Ligation of Integrin $\alpha_3\beta_1$ by Laminin 5 at the Wound Edge Activates Rho-dependent Adhesion of Leading Keratinocytes on Collagen

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Wounding of the epidermis signals the transition of keratinocytes from quiescent anchorage on endogenous basement membrane laminin 5 to migration on exposed dermal collagen. In this study, we attempt to characterize activation signals that transform quiescent keratinocytes into migratory leading cells at the wound edge. Previously, we reported that adhesion and spreading on collagen via integrin $\alpha_3\beta_1$ by cultured human foreskin keratinocytes (HFKs) requires RhoGTP, a regulator of actin stress fibers. In contrast, adhesion and spreading on laminin 5 requires integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ and is dependent on phosphoinositide 3-hydroxykinase (Nguyen, B. P., Gil, S. G., and Carter, W. G. (2000) J. Biol. Chem. 275, 31896–31907). Here, we report that quiescent HFKs do not adhere to collagen but adhere and spread on laminin 5. By using collagen adhesion as one criterion for conversion to a “leading wound cell,” we found that activation of collagen adhesion requires elevation of RhoGTP. Adhesion of quiescent HFKs to laminin 5 via integrin $\alpha_3\beta_1$ and $\alpha_6\beta_4$ is sufficient to increase levels of RhoGTP required for adhesion and spreading on collagen. Consistently, adhesion of quiescent HFKs to laminin 5, but not collagen, also promotes expression of the precursor form of laminin 5, a characteristic of leading keratinocytes in the epidermal outgrowth. We suggest that wounding of quiescent epidermis initiates adhesion and spreading of keratinocytes at the wound edge on endogenous basement membrane laminin 5 via $\alpha_3\beta_1$ and $\alpha_6\beta_4$ in a Rho-independent mechanism. Spreading on endogenous laminin 5 via $\alpha_3\beta_1$ is necessary but not sufficient to elevate expression of precursor laminin 5 and RhoGTP, allowing for subsequent collagen adhesion via $\alpha_3\beta_1$, all characteristics of leading keratinocytes in the epidermal outgrowth.

The migratory process of wound repair involves changes in cell adhesion from a laminin 5-based stable anchoring structure via integrin $\alpha_3\beta_2$ to a dynamic transient adhesion system involving $\beta_1$ integrins. Quiescent epidermal keratinocytes are anchored to laminin 5 in the basement membrane (BM) via integrin $\alpha_3\beta_1$ in hemidesmosomes (HDs) (1, 2). Wounding the quiescent epidermis (see Transition A below) generates an initial activation (see Transition B) of keratinocytes at the wound edge that still adhered to endogenous laminin 5. In response to initial wound activation, keratinocytes at the wound edge transform into an epidermal outgrowth composed of leading and following subpopulations of keratinocytes (2, 3).

Wounding response
Quiescent epidermis $\rightarrow$ initial activation $\rightarrow$ migration
(A) (B) (C)

TRANSITIONS A–C

Active leading keratinocytes are distinct from either the quiescent keratinocytes in epidermis or activated following keratinocytes in the outgrowth. Leading keratinocytes express elevated levels of a precursor form of laminin 5, not expressed by quiescent or following keratinocytes. The leading cells migrate over exposed dermal collagen and deposit precursor laminin 5 as a provisional BM (6–9). Surprisingly, expression of precursor laminin 5, but not other BM components including laminin 10/11, heparan sulfate proteoglycan, or type VII collagen, is elevated in leading cells as one of the earliest activation responses even before migration on collagen is detected (6, 9, 10). The precursor form of the laminin 5 heterotrimer ($\alpha_3\beta_2\gamma_2$), expressed by leading cells, contains a 200-kDa $\alpha_3$ chain with a carboxyl-terminal, heparin-binding subdomain. After secretion, the carboxyl-terminal subdomain is proteolytically removed to generate the mature $\alpha_3$ chain present in BM of quiescent epidermis (2, 7–9). Goldfinger et al. (8) have suggested that the precursor form of laminin 5 preferentially interacts with $\alpha_3\beta_1$ to mediate migration, whereas the processed form interacts with $\alpha_3\beta_4$. The processed form appears to be the major adhesive ligand in the quiescent epidermis (2). However, cells expressing $\alpha_3\beta_1$ readily adhere, spread, migrate, and scatter on the processed form of laminin 5 in vitro (9, 11, 12). This suggests that the activation state of keratinocytes, not just the processing of laminin 5, determines whether $\alpha_3\beta_1$ interacts with laminin 5 in the BM. Conceivably, initial activation of keratinocytes at the wound edge would allow them to adhere...
and spread on the processed form of laminin 5 in the BM before they up-regulate expression of precursor laminin 5 as a downstream response.

In vitro adhesion and migration studies with antibodies against integrins as well as targeted disruptions of α9 in mice (13, 14) have established that integrins α9β1 and α9β2 mediate adhesion and motility on laminin 5 and collagen, respectively. Leading keratinocytes utilize integrin α9β1 and RhoGTP, a regulator of actin stress fibers, to adhere and spread on collagen (9). Adhesion and spreading of HFKs on laminin 5 via integrins α9β1 and α9β2 is dependent on phosphoinositide 3-hydroxykinase (PI3K) and is resistant to toxin B, a bacterial inhibitor of Rho, that blocks adhesion of leading cells on collagen (9). In quiescent tissue, integrins α9β1 and α9β2, and α9β1 are expressed on the cell surface. In contrast, integrin α9β1 is not expressed in quiescent epidermis and requires synthesis and expression for adhesion to dermal fibronectin (15).

