Raf-1 regulates Rho signaling and cell migration

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Introduction

Cell migration is a complex and highly coordinated process that plays a vital role in many physiological and pathological situations, such as embryonic development, wound healing, angiogenesis, and tumor metastasis. Coordinated movement is the result of the ability of a cell to polarize, extend protrusions and form adhesions at the leading edge, translocate the cell body, and finally detach from the substrate at the trailing edge (Ridley et al., 2003). Cytoskeletal remodeling plays a paramount role in migration. In particular, the transition from stationary to motile cell edges entails the disassembly of peripheral actin bundles and their replacement by protrusive actin networks in lamellipodia (Omelchenko et al., 2003). The small GTPases of the Rho family are key regulators of these cytoskeletal dynamics and of migration. At the leading edge, CDC42 is involved in establishing polarity by recruiting the polarity protein PAR6 (Macara, 2004), whereas Rac stimulates the formation of lamellipodia through the activation of the Wave complex (Smith and Li, 2004) and, possibly, via the activation of the PAK serine/threonine kinases (Bokoch, 2003). The role of Rho in migration is at least twofold: it induces the assembly of stable focal adhesions, which may increase adhesion and therefore decrease motility; and it promotes actomyosin contractility, necessary for the translocation of the cell body. Rho induces actin reorganization via the downstream effectors Rok-α and mDia, which cooperate in the formation of stress fibers (Watanabe et al., 1999), but antagonize each other in Rho-dependent Rac activation (Tsuji et al., 2002). To establish and maintain directional movement, a gradient of GTPase activity must be formed, with CDC42/Rac most active at the leading edge, Rho at the cell rear and sides. This is thought to be accomplished by the regulation and localization of GEF and GAP activity (Etienne-Manneville and Hall, 2002; Ridley et al., 2003), but also via alternative mechanisms, like ubiquitin-mediated Rho degradation at the leading edge (Wang et al., 2003).

The Raf serine/threonine kinases relay signals inducing cell proliferation, differentiation, and survival. Raf-1 has been most intensively studied as the downstream effector linking Ras activation to the MEK/ERK module. However, among the three Raf kinases (A-Raf, B-Raf, and Raf-1) B-Raf is the most efficient in interacting with Ras (Marais et al., 1997; Weber et al., 2000) and in activating the MEK/ERK module (Pritchard et al., 1995, 2004; Wojnowski et al., 2000). In addition, gene ablation experiments have shown that ERK activation and proliferation proceed normally in the absence of Raf-1, whereas

af kinases relay signals inducing proliferation, differentiation, and survival. The Raf-1 isoform has been extensively studied as the upstream kinase linking Ras activation to the MEK/ERK module. Recently, however, genetic experiments have shown that Raf-1 plays an essential role in counteracting apoptosis, and that it does so independently of its ability to activate MEK. By conditional gene ablation, we now show that Raf-1 is required for normal wound healing in vivo and for the migration of keratinocytes and fibroblasts in vitro. Raf-1—deficient cells show a symmetric, contracted appearance, characterized by cortical actin bundles and by a disordered vimentin cytoskeleton. These defects are due to the hyperactivity and incorrect localization of the Rho-effector Rok-α to the plasma membrane. Raf-1 physiologically associates with Rok-α in wild-type (WT) cells, and reintroduction of either WT or kinase-dead Raf-1 in knockout fibroblasts rescues their defects in shape and migration. Thus, Raf-1 plays an essential, kinase-independent function as a spatial regulator of Rho downstream signaling during migration.

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Abbreviations used in this paper: KC, kinase-competent; KD, kinase-dead; KGM, keratinocyte growth medium; KO, knockout; MYPT1, regulatory subunit of the myosin light chain phosphatase; WT, wild-type.

