhesion. Data shown is the average and standard deviation of three independent experiments. *Significantly different from nt value, *P*<0.01; Student's *t*-test.

Deposition of laminin 5 is distinct from secretion into the medium

New incorporation of laminin 5 into the extracellular matrix was measured by ELISA assay. Equivalent numbers of activated keratinocytes were adhered and allowed to migrate on exogenous laminin 5 for 4 hours. Precursor laminin 5 deposition was dramatically reduced in cells treated with nocodazole, taxol and rottlerin (Fig. 8A) even though cells could adhere and spread on laminin 5 in the presence of these drugs and could migrate in the presence of nocodazole and taxol but not rottlerin (Fig. 6). Surprisingly, this block was not at the level of secretion. Laminin 5 was immunoprecipitated from ^{35}S-labeled cell culture medium using anti-laminin α3 chain mAb C2-5 and anti-laminin γ2 chain mAb B4-6. Secretion of the assembled trimer was not impaired by nocodazole (Fig. 8B) or by taxol or rotterlin (data not shown). This secreted laminin 5 was active because is supported adhesion when immobilized to a surface coated with anti-laminin 5 antibodies (data not shown). Secretion of fibronectin and thrombospondin was not affected by nocodazole or taxol (data not shown). The selective effect of nocodazole and taxol on deposition of laminin 5 onto the substratum but not
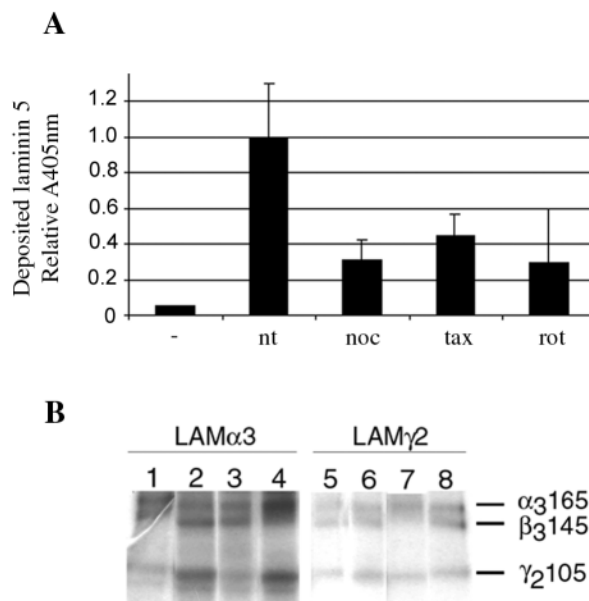

Fig. 8. The deposition of laminin 5 is regulated independently of secretion. (A) ELISA assay for laminin 5 deposits confirmed loss of deposition. Cells were culture for 4 hours on laminin 5 then removed with 5 mM EDTA in PBS for 30 minutes at 37°C and new laminin 5 deposits were quantified by ELISA. Signals were normalized relative to D2-1, DMSO=1.0. No cells (−), DMSO treated cells (nt), 1 µM nocodazole (noc), 1 µM taxol (tax) or 6 µM rottlerin (rot). Presented are the average and standard deviation of three wells from a representative experiment. (B) Secretion of laminin 5 into the culture medium was not blocked by nocodazole. Keratinocytes labeled with [^{35}S]methionine were adhered to exogenous laminin 5 for 3 hours in the presence of 0, 0.1, 0.5 or 1.0 µM nocodazole (lanes 1-4 and lanes 5-8) and the conditioned media were collected. Immunoprecipitation of secreted laminin 5 from cell culture media was performed in RIPA buffer using mAb C2-5 against the laminin α3 chain (lanes 1-4) or mAb B4-6 against the laminin γ2 chain (lanes 5-8). Molecular masses (in kDa) of the three chains of the laminin 5 trimer are indicated.

secretion of soluble laminin 5 into the medium suggests that deposition proceeds via a regulated secretory pathway (Blazquez and Shennan, 2000).