Different factors may contribute to the initial activation at the wound edge. Wounding of confluent quiescent epidermis results in disruption of cell-cell adhesions, influx of soluble growth factors and cytokines, stress responses, and changes in substrate adhesion (4, 5). Each of these wound-induced changes may contribute to initial activation (B) and transition of the quiescent epithelium at the wound margin (A) to the activated leading keratinocytes in the outgrowth (C). Many of the characteristics of quiescent epidermis in vivo are mimicked by culture of keratinocytes at confluent cell densities (16). Furthermore, wounding of the confluent cultures or suspension and readhesion at sparse cell densities duplicate the wound-induced changes and promote characteristics of the leading keratinocytes in the outgrowth. For example, culture of Madin-Darby canine kidney epithelial cells or keratinocytes at confluence for multiple days promotes assembly of calcium-independent desmosomes that do not disassemble in the presence of calcium chelators (16). Wounding of the confluent sheet converts the calcium-independent desmosomes to calcium dependence both adjacent to and distant to the wound edge. Similarly, wounding of epidermis disrupts HDs (17–19) both in vivo and in vitro. Transcription, translation, and deposition of laminin 5, keratins 16 and 17 (21, 22) is not detectable in quiescent epidermis or confluent cultures of keratinocytes. However, injury of the confluent epithelium in vivo or in vitro elevates their expression both adjacent to and distant to the wound margin.

We have shown previously that deposition of laminin 5 by leading keratinocytes over exposed dermal collagen changes substrate adhesion of the following keratinocytes from collagen and RhoGTP dependent to laminin 5 and PI3K-dependent (9). We characterized changes in adhesion and signaling that transform quiescent epidermis at the wound edge into the leading keratinocytes in the epidermal outgrowth. We observed that confluent cultures of human foreskin keratinocytes (HFKs) fail in Rho-dependent adhesion to collagen but still adhere and spread on laminin 5, independent of Rho. However, HFKs can readily adhere to collagen via α9β1 when cultured at subconfluent cell densities (23). This suggested that the quiescent keratinocytes (A in Reaction A–C) may be able to adhere and spread on endogenous laminin 5 in the BM prior to adhesion to collagen. Only after initial wound activation (B) and subsequent up-regulation of collagen adhesion and expression of precursor laminin 5 would the keratinocytes at the wound margin become leading keratinocytes and migrate over the exposed dermis (C). We hypothesized that quiescent keratinocytes anchored via α9β1 to endogenous BM laminin 5 at the wound edge would spread via α9β1 as an initial activation event. Rho-independent spreading mediated by integrin α9β1 on endogenous laminin 5 may increase RhoGTP required for subsequent adhesion of leading keratinocytes on collagen via α9β1. To test this hypothesis, we determined whether cell-substrate adhesion and spreading on endogenous processed laminin 5 was necessary to provide initial adhesion-dependent signals required for expression of the characteristics of leading cells. The characteristics used to define leading cells are elevated levels of RhoGTP, collagen adhesion, and expression of precursor laminin 5. Based on these findings, we suggest that adhesion and spreading of keratinocytes at the wound edge via integrins α9β1 and α9β2 on endogenous BM laminin 5, respectively, are necessary to increase levels of RhoGTP, collagen adhesion, and expression of precursor laminin 5 that define the migratory keratinocytes in the epidermal outgrowth.

**MATERIALS AND METHODS**

**Cells and Cell Culture**—Keratinocytes (unpassaged P0 cells) were isolated fresh from normal human foreskins (HFKs) as described previously (24) by digestion with dispase to separate epidermis from dermis, followed by digestion with trypsin/EDTA to separate basal cells from the epidermis. Freshly isolated HFKs were plated onto culture dishes (6 cm) and grown for 3–5 days as subconfluent P1 HFKs. P1 HFKs were passaged with trypsin/EDTA at different seeding densities from 1 × 10⁵ cells per plate for sparse cells to 1 × 10⁶ cells per plate for confluent cells. The cells were grown for 3 days after seeding at the different cell densities. This corresponded to 3 days of culture at 100% confluence at the highest seeding density. P1 and P2 cultures were maintained in serum-free keratinocyte growth media (KGM, Clonetics Corp., San Diego, CA). Swiss 3T3 cells used as negative base-line controls were grown in RPMI 1640 + 10% fetal bovine serum.

**Cell Adhesion Assays**—Adhesion of HFKs to cryostat sections of epidermis was performed as described previously (9, 10). Adhesion assays for HFKs on surfaces coated with 10 μg/ml human type I collagen or laminin 5 or immobilized anti-integrin antibodies were performed as described previously (25) and as follows. Keratinocytes isolated fresh from neonatal foreskin or from culture plates were labeled with 0.5 μM calcein-AM (Molecular Probes, Eugene, OR) and then added to the different adhesion surfaces (in triplicates per experiment). Cells were allowed 1 h to adhere at 37 °C. Non-adherent cells were removed by washing twice with PBS. Fluorescent readings of pre-wash and post-wash were performed. Fluorescent readings of pre-wash and post-wash were performed. Adherent cells were then calculated as described previously (9).

**MATERIALS AND METHODS**

**ImmunobLOTS**—HFKs were washed once with PBS and solubilized directly with 1% v/v Triton X-100 in PBS containing 5 mM EDTA, 50 μM VO₄, 10 mM NaF, and protease inhibitors (2 mM phenylmethane sulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 1 μg/ml leupeptin). Detergent-soluble extracts were quantitated and separated on SDS-PAGE gels (26). Proteins were blotted onto nitrocellulose, blocked in 2% nonfat milk, and incubated with the following antibodies: anti-phosphotyrosine (PY-20, ICN), anti-PI3K (Upstate Biotechnology, Inc.), antikeratin 5 (Santa Cruz Biotechnology), or anti-p120 catenin (BD Transduction Laboratories). Blots were then washed four times in PBS + 0.1% Triton X-100 and incubated with secondary antibody (donkey anti-rabbit (Jackson Laboratories) or rabbit anti-mouse peroxidase-conjugated (Dako) 1:1 h. Blots were detected with ECL chemiluminescence (Amersham Pharmacia Biotech) and direct exposure to Hyperfilm MP (Amersham Pharmacia Biotech).