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Raf-1 is required to restrain apoptosis during embryonic development (Huser et al., 2001; Mikula et al., 2001). Raf-1 appears to counteract the activation of caspases, proteases which dismantle the cell during apoptosis but also play a role in the terminal differentiation of keratinocytes and erythroblasts (Algeciras-Schimnich et al., 2002). Raf-1 can restrain caspase activity both in the context of apoptosis induced by specific stimuli (Jesenberger et al., 2001; Mikula et al., 2001), and in the context of erythroblast differentiation (Kolbus et al., 2002).

The role of Raf-1 in migration in vivo has never been addressed. However, Raf-1 has been implicated in the control of motility in COS cells downstream of the small GTPase Rac (Leng et al., 1999). Rac can control Raf-1 activation via its downstream target PAK, which phosphorylates serine 338 on Raf-1 (King et al., 1998; Li et al., 2001; Zang et al., 2002). A role for Rho in Raf-1 activation has also been described, but the effectors involved are unknown (Li et al., 2001).

Epidermal development, homeostasis, and repair are complex processes requiring the exact orchestration of cell proliferation, migration, and differentiation. Several of the signals essential for epidermal development and repair, most prominently those transduced by the EGFR and by integrins (Fuchs and Raghavan, 2002; Watt, 2002), reportedly activate Raf-1 downstream of the EGFR ligands by epidermal cells (Schulze et al., 2001) as well as in integrin expression (Woods et al., 2001) and their affinity for the matrix (Hughes et al., 1997). In addition, activation of the Ras–Raf–ERK pathway has been found associated with skin proliferation in physiological as well as pathological conditions (Haase et al., 2001; Vasioukhin et al., 2001; Dajee et al., 2002). In contrast, others have reported that constitutive Ras/Raf activation arrests growth and induces features of terminal differentiation in cultured murine keratinocytes (Lin and Lowe, 2001; Roper et al., 2001). To elucidate the role of Raf-1 in epidermis, we performed tissue-restricted ablation of the gene by generating mice carrying a homozygous c-raf-1loxP/loxP (ffff) allele (Jesenberger et al., 2001) and the K5Cre transgene (c-raf-1loxP/loxP, deleted in epidermis) showed complete conversion of the c-raf-1loxP to the c-raf-1f allele by PCR and lacked Raf-1 protein (Fig. 1, A and B). c-raf-1loxP mice were viable, fertile, and healthy. At 4 wk old, they displayed curled whiskers and a wavy fur (Fig. 1 C), a phenotype lost after the first hair cycle. The architecture of the epidermis (Fig. 1 D) as well as the expression of basal keratin and differentiation markers in the epidermis and hair follicles was indistinguishable in c-raf-1loxP/loxP mice and control littermates (not depicted). Thus, Raf-1 is not essential in epidermal development and homeostasis. Raf-1 ablation, however, markedly affected wound healing. Control mice closed and reepithelialized full thickness wounds (6-mm Ø) by day 9 after wounding, whereas >40% of the wound was still open in c-raf-1loxP mice at this time (not depicted). The wounds were not yet closed on day 12, when control mice had already completed both reepithelialization and clearance of cell debris underneath the wound crust (Fig. 2 A). c-raf-1loxP/loxP mice healed the wounds 4–5 d later, and the healed skin was indistinguishable from that of fff mice (not depicted). The secondary responses of dermal components (granulation, inflammation, and neo-vascularization) were not affected by Raf-1 ablation in keratinocytes. The number of proliferating keratinocytes around the wound site was similarly unaffected (31 ± 10.8 K67+ cells in fff, 32.5 ± 8.8 in ΔΔΔ), and TUNEL staining of the migrating epithelial sheets did not show any apoptotic cells in c-raf-1loxP/loxP or control wounds (not depicted). Thus, lack of Raf-1 significantly delayed wound healing in the absence of proliferation or survival defects, suggesting that Raf-1 might be needed for keratinocyte migration.

Indeed, in a modified Boyden chamber assay, keratinocyte growth medium (KGM), EGF, or KGF efficiently promoted mi-

![Figure 1. Keratinocyte-specific disruption of c-raf-1 by the Cre-loxP system.](Image)
Raf-1 and Migration

Raf-1 ablation affects wound healing in vivo and keratinocytes motility, adhesion, and shape in vitro.