Collagen digestion and laminin 5 deposition switch integrin usage to stabilize cell polarization

While we have shown that newly deposited laminin 5 promoted migration on collagen surfaces, it has also been shown that migration of keratinocytes on collagen surfaces requires digestion of collagen by MMP-1 (Pilcher et al., 1997). We observed that fluorescently tagged collagen was removed from migration tracks of keratinocytes (Fig. 9A). The precise removal of collagen aligned with the distinct pattern of laminin 5 deposits (Fig. 9B). Immunofluorescence using anti-collagen antibodies also showed that the collagen was removed from the surface (data not shown). The broken pattern of laminin 5 deposits and collagen removal suggested that these events occurred not under the leading edge of the lamellipodium but concentrated at the outer rear flanks of the cell, the region where focal complexes were concentrated (Fig. 4). We did not see evidence for removal of exogenous laminin 5 by migrating cells (data not shown). To determine the sites of interaction between the new deposits of laminin 5 and integrins, we used the cell-impermeable cross-linker BS3 (DiPersio et al., 1995) to cross-link integrin-laminin 5 interactions thus making the integrins resistant to detergent extraction. Keratinocytes were plated on collagen surfaces to induce migration. Anti-laminin 5 mAb D2-1 was added for 15 minutes prior to BS3. After cross-linking for 10 minutes with BS3, the cells were extracted with Triton X-100 and fixed with formaldehyde. The cells were then processed for immunofluorescent staining of integrin α2

and α3 as well as laminin 5. This approach detected only the newly deposited laminin 5 and excluded the intense cytoplasmic laminin 5 signal. Laminin 5 deposits were not found at the leading edge of the cell as previously reported (deHart et al., 2003) but at the rear flanks of the cell, as suggested by the migration tracks (Fig. 8D,G). However, when non-migratory cells at the edges of colonies or non-polarized cells were examined, deposits were seen extending from under the cell body to the leading edge of the cell. Similar results were observed in cells that were fixed, permeablized with Triton X-100 then stained with D2-1 and imaged using confocal microscopy. Integrin α3 was not cross-linked in focal complexes under the lamellipodium but was found associated with the punctate laminin 5 deposits in the migration tracks, demonstrating interaction in deposition contacts. In contrast, integrin α2 was found in small focal complexes under the leading lamellipodium but was absent from the migration tracks. While integrin α2 stabilizes contacts under the leading lamellipodium, digestion of collagen and deposition of laminin 5 induce a switch of integrin usage to integrin α3β1 at the rear of the cell.

Discussion

The role of extracellular matrix in regulating migration of cells is typically viewed as an exogenous matrix presented to the cell with little consideration paid to the role of endogenous deposits. Our work demonstrates the importance of laminin 5 deposits in processive migration in human primary keratinocytes. The hallmarks of processive migration, summarized in Fig. 10, were deposition of laminin 5 in contact sites at the rear of the cell, stable cell polarization in the
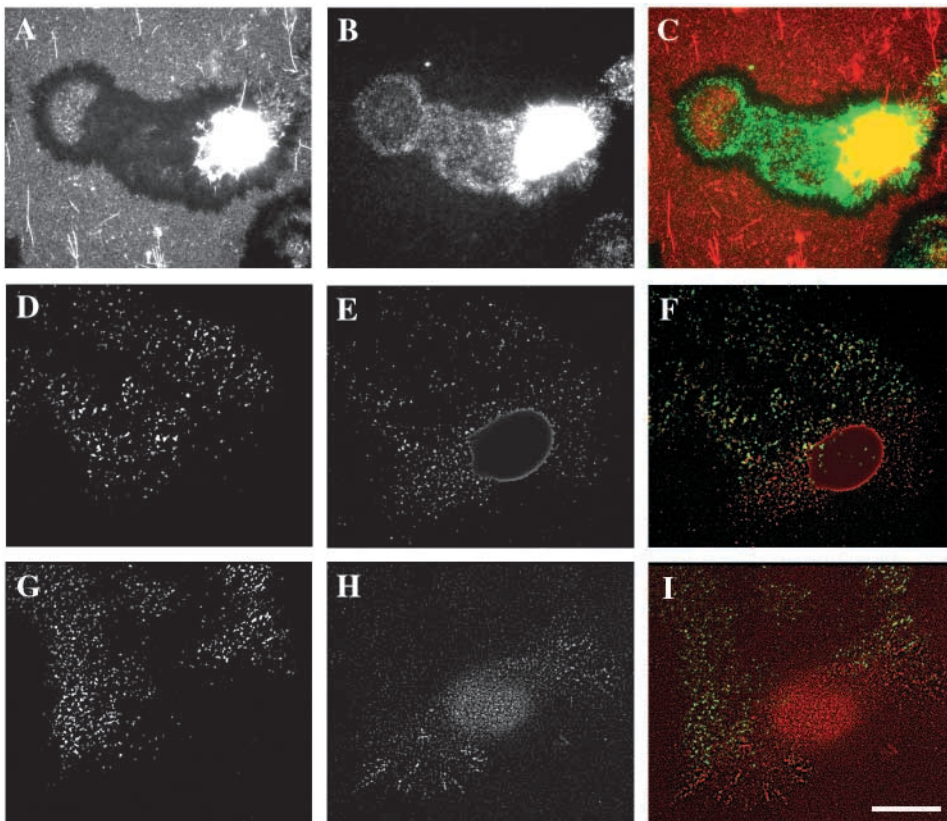