**Immunoprecipitation**—Sparse and confluent HFKs were washed once with PBS and incubated for 1 h in KGM-methionine- and cysteine-free media (Clonetics). 100 μCi/ml of [35S]methionine and [35S]cysteine were added to the media, and cells were labeled for 8 h. Alternatively, sparse and confluent HFKs were surface-biotinylated with N-hydroxysuccinimide ester of Biotin (Pierce) for 10 min at 4 °C. Labeled cells were washed twice with cold PBS and solubilized directly with 1% Triton.
X-100 in PBS. Detergent-soluble extracts were immunoprecipitated with mAbs as follows: P1B5 (anti-\(\alpha_3\)), P1E6 (anti-\(\alpha_2\)), P4C10 (anti-\(\beta_1\)), or B4–6 (anti-\(\gamma_2\) chain of laminin 5). Precipitates of 35S-labeled HFKs were fractionated on 8% SDS-PAGE gels, dried onto blotting paper, and exposed to Hyperfilm (Amersham Pharmacia Biotech). Precipitates of biotinylated proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, blocked in 0.5% BSA/0.1% Triton/PBS for 1 h, and then incubated with peroxidase-conjugated streptavidin (Zymed Laboratories Inc.) for 1 h. Blots were developed with ECL reagent (Amersham Pharmacia Biotech). The gels were then directly exposed to Hyperfilm.

Northern Analysis of mRNA Levels for c-Myc and the \(\alpha_3\) Chain of Laminin 5—HFKs were grown at sparse and confluent cell densities for 3 days, suspended with trypsin/EDTA, and re-plated at equal cell numbers onto surfaces coated with laminin 5 or mAbs against integrins anti-\(\alpha_3\) (P1B5) or anti-\(\alpha_2\) (P1H5), or kept in suspension. Total RNA was isolated using TRizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Northern blots were obtained by electrophoresis of total RNA, 20 \(\mu\)g per lane, in formaldehyde-agarose gels (1% agarose, 6.6% formaldehyde, 20 mM MOPS, pH 7.0, 2.5 mM sodium acetate, 1 mM EDTA). Gels were blotted to Zeta-Probe nylon membranes (Bio-Rad) by capillary action using 20× SSC. Blots were hybridized in 0.25 M NaCl, 7% SDS, 1 mM EDTA, 0.25 M sodium phosphate, pH 7.0, 150 mg/ml salmon sperm DNA, 50% formamide at 48°C. Filters were washed at 55°C with 0.2× SSC, 0.1% SDS. cDNA clone Epi, specific for the \(\alpha_3\) chain of laminin 5 (provided by Dr. Maureen Ryan, Seattle, WA), and cDNA clone specific for c-Myc (provided by Dr. Bob Eisenman, Seattle, WA) were radiolabeled using the Prime-it RmT random primer labeling kit (Stratagene) and used as a hybridization probe for Northern analysis.

Rho Activity Assay—Determining levels of GTP-bound Rho for activity was done as described previously (27) with the following variation. Sparse and confluent grown HFKs were plated onto immobilized mAbs or onto laminin 5-coated dishes for 1 h. Cells were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1%...
Rho-dependent Adhesion

RESULTS

Quiescent Keratinocytes Isolated Fresh from Skin Attach and Spread on Laminin 5 but Not Collagen—The collagen receptor, integrin α3β1, is expressed at the cell surface of basal keratinocytes in quiescent epidermis (23, 28, 29). We determined whether quiescent HFKs isolated fresh from epidermis were able to adhere and spread on collagen via their endogenous α3β1. Quiescent HFKs were isolated fresh from neonatal foreskin (passage 0 or P0 HFKs) and assayed for cell adhesion and spreading on exogenous laminin 5 or collagen. Equal numbers of P0 HFKs were plated and photographed (Fig. 1A, pre-wash). Cells were allowed 1 h to adhere and spread. Non-adherent cells were then washed away (post wash). Freshly isolated P0 HFKs adhered and spread on laminin 5 (Fig. 1A, panel d) but not to collagen (Fig. 1A, panel b). In control experiments, P0 HFKs were also adhered to immobilized anti-α3 or anti-α3 integrin antibodies. P0 HFKs were able to adhere to both antibody-coated surfaces but spread only on anti-α3 antibody (data not shown). This confirmed that P0 HFKs, like HFKs in epidermis (23, 28, 29), express both α3β1 and α3β1. Adhesion and spreading of passage 1 cultured HFKs (P1) grown at sub-confluence were compared with P0 HFKs. P1 HFKs were able to adhere and spread on either laminin 5 or collagen (Fig. 1B), whereas P0 HFKs were only able to adhere and spread on laminin 5. Thus, α3β1 is expressed on the cell surface of freshly isolated P0 HFKs, but these cells adhere poorly to collagen. Activation of the P0 HFKs in culture to generate P1 HFKs is sufficient to increase adhesion to collagen via integrin α3β1.

HFKs Cultured at Confluence Adhere to Laminin 5 but Not Collagen—We wished to understand how suspension and re-attachment of the P0 HFKs could activate adhesion to collagen. Therefore, we attempted to establish an in vitro culture model for the P0 HFKs. We determined whether sparse cultures of P1 and P2 HFKs that can adhere to collagen would reduce collagen adhesion when maintained at confluent cell densities, mimicking the P0 HFKs (Fig. 1). For comparison, we cultured P1 HFKs at both sparse (Fig. 2A, panel a) and confluent cell densities (Fig. 2A, panel b). The sparse HFKs display charac-
Characteristics of activated leading edge keratinocytes in epidermal wounds. Sparse HFKs transcribe elevated levels of mRNA encoding the \( \alpha_3 \) chain of laminin 5 and \( \text{c-Myc} \) (Fig. 2B, lane S) (6). In contrast, HFKs cultured at confluence do not transcribe mRNA for \( \alpha_3 \) laminin 5 or \( \text{c-Myc} \) (Fig. 2B, lane C) similar to quiescent keratinocytes in epidermis. We compared sparse and confluent HFKs for adhesion to exogenous laminin 5 or collagen-coated assay plates. Sparse HFKs can adhere and spread on either laminin 5 (Fig. 2C, black bar) or collagen (Fig. 2C, striped bar). Culturing HFKs at increasing cell densities inhibits adhesion to collagen (Fig. 2C, striped bars), whereas adhesion and spreading on laminin 5 (solid bars) remain unaffected. Thus, cultures of P1 and P2 HFKs maintained at sparse cell densities mimic the adhesion, spreading, and transcription characteristics of leading keratinocytes of an epidermal outgrowth in wounds. Cultures of P1 and P2 HFKs maintained at confluence mimic the adhesion and transcription characteristics of quiescent P0 HFKs isolated fresh from epidermis. The transition of keratinocytes from sparse to confluence blocks adhesion and spreading on collagen via endogenous \( \alpha_3 \beta_1 \) but not adhesion and spreading on laminin 5 via \( \alpha_3 \beta_1 \) and \( \alpha_4 \beta_1 \).

