**Gα\textsubscript{12/13} Is Essential for Directed Cell Migration and Localized Rho-Dia1 Function**

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Scratch-wound assays are frequently used to study directed cell migration, a process critical for embryogenesis, invasion, and tissue repair. The function and identity of trimeric G-proteins in cell behavior during wound healing is not known. Here we show that Gα\textsubscript{12/13} but not Gα\textsubscript{6/11} or Gα\textsubscript{i} is indispensable for coordinated and directed cell migration. In mouse embryonic fibroblasts endogenous Rho activity is present at the rear of migrating cells but also at the leading edge, whereas it is undetectable at the cell front of Gα\textsubscript{12/13}-deficient mouse embryonic fibroblasts. Spatial activation of Rho at the wound edge can be stimulated by lysophosphatidic acid. Active Rho colocalizes with the diaphanous-related formin Dia1 at the cell front. Gα\textsubscript{12/13}-deficient cells lack Dia1 localization to the wound edge and are unable to form orientated, stable microtubules during wound healing. Knock down of Dia1 reveals its requirement for microtubule stabilization as well as polarized cell migration. Thus, we identified Gα\textsubscript{12/13} proteins as essential components linking extracellular signals to localized Rho-Dia1 function during directed cell movement.

Directional cell migration is a fundamental mechanism in wound repair and embryogenesis. In collective cell migration modes such as during embryogenesis, wound healing or invasion, cells dynamically regulate their cytoskeleton and become polarized toward direction of movement (1, 2). Many of these cytoskeletal changes are brought about by Rho-family GTPases, which are activated through cell surface receptors that in turn regulate specific guanine-nucleotide exchange factors (GEFs) (3). In wound healing assays with mammalian cells, the polarized morphology develops 1–6 h after injury of the cell monolayer and is characterized by formation of protrusions at the leading edge containing lamellipodia and filopodia, reorientation of the Golgi and the centrosome, as well as formation of stable and reoriented microtubules (3, 4). Integrin signaling has been identified as a critical process for polarized cell migration in scratch-wound assays of cell monolayers (5). The potential role of heterotrimeric G-protein-dependent mechanisms during wound healing is less well understood. G\textsubscript{i}-proteins have been implicated as signaling intermediates particularly in cell polarization during chemotactant-guided motility of Dictyostelium discoideum amoeboae or leukocytes (6–8). The G\textsubscript{12/13} family of G-proteins have been implicated in various cellular processes such as Rho-mediated organization of the cytoskeleton and subsequently the cell shape (9). G\textsubscript{12/13} Proteins appear to exert essential functions as mice double-deficient for G\textsubscript{12} and G\textsubscript{13} die at embryonic day E 8.5 (9). Coexpression of dominant negative versions of G\textsubscript{12} and G\textsubscript{13} reduced chemotactic polarity of differentiated HL-60 cells, suggesting that G\textsubscript{12/13} proteins may function during directional cell migratory processes (8). However, their potential role for wound induced cell migration and tissue repair has not been investigated.

Studies on wounding induced behavior of NIH3T3 fibroblasts revealed that addition of serum or lysophosphatidic acid (LPA)\textsuperscript{3} promotes the formation of a subpopulation of orientated microtubules known as stable, detyrosinated microtubules (Glu-MTs) (10). Overexpression of dominant active versions of RhoA and the diaphanous-related formins have been shown to induce microtubule stabilization (11), but the upstream signaling components await to be defined. Although active mutants of RhoA or diaphanous-related formins can induce stable microtubule formation, it is not apparent whether they are indeed essential for this process. Rho-induced stabilization of Glu-MTs has been characterized as an early event in the generation of cellular asymmetry in wound healing assays and appears to precede the onset of cell migration (10, 12). Nevertheless, regulation of RhoA activity during changes in cell-cell contact inhibition and directional migration such as in wound repair has not yet been analyzed directly. Although it has been suggested that Rho is involved in cell migration and is believed to be required for rear end retraction, its precise endogenous localization of activation such as in epithelial or mesenchymal cells is not known (13).