Fig. 9. Deposition of laminin 5 is restricted to the rear of the cell and colocalizes with sites of collagen removal. (A-C) Removal of collagen colocalized with sites of laminin 5 deposition. Migrating keratinocyte on Rhodamine-conjugated human placental type-I collagen (A), stained by immuofluorescence with mAb D2-1 for precursor laminin 5 (B). Images overlayed in C. (D-I) Deconvoluted optical sections at the substratum interface of migrating cells on collagen. mAb D2-1 was added for 15 minutes prior to cross-linking for 10 minutes with 0.4 mM BS3. The cells were then extracted with 1% Triton X-100 for 10 minutes, fixed with formaldehyde and processed for immunofluorescent staining for laminin 5 (D2-1; D,G), integrin α3 (E) and α2 (H). The nucleus is visible in E,H as a gray area. F,I merged images of D,E and G,H, respectively. Scale bar, 10 μm.
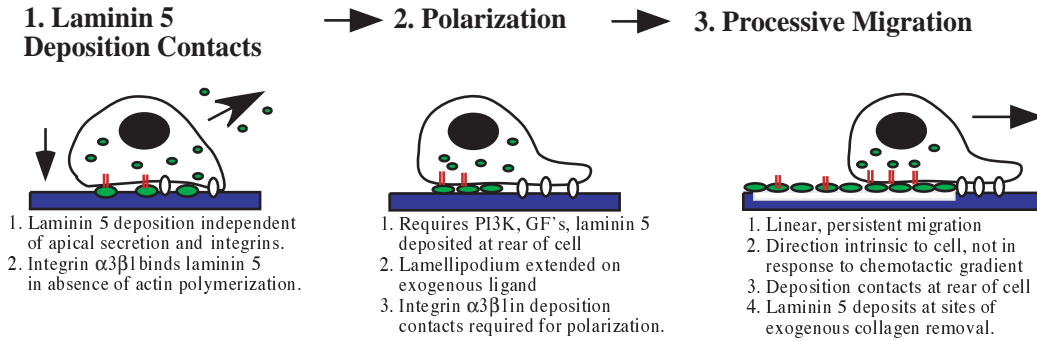
Fig. 10. Laminin 5 deposits direct processive migration on collagen. *Step 1.* Deposition contacts: rapid switch of adhesion from exogenous collagen (blue) and integrin α2 (open ovals) to laminin 5 deposits (green ovals) and integrin α3β1 (red bars). Deposition of laminin 5 down onto the substratum is regulated differently than apical secretion of laminin 5, fibronectin, laminin 1 and thrombospondin into the culture medium. *Step 2.* Polarization: integrin α3-laminin 5 interactions mediate polarization. Focal complexes form under the lamellipodia with exogenous ligand while laminin 5 deposits are restricted to the rear of the cell. *Step 3.* Processive migration removes exogenous collagen at precise sites of laminin 5 deposits that are restricted to the rear of the cell. Persistence of linear migration is not in response to a chemotactic gradient.