(A) Hematoxylin/eosin-stained full thickness skin section, 12 d after wounding. The wound margins (arrows) have not yet met in the c-raf-1Δ/Δ mice animals. C, crust; E, newly formed epidermis; D, dermis.

(B) Impaired migration in Raf-1 KO keratinocytes. Migration was assayed in a modified Boyden chamber assay using KGM (10 h), EGF (25 ng/ml, 15 h), or KGF (20 ng/ml, 15 h) as chemoattractants. (C) The percentage of cells adhering to different ECM components was determined in a 15-min adhesion assay (Fn, fibronectin; Ln, laminin; C1 and C4, collagens I and IV). Integrin-independent adhesion on BSA-coated surfaces was subtracted from the values plotted. Values are means (±SD, vertical bars) of three individual experiments. (D) Morphology of keratinocytes stained to visualize the actin cytoskeleton (rhodamine-conjugated phalloidin). Bar, 15 μm.

The defects in motility, adhesion, and shape were even more dramatic in Raf-1 KO fibroblasts. These performed consistently worse than WT cells in an in vitro wound healing assay, in the presence or absence of motogenic signals (Fig. 3 A), and in a short-term adhesion assay, likely due to a delay in spreading (Fig. 3 B). Raf-1 KO fibroblasts also displayed a symmetric, contracted morphology. Longitudinal stress fibers were markedly reduced, and the actin was organized in tight cortical bundles (Fig. 3 C) reminiscent of those observed in Raf-1 KO keratinocytes. This redistribution of actin fibers to the cell cortex was not accompanied by an alteration in actin treadmilling, as the levels of F- and G-actin detected in WT and KO fibroblasts were comparable (not depicted). Thus, cell-autonomous defects in shape, spreading, and migration were a common feature of Raf-1 KO keratinocytes and fibroblasts.

Rho signaling is deregulated in Raf-1 KO cells

The small GTPase Rho controls cell shape and motility by increasing intracellular tension and contractility (Etienne-Manneville and Hall, 2002). The shape of Raf-1 KO cells, the defects in spreading, and their failure to migrate all suggested a possible hyperactivation of Rho. Rho downstream targets such as the regulatory subunit of the myosin light chain phosphatase (MYPT1; pT696), ezrin (pT567), paxillin (pY118), and FAK (pY397; Fig. 4 A) were hyperphosphorylated in KO cells. Yet, basal Rho activity was not elevated in Raf-1 KO cells, and migration induced Rho-activation was in fact delayed (Fig. 4 A). Activated paxillin (Tsubouchi et al., 2002) and ezrin (Speck et al., 2003) can reportedly inhibit Rho activity, and are likely responsible for the decreased Rho stimulation. Phosphorylation (and inactivation) of MYPT1 results in the phosphorylation of myosin light chain (Kimura et al., 1996) and ezrin (Fukata et al., 1998). Ezrin phosphorylation, in turn, relieves intramolecular inhibition and enables ezrin to act as an actin filament/plasma membrane cross-linker (Bretscher et al., 2002). Together, MYPT1 and ezrin phosphorylation explain the contracted appearance and the tight cortical actin network observed in KO cells. Indeed, expression of a dominant-negative ezrin mutant (DN-Ez-eG, ezrin1-310 fused to eGFP) corrects these defects (Fig. 4 B, compare transfected and untransfected cell). The Rho effector Rok-α phosphorylates MYPT1 directly (Kawano et al., 1999) and regulates ezrin both by direct phosphorylation and by inactivation of MYPT1 (Fukata et al., 1998; Matsui et al., 1998), and further induces paxillin and FAK hyperphosphorylation (Sinnert-Smith et al., 2001; Tsuji et al., 2002). It also phosphorylates vimentin, thereby causing the collapse of the vimentin filaments into a juxtanuclear aggregate (Sin et al., 1998), an additional characteristic feature of Raf-1 KO fibroblasts (see...
Raf-1 ablation affects fibroblasts motility, adhesion, and shape in vitro. (A) Fibroblast motility assayed by in vitro wound healing in serum-free medium. Wound closure was photographed after 18 h. Arrows, wound margins at time 0. The percent wound closure at different times is plotted on the left. The values represent the means (±SD, vertical bars) of three independent experiments. (B) The percentage of cells adhering to different ECM components was determined in a 30-min adhesion assay as described in the legend to Fig. 2. Integrin-independent adhesion on BSA-coated surfaces was subtracted from the values plotted. Values are means (±SD, vertical bars) of five individual experiments. (C) Morphology of fibroblasts stained to visualize the actin cytoskeleton.