The present study was undertaken to investigate whether trimeric G-protein function is principally required for wound healing in mammalian cells. We used previously established MEF cell lines that are double deficient for either G\textsubscript{6/11} or G\textsubscript{12/13} in addition to the usage of pertussis toxin to inactivate G\textsubscript{i} (14). Additionally, we set out to systemically analyze the downstream signaling of Rho-family GTAPases and addressed the role of localized RhoA activation in wounded induced cell migration using a novel GFP-tagged Rho probe combined with an optimized in situ Rho assay approach that was previously reported (15, 16). Our analysis provides evidence that G\textsubscript{12/13} is absolutely essential for directional cell migration and wound healing involving Rho activity at the leading edge, which may control the formation of stable microtubules via Dia1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibody rabbit α-Glu-tubulin was provided by Dr. G. Gundersen or was from Chemicon; antibodies α-myc-TRITC and α-RhoA were from Santa Cruz; α-Ras, α-Rac, α-Cdc42, and α-p140mDia1 were from BD Transduction Laboratories; α-Flag-M2,

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α-α tubulin, and α-vinculin (mouse) were from Sigma-Aldrich. For reagents and plasmids, pGEX-2T encoding TAT-C3 was provided by Dr. E. Sahai; rhodamine-phalloidin, Alexa® 350-phalloidin was from Molecular probes; pertussis toxin and Y27632 were from Calbiochem; lysophosphatidic acid was from Biomol; Transfectin was from Bio-Rad. The purified CRIB peptide was obtained from Leo S. Price (UMC, Utrecht). pEF-Flag-mDia1-ΔDAD (codons 1–1180) was generated by PCR using the primers 5′-gccgcagatgtagacgtgcccgggcccggg-3′ and 5′-gcgcgcagatgtagacgtgcccgggcccggg-3′ and the primers forward 5′-gagctctggctatatatttgcgctgtcact-3′, introducing an NcoI and BamHI site, respectively.

The PCR products were ligated into the H10GFPspacers-MCS1 vector. Bacterial fusion proteins were expressed in the presence of 1 mM isopropyl 1-thio-β-D-galactopyranoside at 30 °C for 4 h in Luria-Bertani medium. His6-tagged fusion proteins were purified under native conditions according to the manufacturer (The QIAexpressionist™, Qiagen) on nickel-nitrilotriacetic acid-agarose (Qiagen). Proteins were eluted from beads with four incubations for 15 min at 4 °C using elution buffer (50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole). Fusion proteins were dialyzed overnight in dialyzing buffer (25 mM Tris, 10 mM MgCl2, 50 mM NaCl, 5% glycerol, 1 mM diethiothreitol). GFP-RBD purifications were tested for in vitro activity by recoupling an aliquot to nickel-beads for pull-down experiments. For in situ Rho[GTP] affinity assays cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.3% Triton/PBS. After blocking (5% FBS, 3% bovine serum albumin, 0.1% glycine in PBS, 45 min) cells were incubated for 2 h at 4 °C with soluble GFP or GFP-RBD (0.02 μg/μl) in 5% FBS/PBS. Samples were washed and mounted using MOWIOl. Images were obtained by fluorescence microscopy using a Leica DMIRE 2 microscope and a CCD camera (Leica, Germany).

**RNA Interference-mediated Knockdown of Mouse Dia1—siRNA corresponding specifically to mDia1 RNA sequences were obtained from Qiagen and chosen according to Arakawa et al. (40). Transfection of MEFs with siRNA duplexes was efficiently performed using magnet-assisted transfection (MATra) as recommended by the manufacturer (IBA). Briefly, 2 μl of MATra-A reagent containing magnetic nanoparticles was Complexed with siRNA (20 μM, 6 μl) in 6-well plates, and magnet force transfection was performed for 15 min on a magnetic plate at 37 °C and 10% CO2. Cells were analyzed 48–72 h after siRNA transfection. For complementation assays RNA interference-resistant mDia1, mDia1-compl, containing two silent mutations at nucleotide positions 10 and 13 in the siRNA target sequence (A711G and C714A, mDia1 cDNA), was generated by overlap PCR using the primers 5′-cagctgctgagctgctgagagcaggtact-3′, 5′-cagctgctgagctgctgagagcaggtact-3′, 5′-cagctgctgagctgctgagagcaggtact-3′, and 5′-cagctgctgagctgctgagagcaggtact-3′. The full-length mDia1 complementation mutant was inserted via NotI and XhoI into the pcDNA3-Flag vector. Mutations were confirmed by DNA sequencing.