We were concerned that native collagen in tissue, as opposed to purified collagen, might support adhesion of confluent HFKs similar to laminin 5. Therefore, we labeled sparse and confluent cultured HFKs with calcein (a green fluorescent dye) and adhered the HFKs to native dermal collagen. Cryostat sections of frozen, split foreskin tissue were used as our assay tissue to which adhesion was scored (9, 10). Sparse HFKs were able to adhere both to laminin 5 in the BM and to interstitial collagen found in the dermis (Fig. 3, a and c). Confluent HFKs could adhere to laminin 5 in the BM but had reduced adhesion to dermal ligands including interstitial collagens and fibronectin (Fig. 3, b and d). This result raises the surprising possibility that BM laminin 5 promotes adhesion and spreading of quiescent confluent keratinocytes, whereas dermal ligands are not able to promote adhesion and spreading of quiescent HFKs. Thus, activation of adhesion to dermal matrices must occur before quiescent HFKs can adhere, spread, or migrate from the wound edge onto the exposed dermal collagen.

**Confluent Keratinocytes Express Reduced RhoGTP and Rho-dependent Adhesion to Collagen**—We wished to understand how confluent cultured HFKs adhered and spread on laminin 5 but were blocked in collagen adhesion. Furthermore, we wished to understand what wound events are sufficient to activate collagen adhesion. Wounding of epidermis or suspension of confluent keratinocytes with trypsin/EDTA (Figs. 1–4) disrupts cell-cell interactions, changes substrate adhesion, and promotes an influx of growth factors and cytokines that may individually or in combination promote collagen adhesion (5). Significantly, suspension of confluent HFKs by trypsin/EDTA was not sufficient to activate collagen adhesion, whereas the same HFKs did adhere and spread on laminin 5. Therefore, we focused on substrate adhesion on laminin 5 as a possible activator of collagen adhesion. First, we confirmed that confluent cultures of HFKs expressed cell surface integrin \( \alpha_3 \beta_1 \) collagen receptor and the endogenous \( \alpha_3 \beta_1 \) could transmit a signal for spreading when compared with sparse HFKs. Sparse (Fig. 4, a and b) and confluent HFKs (c and d) were suspended with trypsin/EDTA and were plated onto immobilized monoclonal antibodies against \( \alpha_3 \) (a and c) or \( \alpha_3 \beta_1 \) integrin (b and d) at equal cell numbers and were allowed to adhere and/or spread for 1 h. Cells were then fixed in 2% formaldehyde and stained with rhodamine-conjugated phalloidin to visualize F-actin and cell spreading. Sparse-derived HFKs adhered and spread on both \( \alpha_3 \) and \( \alpha_3 \beta_1 \) whereas confluent-derived HFKs adhered and spread on \( \alpha_3 \) but did not spread on \( \alpha_3 \beta_1 \).
**Fig. 5. Integrins α3β1 and α2β1 are expressed in both sparse and confluent HFKs.** HFKs were cultured at sparse or increasing cell densities to confluence for 3 days and then either metabolically labeled with [35S]methionine or cell-surface-labeled with N-hydroxysuccinimide-biotin. Labeled HFKs were extracted with Triton X-100 detergent and soluble extracts immunoprecipitated with the indicated antibodies and the labeled precipitate fractionated on SDS-PAGE gels. A, immunoprecipitation of integrins α3, α2, β1, or laminin 5 (lam5, α3–200, etc.), indicates the different chains of the laminin 5 complex from [35S]methionine-labeled extracts of sparse (S) HFKs or cells grown at increasing cell densities to confluence (C). B, immunoprecipitation of surface-biotinylated integrins from extracts of sparse (S, lanes 1, 3, 5, and 7) or confluent (C, lanes 2, 4, 6, and 8) HFKs with negative control (SP2, lanes 1 and 2), anti-α3 (P1B5, lanes 3 and 4), anti-β1 (P4C10, lanes 5 and 6), or anti-α2 (P1E6, lanes 7 and 8) antibodies. Precipitated biotinylated antigens were detected with peroxidase-conjugated streptavidin.

**Fig. 6. Ligation of endogenous α3β1 in confluent HFKs promotes signaling but not spreading.** Sparse (S) and confluent (C) HFKs were trypsin-suspended (susP) and plated onto immobilized antibodies against integrin α3 (P1B5), α2 (P1H5), or β1 (P4C10) for 1 h. Cells were lysed with 1% Triton X-100, and detergent-soluble protein extracts were separated on SDS-PAGE gel and blotted to nitrocellulose. Blot was then detected with PY20 antibody against tyrosine-phosphorylated proteins. Tyrosine-phosphorylated bands corresponding to focal adhesion kinase are marked (FAKp). Note that cells that attached to immobilized α2α3 antibodies did not spread but were able to tyrosine-phosphorylated proteins similar to cells that adhered and spread on anti-α3.

when compared with α3β1. Therefore, we determined if the levels of endogenous α3β1 in confluent cultured HFKs were sufficient to mediate transmembrane signaling in addition to mediating adhesion to immobilized anti-α3 (Fig. 4). Confluent HFKs were treated as in Fig. 4 by adhesion on immobilized anti-α3 mAb to induce adhesion and spreading or on anti-α2 mAb to induce adhesion without spreading. Extracts were prepared from the adherent cells, fractionated by SDS-PAGE, and

immunoblotted with anti-phosphotyrosine antibody (PY20). Ligation of either endogenous α3β1 or α2β1 induced phosphorylation of tyrosine residues on comparable populations of proteins (Fig. 6) Thus, Figs. 4–6 indicate that ligation of the endogenous α3β1 expressed on the cell surface of confluent HFKs is sufficient to mediate cell adhesion and transmembrane signaling but not cell spreading.

