Deposition of laminin 5 over exposed dermal collagen in epidermal wounds is an early event in repair of the basement membrane. We report that deposition of laminin 5 onto collagen switches adhesion and signaling from collagen-dependent to laminin 5-dependent. Ligation of laminin 5 by integrin \(\alpha_6\beta_1\) activates phosphoinositide 3-OH-kinase (PI3K) signaling. This activation allows for adhesion and spreading via integrin \(\alpha_6\beta_1\) on laminin 5 independent of RhoGTPase, a regulator of actin stress fibers. In contrast, adhesion and spreading on collagen via \(\alpha_6\beta_1\) is Rho-dependent and is inhibited by toxin B, a Rho inhibitor. Deposition of laminin 5 and ligation of \(\alpha_6\beta_1\) increases PI3K-dependent production of phosphoinositide di- and triphosphates, PI3K activity, and phosphorylation of downstream target protein c-Jun NH2-terminal kinase. Conversely, blocking laminin 5-deposition with brefeldin A, an inhibitor of vesicle transport, or with anti-laminin 5 monoclonal antibodies abolishes the PI3K-dependent spreading mediated by \(\alpha_6\beta_1\) and phosphorylation of c-Jun NH2-terminal kinase. Studies with keratinocytes lacking \(\alpha_6\beta_4\) or laminin 5 confirm that deposition of laminin 5 and ligation by \(\alpha_6\beta_4\) are required for PI3K-dependent spreading via \(\alpha_6\beta_1\). We suggest that deposition of laminin 5 onto the collagen substrate, as in wound repair, enables human foreskin keratinocytes to interact via \(\alpha_6\beta_4\) and to switch from a RhoGTPase-dependent adhesion on collagen to a PI3K-dependent adhesion and spreading mediated by integrin \(\alpha_6\beta_1\) on laminin 5.

Laminin 5 is a major adhesive component of many epithelial basement membranes (BMs)\(^1\) and is secreted by epithelial cells in tissue and in culture. Injury of epidermis activates transcription and deposition of laminin 5 into the provisional BM by the leading keratinocytes of the epidermal outgrowth that migrates into the wound bed (1–4). The activated expression of laminin 5 occurs within hours after injury and prior to expression of laminin 10/11 or type VII collagen, attesting to the import of laminin 5 for tissue repair. Deposition of laminin 5 over the exposed collagen and fibronecstin is required for the repair of the BM and for re-establishing anchorage of the epithelium to the BM via integrin \(\alpha_6\beta_4\) in hemidesmosomes (5–9). Here, we have examined the function of laminin 5 deposited onto exposed dermal collagen in regulating adhesion and signaling in epidermal outgrowths.

Based on \textit{in vivo} and \textit{in vitro} studies, keratinocyte interactions with laminin 5 are mediated by integrins \(\alpha_6\beta_1\) and \(\alpha_6\beta_3\); interactions with \(\alpha_6\beta_4\) mediate anchorage, whereas interactions with \(\alpha_6\beta_1\) mediate motility (9–11). Laminin 5, composed of \(\alpha_5\), \(\beta_1\), and \(\gamma_1\) chains, also interacts with type VII collagen to link the epidermis to the dermis (12–14). Laminin 5 is synthesized and secreted by cultured keratinocytes in a precursor form containing a 200-kDa \(\alpha_5\) chain (\(\alpha_5\beta_2\gamma_2\)) and a 140-kDa \(\gamma_2\) chain (\(\gamma_2\gamma_3\)) (15). Precursor laminin 5 is deposited into the provisional BM of the wound but is absent from the mature BM (2, 10). Proteolytic processing of \(\gamma_2\gamma_3\) by MT1-MMP, a membrane-bound protease, is suggested to increase migration on laminin 5 (16). Similarly, proteolytic processing of \(\alpha_5\beta_2\) occurs extracellularly (15, 17). Cleavage by plasmin has been suggested to convert precursor laminin 5 from a migration ligand to an anchorage ligand in hemidesmosomes (17). However, processed laminin 5 is also reported to promote motility or scatter via integrin \(\alpha_6\beta_1\) (18, 19). Alternatively, proteolytic processing of laminin 5 may regulate deposition of laminin 5 instead of regulating interactions with integrins. Related to this, the carboxyl-terminal end of the \(\alpha_1\) chain of laminin 1 and the \(\alpha_2\) chain of laminin 2 contain heparin-binding subdomains consisting of laminin globular (LG) repeats 4 and 5 (LG4/5). Interactions of LG4/5 with dystroglycan mediates assembly of laminins 1 and 2 in BM (for review, see Ref. 20). It is not clear if the LG4/5 subdomain of laminin 5 \(\alpha_5\beta_2\) interacts with heparin or other cellular or dermal components to facilitate deposition of laminin 5.

Interactions of integrin \(\alpha_6\beta_4\) with laminin 5 generate both an anchorage function and a signaling function (11). Inherited defects in expression of laminin 5 or \(\alpha_6\beta_4\) inhibit assembly of mature hemidesmosomes and generate severe or lethal blistering of quiescent epithelium in individuals with junctional epidermolysis bullosa (5, 21–24) and junctional epidermolysis bullosa combined with pyloric atresia (JEB-PA). Therefore, the anchorage function via \(\alpha_6\beta_4\) to laminin 5 is physiologically significant. The signaling function of \(\alpha_6\beta_4\) regulates the cell cycle and cell invasion (for reviews, see Refs. 25 and 26). Ligation of \(\alpha_6\beta_4\) activates the Ras-ERK and Rac-JNK signaling pathways through Shc (27). In addition, \(\alpha_6\beta_4\) signals through PI3K, a target effector of Ras that activates Rac-JNK signaling pathways through Rac. It has been suggested that \(\alpha_6\beta_4\) mediates invasion of carcinoma cells via both its adhesive and signaling functions through PI3K (28).
However, in normal human keratinocytes, α6β4 plays a primary anchorage role that is independent of actin or FAK phosphorylation (9, 11, 30). Conceivably, α6β4 may have different adhesion and signaling functions in normal keratinocytes as compared with carcinoma cell lines.

The primary mediator of motility on laminin 5 in normal keratinocytes is integrin α6β4, not α6β1 (9, 11, 18, 31, 32). Adhesion dependent signals mediated by β1 integrins are transmitted via tyrosine phosphorylation, changes in intracellular pH, activation of protein kinases and matrix-activated protein kinase pathways, and recruitment of regulators involved in focal adhesion formation such as focal adhesion kinase (FAK). Focal adhesion formation and phosphorylation of FAK are downstream of RhoGTP activity (33, 34). Curiously, integrin α6β4 in keratinocytes is not readily detectable in focal adhesions on laminin 5 in contrast to the robust localization of α6β1 in focal adhesions on collagen (35, 36). This suggests that interactions of keratinocytes with laminin 5 differ from interactions with collagen and the differences may be attributable to signaling through Rho that affects assembly of focal adhesions.