**DNA Microinjections—siRNA-treated wild type or Gα12/13-deficient MEF monolayers were wounded with 60 min before cells at the wound edge were microinjected into nuclei using an Eppendorf Femtotjet and Injectman micromanipulator attached to a Zeiss Axiovert 135 microscope. Pressure was adjusted to 75 hPa for 0.5 s using an Eppendorf femtotip needle, and plasmids were microinjected at 0.05 μg/μl. Wounding induced migration was continued for additional 2 h before fixation and immunofluorescence analysis.

**RESULTS**

**Gα12/13-proteins Are Essential for Directed Cell Movement during Wound Healing**—To test whether G-protein-mediated signaling pathways are principally required for wound healing in the presence of serum we compared MEF lines generated from wild type embryos or from embryos double deficient for either Gα12/Gα13, or Gα12/Gα13, or MEFs treated with pertussis toxin to inhibit the G i family of G-proteins (14, 17). MEF migration was initiated in the presence of 10% serum in a monolayer using the scratch-induced wound healing assay, and multiple time lap recordings were performed in parallel for 18 h. MEFs migrated into the wound as sheets to reform a tight monolayer within 7–8 h (250–300-μm wound width) (Fig. 1, A and C and supplemental...
**Go12/13-dependent Cell Migration by Rho and Dia1**

**FIGURE 1.** Go12/13 is essential for wounding-induced cell migration. A, shown are statistics from wound distances in scratch-wound assays from 30-min interval live cell recordings of wild type MEFs without (WT) or with pertussis toxin pretreatment (WT+PTX). Go12/13-deficient MEFs, Go12/13-deficient MEFs, and Go12/13-deficient MEFs rescued by cotransfection with GFP, Go12 and Go13 (Rescue) as indicated. For Goq11-deficient MEFs and Go12/13-deficient MEFs data were acquired and summarized from two different clonal cell lines each. Wound distance data are shown from at least four independently performed experiments. In each setup, four movies of four different cell lines or cell treatments were recorded simultaneously. B, cell extracts from Go12/13-deficient MEFs (Go12/13-def) and Go12/13-deficient MEFs cotransfected with GFP, Go12, and Go13 (rescue) were immunoblotted for the indicated proteins. C, representative phase contrast images from 18-h live cell recordings of wounded wild type MEFs (upper panel), Go12/13-deficient MEFs (middle panel), or Go12/13-deficient MEFs that were FAC-sorted for GFP after transfection with plasmids encoding for GFP, Go12, and Go13 (2-µg each/10-cm dish, 15 dishes/FACS sorting) (lower panel), at 15 min, 5 h, and 10 h after wounding.

movie 1). The average velocity was 0.84 ± 0.1 µm/min. Preincubation of MEFs with 100 ng/ml pertussis toxin only moderately reduced wound healing, indicating that Go-proteins are not critically involved in mesenchymal cell migration (Fig. 1A) in contrast to their role in leukocyte motility (8). Goq11-deficient fibroblasts required longer time periods as compared with wild type MEFs but also efficiently reformed an intact monolayer (Fig. 1A and supplemental movie 2). Surprisingly, Go12/13-deficient cells completely lacked the ability to close the scratch wound (Fig. 1A and C and supplemental movie 3). This defect was not due to impaired proliferation over the monitored time period (18 h) because Go12/13-deficient cells proliferated at a slightly faster rate as wild type MEFs, or Goq11-deficient MEFs judged by cell countings performed in parallel (data not shown). The wound healing defect was confirmed using two different Go12/13-deficient MEF clones to exclude clonal variation.