absence of a chemotactic gradient, and persistent, linear migration. Thus, deposition of laminin 5 by the leading cells promotes assembly of the provisional basement membrane that affects adhesion and signaling of the following cell population (Nguyen et al., 2000a). The stable polarization of leading cells in epithelial sheets was preserved in our single-cell model, suggesting polarization is an autonomous response dependent on laminin 5 deposition. We have previously shown that leading cells, while mechanically coupled to neighboring cells, do not signal through gap junctional communication (Lampe et al., 1998). It remains to be shown how this isolated leading cell population integrates with the tightly coupled following cell population.

Deposition contacts

It is evident from our studies that the pattern of laminin 5 deposition is tightly coupled to the movement of the cell. Does the pattern of deposition regulate the movement of integrin or does the movement of integrin regulate the deposition? We show that inhibiting interactions with deposits of laminin 5 blocked processive migration and that keratinocytes from laminin 5 null mice fail to migrate towards an EGF gradient (Nguyen et al., 2000b). Studies of integrin null animals show laminin 5 can still be deposited into basement membrane independent of integrin α6β4, α3β1 or α2β1 (DiPersio et al., 1997; DiPersio et al., 2000; Raghavan et al., 2000). However, disruption of integrin α3β1 inhibits movement and therefore affects the pattern of deposition but not the deposition itself (deHart et al., 2003). While known integrins are not directly required for deposition, integrin interactions with the exogenous substratum may indirectly affect the pattern of deposition by bringing the cell surface into close apposition with the substratum, allowing for laminin 5 deposition. It is not clear what mechanisms and components regulate the deposition of laminin 5.

We define deposition contacts as interactions between integrin α3β1 and newly deposited laminin 5 at the rear of the cell. We observed laminin 5 in deposition contacts at the rear flanks of the cell and not at focal complexes at the leading edge. The punctate deposits of laminin 5 may not provide a high

enough density of ligand to promote integrin α3 clustering, as has been proposed for integrin α6β4 in hemidesmosome formation (Geuijen and Sonnenberg, 2002; Tsuruta et al., 2003). Consistent with this model, processively migrating keratinocytes did not cluster integrin α6β4 at the basal surface nor was integrin α6β4 required for processive migration or adhesion to BSA. When cells in our assay stopped migrating, α6β4 clustered in hemidesmosome-like stable anchoring contacts (SACs).

Polarization and processive migration

The function of integrin α3β1 in deposition contacts was distinct from its function in focal complexes under lamellipodia. Cross-linking of integrins upon adhesion to matrix has been shown to induce localized membrane targeting of Rac and cdc42 at the cell edge to promote formation of lamellipodia and filopodia (Del Pozo et al., 2002; del Pozo et al., 2000). Consistently, focal complexes containing integrin α3 on exogenous laminin 5 or integrin α2 on exogenous collagen stabilized the leading lamellipodia. Our observations demonstrated that integrin α3 did not need to be activated through cross-linking in focal adhesions in order to bind laminin 5: cytochalasin D, which blocks integrin cross-linking and FAK phosphorylation (Xia et al., 1996), did not block the binding of integrin α3 to laminin 5 deposits in the BSA adhesion assay. We therefore hypothesize that the lack of integrin α3 actin-dependent cross-linking at deposition contacts would prevent the localization of Rac and lamellipodia formation at the rear of the cell.

The sustained morphology of processive migration requires efficient disassembly of substratum contacts to promote retraction of the rear. Processive migration resembles the gliding migration seen in fish keratocytes, where rolling and translocation of the spindle-shaped cell body remove focal complexes from the substratum and lateral contacts stabilize traction of the cell body (Lee and Jacobson, 1997; Small et al., 1996). It has been shown that adhesion to laminin 5 by integrin α3β1 and α6β4 uses signaling pathways distinct from adhesion by integrin α2β1 to collagen. When RhoGTPase is blocked, keratinocytes fail to adhere to collagen but can adhere and