Recently, we showed that laminin 5 interactions with α6β1 promote assembly of gap junctions and increase intercellular communication in basal keratinocytes (2). Collagen or fibronectin adhesion via α6β1 and α6β4, respectively, does not promote gap junction intercellular communication. Ligation of α6β4 by collagen, however, promotes expression of matrix metalloproteinase 1 (MMP1) that is necessary for migration on collagen in the wound bed (37–40). Therefore, BM laminin 5 provides instructional signals that are distinct from those of dermal collagen. The primary mediator of motility on laminin 5 in normal keratinocytes at the leading edge of the wound outgrowth is integrin α6β1-collagen ligation has yet to be characterized.

We wished to determine if deposition of laminin 5 onto collagen during wound repair changes signaling from collagen-dependent to laminin 5-dependent and if signaling pathways required for adhesion and spreading on laminin 5 were different than on collagen. We found that laminin 5 is deposited as a precursor form by a subpopulation of keratinocytes migrating at the leading edge of the wound outgrowths. Further, laminin 5 requires and stimulates signaling pathways for adhesion and spreading of keratinocytes that are different then collagen. Deposition of laminin 5 that interact with integrin α6β4 are required for keratinocyte spreading on laminin 5 in the absence of RhoGTPases. We suggest that deposition of laminin 5 by keratinocytes at the leading edge of the wound outgrowth determines which signaling pathways are used by keratinocytes for motility. The advantage of dual signaling pathways for migration of keratinocytes on laminin 5 are discussed in relation to epithelial cell interactions and wound repair.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**Primary keratinocytes from normal human foreskins (HFKs) and keratinocytes from individuals with junctional epidermolysis bullosa-pyloric atresia (JEB-PA; inherited defect in the ITGB4 gene) or from individuals with junctional epidermolysis bullosa-gravis (JEB-G; inherited defect in the LAMB3 gene) were prepared as described by Boyce and Ham (41). Primary and secondary keratinocyte cultures were maintained in serum-free keratinocyte growth media (KGM; Clonetics Corp., San Diego, CA). JEB-PA and JEB-G keratinocytes were infected with an LXSN retrovector (42) expressing E6 and E7 oncoproteins from human papilloma virus to extend life in culture. When JEB-PA and JEB-G keratinocytes were compared with HFKs (Figs. 4 and 5), the HFKs were also infected with the same retrovector vector. Early passages (P1–15) were compared to avoid possible changes in adhesion or signaling due to later passages. JEB-PA individual JF/VSV9-3-96 has premature termination mutations in both alleles of the ITGB4 gene encoding the β1 integrin subunit (JF/VSV9-3-96 is proband from family two in Ref. 43). Three other individuals suspect for defects in ITGB4 were also examined. They are classified clinically as JEB-PA based on blistering phenotype, immature hemidesmosome structures, defective anchorage on laminin 5 in assays specific for α6β4, and decreased expression of β4 in tissue and culture. Causal mutations are currently under investigation. The individual with JEB-G does not deposit laminin 5 in culture and has homozgyous premature termination mutations in both alleles of the LAMB3 gene encoding the β3 chain of laminin 5.

**Explant Assays**—Explant assays were performed as described previously (2) and used as a wound model. The collagen layers were prepared by drying rat tail collagen on Petri dishes (Collaborative Research, acid-soluble, diluted to 100 μg of collagen/ml of water).

**Characterization of the Precursor Laminin 5—**Five- to eight-week-old BALB/c mice were sacrificed, and skin was prepared in the lab from human placenta as described (47) and coated onto 12-well culture plates overnight at 4 C in the concentration of 10 μg/ml. Laminin 5-coated plates were prepared as described previously (11) and briefly as follows: HFKs were grown on TC plates, then detached by brief digestion with 0.5% trypsin (w/v)/EDTA (Sigma). Laminin 5 remaining on the TC surface was blocked with 0.5% w/v heat-denatured BSA/PBS for 30 min and used for adhesion studies. Cell adhesion to these laminin 5 plates was completely inhibited with anti-laminin 5 mAb C2-9 (11). To generate laminin 5-conditioned collagen, HFKs were plated onto collagen-coated assay plates for 24 h. This allowed the cells to deposit laminin 5 onto the collagen substratum. The cells were trypsinized off, leaving the substrata for adhesion assay. Coated plates were blocked with 0.5% w/v heat-denatured BSA/PBS for 30 min. Cultured cells were either not treated for controls or pretreated with 50 ng/ml toxin B (gift from Laurie Neville, Tech Labs, Inc.), 50 nm epidermal growth factor (EGF, Y294092, Sigma), or 5 μm wortmannin (Sigma) were then suspended with trypsin (w/v)/EDTA and labeled with 0.5 μm calcein-AM (Molecular Probes, Eugene, OR). Labeled cells were then plated onto ligand-coated plates in the presence or absence of inhibitors (e.g. anti-α5 antibody, GOH3) for 1 h as indicated. Total cell fluorescence (prewash) was read on CytoFluorII fluorescence plate reader (PerSeptive Biosystems, MA). Cells were washed three times with PBS, and baselining cell fluorescence (post-wash) was again read. An empty well containing just buffer medium where no cells were added was read to give base-line fluorescence that was subtracted from raw data. In every adhesion experiment, triplicates of each condition were done. Percent fluorescent cells adhered was calculated as: % fluorescent cell adhered = (post-wash fluorescence reading – base-line fluorescence)/ (total (prewash) fluorescence reading – base-line fluorescence). Cells were then fixed with 2% formaldehyde in 0.1 M sodium cacodylate/0.1 mM sucrose for 20 min and viewed under phase microscope for documentation of cell spreading.

**In Vitro Tissue Adhesion Assay—**The in vitro tissue adhesion assay was performed as a variation of the assay described (5–9). In brief, epidermis of neonatal foreskin was separated from the dermis by incubation with EDTA/NaCl to expose the underlying BM containing laminin 5. The dermis is abundant in interstitial collagen (types I and III) and fibronectin. Tissue sections (6-μm thick) were cut from OCT-embedded frozen split skin and adhered onto lids of 35-mm Petri dishes, washed twice in PBS, and blocked in 0.5% heat-denatured BSA/PBS for 30 min. HFKs were untreated or pretreated with toxin B (50 ng/ml) for 3 h prior to assay. Calcein-labeled HFKs were plated onto the immobi-

