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The Rac activator Tiam1 is required for α3β1-mediated laminin-5 deposition, cell spreading, and cell migration

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The Rho-like guanosine triphosphatase Rac1 regulates various signaling pathways, including integrin-mediated adhesion and migration of cells. However, the mechanisms by which integrins signal toward Rac are poorly understood. We show that the Rac-specific guanine nucleotide exchange factor Tiam1 (T-lymphoma invasion and metastasis 1) is required for the integrin-mediated laminin (LN)-5 deposition, spreading, and migration of keratinocytes. In contrast to wild-type keratinocytes, Tiam1-deficient (Tiam1−/−) keratinocytes are unable to adhere to and spread on a glass substrate because they are unable to deposit their own LN5 substrate. Both Tiam1 and V12Rac1 can rescue the defects of Tiam1−/− keratinocytes, indicating that these deficiencies are caused by impaired Tiam1-mediated Rac activation. Tiam1−/− cells are unable to activate Rac upon α3β1-mediated adhesion to an exogenous LN5 substrate. Moreover, Tiam1 deficiency impairs keratinocyte migration in vitro and reepithelialization of excision wounds in mouse skin. Our studies indicate that Tiam1 is a key molecule in α3β1-mediated activation of Rac, which is essential for proper production and secretion of LN5, a requirement for the spreading and migration of keratinocytes.

Introduction

Cell adhesion to the ECM plays a central role in development and morphogenesis (Geiger et al., 2001). The ECM regulates various biological processes, including the proliferation, differentiation, and apoptosis of cells. In addition, the ECM influences the remodeling of the cytoskeleton required for the adhesion, spreading, and migration of cells (Geiger et al., 2001). The importance of the ECM for cellular behavior is illustrated by the interaction of basal keratinocytes to underlying basement membrane, which consists predominantly of collagen IV (Col IV) and two laminin (LN) isoforms, LN5 and LN10. This interaction not only supports skin architecture but is also required for the growth, migration, and differentiation of keratinocytes. The major adhesive component in the basement membrane is LN5, which is produced and secreted by keratinocytes (Carter et al., 1991). Cells interact with the basement membrane through several receptors, including syndecans, dystroglycans, and integrins. In vivo and in vitro studies have both shown that the adhesion of basal keratinocytes to LN5 is mediated by two integrins: α3β1 and α6β4 (Georges-Labouesse et al., 1996; van der Neut et al., 1996; DiPersio et al., 2000). The α3β1 integrin is linked to the actin cytoskeleton (Hodivala-Dilke et al., 1998) and thereby regulates adhesion and spreading of keratinocytes on LN5 (Carter et al., 1991). Proteolytic processing of the precursor LN5 to a mature form is believed to provide a specific ligand for subsequent α6β4 binding (DeHart et al., 2003). The α6β4 integrin binds to LN5 in hemidesmosomes, structures which connect the ECM to the less flexible keratin intermediate filaments, anchoring the epidermal cells to the basal membrane and underlying dermis (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). The distinct functions of the α3β1 and α6β4 integrins are reflected in the different skin phenotypes of mice deficient for the α3 or α6 integrin subunits. Mice that lack α3β1 (DiPersio et al.,

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Abbreviations used in this paper: Col IV, collagen IV; ERK, extracellular-signal regulated kinase; FN, fibronectin; GEF, guanine nucleotide exchange factor; LN, laminin; NPAG, p-nitrophenyl *N*-acetyl-β-D-glucosaminide; PAK, p21-activated kinase; PLL, poly-L-lysine; Tiam1, T-lymphoma invasion and metastasis 1; Tiam1−/−, Tiam1-deficient; VN, vitronectin; WT, wild-type.

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1997) or α6β4 (Georges-Labouesse et al., 1996; van der Neut et al., 1996), and also those that are null for their ligand LN5 (Kuster et al., 1997; Ryan et al., 1999; Meng et al., 2003), die shortly after birth. The LN5- and α6β4-deficient mice display much more extensive epidermal detachment than the α3β1 knockout mice, which occasionally develop relatively small blisters only at sites subjected to mechanical stress. Apparently, in α3β1-deficient mice the attachment of the epidermis to LN5 can still be maintained by α6β4.

Small GTPases of the Rho family are involved in downstream signaling of a large number of growth factor receptors and adhesion molecules, including integrins. Integrin-induced activation of Rho proteins regulates many different processes, ranging from cell survival, adhesion, and spreading to the secretion of matrix proteins and their assembly into a basement membrane. The linkage of the ECM receptors to the actin cytoskeleton is crucial for cell matrix assembly (Bishop and Hall, 2000). Through Rho protein–mediated cytoskeletal remodeling and contraction, cells are able to remodel the ECM (Chiquet et al., 1996; Wierzbicka-Patynowski and Schwarzbauer, 2003). In fibroblasts, Rho and Rho kinase activation are necessary for the proper organization of a fibronectin (FN) matrix (Danen et al., 2002), whereas Rac has been implicated in LN1 assembly and in apical pole orientation in epithelial cells (O'Brien et al., 2001; DeHart et al., 2003).

Previously, we identified the Rac activator Tiam1 (T-lymphoma invasion and metastasis 1), which plays an important role in Rac-mediated E-cadherin–based cell–cell adhesions (Michiels et al., 1995; Hordijk et al., 1997; Sander et al., 1998; Malliri and Collard, 2003). We have investigated whether Tiam1 is involved in Rac-mediated cell-matrix signaling. We found that both wild-type (WT) and Tiam1-deficient (Tiam1−/−) keratinocytes adhere to and spread on various exogenous ECM components. However, Tiam1−/− keratiocytes are unable to spread properly on an inert glass substrate, on which these cells have to deposit their own LN5 substrate. Our studies identify Tiam1 as a key molecule in α3β1-mediated activation of Rac, which is essential for proper production and secretion of LN5, a requirement for spreading and migration of keratinocytes.

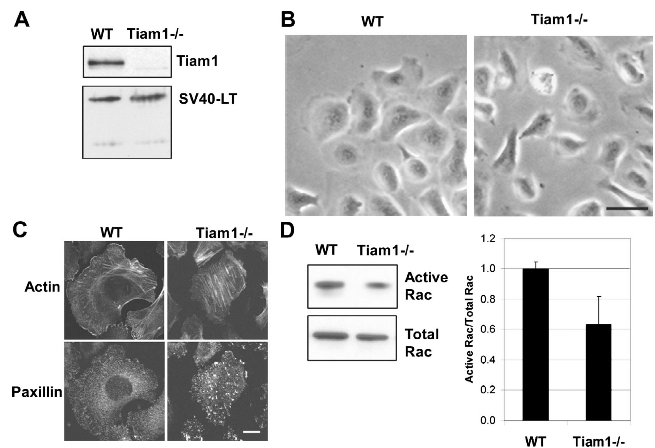

Figure 1. **Tiam1−/− keratinocytes spread less well on exogenous Col IV, show more stress fibers, larger focal adhesions, and less Rac activity than WT cells.** (A) Lysates were subjected to immunoprecipitation and analyzed for Tiam1 expression. SV40 LT expression was determined by immunoblotting the same lysates. (B) Cells were seeded on a Col IV matrix for 24 h. Bar, 20 μm. (C) Cells were seeded on Col IV–coated coverslips for 48 h, fixed, and stained for F-actin fibers (phalloidin) and focal adhesions (paxillin). Bar, 10 μm. (D) Cells were seeded on a Col IV matrix for 48 h and lysed, and Rac activity was determined. The histogram represents the average Rac activation (relative to the total Rac levels) in both WT and Tiam1−/− cells determined in four independent experiments. Error bars represent the SD.