spread on laminin 5 by a PI 3-kinase-dependent mechanism (Nguyen et al., 2000a). One possible explanation for the higher percentage of polarization on laminin 5 is the ability of laminin 5 to activate PI 3-kinase by this distinct pathway. Similarly, signaling by integrin α3β1 on laminin 10/11 preferentially activates Rac while adhesion to fibronectin via integrin α5β1 promotes stress fiber formation and focal contacts (Gu et al., 2001). The formation of more tightly organized focal complexes with integrin α2β1 on collagen may have contributed to the lower percentage of cells able to migrate processively. We observed that keratinocytes removed collagen from the substratum during processive migration. Site-specific digestion of type I collagen by MMP-1 loosens tight contacts with α2β1 under the leading lamellipodia of keratinocytes to promote migration (Pilcher et al., 1997). Significantly, laminin 5 was deposited precisely into the sites of collagen removal. This suggests that dermal remodeling may be linked to assembly of the provisional basement membrane.

Laminin 5 deposition

In addition to the role of microtubules in establishing polarity and promoting disassembly of focal adhesion (Ballestrem et al., 2000; Bershadsky et al., 1991; Kaverina et al., 2002; Waterman-Storer et al., 1999; Worthylake et al., 1999), we show that supressors of microtubule dynamics (nocodazole and taxol) selectively blocked laminin 5 deposition but not constitutive secretion. Post-translational glycosylation (Carter et al., 1991) and possibly assembly of the laminin 5 trimer would require processing through the Golgi complex. Drugs that disrupt the microtubule cytoskeleton may interfere with transport to the Golgi complex (Lippincott-Schwartz, 1998) and prevent specific processing of laminin 5 that is required for deposition. Rottlerin, an inhibitor of PKC-δ, also selectively blocked laminin 5 deposition. PKC-δ has been implicated in migration of keratinocytes on collagen (Li et al., 2002). Our results suggest defects in migration were due to the loss of laminin 5 deposition in addition to, or possibly as a result of increased α6β4 in hemidesmosome-like SACs. The deposition of laminin 5 into the remodeled collagen dermis would then facilitate the formation of anchoring hemidesmosomes.

Laminin 5 (α3β3γ2 chains) is deposited as an immature heterotrimer that is proteolytically processed during maturation of the basement membrane (Aumailley et al., 2003; Lampe et al., 1998; Nguyen et al., 2000b). The G4-G5 subdomain (LG4/5) is removed from the carboxyl terminus of the 200 kDa laminin α3 chain precursor (Goldfinger et al., 1999; Goldfinger et al., 1998; Marinkovich et al., 1992; Nguyen et al., 2000b). This cleavage of laminin $\alpha3_{200}$ chain by plasmin inhibits migration and promotes hemidesmosome formation (Goldfinger et al., 1998). This point is controversial because cleaved laminin 5 functions as a scatter factor (Kikkawa et al., 1994; Miyazaki et al., 1993), so it clearly promotes migration via α3β1. We have not seen a difference in processive migration on substrata with precursor laminin $\alpha3_{200}$ or cleaved laminin $\alpha3_{165}$ (D.F., unpublished observations). Engineered laminin α2 chain lacking G4/5 fails to assemble in the basement membrane (Li et al., 2003; Smirnov et al., 2002). This suggests that G4/5 of laminin α3 may have a similar function. We have observed that deposition of precursor laminin 5 is dependent on LG4/5 and defects in LG4/5 inhibit

deposition and affect integrin interactions (R. Sigle and W.G.C., manuscript in preparation). Furthermore, binding of the LG4 module of laminin α3 chain to syndecan induces MMP-1 expression in keratinocytes (Utani et al., 2003).

Our studies have shown that polarization and processive migration displayed by leading cells in an epidermal outgrowth can be intrinsically replicated in a single-cell population and that these events are dependent on the deposition of laminin 5. Since deposition of laminin 5 was regulated differently from secretion, it is likely that there will be other membrane-bound signaling components directed to sites of deposition contacts. Further studies of the mechanism by which laminin 5 deposition directs integrin contacts to orchestrate polarization and linear migration of leading cells will provide insight into the process of wound healing.

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