**In Vitro Cell Adhesion Assay—**Human placental collagen type I was prepared in the lab from human placentas as described (47) and coated onto 12-well culture plates overnight at 4 C in the concentration of 10 μg/ml. Laminin 5-coated plates were prepared as described previously (11) and briefly as follows: HFKs were grown on TC plates, then detached by brief digestion with 0.5% trypsin (w/v)/EDTA (Sigma). Laminin 5 remaining on the TC surface was blocked with 0.5% w/v heat-denatured BSA/PBS for 30 min and used for adhesion studies. Cell adhesion to these laminin 5 plates was completely inhibited with anti-laminin 5 mAb C2-9 (11). To generate laminin 5-conditioned collagen, HFKs were plated onto collagen-coated assay plates for 24 h. This allowed the cells to deposit laminin 5 onto the collagen substratum. The cells were trypsinized off, leaving the substrata for adhesion assay. Coated plates were blocked with 0.5% w/v heat-denatured BSA/PBS for 30 min. Cultured cells were either not treated for controls or pretreated with 50 ng/ml toxin B (gift from Laurie Neville, Tech Labs, Inc.), 50 μM epidermal growth factor (EGF, Y294092, Sigma), or 5 μM wortmannin (Sigma) were then suspended with trypsin (w/v)/EDTA and labeled with 0.5 μM calcein-AM (Molecular Probes, Eugene, OR). Labeled cells were then plated onto ligand-coated plates in the presence or absence of inhibitors (e.g. anti-α5 antibody, GOH3) for 1 h as indicated. Total cell fluorescence (prewash) was read on CytoFluorII fluorescence plate reader (PerSeptive Biosystems, MA). Cells were washed three times with PBS, and baselining cell fluorescence (post-wash) was again read. An empty well containing just buffer medium where no cells were added was read to give base-line fluorescence that was subtracted from raw data. In every adhesion experiment, triplicates of each condition were done. Percent fluorescent cells adhered was calculated as: % fluorescent cell adhered = (post-wash fluorescence reading – base-line fluorescence)/ (total (prewash) fluorescence reading – base-line fluorescence). Cells were then fixed with 2% formaldehyde in 0.1 M sodium cacodylate/0.1 mM sucrose for 20 min and viewed under phase microscope for documentation of cell spreading.

**Spreading Assay on Immobilized Antibodies—**Antibody-coated surfaces were prepared as described previously (2). Cells were untreated or pretreated for 3 h with toxin B (50 ng/ml), wortmannin (50 μM), or the
combination, then plated onto immobilized anti-\( \alpha_1 \) (P1B5), anti-\( \alpha_2 \) (P1H5), or anti-\( \alpha_6 \) (GOH33) mAbs for 1 h. Cells were fixed, and phase images were taken to document cell spreading.

**Immunofluorescence Staining**—Cells were plated onto surfaces coated with immobilized anti-\( \alpha_2 \) antibodies or laminin 5 for 30 min. Cells were then treated + toxin B (200 ng/ml) or + wortmannin (50 nM) for 3 h. Cells were fixed for 20 min with 2% formaldehyde in 0.1 M sucrose, 0.1 M sodium cadoxylate (pH 7.2), permeabilized with 0.5% Triton X-100 for 10 min, and then blocked in 0.5% heat-denatured BSA in PBS for 30 min. P1F2 (anti-\( \alpha_2 \)), VIN-11 (anti-vinculin, Sigma), and anti-FAK (ICN) were incubated with the cells for 1 h at room temperature. Cells were washed, and bound antibody was reacted with fluorescein isothiocyanate-conjugated, goat anti-mouse antibody (Cappel). Rhodamine-conjugated phalloidin (Molecular Probes) was incubated for 10 min and washed. For laminin 5 staining, attached cells were extracted with Triton, then the matrix was stained with biotinylated anti-laminin 5 mAb (P1E1). Bound biotinylated mAb was detected with rhodamine-avidin. Immunofluorescence was visualized using a Zeiss microscope equipped with epifluorescence. Images were photographed with TMAX 400 ASA film.

**Immunoblotting**—HFKs were untreated or pretreated with 50 nM wortmannin for 3 h, trypsin-suspended, and plated onto laminin 5-, collagen-, or immobilized antibody-coated surfaces for 1 h in the absence or presence of inhibitory anti-laminin 5 antibody (C2-9). Cells were solubilized directly with 1% Triton X-100 in PBS containing 5 mM EDTA, 50 \( \mu \)M NaN\(_3\)Y0, 10 mM NaF, and protease inhibitors (2 mM phenylmethysulfonyl fluoride, 1 mM N-ethylemaleimide, 1 \( \mu \)g/ml pepstatin, 10 \( \mu \)g/ml aprotinin, 1 \( \mu \)g/ml leupeptin). Detergent-soluble extracts were quantitated and separated on SDS-polyacrylamide gels (48). Proteins were blotted onto nitrocellulose, blocked in 1% nonfat milk, and incubated with primary antibodies: anti-phosphorysorine (PY20; ICN), anti-phosphorylated JNK (JNKp(G7); Santa Cruz), anti-JNK all (JNKc17; Santa Cruz), anti-ERK2 (Transduction Laboratories), anti-keratin (K8.13, Sigma), anti-laminin 5 (D21, C25, R9883). Primary antibodies were reacted with peroxidase-conjugated rabbit anti-mouse IgG (RAMP; Dako) or goat anti-rabbit IgG (ICN) for 1 h. Blots were developed with ECL chemiluminescence (Amersham Pharmacia Biotech) and direct exposure to Hyperfilm MP (Amersham Pharmacia Biotech). Densitometry was performed as described previously (1–4).

**Steady-state Cellular Phosphoinositide Levels**—Phospholipid labeling was performed as described previously (44). Briefly, phosphate-free Dulbecco's modified Eagle's medium containing \(^{32}P\)Pi (250 \( \mu \)Ci/ml) was incubated for 1 h with HFKs that were either adherent (\( \lambda \)), trypsin-suspended (\( \lambda \)), or plated onto 35-mm dishes coated with laminin 5 (Lam5) or collagen type I (Col, 10 \( \mu \)g/ml). Radioactive culture supernatants were discarded, cells were washed, then extracted with MeOH:CHCl\(_3\) (2:1) +0.63 mg/ml butylated hydroxytoluene, 10 \( \mu \)g/ml phosphatidylinositol carrier. The organic extracts containing labeled lipids were mixed with CHCl\(_3\):MeOH (2:1 v/v) for thin layer chromatography using 20 x 20-cm oxalate-coated silica gel plates and developed with CHCl\(_3\):acetone:MeOH:acetic acid:H\(_2\)O (80:30:26:24:14 v/v/v/v/v). Phospholipid standards were detected with iodine vapor and labeled products with exposure to Hyperfilm.

**PI3K Assay**—PI3K enzymatic activity was assayed as described previously by (49). HFKs were suspended, or adhered on immobilized anti-\( \alpha_3 \) (P1H5), anti-\( \alpha_2 \) (P1B5), or anti-\( \alpha_6 \) (GOH33) integrin antibodies for 1 h, then extracted 1% v/v Nonidet P-40 detergent in kinase buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1 mM sodium orthovanadate, 20 mM MgCl\(_2\)). PI3K proteins were immunoprecipitated from the extract with rabbit polyclonal anti-PI3K (Upstate Biotechnology, Inc.). Immunoprecipitates were incubated with 20 \( \mu \)g of phosphatidylinositol 4,5-bisphosphate (Avanti Polar Lipids, Inc.) and \( ^{32}P\)ATP (30 \( \mu \)Ci) in kinase buffer for 15 min at 37 °C. Immunoprecipitates were removed by centrifugation; supernatant solutions were extracted for lipids, spotted onto TLC silica gel 60 plates precoated with 5% oxalate acid, and developed in propanol plus 2 \( \mu \) acetic acid 65:35 (v/v).