Results

Tiam1 deficiency reduces keratinocyte spreading on a Col IV substrate

To investigate the role of Tiam1 in Rac-dependent adhesion and cell spreading, we isolated keratinocytes from WT and Tiam1−/− mice. The absence of Tiam1 in cells derived from the knockout mice was confirmed by immunoprecipitation (Fig. 1 A). Primary, as well as immortalized WT and Tiam1−/−, keratinocytes were used in further studies.

WT keratinocytes grow in colonies on a Col IV substrate (Fig. 1 B), whereas Tiam1−/− keratinocytes grow dispersed (Fig. 1 B and not depicted). In addition, WT keratinocytes spread ~30% more than Tiam1−/− cells and extended large lamellae with ruffles (Fig. 1, B and C). Actin staining revealed a fine network of filaments at the ridge of the lamellae and throughout the cell (Fig. 1 C). In contrast, in Tiam1−/− keratinocytes lamellae sprouted from the edges of cells but remained small, and the actin cytoskeleton was organized in thick and short actin stress fibers (Fig. 1 C).

F-actin stress fibers terminate at focal adhesion sites, at which integrins connect cells to the ECM. To visualize the distribution of the focal adhesions, cells were stained with a paxillin antibody. Consistent with the F-actin distribution, WT keratinocytes showed many small adhesion complexes and only few focal adhesions at the ends of actin cables (Fig. 1 C). In contrast, Tiam1−/− keratinocytes displayed fewer, but larger, focal adhesions at the end of stress fibers.

Because Rac activity is involved in the regulation of cell spreading, we analyzed the effect of Tiam1 deficiency on the level of Rac activity in the keratinocytes grown on a Col IV matrix. Consistent with the phenotypic differences on a Col IV substrate, RacGTP levels were reproducibly lower (30–50%) in Tiam1−/− than in WT keratinocytes (Fig. 1 D).

From these studies we concluded that Tiam1 deficiency leads to reduced basal Rac activity and reduced cell spreading of keratinocytes when seeded on a Col IV substrate. This reduction in cell spreading is accompanied by the appearance of a large number of thick focal adhesions and actin stress fibers and a decrease in lamellipodial extensions.

Tiam1 is essential for keratinocyte adhesion to, and spreading on, a glass surface

The capacity of WT and Tiam1−/− keratinocytes to adhere to a LN5-, FN-, vitronectin (VN)-, or Col IV-coated surface was not significantly different (Fig. 2, A–C), although spreading of

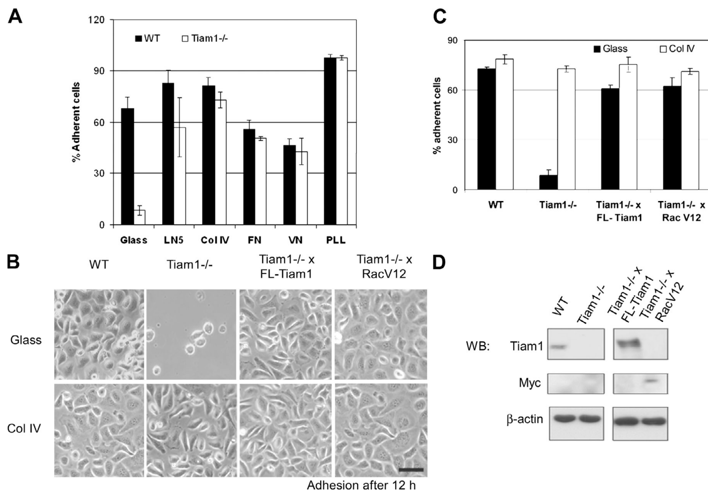

Figure 2. **Tiam1 is required for cell spreading when seeded on an inert surface.** (A) Wt and Tiam1−/− keratinocytes were seeded on glass coverslips or on LN5-, Col IV-, FN-, VN-, or PLL-coated coverslips. After 8 h, the number of adherent cells was quantified with an enzymatic assay using NPAG as a substrate. The values in the histogram are means ± SD. (B) Wt, Tiam1−/−, and Tiam1−/− keratinocytes stably expressing full-length Tiam1 or RacV12 were seeded for 12 h on glass coverslips and on coverslips coated with Col IV. Cells were washed and phase-contrast images were taken. Bar, 50 μm. (C) Cells were seeded as in B and the number of adherent cells was quantified with an enzymatic assay using NPAG as a substrate. The values in the histogram are means ± SEM ($n = 3$). (D) Cell lysates of WT, Tiam1−/−, and Tiam1−/− cells infected with full-length Tiam1 or RacV12 viruses were immunoblotted for Tiam1 and myc-tagged RacV12.

Tiam1−/− cells was consistently found to be slightly reduced (Fig. 1 C and not depicted). If no exogenous matrix is available, adhesion and spreading of keratinocytes depends on the ability of these cells to secrete and deposit their own LN5 matrix. To investigate how the loss of Tiam1 affects keratinocyte adhesion under such conditions, Tiam1−/− and WT cells were seeded on an inert glass surface. After 12 h, 75% of the WT cells had adhered to and spread on the glass. In contrast, only 5–10% of the Tiam1−/− keratinocytes had adhered to the glass (Fig. 2, B and C). The few adherent cells were rounded and refractile, as if they were blocked in an early stage of spreading (Fig. 2 B). Similar results were found when cells were seeded on plastic (not depicted).

To confirm that the observed defect in adhesion and spreading was because of the absence of Tiam1-mediated Rac activation, we introduced full-length Tiam1 and constitutively active Rac1 (V12 or L61 mutants) into Tiam1−/− keratinocytes. The moderate expression level of the transduced proteins was confirmed by Western blotting (Fig. 2 D). Adhesion assays revealed that both Tiam1 and V12Rac were able to rescue the adhesion and spreading defect of Tiam1−/− cells (Fig. 2, B and C).

We next investigated the pathways controlled by Rac that are responsible for the adhesion and spreading defect. We used effector loop mutants of GTPases previously shown to differentially bind and activate downstream effectors (Lamarche et al., 1996). The constitutively active L61Y40C mutant of Rac1 has lost its ability to interact with p21-activated kinase (PAK)–1 and is unable to activate c-Jun NH_2-terminal kinase activity, but it still induces F-actin polymerization and membrane ruffling. Conversely, the L61F37A mutant of Rac1 is unable to remodel the cytoskeleton, but interacts with $p65^{PAK}$ and activates c-Jun NH_2-terminal kinase. As shown in Fig. 3, the expression of the L61Y40C mutant of Rac1, but not that of the L61F37A mutant, strongly increased the number of Tiam1−/− cells adhering to and spreading on glass.

Together, these data indicate that Tiam1 is essential for spreading of keratinocytes after their initial adhesion to glass, on which these cells have to deposit their own LN5 matrix. The finding that both Tiam1 and constitutively active RacL61C40 could rescue the spreading defect of Tiam1−/− keratinocytes suggests that this defect is caused by impaired Tiam1-mediated Rac signaling toward the actin cytoskeleton.