To verify that Go12/13-proteins are indeed required for wound healing in MEF monolayers Go12/13-deficient fibroblasts were transfected with plasmids encoding Go12, Go13, and GFP, sorted for GFP-positive cells (3–7% transfection efficiency), and replated for wound experiments. Reexpression of Go12 and Go13 restored the ability of Go12/13-deficient MEFs to efficiently close the gap within 7–8 h (Fig. 1 and supplemental movie 4), clearly demonstrating that Go12/13 are essential for wound-induced cell migration. Expression of either Go12 or Go13 alone only partially rescued wound healing of Go12/13-deficient MEFs (data not shown).

**Cytoskeletal Characteristics in Wounded Go12/13-deficient MEF Monolayers**—Cell migration requires coordinated changes in cytoskeletal dynamics, which govern the protrusion of actin filament (F-actin) and microtubule-containing lamellipodia and filopodia at the front, retraction at the back, and constant assembly and disassembly of focal adhesions (18). Hence, we analyzed the morphology of focal adhesions, F-actin, and microtubules of Go12/13-deficient MEFs after wounding. Visualization of vinculin revealed that the general appearance of focal adhesions in Go12/13-deficient cells was comparable with those of wild type MEFs over the entire wound-healing period (Fig. 2A). Comparison of F-actin between wild type and Go12/13-deficient cells showed only moderate differences; leading edge Go12/13-deficient MEFs appeared to have slightly lower F-actin contents, as judged by phalloidin staining (Fig. 2A). Strikingly although and in contrast to wild type MEFs, which adopted an elongated shape with long microtubule-containing protrusions toward the wound, Go12/13-deficient MEFs displayed a microtubule meshwork that was not orientated and appeared disorganized (Fig. 2A). This phenotype was observed throughout scratch-induced migration, demonstrating that microtubule morphology and rearrangement were altered in cells lacking Go12/13.
FIGURE 2. Cytoskeletal characteristics and Rho-GTPase signaling during directed migration of Gα12/13-deficient MEFs. A, scratch-induced migration was initiated in confluent monolayers of wild type (wt) or Gα12/13-deficient (G12/13-def.) MEFs for 4 h prior fixation and permeabilization. Cells were stained either for focal adhesions using vinculin antibodies, for F-actin using phalloidin, or for visualization of microtubules using α-tubulin antibodies as indicated. The direction of wound is indicated by a dotted white line. Scale bar, 10 μm.

B–E, for GTPase activity lysates from scratch-wounded MEF monolayers were subjected to pull-down assays as described under “Experimental Procedures.” Times after wounding before cell lysis are indicated. Cdc42 (B) or Rac (C) pull-down assays were performed simultaneously to either Ras (D) or RhoA (E) assays by adding biotinylated CRIB peptide to the lysis reaction. Densitometric quantifications of 3–5 independently performed GTPase pull-down assays ± S.E. are shown together with representative Western blots using the indicated antibodies.
Role of Rho GTPases in $\alpha_{12/13}$-mediated Wound Healing—Several trimeric G-proteins are known to regulate the functions of Rho GTPases that control F-actin, microtubule, and focal adhesion dynamics. Therefore, we analyzed scratch-induced activation of Cdc42, Rac, Ras, and RhoA in $\alpha_{12/13}$-deficient MEFs under the applied wound assay conditions. Using immobilized PAK-CRIB peptides as a probe (19), we found that the activities of Cdc42 and Rac were not significantly reduced during migration of wild type or $\alpha_{12/13}$-deficient MEFs (Fig. 2, B and C). We also directly assessed the involvement of Ras as it cooperates with RhoA to control focal adhesion turnover (20). Ras became rapidly activated upon wounding induced migration in both wild type and $\alpha_{12/13}$-deficient monolayers (Fig. 2D). These data show that wounding induced Cdc42, Rac, or Ras activation does not depend on $\alpha_{12/13}$.