### RESULTS

**Leading Keratinocytes in Epidermal Outgrowths Express Laminin 5 and Are Sensitive to Toxin B**—We used skin explants placed in organ culture to study keratinocyte migration over collagen and the effects of deposited laminin 5. Biopsies of skin were placed onto collagen-coated surfaces, allowing keratinocytes to migrate out as a continuous epithelial outgrowth. Keratinocytes at the leading edge of the outgrowth are in direct contact with collagen, and express elevated cytoplasmic levels of a precursor form of laminin 5 recognized by mAb D2-1 (45, 46). This distinguishes the leading keratinocytes from the following cells that express little cytoplasmic laminin 5 (Fig. 1a; arrowheads indicate leading cells, arrows identify following cells). Precursor laminin 5 is deposited by the leading cells onto the collagen substratum over which the following cells migrate. Extraction of the epithelial outgrowth with Triton X-100 detergent allows antibody access to the substrate and exposes the deposited laminin 5 under the following cells (Fig. 1b, arrow indicates direction of migration). Note that the extraction also removes the cytoplasmic precursor laminin 5 from the leading cells (dotted line demarks leading edge).

mAb D2-1 was used to characterize the precursor form of laminin 5 (\( \alpha_3\beta_2\gamma_2 \)) synthesized and deposited by leading cells. Results in Fig. 2 establish three points. (i) Precursor laminin 5 immunoprecipitated with anti-laminin 5 mAbs (D2-1, C2-5, B4-6) from the cell layer of culture HFKs is composed of chains \( \alpha_3\cdot200 + \beta_1\cdot140 + \gamma_2\cdot140 \) (Fig. 2A; panel CELLS). mAb D2-1 reacts with the 200-kDa form of the \( \alpha_3 \) chain of precursor laminin 5 (\( \alpha_3\cdot200 \)), and two peptides \( \alpha_3\cdot35 \) and \( \alpha_3\cdot37 \) (\( \alpha_3\cdot35/37 \); Fig. 2A, panel MEDIAN). Rabbit polyclonal Ab R9882 specific for the carboxyl-terminal LG 5 subdomain of \( \alpha_3\cdot200 \) reacts with \( \alpha_3\cdot35/37 \) establishing the origin of the peptide from the carboxy terminus of \( \alpha_3\cdot200 \) (Fig. 2A, IP:BLOT). (ii) After secretion into the culture medium, precursor laminin 5 is proteolytically processed into mature laminin 5 composed of chains \( \alpha_3\cdot165 + \beta_1\cdot140 + \gamma_2\cdot100 \) (Fig. 2A, MEDIUM). Pulse-chase studies with D2-1 (results not shown) indicated that proteolytic processing of \( \alpha_3\cdot200 \) in the medium occurs as a post-secretory event and

FIG. 1. Leading keratinocytes in epidermal outgrowths express laminin 5 and are toxin B-sensitive. Skin explants were cultured on a collagen surface. a, immunofluorescence staining of precursor laminin 5 with mAb D2-1 shows positive cytoplasmic expression in leading cells (white arrowheads) compared with following confluent cells (white arrows). b, epidermal outgrowth was extracted with 0.5% Triton X-100 and the exposed substrata was stained for laminin 5 with mAb D2-1 to show deposited laminin 5 beneath confluent following cells. White arrow indicates direction of epithelial cell outgrowth. White dashed lines indicate the leading front. c, phase image of an epidermal outgrowth showing leading edge keratinocytes migrating on collagen and the following confluent population migrating on deposits of laminin 5. Black arrow is used as a point of reference. d, the same field as in panel c was treated with 50 ng/ml toxin B for 10 min then re-photographed. e, 2-fold magnification of panel d to show rounding of leading cells (black arrow). Toxin B caused rounding of the leading cells on collagen but not the following cells on the deposited laminin 5.
Laminin 5 Regulates Integrin Adhesion and Signaling

We examined signaling differences in leading and following keratinocytes that interact with collagen and deposited laminin 5, respectively. We compared the sensitivities of leading and following keratinocytes to toxin B, an inhibitor of Rhophilase that regulates actin stress fibers. Toxin B glucosylates Thr37 of Rho proteins, thereby rendering them inactive (45, 46). Toxin B (50 ng/ml) was added to cultures of skin explants grown on collagen, and the epidermal outgrowth was photographed at the zero time point (Fig. 1c, black arrow indicates leading edge) and also at 10 min (Fig. 1, d and e; panel e is a 2-fold magnification of d to better show cell rounding at the leading edge). Toxin B selectively rounded the leading cells, whereas following cells remained adherent and spread. Thus, adhesion and spreading of leading keratinocytes on collagen is toxin B-sensitive, whereas adhesion and spreading of following cells on deposits of laminin 5 is toxin B-insensitive.

Integrin α6β1 Interaction with Collagen Is Toxin B-sensitive Compared with α6β1-Laminin 5—We compared toxin B sensitivity of HFKs adherent on exogenous collagen via α6β1 to cells adherent on laminin 5 via α6β3 and α6β1. Cultured HFKs were plated onto exogenous laminin 5 (Fig. 3, a and c) or collagen (Fig. 3, b and d) for 30 min. Toxin B (50 ng/ml) was added, and cells were photographed at time 0 (a and b) and at 30 min (c and d) and was stained with rhodamine-conjugated phalloidin for actin. HFKs in the presence of toxin B on collagen, similar to the epibole model, rounded up while cells on laminin 5 retained their spread morphology.

We used a tissue adhesion assay to determine whether native collagen in tissue could support adhesion of HFKs in the
presence of toxin B (45, 46). HFKs were treated with toxin B (50 ng/ml for 3 h), fluorescently labeled with calcein, and then assayed for adhesion on cryostat sections of skin (Fig. 4C). For these studies, the epidermis was separated from the skin by incubation with EDTA/NaCl in order to expose the underlying BM containing laminin 5. The dermis is rich in interstitial collagens, including types I and III, and fibronectin. Untreated control cells adhered to both laminin 5 in the BM and to the dermis (Fig. 4C, panels a and c). In contrast, toxin B-treated HFKs only adhered to the BM and not to the dermis (Fig. 4C, panels b and d). Thus, α3β1 failed to mediate adhesion in the presence of toxin B even on native collagen in dermis. In contrast, toxin B had no observable inhibitory effects on adhesion to laminin 5 in the BM.