Tiam1−/− keratinocytes do not migrate in a scrape wound-healing assay because of their inability to deposit LN5

Next, we studied the migration of WT and Tiam1−/− keratinocytes into a scrape wound, a process also dependent on the ability of keratinocytes to produce and secrete LN5. Confluent

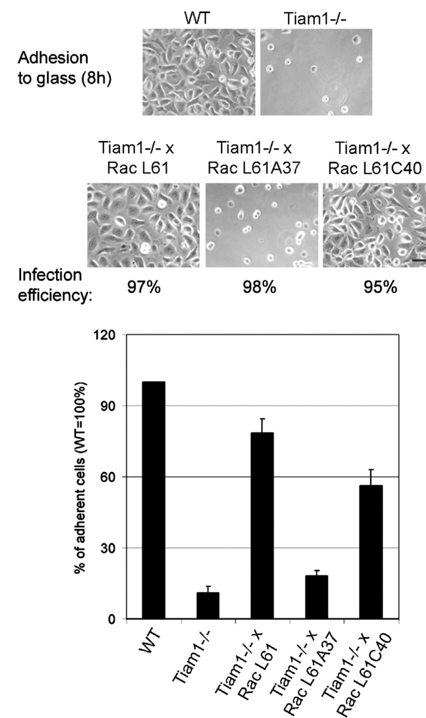

Figure 3. **Rescue of Tiam1 deficiency by Rac effector mutants.** Wt, Tiam1−/−, and Tiam1−/− keratinocytes expressing RacL61, RacL61A37, or RacL61C40 were seeded on glass coverslips. After 8 h, cells were washed and phase-contrast images were taken. Bar, 50 μm. The number of adherent cells was quantified in an enzymatic assay using NPAG as a substrate. The values in the histogram are means ± SD.

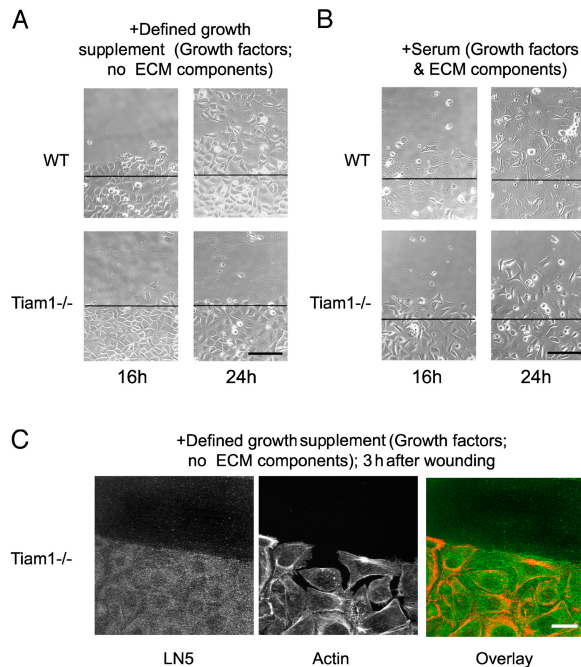

Figure 4. **Tiam1−/− keratinocytes and cell migration.** (A) Cells were seeded on Col IV–coated dishes and grown to confluency. The cultures were band stripped and allowed to migrate into the wounded area for 24 h in keratinocyte medium with defined growth supplement. Photographs show representative examples. The line indicates the wound edge at the start of the experiment (t = 0 h). Bar, 200 μm. (B) Cells were seeded and grown as described in A, band stripped, and allowed to migrate into the wounded area for 24 h in keratinocyte medium with defined growth supplement and 10% chelexed fetal calf serum. Photographs show representative examples. The line indicates the wound edge at the start of the experiment (t = 0 h). Bar, 200 μm. (C) Tiam1−/− keratinocytes were seeded on Col IV–coated dishes and grown to confluency. The cultures were band stripped and allowed to migrate into the wounded area for 3 h in keratinocyte medium with defined growth supplement. Cells were fixed and stained for F-actin and the LN5 γ2 chain. Bar, 40 μm. Note the removal of the LN5 substrate by scratching.

monolayers of WT and Tiam1−/− keratinocytes, cultured on Col IV–coated surfaces in keratinocyte medium with defined growth factors but without ECM components, were scrape wounded and the migration of keratinocytes was investigated. As expected, WT keratinocytes migrated into the denuded area and closed the wound within 24 h (Fig. 4 A). In contrast, Tiam1−/− keratinocytes did not migrate into the wound (Fig. 4, A and C), where the LN5 and Col IV coating was removed by scraping (Fig. 4 C). However, when ECM components were provided by the addition of chelated fetal calf serum to the medium after scraping, the Tiam1−/− cells did migrate, albeit less efficiently than WT keratinocytes (Fig. 4 B). These data are consistent with our earlier conclusion that Tiam1−/− keratinocytes are unable to produce and secrete sufficient amounts of LN5 substrate, resulting in their inability to spread and migrate onto an uncoated surface.

Expression and function of the α3β1 integrin in Tiam1−/− keratinocytes

It has been well established that the α3β1 integrin, and not the α6β4 integrin, is essential for cell spreading on LN5. Keratinocytes expressing α6β4 that were isolated from α3-null mice retained their ability to attach to LN5, but did not spread properly (DiPersio et al., 1997; Hodivala-Dilke et al., 1998). This suggests that Tiam1 acts downstream of α3β1 rather than of α6β4. Indeed, α3-deficient cells contain more thick actin bundles than WT keratinocytes and display robust peripheral focal adhesions (Hodivala-Dilke et al., 1998). Both phenotypes are also found in Tiam1−/− keratinocytes, suggesting a role for Tiam1 in α3β1-mediated adhesion and signaling, rather than in the mechanisms of α6β4. To further substantiate this hypothesis, WT and Tiam1−/− keratinocytes were seeded on LN1, which is a ligand for α6β4, but not for α3β1 (Delwel et al., 1993; Rousselle and Aumailley, 1994). As expected, both WT and Tiam1−/− keratinocytes adhered to LN1, but they were unable to spread on this substrate (Fig. 5 A). In general, Tiam1−/− cells adhered even better to LN1 than WT cells, indicating that adhesion through α6β4 is not impaired in Tiam1−/− cells (Fig. 5 A). In accordance with the notion that adhesion of keratinocytes to LN5 is mediated by both α3β1 and α6β4, both genotypes adhered much better to LN5 than to LN1 (Fig. 5 A). The cells spread on LN5 using α3β1, although the spreading of the Tiam1−/− keratinocytes was still reduced when compared with that of WT keratinocytes. Consistent with the adhesion results, Western blotting and FACS analysis revealed a similar level of expression of the α3 and β1 integrin subunits in the keratinocytes of both genotypes (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509172/DC1). In addition, no differences were found in the expression level of the α6β4 integrin in WT and Tiam1−/− cells, although a proportion of the WT cells lacked α6β4. These results confirm that the observed defects in adhesion and spreading of Tiam1−/− cells are not caused by changes in the expression of the LN5-binding integrins.

Analysis of signaling pathways activated by α3β1

Next, we investigated which α3β1-regulated pathways could be involved in the regulation of cell spreading on an exogenous LN5 matrix deposited by Rac-11P cells (Delwel et al., 1993). Because integrins can modulate growth factor signaling (for review see Damsky and Ilic, 2002), the experiments were performed in the absence of growth factors. WT and Tiam1−/− keratinocytes were seeded on an exogenous LN5 matrix for various periods of time and cell lysates were subsequently analyzed using phosphorylation-specific antibodies. In both WT and Tiam1−/− keratinocytes, the FAK was rapidly phosphorylated after adhesion to the LN5 matrix (Fig. 5 B). In addition, other downstream targets of integrin signaling such as Src, extracellular-signal regulated kinase (ERK)1/2, and Stat3 were equally well phosphorylated upon adhesion to exogenous LN5 in WT and Tiam1−/− keratinocytes (Fig. 5 C), suggesting that interference with these pathways was not responsible for the differences in LN5 deposition.