To determine total RhoA activity we performed biochemical pull-down experiments using the Rho-binding domain (RBD) of rhotekin as a probe (21). Interestingly, wounding of MEF monolayers caused a down-regulation of total RhoA activity (Fig. 2E), demonstrating that total RhoA activity is inversely regulated as compared with Cdc42, Rac, or Ras (Fig. 2). However, low levels of active RhoA appeared to be maintained during wound healing in wild type MEFs (Fig. 2E). In contrast, RhoA activity levels were found to be significantly reduced or in some cases even undetectable in $\alpha_{12/13}$-deficient MEFs during wounding (Fig. 2E). These results showed that wild type MEFs maintained higher levels of total active RhoA during migration, when compared with MEFs lacking $\alpha_{12/13}$.

Specificity of a Recombinant Rho[GT]P Affinity Probe—To be able to investigate endogenous RhoA activity in more detail we performed a modified in situ Rho[GT]P affinity assay (15, 16) for which we have generated a His-tag-purified GFP-RBD fusion protein as a probe in which the Rho-binding-domain of rhotekin was coupled to GFP and produced recombinantly. To characterize the binding properties of this fusion protein we performed in vitro pull-down assays by recoupling purified GFP-RBD to nickel beads. Starved cells were serum stimulated for 5 min and endogenous active RhoA was efficiently precipitated by GFP-RBD as compared with untreated cells (Fig. 3A), clearly demonstrating the specificity of the probe for GTP-RhoA in vitro.

We then studied the ability of the GFP-RBD to detect active RhoA in situ. Incubation of fixed and permeabilized cells with GFP alone as a control resulted in a background signal labeling the nuclei (Fig. 3B). To control for the specificity of GFP-RBD for in situ fluorescence applications we transfected fibroblasts either with dominant negative RhoA (N19RhoA) or with a constitutively active mutant for RhoA (V14RhoA). Cells were transfected at subconfluency and serum-starved in 0.5% FBS. As expected, cells that expressed V14RhoA displayed excessive stress fiber formation and cell body contraction (Fig. 3C). As shown in Fig. 3, cells expressing V14RhoA displayed a robust increase in signal intensity for GFP-RBD, whereas cells expressing dominant negative N19RhoA did not (Fig. 3C). In contrast, cells expressing active V14RhoA that were incubated with GFP alone displayed a nonspecific background signal labeling the nucleus as compared with non-transfected cells or cells that were maintained in 10% serum conditions (Fig. 3B). To further verify that the GFP-RBD probe only recognizes the GTP-bound conformation of RhoA, fibroblasts were cotransfected with V14RhoA and C3 exoenzyme to inactivate Rho proteins (22). This resulted in a reduction of the GFP-RBD signal almost to background detection levels as compared with starved, non-transfected cells (Fig. 3C). We also transfected cells with the active form of Cdc42, V12Cdc42. Cells expressing V12Cdc42 were not detected by GFP-RBD again demonstrating the specificity of this probe for active Rho (Fig. 3C). In contrast and as an additional control, overexpression of V12Cdc42 could be strongly visualized by the addition of purified GFP-WASpGBD, a probe which we have generated to detect active Cdc42 (Fig. 3C).

Absent Rho Activity at the Leading Edge of Wounded Fibroblasts Lacking $\alpha_{12/13}$—Because $\alpha_{12/13}$-deficient MEFs had reduced levels of Rho activity we decided to visualize the regulation of this GTPase in a spatial manner. Hence, to study the localization of endogenous RhoA activity during cell migration we performed in situ Rho[GT]P affinity assays on scratch-wounded MEFs. Using GFP-RBD as a probe Rho activation could be visualized in the cytoplasm and tail regions of wounded wild type MEFs with signal intensities declining toward the lamellipodial front (Fig. 4, A and D). Interestingly, a distinct signal could be detected at the leading edge and along F-actin-containing protrusions (Fig. 4, A, B, and D), indicating that active Rho is present in these structures. Rho activation at the leading edge was readily observed within the first hour after wounding and slowly declined over the next 2–3 h (Fig. 4E), showing that frontal Rho activity is temporally regulated during wounding induced MEF migration. In contrast, in wounded $\alpha_{12/13}$-deficient fibroblasts overall Rho activity appeared to be lower, and no significant increase of active Rho could be observed at the cell front over wounding times (Fig. 4), suggesting a specific role for Rho activity at the leading edge of migrating fibroblasts. Thus, we identified $\alpha_{12/13}$-proteins as essential components for localized RhoA activation at the leading edge during wounding induced cell migration.