We reported previously (9) that adhesion and spreading of HFKs on collagen via α3β1 is dependent on Rho signaling, whereas adhesion and spreading on laminin 5 via α3β1 and α2β1 is Rho-independent. Therefore, we assayed for the levels of active RhoGTP in sparse and confluent HFKs utilizing the Rhotekin binding assay (27). Rhotekin is a Rho effector protein that preferentially binds RhoGTP. Rhotekin beads were used to precipitate RhoGTP from cell extracts, and precipitated Rho was quantitated by immunoblotting with anti-Rho mAb. Bar graphs display bound RhoGTP normalized to total Rho in the cell lysate (Fig. 7A). As our negative control, we used serum-starved Swiss 3T3 cells that have been shown to have levels of RhoGTP activity inadequate to support focal adhesion formation (27). RhoGTP levels in confluent HFKs were comparable

**Fig. 7. Confluent HFKs have decreased RhoGTP activity.** HFKs were cultured at sparse (S) or confluent (C) cell densities for 3 days. Extracts were prepared and assayed as follows. A, levels of RhoGTP in HFKs were compared with serum-starved Swiss 3T3 cells (negative control). RhoGTP in the extracts was determined by precipitation with GST-Rhotekin beads, and bound RhoGTP was blotted with anti-Rho antibodies and quantitated. Graph is representative of three separate experiments, and error bars represent the S.D. of triplicate samples per experiment. B, equal protein (75 μg) in extracts from sparse (S) or confluent (C) HFKs were separated by SDS-PAGE and blotted with antibodies against PI3K, RhoA, or p120 catenin. Sparse HFKs contain more active RhoGTP than confluent HFKs, but confluent HFKs contain more total Rho protein and p120 catenin than sparse HFKs.
with our negative 3T3 control, whereas sparse HFKs had elevated levels. The error bars are the S.D. of triplicate samples per experiment, and the graph is representative of three separate experiments. The low levels of RhoGTP in confluent HFKs were not due to low expression of Rho protein (Fig. 7B). Levels of Rho protein were higher in confluent HFKs by at least 5-fold compared with the sparse cells. As a possible explanation for the low RhoGTP but high Rho protein, we found that expression of p120 catenin, a reported inhibitor of Rho (30, 31), was also increased in confluent HFKs (Fig. 7B). For comparison, levels of PI3K were similar in sparse and confluent HFKs. Thus, low levels of active RhoGTP in confluent HFKs may explain why confluent HFKs do not adhere or spread on collagen while maintaining Rho-independent adhesion and spreading on laminin 5.

Spreading via $\alpha_3\beta_1$ in Confluent-derived HFKs Increases Levels of RhoGTP and Adhesion to Collagen—Leading keratinocytes in the epidermal outgrowth utilize Rho-dependent ligation of integrin $\alpha_3\beta_1$ for adhesion and spreading on collagen. Consistently, toxin B, a Rho inhibitor, selectively induces cell

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**Fig. 8.** Adhesion of confluent-derived HFKs to laminin 5 via $\alpha_3\beta_1$ activates subsequent $\alpha_6\beta_4$-collagen adhesion. A, sparse or confluent (Confl) HFKs were adhered to laminin 5 (black bars) or collagen (striped bars) at equal cell numbers. Confluent HFKs (that do not adhere to collagen) were stimulated by adhesion to laminin 5 for 30 min (Confl. Stimulated) and then trypsinized and assayed for subsequent adhesion to laminin 5 (Lam5) or collagen (Col I). B, phase images of sparse (panels a and b), confluent (panels c and d), or confluent-stimulated (panels e and f) HFKs that were adhered and spread on laminin 5 (panels a, c, and e) or collagen (panels b, d, and f). C, adhesion to laminin 5 (black bars) or collagen (striped bars) of sparse HFKs were compared with confluent and confluent-stimulated HFKs. Confluent-derived HFKs were stimulated on laminin 5 for 1 h in the presence of no antibodies (none), negative control antibody (SP2), inhibitory anti-$\alpha_6$ integrin antibody (G0H3; cells are still spread), or inhibitory anti-$\alpha_3$ antibody (P1B5; cells adhered to laminin 5 but are round). These cells were then trypsinized off and assayed for adhesion to collagen (striped bars, panel a). As a control (panel b), confluent-derived HFKs stimulated on laminin 5 without any antibody treatments were assayed onto collagen in the presence of inhibitory anti-$\alpha_3$ antibody (P1H5), anti-$\alpha_6$ antibody (P1B5), or anti-$\alpha_3$ antibody (G0H3). Stimulation of confluent HFKs by adhesion to laminin 5 via $\alpha_3\beta_1$ is sufficient to activate adhesion and spreading on collagen via $\alpha_3\beta_1$. 