We then studied the activation of Rac upon adhesion of keratinocytes to exogenous LN5 substrate. In WT cells, a small increase in active GTP-bound Rac could be measured 5 min after seeding, and this increase was much more pronounced after

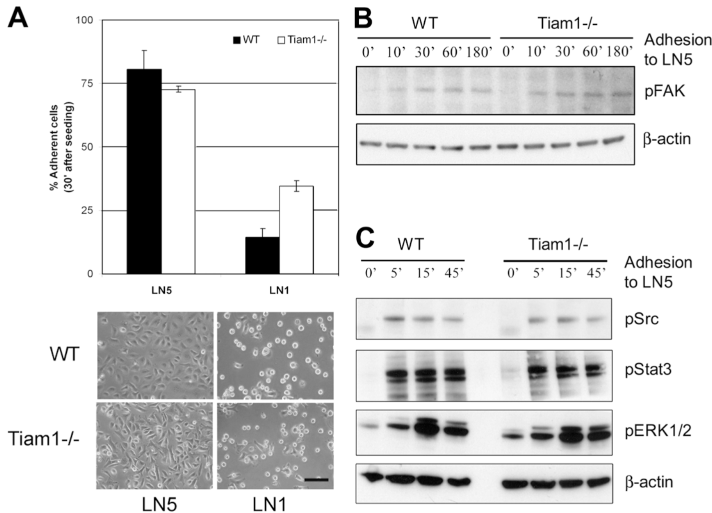

Figure 5. **Adhesion and signaling of WT and Tiam1−/− keratinocytes.** (A) Keratinocytes were seeded on an exogenous LN5 or LN1 matrix. After 30 min, the number of adherent cells was quantified in an enzymatic assay. Phase-contrast images were taken before cell lysis. The values in the histogram are means ± SD. Note that both WT and Tiam1−/− keratinocytes hardly adhere to and spread on LN1. Bar, 50 μm. (B) Growth factor–starved WT and Tiam1−/− keratinocytes were detached from the culture dish and reseeded on a LN5-coated surface in growth factor–free medium. A sample of the cells in suspension was lysed and the attached cells were lysed after 10, 30, 60, and 180 min. Lysates were immunoblotted for phosphoY397-FAK. β-Actin was used as a loading control. (C) Cells seeded as described in B were lysed after 5, 15, and 45 min. Lysates were immunoblotted for phospho-Src, -Stat3, and -ERK1/2. β-Actin was used as a loading control.

30 min (Fig. 6 A). Rac activity remained elevated for at least 1 h and returned to basal levels within 3 h (Fig. 6 B). In contrast, in Tiam1−/− keratinocytes we did not detect any Rac activation upon adhesion to LN5 after 5 and 30 min, or even after 3 h (Fig. 6, A and B), indicating that α3β1-mediated Rac signaling is impaired in Tiam1−/− cells.

In addition, we analyzed the activation of Rac after seeding keratinocytes on other exogenous substrates, such as FN-, VN-, or Col IV–coated surfaces, on which cells with either genotype adhered equally well (Fig. 2 A). On all other substrates tested, the degree of activation of Rac in Tiam1−/− keratinocytes was comparable to that in WT cells 1 h after adhesion (Fig. 6 C). Moreover, we determined the Rac activation upon adhesion to Col IV, poly-L-lysine (PLL), and FN at different time points and again found no differences between WT and Tiam1−/− cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200509172/DC1). This indicates that the moderate activation of Rac as a result of adhesion to FN, VN, or Col IV is independent of Tiam1 and is most likely regulated by other Rac–guanine nucleotide exchange factors (GEFs).

Although Tiam1−/− keratinocytes spread less well on exogenous LN5 matrix than WT cells (Fig. 7 A), they are able to spread without any detectable change in Rac activity. Earlier studies have demonstrated that cross talk exists between Rac and Rho GTPases, and that changes in the balance between the activities of these proteins can influence cell morphology (van Leeuwen et al., 1997; Kodama et al., 1999; Sander et al., 1999). Therefore, we hypothesized that spreading of Tiam1−/− keratinocytes might be regulated by a change in Rho activity, rather than Rac activity. In WT cells, a small decrease (10–15%) in Rho activity could be detected 5, 30, and 60 min after seeding on LN5 (Fig. 7, B and C). However, in Tiam1−/− cells a much larger decrease in Rho activity was found 5, 30, and 60 min after seeding (45, 55, and 60%, respectively). In both WT and Tiam1−/− cells, the decrease in Rho activity was observed during at least 1 h and returned to basal levels within 3 h (Fig. 7, B and C). This suggests that spreading of Tiam1−/− keratinocytes is caused by a substantial decrease in Rho activity, which leads to cytoskeletal relaxation. In this manner, in

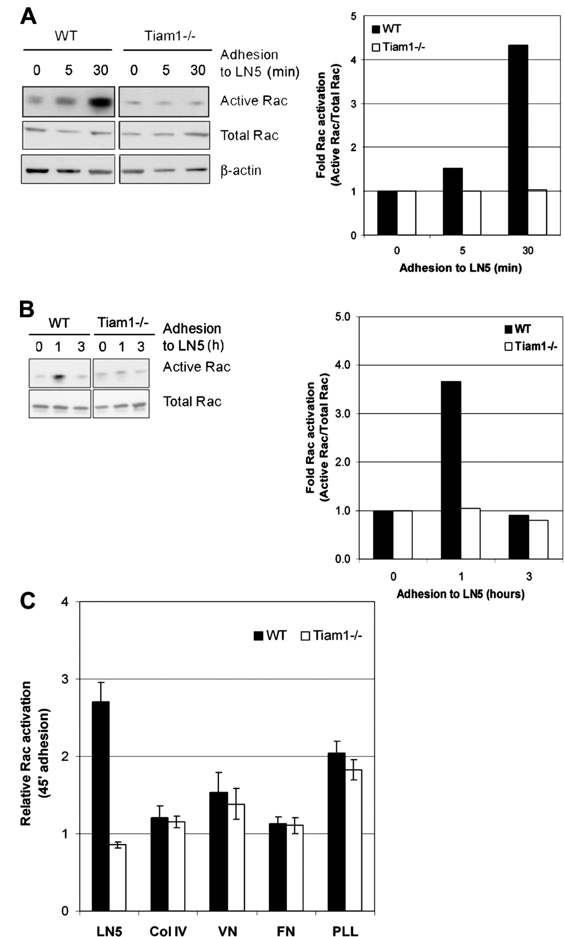

Figure 6. **Tiam1 is required for integrin induced Rac activation upon adhesion to LN5.** (A) Growth factor–starved keratinocytes were seeded on a LN5 matrix. After 0, 5, and 30 min, cells were lysed and Rac activity was determined. A representative experiment is shown and quantified in the histogram, which represents the Rac activation (relative to the total Rac levels) in WT and Tiam1−/− cells. (B) Growth factor–starved keratinocytes were seeded on a LN5 matrix. After 0, 1, and 3 h, cells were lysed and Rac activity was determined and quantified as described in A. (C) Growth factor–starved keratinocytes were seeded on LN5-, FN-, VN-, or PLL-coated plates for 45 min. Subsequently, cells were lysed, and Rac activity was determined. The histogram represents the average Rac activity after 45 min in both WT and Tiam1−/− cells determined in three independent experiments. Error bars represent the SD.