Fig. 5 C). Thus, the defects observed in KO cells were consistent with deregulation of Rok-α enzymatic activity.

The expression of Rok-α was slightly (1.5/2-fold) reduced in Raf-1 KO cells (Fig. 4, A and G). However, both basal and serum-stimulated Rok-α activity was elevated in Raf-1 KO fibroblasts (Fig. 4 C). ADP-ribosylation of the Rho GTPase by cell-permeant C3 exoenzyme (Barth et al., 1998) reduced the hyperphosphorylation of ezrin detected in Raf-1 KO fibroblasts, indicating that Rok-α activation still required basal Rho activity in these cells (Fig. 4 D).

To assess whether Rok-α deregulation caused the defects in shape and migration, Raf-1 KO cells were treated with the Rok inhibitor Y-27632. The inhibitor corrected MYPT1 and paxillin hyperphosphorylation (Fig. 4 E) as well as the redistribution of vimentin to a perinuclear location in KO fibroblasts (Fig. 5 C), and significantly improved the migratory ability and shape of Raf-1 KO fibroblasts (Fig. 4 F and Fig. 5 C) and keratinocytes (Fig. 4 H; and not depicted). Transfection with KD Rok-α had an even more profound impact than Y-27632 on both WT and KO fibroblasts, ruling out possible Rok-α–independent effects of the inhibitor. Raf-1 KO fibroblasts transfected with KD Rok-α lost their characteristic tight cortical actin bundles and contracted shape, formed stress fibers and vimentin filaments similar to those of untreated WT fibroblasts (Fig. 5 D), and migrated as efficiently as untransfected WT cells in a transwell assay (Fig. 5 E). In contrast, the expression of KD Rok-α had a deleterious effect on both the shape (Fig. 5 D) and the migratory ability (Fig. 5 E) of WT cells. This is in line with a requirement for Rok-α in the maintenance of cell shape and migration of normal fibroblasts. The opposite effects of KD Rok-α on WT and KO cells strongly argue in favor of Rok-α hyperactivity as the molecular basis of the defects of Raf-1 KO cells.

The physical presence of Raf-1, but not its kinase activity, are required for efficient migration

The data above support the hypothesis that Raf-1 restrains Rok-α signaling during migration. As Raf-1 was activated during early in vitro wound healing, albeit not to the extent induced by growth factor stimulation (Fig. 5 A), we investigated whether kinase activity was necessary for this function by generating stable KO fibroblast lines expressing full-length kinase-competent (KC) Raf-1 or a KD version of the protein at nearly endogenous levels (Fig. 5 B). Vector-transfected clones (V) were morphologically indistinguishable from Raf-1 KO fibroblasts (Fig. 5 C), and migrated just as poorly (Fig. 5 B). Both KC and KD Raf-1 rescued cell morphology (Fig. 5 C) as well as the ability of cells to adhere (not depicted) and to migrate in the in vitro wound assay (Fig. 5 B). Re-expression of both KC and KD Raf-1 also rescued vimentin filaments (Fig. 5 C). Thus, the physical presence of Raf-1, but not its kinase activity, is required to regulate Rho downstream signaling and to support migration.