G-protein-regulated Localization of Active Rho and Dia1 to the Leading Edge—Next, we asked if serum or LPA stimulation could activate Rho at the wound edge of MEFs. Monolayers of wild type MEFs were serum-starved in 0.5% FBS, wounded, and stimulated with 100 nM LPA for 1 h before fixation and incubation with GFP-RBD. Serum-starved cells showed low signal intensities for GFP-RBD, whereas LPA-treated MEFs displayed a general increase in GFP-RBD staining (Fig. 5A). Additionally, ~61% of LPA-treated cells exhibited active Rho at the leading edge as compared with ~19% cells maintained under serum-free conditions (Fig. 5, A and B). LPA-stimulated localization of Rho activity to the wound edge was almost completely blocked by preincubation with the cell-permeable Rho inhibitor TAT-C3 (15) (Fig. 5, A and B). TAT-C3-treated cells consistently showed almost undetectable levels of GFP-RBD in the cytoplasm and in most cases displayed strongly reduced F-actin staining characteristic for inactivation of RhoA (Fig. 5A).

The presence of Rho activity at the leading edge led us to investigate the involvement of Rho effectors in wounding induced cell migration. Inhibition of Rho-kinase (ROCK) has been previously reported not to interfere with cell motility in fibroblast scratch-wound assays (23). We therefore investigated whether Dia1 and active RhoA colocalize to the leading edge of wounded wild type MEFs using immunostaining. Endogenous Dia1 was distributed diffusely throughout the cytoplasm, and no localization of Dia1 at the wound edge could be observed in cells under serum-free conditions (Fig. 5A). In contrast, in MEFs treated with LPA we readily detected Dia1 staining at the leading edge along with active Rho (Fig. 5, A and C). Similar results were observed when cells were stimulated with 10% FBS (not shown).

We also compared endogenous Dia1 localization between wild type and $\alpha_{12/13}$-deficient MEFs under normal wound assay conditions and found that Dia1 was mostly absent from the leading edge of migrating cells lacking $\alpha_{12/13}$ apart from a moderate increase to ~24% cells that showed leading edge Dia1 at 2 h postwounding (Fig. 5, D and E), a fact which is also reflected by the correlative increase of frontal GFP-RBD signals in $\alpha_{12/13}$-deficient MEFs (Fig. 4E).
stitutively active V14RhoA into wounded Gα12/13-deficient MEFs to restore Dia1 recruitment to the leading edge, but, because of increased stress fiber formation and shape change of V14RhoA-expressing cells we were unable to detect any specific Dia1 signal at the cell periphery (data not shown). Our data demonstrate that localization of active Rho and Dia1 to the leading edge of wounded fibroblasts is regulated dynamically and depends on Gα12/13.

Dia1 Is Required for Polarized Cell Migration—So far our analysis revealed that wounded Gα12/13-deficient MEFs had overall reduced RhoA activity and undetectable levels of active Rho at the leading edge (data not shown). Our data demonstrate that localization of active Rho and Dia1 to the leading edge of wounded fibroblasts is regulated dynamically and depends on Gα12/13.

Dia1 is required for polarized cell movement. Therefore, we knocked down Dia1 expression by RNA interference prior to induction of scratch-wound assays (Fig. 6A). Analysis of wild type MEFs after Dia1 knock-down showed that scratch-induced cell migration was efficiently blocked (supplemental movies 5 and 6, and Fig. 6B), whereas preincubation with 5 μM Y27632 to inactivate ROCK only moderately reduced wound healing by ~1 h (Fig. 6B). These data clearly show that Dia1 plays an essential role in polarized cell migration.