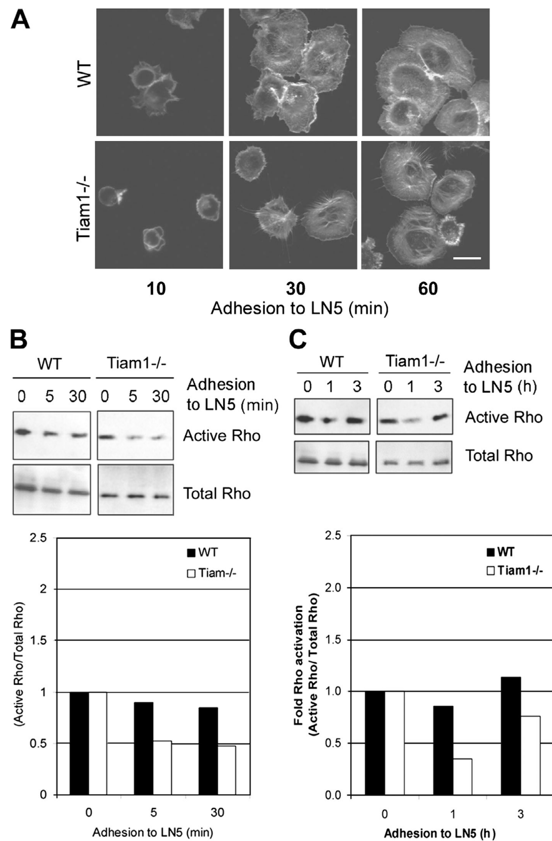

Figure 7. **Rho activity upon adhesion to LN5.** (A) Wt and Tiam1−/− keratinocytes were seeded on LN5-coated coverslips. After 10, 30, and 60 min, cells were stained with phalloidin. Bar, 20 μM. (B) Growth factor–starved keratinocytes were seeded on a LN5 matrix. After 0, 5, and 30 min, cells were lysed and Rho activity was determined. A representative experiment is shown and quantified in the histogram, which represents the Rho activation (relative to the total Rho levels) in WT and Tiam1−/− cells. (C) Growth factor–starved keratinocytes were seeded on a LN5 matrix as described in B. After 0, 1, and 3 h, cells were lysed and Rho activity was determined and quantified as described in B.

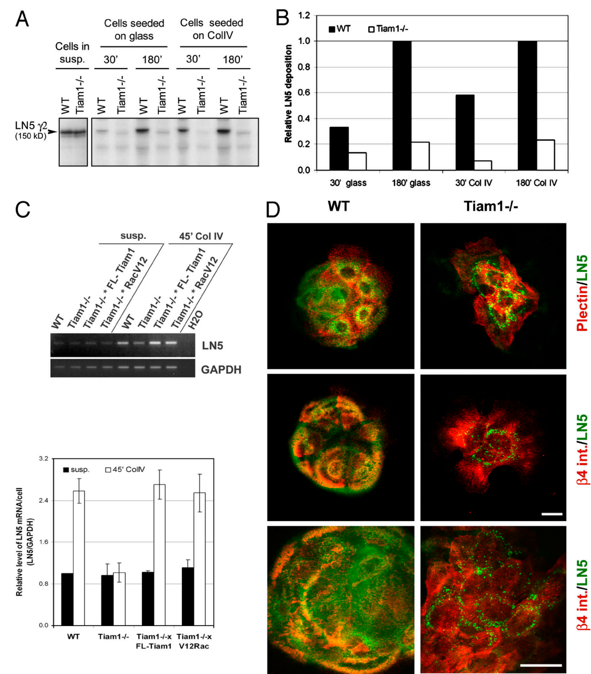

Figure 8. **Production and secretion of LN5 by WT and Tiam1−/− keratinocytes.** (A) Suspended WT and Tiam1−/− keratinocytes were seeded on a glass surface or on a Col IV–coated surface. After 30 or 180 min, attached cells were detached with 10 mM EDTA and secreted LN5 was detached from the surface with SDS–sample buffer. As a control, suspended cells were lysed. The samples were subjected to Western blotting and probed for LN5 (γ2 chain). (B) The amounts of secreted LN5 (γ2 chain) levels in the Western blot shown in A were quantified relative to levels in the 180-min WT sample (=100%) with Image J. (C) Suspended WT, Tiam1−/−, and Tiam1−/− keratinocytes stably expressing full-length Tiam1 or RacV12 were seeded on a Col IV–coated surface. A sample of the cells in suspension was used for mRNA isolation. After 45 min, adherent cells were detached from the surface and lysed for mRNA isolation. An RT-PCR reaction was performed to determine the amount of LN5 γ2 chain mRNA present in the samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control. The histogram represents the increase in LN5 mRNA levels in both WT and Tiam1−/− cells determined in two independent experiments. Error bars represent the SD. (D) Cells were seeded on Col IV–coated coverslips for 16 h, fixed, and stained for plectin, the integrin β4 subunit, and the LN5 γ2 chain. Note the accumulation of LN5-containing vesicles in the Tiam1−/− cells. Bars, 20 μm.

Tiam1−/− keratinocytes Rac activity can be relatively increased, as compared with Rho activity, upon adhesion to exogenous LN5, thereby allowing cell spreading.

Tiam1-Rac signaling regulates deposition of a LN5 matrix

Spreading of keratinocytes on glass requires the secretion and deposition of LN5 for initial adhesion. Keratinocytes bind to LN5 through the α3β1 integrin and increase their LN5 production and secretion during cell spreading. We first analyzed whether a lack of Tiam1 affected the intrinsic capacity of keratinocytes to produce LN5. In the absence of adhesive stimuli (suspended cells), an equal amount of protein (Fig. 8 A) and mRNA (Fig. 8 C) of LN5 (γ2 chain subunit) was found in WT and Tiam1−/− keratinocytes, indicating that the loss of Tiam1 expression did not affect the transcription and translation of LN5. We also analyzed the requirement of Tiam1 for the stimulation of LN5 production and secretion after initial adhesion. WT and Tiam1−/− keratinocytes were seeded on glass and the secreted matrix was scraped off the plates, size-separated by SDS-PAGE, and immunoblotted with a γ2-specific antibody. As shown in Fig. 8 (A and B), the amount of LN5 secreted by Tiam1−/− cells on glass for 30 and 180 min was strongly reduced as compared with WT cells. We also seeded these cells on an exogenous Col IV substrate to allow cell spreading. In WT cells, a consistent increase in the amount of LN5 mRNA (Fig. 8 C) was found after adhesion to Col IV. In contrast, no increase in LN5 mRNA was detected in Tiam1−/− keratinocytes (Fig. 8 C). Moreover, the amount of secreted LN5 on a Col IV matrix was strongly reduced in Tiam1−/− cells similarly as found when seeding these cells on glass (Fig. 8, A and B). To confirm that the increase in LN5 mRNA levels was because of Tiam1-mediated Rac activation, we analyzed Tiam1−/− keratinocytes that were reconstituted with Tiam1 and constitutive active V12Rac1 (Fig. 2). RT-PCR analysis revealed that both Tiam1 and V12Rac were able to rescue the increase in LN5 mRNA in Tiam1−/− cells to the level of WT cells (Fig. 8 C).