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A

B

C

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- **A**
- **B**
- **C**

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Fig. 9. Ligation of αβ3, in HFKs derived from confluent cultures activates RhoGTP and laminin 5 transcription. A, RhoGTP was quantitated in sparse or confluent HFKs or confluent HFKs stimulated by adhesion to laminin 5, immobilized anti-α3 (P1B5), or anti-α2 (P1H5) antibodies for 1 h. RhoGTP was pulled down with GST-Rhotekin beads and blotted with anti-RhoA antibody for quantitation. Duplicates of each sample were assayed in three separate experiments with similar results. Graph depicts one of three experiments. B, sparse (S) (lanes 1, 3, 5, 7, and 9) or confluent (C) (lanes 2, 4, 6, 8, and 10) HFKs were plated onto immobilized anti-α3 (lanes 1 and 2), anti-α2 (lanes 3 and 4), or laminin 5 (Lam5) (lanes 9 and 10) compared with parental cells (lanes 5 and 6) and cells in suspension (lanes 7 and 8). Total RNA was isolated, and Northern blots were probed with α3 chain laminin 5-specific cDNA or c-Myc cDNA. Ethidium bromide staining of 28S RNA was included to visualize sample loading. Ligation of αβ3, but not αβ1, in confluent HFKs activates RhoGTP and transcription of laminin 5 and c-Myc.

As seen in Fig. 2, and as reported previously (3, 6), sparse cultures of HFKs, like leading keratinocytes in the epidermal outgrowth, express elevated levels of mRNA transcripts for laminin 5 and c-Myc in addition to being Rho-dependent and able to adhere to collagen. We determined whether ligation of αβ1, to laminin 5 in confluent HFKs was sufficient to activate transcription of Myc and laminin 5 mRNAs in addition to activating RhoGTP. Sparse and confluent HFKs were plated onto immobilized anti-α3 (P1B5) and anti-α2 (P1H5) antibodies or onto exogenous laminin 5. mRNA was isolated from the adherent cells after 1 h. A Northern blot was prepared, and the fractionated mRNAs were probed with cDNAs corresponding to the α3 chain of laminin 5 and c-Myc. In confluent HFKs, ligation of αβ3, by laminin 5 or antibody, but not αβ1, increased equilibrium levels of mRNAs for both laminin 5 and c-Myc (Fig. 9B). Thus, adhesion and spreading of confluent HFKs on laminin 5 via αβ3, but not on collagen via αβ1, is sufficient to activate RhoGTP, collagen adhesion, and transcription of c-Myc, elevated in wounds (32, 33), and laminin 5, a characteristic of activated leading edge keratinocytes in the epidermal outgrowth of wounds.

DISCUSSION

HFKs at the Wound Edge Spread on Endogenous BM Laminin 5 via αβ3 and Promote Leading Cells—Quiescent keratinocytes isolated fresh from skin or grown at confluence in

rounding of leading cells but not the confluent following keratinocytes (9). Confluent following cells in the outgrowth utilize integrins αβ3, αβ4, and a PI3K pathway for adhesion and spreading on the path of laminin 5 deposited by the leading keratinocytes. As seen in Fig. 3, confluent HFKs failed to adhere to dermal ligands but readily adhered to BM laminin 5. We hypothesized that confluent HFKs can ligate αβ3 to laminin 5 in a PI3K-dependent adhesion, and this Rho-independent adhesion may increase levels of RhoGTP critical for subsequent adhesion to collagen. Because laminin 5, but not dermal ligands, promotes adhesion of confluent cultured keratinocytes, we determined whether Rho-independent adhesion to laminin 5 was sufficient for activation of RhoGTP and subsequent adhesion to collagen. Confluent HFKs adhered and spread on laminin 5 (Fig. 8A, black bars, and B, panel c) but did not adhere to collagen (Fig. 8A, striped bar, and B, panel d). However, confluent HFKs that were first stimulated by adhesion on laminin 5 for 30 min (see “Materials and Methods”) were then able to adhere and spread on collagen (Fig. 8A, striped bar, confl stimulated, and B, panel f). In controls, maintenance of the HFKs in suspension failed to increase adhesion to collagen and indicated that substrate adhesion was required for the activation (data not shown). Anti-α3 integrin antibody (P1H5) blocked the adhesion of stimulated-confluent HFKs on collagen (Fig. 8C, panel b, anti-α2). Interestingly, preventing αβ3 ligation to laminin 5 during the stimulation step using inhibitory anti-α3 mAb (P1B5), but not anti-α6 mAb (G0H3), abolished subsequent adhesion of stimulated-confluent HFKs to collagen (Fig. 8C, panel a, compare anti-α3 to SP2 or anti-α6). This suggests that adhesion and/or spreading via αβ3 on laminin 5 during the stimulation step activates the αβ3 collagen receptor in confluent HFKs.