**Laminin 5 Is Required for Spreading via α3β1 in the Presence of Toxin B**—As seen in Figs. 1, 3, and 4, HFKs interacting with laminin 5 can spread via α3β1 in the presence of toxin B. We evaluated the import of laminin 5-deposition on this toxin B-insensitive spreading. HFKs were plated onto immobilized anti-α6 antibody surfaces. The cells adhered, spread, and deposited laminin 5 onto the anti-α6 substratum in the absence (Fig. 5, A (Control, panel a) and B (panel a)) or in the presence of toxin B (Fig. 5, A (ToxB, panel d) and B (panel b)). Blocking laminin 5 deposition with brefeldin A (Fig. 5B, panel d) inhibited HFK spreading in the presence of toxin B (Fig. 5A, compare panels b and e). Keratinocytes from...
an individual with JEB-G with inherited defects in laminin 5 expression (50) were unable to spread on anti-α5 immobilized surface in the presence of toxin B (Fig. 5A, panel f compared with untreated control panel c). These JEB-G keratinocytes do not express nor deposit laminin 5 in culture (data not shown). However, addition of exogenous laminin 5 allowed JEB-G keratinocytes to spread in the presence of toxin B (Fig. 5A, panel h). Therefore, either endogenous or exogenous laminin 5 on the substrate is required for the toxin-B-insensitive cell spreading via α5β1.

Toxin B-insensitive Spreading via α5β1 on Laminin 5 Requires α6β4 and PI3K—Since α6β4 also contributes to adhesion to laminin 5, we determined if interaction of α6β4 with laminin 5 participates in the toxin B-insensitive spreading via α5β1. Individuals with JEB-PA bear mutations in the ITGB4 gene that encodes the integrin β4 subunit (51, 52) and display severe to lethal defects in anchorage function of β4 (13). Keratinocytes cultured from a JEB-PA individual (Fig. 6A, striped bars) attached and spread on exogenous laminin 5 via α6β4 in the absence of toxin B comparable to normal HFK controls (Fig. 6A, solid bars). However, JEB-PA keratinocytes failed to adhere and spread on exogenous laminin 5 in the presence of toxin B (Fig. 6A, + Toxin B, striped bars). Keratinocytes from three additional individuals suspect for JEB-PA were also examined for sensitivity to toxin B. Toxin B inhibited adhesion and spreading on laminin 5 for all three of the suspect JEB-PA keratinocytes (data not shown). Therefore, ligation of α6β4 by laminin 5 is required for keratinocyte spreading via α5β1 in the presence of toxin B.

We wished to identify the signaling pathway involved in the toxin B-insensitive spreading on laminin 5. A panel of known inhibitors of adhesion was examined for effects on HFK adhesion and spreading on laminin 5 in the presence of toxin B. In preliminary studies, wortmannin or LY294002, inhibitors of phosphoinositide 3-OH-kinase (PI3K; Ref 53), selectively inhibited α5β1-mediated spreading on laminin 5 when toxin B was also present. Quantitative experiments were performed (Fig. 6B). HFKs were pretreated with toxin B alone, 50 nM wortmannin alone, or the combination of both, then assayed for adhesion to exogenous laminin 5 (solid bars) or collagen (striped bars). Untreated HFKs adhered and spread on both laminin 5 and collagen. Toxin B (TxB) selectively inhibited collagen adhesion whereas on laminin 5 the cells still adhered and spread. Wortmannin (Wt) by itself did not inhibit adhesion or spreading on either laminin 5 or collagen. However, the combination of toxin B and wortmannin (TxB + Wt) inhibited adhesion and spreading on both laminin 5 and collagen. Note that LY294002 generated results similar to wortmannin (results not shown) and had no inhibitory effects on the deposition of laminin 5 (Fig. 5B, panel c). Note that the assay was done in the presence or absence (data not shown) of anti-α5 mAb (GoH3) to block α5β1 contribution to adhesion. In the absence of anti-α5 mAb spreading was inhibited by toxin B and wortmannin but adhesion occurred. Thus, α6β4 mediates adhesion and spreading on laminin 5 via two pathways; one is PI3K-dependent, and the other is toxin B-sensitive.

Similar results were obtained when we assayed cell spreading on immobilized antibodies (Fig. 6C). We compared normal HFKs to JEB-PA keratinocytes adherent on immobilized anti-α5 antibodies under all assay conditions. JEB-PA keratinocytes spread in the absence but not the presence of toxin B (Fig. 6C, compare panels c and f) and was unaffected by wortmannin (panel i). HFKs spread on anti-α5 in the absence (panel a) or presence of toxin B (panel d) or wortmannin (panel g). HFK spreading was blocked only in the presence of a combination of inhibitors (panel j). Therefore, integrin α5β1 plays a critical role in regulating α5β1 spreading via the PI3K-dependent pathway.

Adhesion of HFKs on immobilized anti-α5 antibody was apparent under all assay conditions but spreading was inhibited in the presence of toxin B or toxin B + wortmannin but not by wortmannin alone (Fig. 6C, panels e, h, and h, respectively). The behavior of HFKs on immobilized anti-α5 is similar to the behavior of JEB-PA keratinocytes on anti-α5.

Inhibition of PI3K Increases Focal Adhesions and FAK Phosphorylation—Laminin 5 interaction with α6β4 is required for cell spreading on laminin 5 via α5β1 in the presence of toxin B (Figs. 5 and 6). Further, PI3K is required for spreading on laminin 5 via α5β1 in the presence of toxin B. We hypothesized that inhibition of PI3K would increase Rho dependence and increase Rho activity. Rho activity is essential for focal adhesion formation when cells interact with ECM ligands via integrin receptors (33, 34). We examined focal adhesions in HFKs plated on laminin 5 after treatment with toxin B or wortmannin. Untreated control HFKs did not efficiently localize α5β1, vinculin (VIN), or FAK into focal adhesion structures (Fig. 7, panels a, d, and g). Consistent with inactivating Rho, toxin B treatment decreased localization of all three components in focal adhesions (panels b, e, and h). Note that toxin B caused disappearance of actin stress fibers; however, the cells retained...
Toxin B-insensitive adhesion and spreading mediated by α3β1 requires α6β4 and PI3K. A, HFKs (solid bars) and keratinocytes from an individual (JF/VS 9-3-96) with JEB-PA (striped bars) lacking integrin α6β4 were treated with (+) or without (−) 50 ng/ml toxin B for 3 h, trypsin-suspended, labeled with calcein, and assayed for adhesion to exogenous laminin 5. B, HFKs were untreated (Control), or treated with toxin B (TxB; 50 ng/ml), wortmannin (Wt; 50 nM), or toxin B + wortmannin combination for 3 h, trypsin-suspended, labeled with calcein, and added to surfaces coated with exogenous laminin 5 (solid bars) or collagen (striped bars). The assay was done in the presence or absence (data not shown) of anti-α6 mAb (G0H3). In either case no spreading occurred in the presence of toxin B + wortmannin. In the absence of anti-α6, adhesion was unaffected but spreading was inhibited with toxin B + wortmannin. C, phase images of HFKs that were untreated (Control, panels a and b), or treated with toxin B (TxB, d and e), wortmannin (Wort., g and h), or the combination of toxin B and wortmannin (j and k) for 3 h, and plated onto
their spread morphology (Fig. 7, compare panels j and k). Inhibiting PI3K with wortmannin caused a major increase in localization of α5β1, vinculin, FAK, and actin into focal adhesion sites (Fig. 7, panels c, f, i, and l). Note that LY294002 shows the same effects (data not shown).