Together these findings indicate that Tiam1-mediated Rac activation is required for the increase in levels of LN5 mRNA and protein upon adhesion of keratinocytes to an exogenous substrate. In addition, our data show that the LN5 secretion of Tiam1−/− keratinocytes upon adhesion to both glass and Col IV is impaired when compared with WT cells. Indeed, immunohistological analysis of WT keratinocytes with LN5 antibodies (γ2 chain subunit) revealed deposition of LN5 into regular archlike structures, partially colocalized with the integrin β4 subunit. In contrast, in Tiam1−/− cells most of the LN5 was retained in vesicles and only a small amount of secreted LN5 could be detected (Fig. 8 D). These data are consistent with the differences found in LN5 production and secretion using biochemical methods (Fig. 8, A–C). Together our findings suggest that Tiam1-Rac signaling regulates the increase in LN5 mRNA and protein levels, as well as the secretion of LN5. These processes are both essential for proper LN5 deposition and spreading of keratinocytes.

Wound repair is impaired in Tiam1−/− mice

In mouse skin, Tiam1 is strongly expressed in basal and suprabasal keratinocytes of the interfollicular epidermis and in hair follicles. Tiam1−/− mice show no major skin phenotype, but are resistant to Ras-induced skin tumors (Malliri et al., 2002). Because of the effect of Tiam1 deficiency in LN5 production and secretion in keratinocytes, we wondered whether defects in the skin of Tiam1−/− mice could be found by a more detailed analysis of intact and wounded skin. Analysis of intact skin revealed no differences in the deposition and processing of LN5, the expression of the α3 and β4 integrins, and the components of the basal lamina between WT and Tiam1−/− mice (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200509172/DC1, and not depicted). Subsequently, we generated full-thickness excision wounds in the skin of WT and Tiam1−/− mice, and wound closure was monitored over time. The repair of such excision wounds is achieved by clot formation followed by extensive migration and hyperproliferation of keratinocytes at the wound rim, which is partly dependent on LN5 secretion. Immunological and histological analysis revealed that the closure of the wound margins was significantly delayed in the Tiam1−/− mice when compared with WT mice (Fig. 9). The reepithelialization process was completed in ∼5–6 d in both WT and Tiam1−/− mice. Although these data do not exclude the possibility that Tiam1 may also influence wound repair by other mechanisms, the delayed reepithelialization of the wounds in the skin of Tiam1−/− mice supports our in vitro findings that Tiam1 plays a role in LN5 deposition that is required for the spreading and migration of keratinocytes.

Discussion

Tiam1-Rac signaling has been shown to control various processes in epithelial cells, such as cell migration, by affecting E-cadherin–based adhesions (Sander and Collard, 1999; Malliri and Collard, 2003; Minard et al., 2004), and cell polarization, by affecting tight junction biogenesis (Mertens et al., 2005).

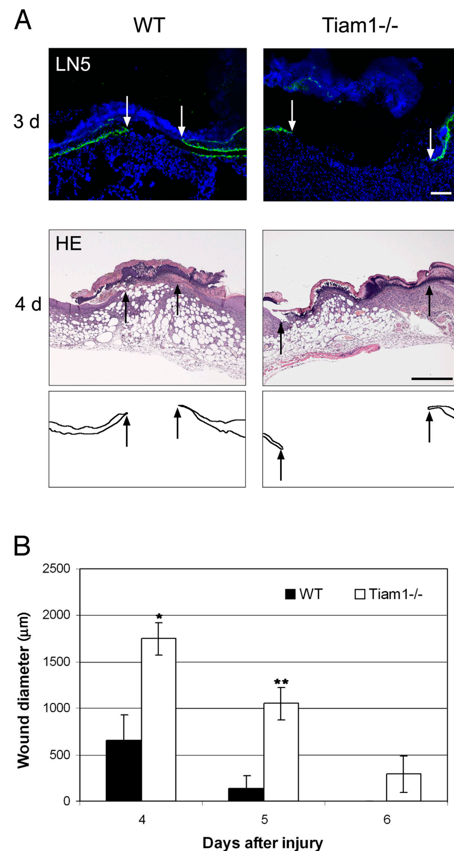

Figure 9. **Tiam1 deficiency delays wound repair in vivo.** (A) Examples of cross sections from 3-d-old wounds from WT and Tiam1−/− mice stained for LN5 (top) and of cross sections from paraffin-embedded 4-d-old wounds stained with hematoxylin and eosin (HE; bottom). Arrows and drawings indicate the position of the advancing epithelial edges. Bar, 250 µm. (B) Histograms of wound diameters (in micrometers ± SEM) measured from 12 wounds in the skin of three mice per group at each time point. *, $P < 0.01$; **, $P < 0.005$.

We have investigated the role of Tiam1-Rac signaling in the adhesion and spreading of mouse keratinocytes. We found that Tiam1−/− keratinocytes adhere to and spread on various exogenous cell substrates, but do not spread on uncoated surfaces such as glass, on which these cells have to make their own LN5 substrate. Both Tiam1 and V12Rac1 can rescue the adhesion and spreading defect of Tiam1−/− keratinocytes on glass, indicating that this defect is the result of impaired Rac signaling. Our studies indicate that Tiam1 is a key molecule in α3β1-mediated activation of Rac, which is required for LN5 production and secretion. We propose that Tiam1 regulates α3β1-mediated Rac activation upon initial adhesion, which controls actin remodeling and increased LN5 production and secretion, which are all necessary for the spreading and migration of keratinocytes (Fig. 10).

In contrast to WT cells, Tiam1−/− keratinocytes were unable to activate Rac in response to interaction with an exogenous LN5 substrate, suggesting that Tiam1 is required for LN5 signaling toward Rac. No differences in Rac activation were found between WT and Tiam1−/− keratinocytes upon seeding these cells on other exogenous substrates such as FN, VN, or Col IV, suggesting that other RacGEFs are used upon adhesion

Figure 10. **Model of the function of Tiam1 in keratinocyte adhesion to and spreading on an inert substrate.** (1) Upon touching the surface, WT cells secrete small amounts of LN5. (2) The cells bind the LN5 with their LN5 integrin (α3β1) and signal to Tiam1, which is necessary for Rac activation and actin remodeling. (3) Tiam1-mediated Rac activation and actin polymerization are required for increased LN5 production, LN5 vesicle secretion, and cell spreading. (1′) Tiam1−/− cells secrete small amounts of LN5 upon touching an inert surface. (2′) Cells fail to activate Rac through the α3β1 integrin as a result of Tiam1 deficiency. (3′) Consequently, Tiam1−/− cells are unable to produce and secrete proper amounts of additional LN5 and, therefore, cell spreading and cell migration is impaired.

to these substrates. Intriguingly, in Tiam1−/− cells we found a significant reduction in Rho activity during spreading on exogenous LN5, suggesting that Tiam1−/− cells compensate for their inability to increase Rac activity by decreasing Rho activity, thereby allowing cell spreading. These observations are consistent with earlier ones, which showed that after initial adhesion Rac and Cdc42 are activated and/or Rho is downregulated, leading to actin polymerization and cell spreading (Price et al., 1998; Ren et al., 1999). Moreover, the results are in accordance with data showing that the balance between Rac and Rho activity determines cellular properties such as the strength of cell–cell adhesions, fibroblast migration, and neurite outgrowth (van Leeuwen et al., 1997; Kodama et al., 1999; Sander et al., 1999).