We determined whether ligation of αβ3 in confluent HFKs was sufficient to activate RhoGTP in addition to activating adhesion to collagen. RhoGTP levels were greater in cultures of sparse HFKs when compared with confluent HFKs (Fig. 9A). Stimulation of confluent HFKs by adhesion on laminin 5 or immobilized anti-α3 (P1B5) antibody, but not anti-α2 (P1H5) antibody, increased RhoGTP levels to those of sparse HFKs (Fig. 9A). Thus, ligation of αβ3 by laminin 5 in confluent HFKs is sufficient to increase levels of RhoGTP and promote collagen adhesion via αβ3.
culture have insufficient levels of RhoGTP to support adhesion to collagen via \( \alpha_3 \beta_1 \). However, the keratinocytes at the wound edge can adhere and spread on endogenous BM laminin 5 via \( \alpha_3 \beta_1 \) and \( \alpha_4 \beta_1 \) utilizing a Rho-independent mechanism. Fig. 10 provides a diagrammatic summary of our results and how these findings fit into the initial events of wound activation. The Rho-independent adhesion and spreading on endogenous laminin 5 via \( \alpha_3 \beta_1 \) and \( \alpha_4 \beta_1 \) are necessary to increase the levels of RhoGTP and promote the subsequent adhesion to collagen. Consistently, ligation of \( \alpha_3 \beta_1 \) by immobilized anti-\( \alpha_3 \) antibody, but not ligation of \( \alpha_4 \beta_1 \) by immobilized anti-\( \alpha_4 \) antibody, also elevates levels of RhoGTP and promotes expression of precursor laminin 5 and c-Myc. We suggest that quiescent keratinocytes at the wound edge can spread on endogenous BM laminin 5 via \( \alpha_3 \beta_1 \) and \( \alpha_4 \beta_1 \). Adhesion and spreading on endogenous BM laminin 5 are sufficient for adhesion-dependent initial activation signals that promote characteristics of leading cells. It is unclear how endogenous laminin 5 may function as both an anchorage ligand for quiescent keratinocytes and as an activation ligand for keratinocytes in wounds. We suggest the following possible explanation. Initially quiescent keratinocytes are adherent to endogenous laminin 5 via \( \alpha_3 \beta_4 \) in HDs (Fig. 10A). At least three events occur in response to wounding to convert quiescent keratinocytes into activated leading cells (Fig. 10C). First, wounding activates a change in cell signaling in keratinocytes at the wound edge. This is probably in response to local factors in the new wound including influx of growth factors, changes in cell-cell interactions, and stress responses. Second, the changes in cell signaling allow both \( \alpha_3 \beta_4 \) and \( \alpha_3 \beta_1 \) to mediate cell adhesion and spreading, respectively, on the endogenous laminin 5 independent of RhoGTP (Fig. 10B). Third, the adhesion and spreading of keratinocytes on endogenous laminin 5 at the wound edge provide adhesion-dependent signals that are necessary but not sufficient to promote downstream characteristics of leading keratinocytes including increased expression of precursor laminin 5, activation of Rho, and activation of collagen adhesion (Fig. 10C). Thus, we suggest that cell signaling in quiescent keratinocytes (Fig. 10A) is different from signaling in wound-activated keratinocytes (Fig. 10B). This difference can explain the distinct adhesion functions of quiescent and activated keratinocytes on endogenous laminin 5. Importantly, the adhesion-dependent signals are necessary for establishing the phenotype of leading cells (Fig. 10C). Below, we will discuss specific factors and mechanisms that may contribute to the initial wound activation.

Based on the current findings, future studies will attempt to clarify the following. What wound-dependent initial activation signals stimulate \( \alpha_3 \beta_1 \)-mediated spreading on the endogenous laminin 5 at the wound edge? What adhesion-dependent signals elevate RhoGTP, collagen adhesion, or precursor laminin 5? Studies will also attempt to integrate signals from growth factors and cell-cell interactions that cooperate with \( \alpha_3 \beta_1 \)-laminin 5 adhesion in regulating wound activation.

**Mechanisms That May Participate in Initial Wound Activation**—We have attempted to identify candidates that participate in adhesion and signaling in initial activation of leading keratinocytes at the wound edge. Confluent HFKs used for these studies are quiescent based on low expression of laminin 5, c-Myc, RhoGTP, and collagen adhesion while maintaining adhesion and spreading on endogenous BM laminin 5 via \( \alpha_3 \beta_4 \) and \( \alpha_3 \beta_1 \) (11, 28). Maintenance of HFKs at confluence promotes other characteristics of quiescent keratinocytes including assembly of HDs or stable anchoring contacts (28) and increased cell-cell adhesions. Detachment and re-adhesion of confluent HFKs elevate RhoGTP, collagen adhesion, and precursor laminin 5 but only when coupled to adhesion and spreading on laminin 5. Specifically, elevation in transcription in the \( \alpha_3 \) chain of laminin 5 and up-regulation of RhoGTP require adhesion to laminin 5. It is not apparent from the studies here if \( \alpha_3 \beta_1 \) is constitutively active in the quiescent epidermis and therefore able to mediate spreading on endogenous laminin 5. Conceivably, an uncharacterized initial activation event induced by wounding may activate \( \alpha_3 \beta_1 \) allowing for spreading of the adherent keratinocytes at the wound edge. It appears (Fig. 9B) that keratinocytes at the wound edge can adhere and spread on the mature proteolytically processed form of laminin 5 present in the BM and that this interaction is sufficient for the adhesion, spreading, and expression of precursor laminin 5, an event that defines leading keratinocytes in the epidermal outgrowth. We have not established the order of events that includes elevation in precursor laminin 5, RhoGTP, and collagen adhesion. Therefore, \( \alpha_3 \beta_1 \)-dependent increases in RhoGTP...
and collagen adhesion may be dependent on interactions with the deposits of precursor laminin 5. Consistent with this idea, by 8 h after injury, precursor laminin 5 is expressed and deposited into the BM of the wound edge prior to migration on exposed dermal collagen (2, 3). Furthermore, elevation of RhoGTP may depend on more complex activation signals in addition to adhesion to laminin 5. Wounding of the quiescent epidermis or confluent cultures of HFKs disrupts cell-substrate and cell-cell junctions that may both contribute to initial activation signals either concurrent with or prior to the adhesion-dependent signals from α6β1-laminin 5. Confluent HFKs express high levels of Rho protein but low RhoGTP (Fig. 7). Low RhoGTP in confluent HFKs may result from the elevated levels of p120 catenin (Fig. 7B). p120 catenin stabilizes strong cell-cell adhesions when associated with classic cadherins (34) but can also bind Vav2, a Rho guanine exchange factor, thus inhibiting Rho (30) and suppressing focal adhesions and stress fibers (31). It remains to be established if ligation of laminin 5 via α6β1 in leading cells regulates p120 catenin or if wound-dependent changes in cell-cell adhesion regulate p120 catenin levels. In attempting to identify initial activation events displayed by keratinocytes at the wound edge, we observed that HD-stable anchoring contacts are disassembled within minutes of ligation of α6β1 but not α6β4.2 The α6β1-dependent disassembly of HD-stable anchoring contacts in confluent HFKs is significant because it occurs coincident to initial spreading of keratinocytes on processed laminin 5 via α6β1 and prior to the α6β1-dependent increase in collagen adhesion. Disassembly of HDs is a characteristic of the keratinocytes that have migrated into the wound (19). Wounding of confluent epithelial cells has also been reported to transform desmosomes at cell-cell junctions from calcium-independent to calcium-dependent both at and adjacent to new wounds (16). Activation of protein kinase Ca also shifts the calcium-independent desmosomes to calcium dependence. Activation of protein kinase Ca has also been reported to mobilize α6β4 of HDs in A431 squamous carcinoma cells that correlate with serine phosphorylation of β4 (35). In another report (36), exposure of 804G cells to orthovanadate, a tyrosine phosphatase inhibitor, increases tyrosine phosphorylation of β4 and also disrupts HDs.