Gα12/13 and Dia1 Are Essential for the Formation of Orientated, Stable Microtubules—It has recently been shown that transient expression of active mutants of RhoA and its effector diaphanous, but not active ROCK, induces the formation of orientated, stable microtubules (24). Orientated Glu-MTs represent a subset of microtubules...
at the leading edge with a long half-life (>1 h) and are thought to be important for the onset of directed cell migration (24). Thus, we decided to analyze Glu-MT formation in Gα₁2/13-deficient MEFs. Comparing scratched monolayers of wild type and Gα₁2/13-deficient MEFs for Glu-MTs using a specific antibody (25), we found that wild type MEFs started to form orientated Glu-MTs within the first 2 h after wounding, whereas in contrast Gα₁2/13-deficient MEFs largely failed to do so (Fig. 6, C and D). Both, orientation toward the wound edge as well as overall formation of polarized Glu-MTs appeared to be diminished.

Although expression of active mutants of diaphanous-related formins are sufficient to promote Glu-MT formation in fibroblasts (11) we tested whether Dia1 is in fact necessary for stable microtubule formation using RNA interference. RNA interference against the RhoA effector mDia1 efficiently and almost completely blocked the formation of orientated Glu-MTs in wounded wild type MEFs (Fig. 6, C and E). Reexpression of an siRNA target sequence resistant mutant mDia1 (Dia1-compl, see “Experimental Procedures”) using microinjection of wound edge MEFs silenced for mDia1 restored Glu-MT formation in these cells (Fig. 6F). Thus, Dia1 appears to be essential for the generation of stable microtubules, clearly indicating that this is a critical function exerted by this formin-related protein.

We then analyzed whether Dia1 is sufficient for Glu-MT formation in Gα₁2/13-deficient MEFs. Therefore, we generated an active Dia1 mutant lacking the C-terminal diaphanous autoregulatory domain (DAD), known to interact with regions in the N-terminal RhoA-binding domain (26). The ΔDAD mutant was microinjected into Gα₁2/13-deficient MEFs at the wound edge. Strikingly, introduction of Dia1-ΔDAD into wound edge cells lacking Gα₁2/13 rescued the formation of Glu-MTs that were polarized toward the leading edge (Fig. 6G), suggesting that Gα₁2/13 functions upstream of Dia1.
To confirm that $\alpha_{12/13}$ is essential for microtubule stabilization we transfected $\alpha_{12/13}$-deficient MEFs with plasmids encoding $\alpha_{12}$, $\alpha_{13}$, and GFP, for FACS sorting. GFP-sorted MEFs were replated, wounded, and analyzed for Glu-MT formation. As shown in Fig. 6H reintroduction of $\alpha_{12}$ and $\alpha_{13}$ restored scratch-induced formation of orientated Glu-MTs in $\alpha_{12/13}$-deficient MEFs. Taken together, these results show that $\alpha_{12/13}$-proteins are critical components for microtubule stabilization in migrating cells.

**DISCUSSION**

In this paper, we have identified $\alpha_{12/13}$ subunits as essential components for directed cell migration. We further showed that global RhoA
activity becomes down-regulated during initiation of migration but that low levels may be maintained to ensure formation of Glu-MTs and hence efficient wound healing. Endogenous active Rho can be detected at the cell front of motile fibroblasts, and this depends on functional \( \alpha_{12/13} \)-proteins. In parallel, the RhoA effector \( \text{mDia1} \) is recruited to the wound edge and is required for directed cell movement during wound repair.

\( \alpha_{12/13} \)-proteins have been suggested to be able to interact with the family of RGS domain-containing RhoGEFs thereby transducing signals toward the activity of the small GTPase RhoA (27, 28). Our results show that under normal serum conditions wild type MEFs maintained higher toward the activity of the small GTPase RhoA (27, 28). Our results show that under normal serum conditions wild type MEFs maintained higher

\[ \text{Cells with Glu-MTs (%)} \]

\[ \begin{align*}
\text{WT} & \quad \text{G12/13-def.} \\
\alpha_{12/13} & \quad 100 \\
\text{G12/13-def.} & \quad 12 \\
\text{mock} & \quad 20 \\
\text{Dia1} & \quad 0 \\
\text{G12/13-def.} & \quad 0
\end{align*} \]