Keratinocytes can bind to LN5 via two integrins, α3β1 and α6β4, and several studies have addressed the mechanism by which keratinocytes adhere to LN5 in vitro. The α3β1 integrin links the ECM to the actin cytoskeleton (Hodivala-Dilke et al., 1998) and thereby triggers adhesion and spreading of keratinocytes (Carter et al., 1991). The α6β4 integrin mediates a long-term stable adhesion by inducing hemidesmosome assembly (Borradori and Sonnenberg, 1999). Keratinocytes isolated from α3-null epidermis (expressing α6β4) retained the ability to attach to LN5, but did not spread properly (DiPersio et al., 1997), indicating that α3β1 and α6β4 have distinct but overlapping functions in keratinocytes. Both integrins can support initial cell attachment to LN5, but α3β1 is required for subsequent cell spreading. Consistent with this conclusion are the findings that the spreading defect on LN5 in β1-deficient cells cannot be rescued by expression of the α6β4 integrin (Kikkawa et al., 2004). We found that Tiam1−/− keratinocytes spread less well on several matrix components. On glass, where keratinocytes have to deposit their own LN5 matrix, initial adhesion occurred, but the subsequent spreading was impaired, suggesting that Tiam1 is required for α3β1-mediated cell spreading. Indeed, no major differences in adhesion between WT and Tiam1−/− cells were found on an exogenous LN1 substrate, for which keratinocytes use the α6β4 integrin only. A role for Tiam1 in α3β1 signaling is further supported by the observation that Tiam1−/− keratinocytes display several defects that are also observed in α3-deficient keratinocytes. Experiments with WT and α3-null keratinocytes showed that both spread on FN. However, WT cells spread approximately two times better and displayed typical, well-extended lamellipodia, whereas α3-null keratinocytes exhibited fewer and smaller lamellipodia (Hodivala-Dilke et al., 1998). This is consistent with the phenotype of the Tiam1−/− keratinocytes on various substrates including Col IV and LN5. In α3-null keratinocytes, cells display thick actin stress fibers and robust peripheral focal adhesions (DiPersio et al., 1997; Hodivala-Dilke et al., 1998), similar to those found in Tiam1−/− keratinocytes.

Cell spreading is initiated by integrin-mediated cell-matrix interaction and requires the activation of multiple signaling pathways including Rac (for review see Schwartz and Shattil, 2000). More than 60 GEFs for Rho GTPases have been identified (Etienne-Manneville and Hall, 2002) but only a few have been implicated in integrin-mediated Rac signaling. Vav1, which is expressed in hematopoietic cells only, was shown to be essential for the spreading of T cells. The closely related GEF Vav2 is widely distributed and has been implicated in the spreading of fibroblasts (Marignani and Carpenter, 2001). An alternative pathway from β1 integrin−ECM ligands to Rac activation has been proposed in human lung adenocarcinoma cells (Gu et al., 2001). This pathway depends on FAK/Cas/Crk-mediated activation of the RacGEF DOCK180 upon adhesion to LN10/11 via α3β1. This might indicate that different

cells are using different GEFs in $\alpha3\beta1$ signaling toward Rac (i.e., DOCK180 or Tiam1). Alternatively, tumor cells might have acquired additional means to activate Rac. In our study, we demonstrate that keratinocytes, which lack the RacGEF Tiam1, have a defect in $\alpha3\beta1$-mediated Rac activation and cell spreading, as a result of decreased LN5 production and secretion.

The in vitro data on adhesion, spreading, and migration of keratinocytes and the results in vivo in skins of WT and Tiam1−/− mice resemble the data on the cells and skin of mice with a null mutation for the α3 integrin subunit (Itga3; DiPersio et al., 1997), although the phenotype of the Tiam1−/− mice is less severe. The $\alpha3\beta1$-deficient mice die shortly after birth because of defects in kidney and lung organogenesis (Kreidberg et al., 1996). However, in contrast to $\alpha6\beta4$ knockout mice that show extensive skin blistering (Georges-Labouesse et al., 1996; van der Neut et al., 1996; DiPersio et al., 2000), the skin of α3 knockout mice develops normally. In these mice, regions were occasionally observed in which the LN matrix was deposited in a disorganized manner, which caused microblistering at sites of the body susceptible to high mechanical stress, such as the feet (DiPersio et al., 1997; Hodivala-Dilke et al., 1998). We did not find that targeted deletion of Tiam1 affects development of the epidermis and its adhesion to the basement membrane. Most likely, various mechanisms are active in vivo that may compensate for the loss of Tiam1. However, we found that Tiam1 expression is important for the reepithelialization of full-thickness excision wounds in the mouse skin. Specifically, Tiam1 deficiency leads to a significant delay in wound closure of the epidermis. This is consistent with the delay in wound closure of Tiam1−/− versus WT keratinocytes in "scratch wound assays" in vitro. It has been well established that migration and proliferation of keratinocytes is crucial for reepithelialization of cutaneous wounds. Recently, a study in mice with an epidermis-specific knockout of β1 integrins showed that cutaneous wounds failed to heal properly owing to a defect in the initiation of cell migration (Grose et al., 2002). Although β1 integrins interact with several potential integrin ligands, the interaction between $\alpha3\beta1$ and LN5 is of particular importance in the impaired keratinocyte migration in K5β1-null mice (Grose et al., 2002). Moreover, other studies have shown that expression of LN5 is required for the reepithelialization of cutaneous wounds (Ryan et al., 1999; Nguyen et al., 2000b) and that the $\alpha3\beta1$ integrin is an important player in the regulation of both basement membrane assembly and cutaneous wound repair (DiPersio et al., 1997; Hodivala-Dilke et al., 1998; Nguyen et al., 2000a,b; Choma et al., 2004). In light of the presented properties of Tiam1, with respect to LN5 secretion, adhesion, and impaired migration of epithelial cells in vitro, it is reasonable to assume that Tiam1 plays a similar role in epidermal wound closure in vivo, although we cannot exclude that Tiam1 may contribute to wound repair by other mechanisms as well.

In summary, our data indicate that Tiam1 is an essential GEF involved in the $\alpha3\beta1$-mediated activation of Rac upon adhesion of keratinocytes to LN5. The Tiam1-mediated Rac activation regulates the further production, secretion, and organized deposition of LN5, which is essential for the spreading and migration of keratinocytes.

Materials and methods

Keratinocyte isolation and immortalization

Primary keratinocytes were isolated from newborn WT or Tiam1−/− mice (Malliri et al., 2002) and cultured in medium containing 0.02 mM $CaCl_2$, using established procedures (Hennings et al., 1980). Dermis and epidermis were separated overnight by trypsin treatment (Invitrogen) and minced in Epilife minimal medium (Cascade Biologics, Inc.), and cells were detached by gently stirring in Erlenmeyer flasks. Cell suspensions were filtered and distributed in Col IV–coated dishes. Isolated keratinocytes were cultured in Epilife keratinocyte medium supplemented with 0.02 mM $CaCl_2$ Epilife defined growth supplement (both Sigma-Aldrich) and 100 IU/ml of penicillin/streptomycin.

To obtain immortalized cells, WT and Tiam1−/− keratinocytes were transduced with supernatant containing pBabe puro SV40 LargeT (LT) antigen viruses. Expression levels of the SV40 LT antigen in WT and Tiam1−/− cells were determined by Western blot analysis (Mertens et al., 2005).

Gene transfer into keratinocytes by retroviral transduction

SV40 LT antigen and the Rac mutants (a gift from L. van Aelst, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were cloned into the LZRS-IRES-neo retroviral vector, whereas the Tiam1 coding sequence was cloned into the LZRS-IRES-blasticidin vector (Michiels et al., 2000). Retroviral constructs were transfected into Phoenix ecotropic packaging cells, and fresh viral supernatants were collected and used for infections, as previously described (Michiels et al., 2000).