Additional signaling events that are dependent on adhesion to laminin 5 via α6β1 and α6β4 include elevation of PI3K enzyme activity and activation of c-Jun amino-terminal kinase (9). Furthermore, detachment of HFKs and reattachment to laminin 5 promote reversible tyrosine phosphorylation and dephosphorylation, respectively, of a membrane-associated 80-kDa protein (11). Thus, signaling events including Rho, PI3K, c-Jun amino-terminal kinase, protein kinase C, and p80 are candidates for initial activation in response to adhesion and spreading of keratinocytes at the edge of a wound on endogenous laminin 5 via α6β1 and α6β4. These activation signals may each contribute to the phenotype of leading keratinocytes at the wound edge that includes expression of precursor laminin 5, elevated RhoGTP and collagen adhesion, disassembly of HDs, and gap junctions.

Laminin 5, α6β1, and α6β4 in Activation and Migration of Leading Keratinocytes—Considerable confusion exists as to the function(s) of α6β1, α6β4, and laminin 5 in adhesion, spreading, and migration of keratinocytes. Adhesion of keratinocytes via α6β1 has been reported to be either inhibitory or required for migration. Kim et al. (37) reported that anti-α6 antibody enhanced migration of keratinocytes on fibronectin and collagen. Furthermore, α6β1 localized in focal adhesion of cells on fibronectin and collagen in addition to laminin 5 (38). They reported that α6β1 did not contribute to initial adhesion and spreading except on laminin 5. Furthermore, disruption of α6β1 in keratinocytes increased stress fibers, focal adhesions, and receptor activity for dermal ligands (39). This suggested that α6β1 is a trans-dominant inhibitor of the function of other β1 integrins in mouse keratinocytes (39) and carcinoma cells lines (40). Decline and Rousselle (41) suggested that α6β1 interactions with deposits of laminin 5 on collagen inhibit migration of transforming growth factor-β-activated keratinocytes, whereas α6β1 interactions with the deposits of laminin 5 are responsible for migration. These results disagree with other results that suggest a crucial role for α6β1 in migration on deposits of laminin 5 (42, 43).

Results here suggest that adhesion of quiescent keratinocytes at the wound edge activates spreading via α6β1 on endogenous laminin 5, elevates RhoGTP, collagen adhesion via α6β1, and expression of precursor laminin 5, defining leading keratinocytes in the epidermal outgrowth (summarized in Fig. 10). Downstream from this activation, we reported previously (9) that deposition of laminin 5 by leading keratinocytes as they migrate over exposed dermal collagen switches the ligand from collagen- to laminin 5-dependent and signaling from Rho- to PI3K-dependent for the following keratinocytes. Apparently, adhesion to endogenous laminin 5 can maintain quiescence of normal keratinocytes or activate keratinocytes at the wound edge or promote quiescence in the following keratinocytes in the wound outgrowth (Fig. 10). How keratinocytes respond to laminin 5 may be as dependent on the cell signaling status of the keratinocytes as it is on the proteolytic processing of laminin 5. For example, keratinocytes at the wound edge activated by disruption of cell-cell junctions interacting with processed laminin 5 may respond differently than quiescent confluent keratinocytes interacting with the same processed laminin 5. Disagreement in published results may stem from the distinct cell responses displayed by different cells or subpopulations of keratinocytes when interacting with the same laminin 5. Furthermore, laminin 5-α6β1 interactions may be distinct from collagen-α6β1 at the wound edge because α6β1 is either constitutively active even at confluence or because it can be activated at the wound edge by local factors. In Fig. 10A, the adhesion status of α6β1 is indicated with a ? because the status is unclear in both normal epidermis and wounds. In contrast to α6β1, both the Rho-dependent α6β2 or α6β1 may be less effective in promoting spreading or migration except in the position of Rho-dependent leading keratinocytes.

Biological Import of Rho-dependent Leading and Rho-independent Following Keratinocytes—Activating Rho-dependent leading keratinocytes in the epidermal outgrowth may be critical to normal wound repair but not desirable in quiescent tissue. Wounding of embryonic epidermis activates assembly of a Rho-dependent actin cable or “purse string” at the wound edge (44). Contraction of the purse string draws the wound together. Similar contractile functions may be executed by the Rho-dependent leading keratinocytes that migrate on collagen. In contrast, Rho-independent adhesion of α6β1 and α6β4 to laminin 5 in the following cells promotes gap junctions that integrate individual following cells into an epithelial sheet (3). Leading cells that do not communicate through gap junctions are isolated from the following cells. Potentially, Rho-dependent substrate adhesion of leading cells on dermal ligands may facilitate migration while isolating them from the rest of the colony. This isolation could limit the potential of the leading cells for invasion of the collagenous dermis by limiting the access to metabolic energy or other resources required for outgrowth. Furthermore, Rho-dependent leading cells may guide the Rho-independent following cells by deposition of a path of

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2. C. A. Dunn, B. P. Nguyen, and W. G. Carter, unpublished data.
laminin 5. Adhesion of leading cells to collagen, but not laminin 5, is suppressed by confluence. At wound closure, confluence can arrest the migration/invasion of both the leading cells by down-regulating RhoGTP and the migration of the following cells by down-regulating the expression of precursor laminin 5.

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Ligation of Integrin $\alpha_3\beta_1$ by Laminin 5 at the Wound Edge Activates Rho-dependent Adhesion of Leading Keratinocytes on Collagen
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