Consistent with immunolocalization studies, FAK phosphorylation was reduced upon toxin B treatment (Fig. 8, A (lane 2) and B (white bar)) compared with untreated control cells (Fig. 8, A (lane 1) and B (black bar)). Wortmannin treatment increased tyrosine phosphorylation of FAK by 2–3-fold over control cells (Fig. 8, A (lane 3) and B (striped bar)). Thus, Rho participation in α5β1 interactions with laminin 5 increases when PI3K is inhibited.

**Adhesion to Laminin 5 Increases Synthesis of Phosphoinositides and PI3K Activity**—We compared steady-state levels of phosphotyidylinositol di-phosphate (PIP$_2$) synthesized by PI3K in HFKs that are adherent on either laminin 5 or collagen (Fig. 9A). Cells were labeled for 1 h with $^{32}$P-in original adherent (A) or in suspension (S) or during re-adhesion to laminin 5 (L5) or collagen (Col) with (+) or without (−) wortmannin. Extracts from the labeled cells were fractionated by thin layer chromatography and levels of PIP$_2$ were quantitated. HFKs adhering to laminin 5 synthesized over 2 times higher levels of PIP$_2$ than cells adhering on collagen (L5:−: 39.35; Col:−: 19.15) and this increased level could be inhibited by wortmannin (L5:+: 18.32; Col:+: 13.74). These results suggest that HFKs adhering to laminin 5 had higher levels of PI3K activity compared with cells on collagen.

We examined the time course of PIP$_2$ and PIP$_3$ synthesis, both known products of PI3K activation, resulting from HFKs adhesion via α5β1 and α6β4 (Fig. 9, B and C). HFKs were labeled for 1 h with $^{32}$P in suspension (0) and then adhered onto immobilized anti-α5 (P1B5) or anti-α6 (P1H5) antibodies. Immobilized anti-integrin α5 (P1B5; a, d, g, and j) or anti-integrin α6 (P1H5; b, e, h, and k) for 1 h of adhesion. HFKs were compared with JEB-PA keratinocytes when adherent on immobilized anti-α5 antibodies either untreated (panel c), or treated with toxin B (panel f), wortmannin (panel i), or the combination of toxin B and wortmannin (panel l).

Samples were taken at 15, 30, 60, and 120 min of adhesion. Adhesion and spreading of HFKs on anti-α5 (Fig. 9B; quantitated in Fig. 9C, open circles) generated a more rapid and higher levels of PIP$_2$ and PIP$_3$ than cells adhering on anti-α6 (Fig. 9, B and C, black squares). This confirms that adhesion to laminin 5 via α5β1 generates a higher level of PI3K activity than adhesion to collagen via α6β4.

Enzymatic activity of PI3K was assayed in HFKs adherent via α5β1, α6β1, or α6β4: PI3K enzyme was immunoprecipitated with anti-PI3K rabbit polyclonal antibody from HFKs that were suspended, or adherent to immobilized anti-α5 (P1H5), anti-α6 (P1B5), or anti-α6 (G0H3) integrin mAbs. Immunoprecipitated PI3K samples were incubated with phosphatidylinositol 4,5-bisphosphate substrate and [$v^{32}$P]ATP. Labeled PI3K product was fractionated by thin layer chromatography and quantitated (Fig. 9, D and E; see “Experimental Procedures”). Compared with suspended cells, PI3K activity was higher in all adherent cells. However, cells adhering to anti-α5 and anti-α6 had higher PI3K activity than cells adherent on anti-α6. This relationship was observed in three separate experiments and suggested that ligation of either α5β1, or α6β4 promoted PI3K activity as well as production of both PIP$_2$ and PIP$_3$.

**Deposition of Laminin 5 Regulates PI3K-dependent JNK Phosphorylation**—To confirm the results from the functional adhesion studies on laminin 5 and collagen and the PI3K...
Fig. 9. Interaction with laminin 5 activates PI3K activity compared with collagen. A, steady-state levels of phosphotyidylinositol diphosphate (PIP2) synthesized by PI3K were compared in HFKs that are adherent on either laminin 5 or collagen. Cells were labeled for 1 h with 32P in original adherent (A) or labeled in suspension (S) or during adhesion to laminin 5 (L5) or collagen (Col) with (+) or without (−) wortmannin. B, time course of PIP2 and PIP3 synthesis resulting from PI3K activation upon cell adhesion via α3β1 and α2β1. HFKs were labeled for 1 h with
Laminin 5 Regulates Integrin Adhesion and Signaling

HFKs were suspended (Fig. 10C, lane 1) and plated onto immobilized anti-α5 (Fig. 10C, lane 2), anti-α6 (lane 3), or anti-α6 (lane 4) antibodies for 1 h. HFKs adherent on either anti-α5 or anti-α6 antibodies induced JNK phosphorylation (Fig. 10, C lanes 2 and 4 and E). In contrast, HFKs adherent and spread on anti-α6 antibody did not induce JNK phosphorylation when compared with suspended controls. Wortmannin inhibited JNK phosphorylation induced by ligation of α5β1 and α6β4 (Fig. 10, D lanes 2 and 4) and E). Therefore, both integrins α5β1 and α6β4 induced JNK phosphorylation and this required a PI3K-dependent pathway.

Adhesion of HFKs to immobilized anti-α5 antibody induced toxin B-resistant spreading but only when laminin 5 was deposited (Fig. 5). Therefore, we hypothesized that deposits of laminin 5 onto either immobilized anti-α3 or anti-α6 mAbs could ligate both α5β1 or α6β4 to signal through PI3K to JNK (Fig. 10C, JNKp, lanes 2 and 4). To test this possibility, HFKs were incubated without (Fig. 10F, control) or with inhibitory anti-laminin 5 mAb (Fig. 10F, +C29) or with wortmannin (Fig. 10F, +wortmannin). Adhesion of HFKs on anti-α5 (Fig. 10F, lane 2) and anti-α6 (lane 4) induced JNK phosphorylation when compared with suspended HFKs (lane 1) or HFKs adherent on anti-α6 (lane 3). Wortmannin treatment blocked this induction. Consistent with results from Fig. 5, anti-laminin 5 antibody (C29) inhibited toxin B-resistant spreading on anti-α5 and also inhibited PI3K-dependent JNK phosphorylation on both anti-α3 and anti-α6 mAbs. In controls, total JNK levels were similar for all control lanes (Fig. 10G) and for other treatment conditions (data not shown). We conclude that deposition of laminin 5 onto immobilized anti-α3 or anti-α6 is sufficient for downstream signaling through α5β1 and PI3K to integrin α5β1 for spreading and JNK phosphorylation.