Coating of dishes with ECM molecules

All ECM proteins except LN5 were coated to culturing dishes overnight at 4°C at the following concentrations: 10 μg/ml FN (isolated from human plasma); 10 μg/ml LN1 (Becton Dickinson); 10 μg/ml VN (Sigma-Aldrich); 20 μg/ml Col I (Vitrogen/Nutacon); and 25 μg/ml Col IV (Becton Dickinson). A LN5 matrix was obtained by culturing Rac-11P cells to confluency (Delwel et al., 1993), after which cells were detached with 10 mM EDTA in PBS containing a mix of protease inhibitors (Complete protease inhibitor cocktail tablets; Roche) at 4°C. Before use, the dishes were washed twice with PBS.

Cell culture

Keratinocytes were grown on a Col IV substrate and maintained in Epilife keratinocyte medium. For experiments, cells were used at a density of 70–80% confluency. NIH 3T3 and Rac-11P cells were cultured in DME supplemented with 10% bovine calf serum. Cells were seeded 24 h before use to obtain a final density of 70% (NIH 3T3) and 100% (Rac-11P), respectively.

Immunoprecipitation and Western blotting

For immunoprecipitation, cells grown in 10-cm Ø dishes were lysed in 1 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, and protease inhibitors. Extracts were clarified by centrifugation and precleared with GammaBind Sepharose beads (GE Healthcare). Precleared lysates were incubated with anti-Tiam1 antibody (C16; Santa Cruz Biotechnology, Inc.) and immune complexes were precipitated using GammaBind Sepharose. After overnight incubation, beads were washed three times and resuspended in SDS–sample buffer.

For Western blotting, cell lysates or samples of precipitated proteins were boiled for 5 min and resolved by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories), blocked with BSA or skimmed milk, and probed using the indicated antibodies. Specific binding was detected using a secondary peroxidase-conjugated antibody (GE Healthcare) followed by chemiluminescence. Anti-Rac1 monoclonal antibody was obtained from Upstate Biotechnology. Activated FAK, ERK1/2, Src, and Stat3 were detected by anti–phospho-FAK (Y397; Biosource), anti–phospho-ERK1/2 (New England Biolabs, Inc.), anti–phospho-Src (Y418; Biosource International), and anti–phospho-Stat3 (Y705; Cell Signaling Technology), respectively. Polyclonal anti-Tiam1 (DH) has been previously described (Habets et al., 1994). Anti–LN γ2 (1109) was a gift from T. Sasaki (Max Planck Institute for Biochemistry, Martinsried, Germany). Anti–β-actin was purchased from Sigma-Aldrich. Anti-Tiam1 (C16), anti-Rho (26C4), anti-myc, and anti–SV40 LT were obtained from Santa Cruz Biotechnology, Inc.

Rac and Rho activity assays

GTPase activity was assayed essentially as previously described (Sander et al., 1999). In brief, after the adhesion of cells to a relevant surface,

cells were washed and lysed with a 1% Nonidet P-40 buffer containing either 2 μg/ml PAK-CRIB peptide (Price et al., 2003) or GST-Rhotekin (Sander et al., 1999). Cell lysates were sheared though an insulin needle and cleared by centrifugation. Active complexes were precipitated with streptavidin–agarose beads (Rac-PAK-CRIB; Sigma-Aldrich) or with glutathione beads (Rho-GST-Rhotekin; Sigma-Aldrich) and solubilized in SDS–sample buffer. Rac and Rho were detected by Western blotting. Anti-Rac1 monoclonal antibody was purchased from Upstate Biotechnology and anti-Rho (26C4) was obtained from Santa Cruz Biotechnology, Inc.

Wound repair in vivo

Adult mice were anesthetized, shaved, and two full-thickness excision wounds (4 mm in diameter) were cut with small scissors on either side of the dorsal midline of each mouse. The four wounds per mouse were left uncovered. For histological and immunofluorescence analysis, the complete wounds including surrounding tissue (~8 mm) of adjacent normal skin were excised at 3, 4, 5, and 6 d after injury. Cryo- and paraffin sections across the middle of the wounds were stained: the cryosections with LN γ2 (1109) and DAPI, and the paraffin sections with hematoxylin and eosin. For each time point, wound diameters (distance between epithelial rims) were determined in three mice.

Phase-contrast microscopy and confocal microscopy

For phase-contrast microscopy, cells were seeded for 24 h onto either plastic coated with LN5 or Col IV or onto glass coverslips, viewed under a microscope (model Axiovert 25; Carl Zeiss MicroImaging, Inc.), and photographed. For immunofluorescence staining, keratinocytes were seeded on glass coverslips, which were either uncoated or coated with Col IV or LN5. After 16 h, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 2% BSA in PBS. Filamentous actin was labeled with 0.2 μM Alexa Fluor 568–phalloidin (Invitrogen) for 30 min. The following primary antibodies were used: a mouse monoclonal against paxillin (BD Biosciences) and plectin (HD1; a gift from K. Owaribe, Nagoya University, Nagoya, Japan); rabbit antibody against mouse LN γ2 (1109); and rat monoclonal antibody against integrin β4 (346-11A; BD Biosciences). Primary antibodies were visualized with appropriate FITC- or Alexa Fluor 568–labeled secondary antibodies (Zymed Laboratories). Images were collected by confocal microscopy (model TCS NT; Leica).

Adhesion assays

24-well microplates were coated overnight at 4°C with 25 μg/ml Col IV or LN5, which were secreted by Rac-11P cells (as described in Coating of dishes with ECM molecules). Plates were washed with PBS and saturated with 1% (wt/vol) BSA (Sigma-Aldrich), for 2 h at 37°C to block nonspecific adhesion. Keratinocytes were detached with EDTA and suspended in supplement-free keratinocyte medium. 6×10^4 cells/well were plated in triplicate and incubated for 6–12 h at 37°C. Nonadherent cells were removed by washing with PBS, and cell adhesion was estimated in a colorimetric assay, based on NPAG (p-nitrophenyl *N*-acetyl-β-D-glucosaminide; Sigma-Aldrich) reaction. Absorbance was measured at 405 nm by using an ELISA reader (Bio-Rad Laboratories).

mRNA isolation and RT-PCR

Total cellular RNA was isolated from 70% confluent 10-cm Ø dishes using RNAzol B (Campro Scientific) and cDNA was synthesized by RT-PCR performed on 1 μg RNA using the Thermoscript RT-PCR system kit (Invitrogen). Specific transcripts were amplified with the following primers (Sigma Genosys): *LN5γ2* (forward, 5′-aaccagcaagtgagttacgg-3′; and reverse, 5′-ccattgtgacagggacatgg-3′) and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-accacagtccatgccatcac-3′; and reverse, 5′-tccaccaccctgttgctgta-3′) using the standard PCR protocol for the Platinum Taq PCR_X DNA polymerase kit (Invitrogen). The PCR products were resolved by electrophoresis on 1.5% agarose gels and viewed after ethidium bromide staining.

Online supplemental material

Fig. S1 shows that both α3β1 and α6β4 are equally expressed in WT and Tiam1−/− keratinocytes. Fig. S2 shows that on various other substrates such as Col IV, PLL, and FN, the degree of Rac activation in Tiam1−/− keratinocytes was comparable to that in WT cells, suggesting that the activation of Rac as a result of adhesion to these substrates is regulated by other RacGEFs. In Fig. S3, analysis of intact skin revealed no differences in the deposition and processing of LN5, the expression of the β1- and β4-integrin subunits, and components of the basal lamina between WT and Tiam1−/− mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200509172/DC1.

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