Raf-1 associates physically with Rok-α and controls its localization within the cell

We next tested whether Raf-1 associates physically with Rok-α. Indeed, Rok-α, but not the closely related kinase Rok-β (not depicted), was present in increasing amounts in endogenous Raf-1 immunoprecipitates obtained from migrating fibroblasts (Fig. 6 A). Rok-α could be coimmunoprecipitated with an antibody recognizing the COOH terminus of Raf-1, but not its NH₂ terminus, suggesting that the latter may be involved in the interaction with Rok-α. To confirm this and to test whether the association could be detected by using an antibody directed against Rok-α instead of Raf-1, we coexpressed full-length Raf-1 as well as the truncated NH₂-terminal (ΔC) and COOH-terminal (ΔN) domain of Raf-1 with HA-tagged Rok-α in COS-1 cells. HA immunoprecipitates from subconfluent COS-1 cells contained full-length and ΔC Raf-1, but not the form lacking the NH₂ terminus (Fig. 6 B). Thus, Raf-1 associates physically with Rok-α via its NH₂ terminus. Whether Raf-1 binds Rok-α directly, or whether a bridging molecule is involved in the interaction, is presently unknown. Raf-1 and Rok-α decorated elongated structures previously identified as vimentin filaments (not depicted; Sin et al., 1998; Janosch et al., 2000). Raf-1 colocalized with Rok-α in both nonmigrating (Fig. 6 C) and migrating WT cells (Fig. 6 D), although Raf-1 distribution was not restricted to the filaments. In migrating cells, both kinases displayed a polarized pattern, being mainly concentrated around the nucleus, in the trailing edge and along the lateral margins of the cells, and essentially excluded from the protrusions.

Lack of Raf-1 induced profound changes in the subcellular distribution of Rok-α. In KO fibroblasts Rok-α was no longer associated with the vimentin cytoskeleton, but was rather localized at the membrane at the cell front. Re-expression of either KC or KD Raf-1 restored this defect (Fig. 7 A).
Similarly, Rok-α was often observed at the membrane in KO keratinocytes, whereas in WT cells the staining was essentially excluded from this location (Fig. 7 B).

**Discussion**

The data described above assign a novel function to Raf-1 in migration. Raf-1 KO fibroblasts and keratinocytes are defective in adhesion and motility, whereby the latter phenotype is more severe and can be observed in vivo as delayed keratinocyte migration during wound healing. The basis of these phenotypes is the deregulation of Rok-α, which interacts physically with Raf-1 in WT cells. Thus, Raf-1 operates as a regulator of Rho-downstream signaling.

**Role of Raf-1 in the epidermis**

The epidermis of c-raf-1 H9004/-H9004 ep mice shows normal thickness and architecture indicating that, in vivo, Raf-1 is not an essential component of the pathways mediating skin proliferation and differentiation. Consistently, the phosphorylation of ERK, which has been established as an important player in skin proliferation (Haase et al., 2001; Vasioukhin et al., 2001; Dajee et al., 2002) and as the main mediator of the transcriptional responses induced by Raf-1 (Schulze et al., 2004), is not reduced in Raf-1 KO keratinocytes. Rok-α immunoprecipitates were prepared from untreated (Unt) and serum-stimulated (FCS, 10%, 1 h) WT and KO fibroblasts. Rok-α kinase activity was assayed as the ability of the immunoprecipitates to phosphorylate recombinant MYPT1. The results plotted are the mean (±SD) of three independent experiments, normalized for the amount of Rok-α present in the immunoprecipitates and expressed as percentage of the activity present in the immunoprecipitates from unstimulated WT cells (Fig. 7 E).

**Raf-1 regulates Rho signaling and migration**

The results discussed above show that Raf-1 regulates Rho signaling at the level of Rok-α localization and activation. We hypothesize that in WT cells, the formation of a Raf-1–Rok-α complex would result in the inhibition of Rho kinase activity, thus impairing the migration of keratinocytes and fibroblasts. This hypothesis is supported by the observation that Y-27632, a Rho kinase inhibitor, reverses the hyperphosphorylation of MYPT1 and paxillin in Raf-1 KO fibroblasts (Fig. 7 F).