**Deposition of Laminin 5 on Collagen Switches HFK Spreading from Toxin B-sensitive to -insensitive**—We have shown that laminin 5 is deposited onto substratum during epithelial migration on collagen (Fig. 1), and that this deposition of laminin 5 is required for toxin B-resistant spreading on immobilized anti-α5 mAbs (Fig. 5) and induction of PI3K-dependent phosphorylation of JNK (Fig. 10F). We sought to determine if deposition of laminin 5 is sufficient to convert cell spreading from toxin B-sensitive to toxin B-resistant. We compared HFK adhesion and spreading on exogenous laminin 5, collagen, and laminin 5-conditioned collagen (see “Experimental Procedures”). HFKs were untreated (Fig. 11, Control) or treated with toxin B (50 ng/ml, TxB), LY294002 (5 nM, LY), or the combination of toxin B and LY294002 (TxB+LY) for 3 h. Cells were then trypsinized and in the presence of inhibitory G0H3 were plated on exogenous laminin 5 (Lam5; black bars), collagen (Col I; striped bars), or laminin 5-conditioned collagen (Lam5 cond; gray bars). Toxin B was unable to inhibit HFK adhesion and spreading on the laminin 5-conditioned collagen, whereas it inhibits on collagen alone. Inhibiting PI3K with LY294002 in conjunction with toxin B blocked cell adhesion and spreading on all surfaces. Thus, laminin 5 deposited over collagen substratum, as in the case of cell migration in epidermal wound, is sufficient to convert HFKs from a toxin B-sensitive to a toxin B-resistant adhesion and spreading.

In summary, α5β1 mediating adhesion and spreading on laminin 5 utilizes either Rho or PI3K. The PI3K pathway in HFKs requires expression of α5β4 and ligation of laminin 5 and

**FIG. 10. Downstream signaling induced by ligation of α5β1, α6β4, and α5β6.** A, trypsin-suspended HFKs (lane 1) were plated onto exogenous laminin 5 (lane 2) or collagen (lane 3) for 1 h. Triton X-100 protein extracts were isolated and blotted with anti-phosphorylated-JNK (JNKp), anti-JNK (JNK), or anti-ERK2 (ERK2) antibody. B, densitometry reading showing relative phosphorylated JNK over total JNK from blot in A. Susp, suspension; Lam5, laminin 5; ColI, collagen. C, HFKs were trypsin-suspended (lane 1) and plated onto immobilized anti-α5 (P1B5; lane 2), anti-α6 (P1H5; lane 3), or anti-α6 (G0H3; lane 4) antibodies for 1 h. Protein extracts were immunoblotted with anti-phosphorylated JNKp (JNKp) antibody. D, densitometry reading showing relative phosphorylated JNK over total JNK from blot in A. Susp, suspension; Lam5, laminin 5; ColI, collagen. E, HFKs adherent on either immobilized anti-α5 (P1B5; lane 2), anti-α6 (P1H5; lane 3), or anti-α6 (G0H3; lane 4) antibodies for 1 h. Protein extracts were prepared and immunoblotted for anti-phosphorylated JNK (JNKp) antibody. E, densitometry reading from blots in C (--wortmannin) and D (+wortmannin) showing relative JNK phosphorylation over total JNK. F, Western blot of phosphorylated JNK from Triton-soluble extracts of HFKs that were untreated controls, pretreated with 50 nM wortmannin for 3 h, or treated for 1 h in the presence of inhibitory anti-laminin 5 antibodies (C29). HFKs were trypsin-suspended (lane 1) or adhered onto immobilized antibodies: anti-α5 (P1B5; lane 2), anti-α6 (P1H5; lane 3), or anti-α5 (G0H3; lane 4). G, immunoblot with anti-JNK of control suspended cells (lane 1) or cells adherent on anti-α5 (lane 2), anti-α6 (lane 3), or anti-α6 (lane 4) to detect all forms of total JNK.

activation assays, we examined downstream signaling responses induced by adhesion to laminin 5 and collagen. We found that adhesion of HFKs to laminin 5 (Fig. 10, A lane 2 and B) induces phosphorylation of Jun kinase (JNK) 2-fold over adhesion to collagen (Fig. 10, A lane 3 and B) when assayed by quantitative immunoblotting. In contrast, HFK adhesion to collagen induced higher levels of phosphorylated ERK2 seen as a slower migrating band (Fig. 10A, lane 3 compared with lane 2). Phosphorylation of ERK2 is inhibited by toxin B (results not shown). This confirmed that adhesion of HFKs on laminin 5 stimulates different signals then collagen. We determined which integrin, α5β1 or α6β4 was responsible for induction of JNK phosphorylation as a result of adhesion to laminin 5.

32P in suspension (○) and then adhered onto immobilized anti-α1 (P1B5) or anti-α4 (P1H5) mAbs. Samples were taken at 0, 15, 30, 60, and 120 min of adhesion. C, densitometry reading of 32P or IP3 bands from B, comparing time course between cells on immobilized anti-α1 (open circles) or anti-α2 (black squares). D, P13K immunoprecipitated from extracts of HFKs that were suspended, or plated onto immobilized anti-α1 (P1B5), anti-α2 (P1H5), or anti-α6 (G0H3) and P3(3,4)IP2 was used as an exogenous substrate (see “Experimental Procedures”). E, densitometry reading of relative ratios of IP3 on TLC from D.
functions to convert cell signaling pathways from toxin B-sensitive to toxin B-insensitive. Finally, this PI3K pathway enables wound keratinocytes to adhere, spread on laminin 5 via αβ4 in the absence of RhoGTPase activity.

DISCUSSION

We report that the deposition of laminin 5 by keratinocytes onto the substrate is a key factor in regulating mechanisms of both cell spreading and signaling. The findings suggest that primary human keratinocytes utilize at least two different signaling pathways when adhering and spreading on ECM. Which pathway is utilized by the keratinocytes is established by the substrate ligand. One pathway is dependent on PI3K signaling and the other on RhoGTPases. Adhesion and spreading via αβ4 on laminin 5 is blocked only when both pathways are inhibited using toxin B and wortmannin. In contrast, adhesion and spreading on collagen via αβ1 is blocked with toxin B alone, suggesting a primary dependence on RhoGTPases. Deposition of laminin 5 onto collagen substratum switches signaling from a Rho-dependent to a PI3K-dependent pathway.