at wound margins containing orientated Glu-MTs were determined. Times after wounding are as indicated. Data are representative of three independent experiments (± S.E.). C, percentage of wild type MEFs (WT) \( \alpha_{12/13} \)-deficient MEFs (G12/13-def.), or MEFs transfected with siRNA against mDia1 (siRNA mDia1) at wound margins containing orientated Glu-MTs were determined. Times after wounding are as indicated. Data are representative of three independent experiments (± S.E.). D, wild type (wt) or \( \alpha_{12/13} \)-deficient MEFs (G12/13-def.) were wounded and subjected for detection of microtubules using \( \alpha \)-tubulin or of stable microtubules using Glu-MT antibodies as indicated. E, wild type MEFs were transfected with mock siRNA as a control (siRNA mock) or siRNA directed against mDia1 (siRNA Dia1). MEF monolayers were scratch-wounded for 2 h, fixed, and subsequently analyzed for microtubules (\( \alpha \)-tubulin) and for Glu-MTs as indicated. Merged images showing microtubules in green and Glu-MTs in red are indicated in the right panels. Shown are wound edges in 40x magnification. F, wild type MEFs were transfected with siRNA directed against mDia1 (siRNA Dia1), and monolayers were scratch-wounded for 60 min before microinjecting Flag-mDia1-complementation expression plasmids (D12-compl) into wound edge cells. 2 h later cells were stained for F-actin using Alexa488 350 phalloidin (blue), for mDia1-compl using Flag-M2 antibodies (green), and for Glu-MTs (red) as indicated. Merged green, red, and blue channels are shown in the panel on the right. G, \( \alpha_{12/13} \)-deficient MEF monolayers were scratch-wounded for 60 min before microinjecting Flag-mDia1-DAD expression plasmids (D12-DAD) into wound edge cells. 2 h later cells were stained for F-actin using Alexa488 350 phalloidin (blue), for mDia1-DAD using Flag-M2 antibodies (green), and for Glu-MTs (red) as indicated. Merged green, red, and blue channels are shown in the panel on the right. H, \( \alpha_{12/13} \)-deficient MEFs were FACs-sorted for GFP after transfection with plasmids (2 μg each/10-cm dish; 10 dishes/FACs sorting) encoding for GFP alone or for GFP, \( \alpha_{12/13} \), and \( \alpha_{13} \) as indicated. After reaching confluence MEF monolayers were wounded for 2 h prior to fixation and Glu-MT staining. The percentage of cells at wound margins containing orientated Glu-MTs were determined (n = 3, mean ± S.E.). Scale bars, 10 μm.

We generated a probe to investigate spatial Rho activity in migrating cells. Indeed and as may be expected, endogenous active RhoA was present at the rear of polarized wild type MEFs, whereas it was reduced at the rear of migrating \( \alpha_{12/13} \)-deficient MEFs, and these findings support the current model that RhoA and ROCK are locally involved in tail retraction (13), probably adding to the overall phenotype during \( \alpha_{12/13} \)-deficient wound healing. However, random cell movement of \( \alpha_{12/13} \)-deficient MEFs was comparable with wild type cells.4

4 P. Goulimari and R. Grosse, unpublished observations.
In addition and to our surprise, we observed spatial Rho activity at the leading edge of motile cells, which depended on \( \text{G}_{12/13} \) proteins. This strongly argues that Rho is involved in additional functions that regulate cell movement apart from cell tail retraction. Interestingly, Wang et al. (32) recently demonstrated that RhoA becomes degraded in filopodia of migrating cells by the E3 enzyme Smurf1, consistent with the previous hypothesis that Rho activity at the leading edge would rather interfere with cell migration (3). Nevertheless, this could also indicate that Rho function is tightly controlled in a spatiotemporal manner and constantly turned over at these structures to fine tune protrusive and adhesive activity at the front of controlled in a spatiotemporal manner and constantly turned over at these structures to fine tune protrusive and adhesive activity at the front of migrating cells. We observed that endogenous active Rho clearly appears in membrane ruffles, indicating that Rho activity indeed stabilizes and cell migration, indicating that this is an important physiological function of the protein. In conclusion, our results clearly identify \( \text{G}_{12/13} \) as essential components to promote localized Rho activity at the leading edge that is likely to control Dia1 function required for microtubule stabilization and directed cell migration.

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