Laminin 5-αβ4 Interactions Regulate Spreading and Signaling Mediated by αβ1—Toxin B-insensitive spreading via αβ1 requires ligation of both αβ4 and αβ1 to laminin 5 deposited by keratinocytes. PI3K signaling is also required. Consistently, PI3K is activated through the ligation of αβ4 to laminin 5. Recent findings indicate that αβ4 signals through PI3K to RacGTPases to mediate migration and invasion of carcinoma cell lines (29). In contrast to carcinoma cell lines, primary HFKs utilize integrin αβ1 for cell spreading, not αβ4, yet at the same time is dependent upon αβ4-laminin 5 signaling. Further, we observed the PI3K-dependent spreading on laminin 5 but not on collagen, suggesting that αβ1 does not respond to signals from PI3K or that ligation of collagen inhibits PI3K-dependent spreading. We have also examined toxin B sensitivity in cell adhesion and spreading in primary human foreskin fibroblasts (HFF, data not shown). HFFs express αβ1 and can interact with exogenous laminin 5 (29). However, HFFs in the presence of toxin B fail to adhere and spread on laminin 5. This characteristic of HFFs is similar to keratinocytes isolated from JEB-PA individuals lacking αβ4 function. This suggests that the toxin B-insensitive spreading on laminin 5 is unique to cells possessing αβ4 signaling. It remains to be established if the expressed αβ4 must also be able to function in hemidesmosomes. In addition, we have also tested keratinocyte cell lines immortalized with E6/E7 oncogenes from human papilloma virus. These cells promiscuously adhered and spread in the presence of toxin B to all tested matrix surfaces (data not shown). Hence, the specific regulation of signaling pathways that differentiate between laminin 5 and collagen is lost in transformed cells. Therefore, collagen invasion by carcinomas or transformed keratinocyte cell lines may originate as a subversion of a normal wound repair pathway that is selective for αβ4 on laminin 5 in normal epithelium.

Links between the Rho-dependent and PI3K-dependent Pathways—Our results establish that integrin αβ1 utilizes two different signaling pathways for adhesion and spreading; Rho-dependent and PI3K-dependent. The PI3K-dependent pathway requires laminin 5 ligation of αβ4. Inhibition of PI3K by wortmannin increased focal adhesions containing αβ1, vinculin, FAK, and actin stress fibers as well as increased phosphorylation of FAK. In this condition, adhesion and spreading mediated by αβ1 becomes dependent upon Rho and can be inhibited by toxin B. Interestingly, keratinocytes from JEB-PA (β4-deficient) individuals also had increased focal adhesions and stress fiber assembly on laminin 5 (data not shown). Consistently, toxin B blocked JEB-PA keratinocyte adhesion to laminin 5, suggesting that αβ4 and PI3K signaling were required for Rho independence of αβ4. Thus, the active PI3K pathway induced by laminin 5 in primary HFKs may serve to suppress the Rho-dependent pathway on laminin 5.

It is not apparent why HFKs in the presence of toxin B can adhere and spread on laminin 5, immobilized anti-α5 but not on collagen or immobilized anti-α2 mAbs. HFKs deposit precursor laminin 5 onto the collagen and presumably interact with the deposits via αβ4 and αβ1. Further, we established that deposited laminin 5-αβ4 interactions signal through PI3K to control spreading through αβ1. On laminin 5, the PI3K signals suppressed FAK phosphorylation and focal adhesion formation that require Rho (Figs. 7 and 8). Conceivably, interaction of collagen with αβ4 and signaling through Rho may be dominant to the PI3K signals resulting from the laminin 5 deposits. However, this dominance must be short-lived; as seen in Fig. 11, extended deposition of laminin 5 on the collagen surface eventually overrides the toxin B-sensitive interactions with collagen making them toxin B-resistant. How HFKs establish these adhesion and signaling preferences remains to be established.

In relation to the above point, laminin 5 is deposited onto the collagen substrate by leading keratinocytes in a precursor form containing the heparin-binding LG4/5 subdomain at the carboxyl terminus of the α2-200 chain. The LG4/5 domain is released by proteolytic processing of the precursor laminin 5 as a post-secretory event generating the mature laminin 5. Hypothetically, the heparin binding activity and/or the proteolytic processing of precursor laminin 5 at either the α3 or γ2 chains may participate in regulating the activity of PI3K and/or Rho. The addition of exogenous processed laminin 5 that lacks the LG4/5 to JEB-G keratinocytes rescued the PI3K signaling pathway (Fig. 5, panel h). Thus, LG4/5 does not appear to be required for toxin B-resistant spreading. Future studies will determine if the precursor form of laminin 5 promotes toxin B-sensitive spreading of HFKs on substratum.

Advantage of the Dual Signaling Pathways during Wound Repair—There are multiple possible advantages for a dual signaling pathway mediating adhesion and spreading on laminin 5 deposits. Stimulation of PI3K activity by laminin 5 may promote survival signals during stress-activated events such as tissue injury. Indeed, in mammary epithelial cells, laminin acts as a survival ligand through β1 integrins and cooperates with...
growth factor signals to suppress apoptosis (54). Several recent reports have shown that FAK signaling to PI3K and Cas is important for matrix survival signals (55, 56).

Laminin 5 dual signals could also regulate communication between cell-substrate and cell-cell adhesions. We recently reported that laminin 5, but not collagen or fibronectin, promoted gap junctional intercellular communication (GJIC; Ref. 54). Our findings suggest that Rho was required for laminin 5 induction of GJIC. RhoGTP and RacGTP are both required for E-cadherin-based cell-cell adhesion in epithelial cells (57–60). During epidermal wound repair, keratinocytes move as an integrated epithelial sheet. Because Rho is required for cell-cell adhesion and GJIC on laminin 5, PI3K may be necessary to substitute for Rho in cell-substrate adhesion. Conversely, leading edge keratinocytes migrating over collagen do not communicate via gap junctions. Thus, leading keratinocytes on collagen depend on RhoGTPases for substrate interactions (Fig. 1). Active RhoGTPases may be a limiting factor in keratinocytes, and regulation may be controlled by cellular localization that is dependent on laminin 5. Collard's group (60) has shown that adhesion to laminin increased adhesion; adhesion to substrate collagen decreased intercellular contacts, and induced cell invasion. Adhesion of keratinocytes in epidermal outgrowths plays a critical role in regulating adhesion, spreading, and downstream JNK signaling as well as PI3K-dependent pathway for adhesion, spreading, and downstream JNK signaling as well as PI3K activation of the PI3K-dependent pathway for adhesion, spreading, and downstream JNK signaling as well as PI3K-dependent pathway for adhesion, spreading, and signaling. Adhesion of HFKs to collagen is sufficient to promote Rho-dependent spreading and deposition of laminin 5 as part of a BM repair process. Interaction of following keratinocytes with this deposited laminin 5 activates the PI3K-dependent pathway for adhesion, spreading, and downstream JNK signaling as well as promotes gap junctional intercellular communication. Thus, instructions from collagen and laminin 5 allow leading and following keratinocytes, respectively, to perform distinct functions necessary for repair of the BM while maintaining the barrier function of a continuous impermeable epidermis.

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