(G) Lysates collected from subconfluent keratinocytes. Protein expression and phosphorylation was determined as in A, except that Raf-1 was detected with an antibody specific for the Raf-1 NH2 terminus. Loading control, Keratin 5. [H] Y-27632 improves migration of KO keratinocytes. Y-27632–treated and –untreated cells were allowed to migrate for 6 h in a modified Boyden chamber assay using KGM as a chemoattractant. Values are means ± SD of three individual samples.
complex limits Rok-α activation and its presence at the leading edge, thereby releasing tension and allowing lamellipodia formation. Rok-α activation by Rho involves the reorganization of the vimentin network, which collapses in a juxtanuclear aggregate and releases Rok-α. Rok-α then translocates to the cell periphery, and this correlates with increased contractility. Rok-α itself phosphorylates vimentin. This phosphorylation decreases the affinity of vimentin for Rok-α and contributes to the collapse of the intermediate filaments. Thus, either autoinduction or a positive feedback loop are apparently involved in Rok-α activation (Sin et al., 1998). How could Raf-1 antagonize this process? At least two possibilities can be envisioned: first, the presence of Raf-1 may increase the affinity of Rok-α for vimentin, thereby limiting Rok-α membrane translocation and activation. Alternatively, Raf-1 might have a more direct role and inhibit Rok-α kinase activity. Both Raf-1 (Cutler et al., 1998) and Rok-α (Amano et al., 1999) are regulated by intramolecular inhibition. In both cases, an autoregulatory region (the NH2-terminal domain in Raf-1, the COOH-terminal domain in Rok-α) binds to the kinase domain and inhibits activity. Upstream activators are thought to disrupt the interaction between the kinase domain and the autoinhibitory region to give rise to an active kinase. Remarkably, the autoregulatory domains of the two kinases contain cysteine-rich, highly ho-
mologous pleckstrin-homology domains (Leung et al., 1996). It is conceivable that Raf-1 may bind to the Rok-α kinase domain once autoinhibition has been released, thereby keeping Rok-α activation in check. Consistent with this model, Rok-α activation in Raf-1 KO cells still depends on a functional Rho GTPase (Fig. 4 D), and Raf-1 binds to Rok-α via its NH₂-terminal regulatory domain (Fig. 6 B). In addition, we have recently demonstrated that transfection with the isolated NH₂-terminal domain of Raf-1 is sufficient to rescue the defects of the Raf-1 KO fibroblasts (unpublished data).

Either of the proposed mechanisms may involve phosphorylation of one or both proteins by upstream kinases. In this context, we have shown that the kinase activity of Raf-1 is moderately stimulated during in vitro wound healing. The same phosphorylation events that stimulate Raf-1 MEK-kinase activity in vitro by relieving autoinhibition might render the NH₂-terminal domain of Raf-1 available to interact with Rok-α; alternatively, they may determine the subcellular localization of the complex. At present, we favor the former hypothesis, as Raf-1 and Rok-α colocalize on the intermediate filaments even in nonmigrating, starved cells. In either case, however, the KD mutant would be phosphorylated by upstream kinases in a manner similar to the WT and hence rescue adhesion/migration.

The discovery of a kinase-independent function of Raf-1 in the control of cell polarity and migration is surprising given the number of Raf-1 phosphorylation targets of this kinase that affect these processes. Activated MEK/ERK, the traditional target of the Raf-1 kinase, plays an important role in the maintenance of cell shape and in migration (Klemke et al., 1997; Cheres et al., 1999; Glading et al., 2001), and can reduce Rok expression and activation (Sahai et al., 2001; Pawlak and Helfman, 2002; Vial et al., 2003). However, as previously described for growth factor–induced activation (Huser et al., 2001; Mikula et al., 2001), Raf-1 is dispensable for ERK activation during migration. Consistently, B-Raf and MEKK1 have been identified as the upstream kinases required for ERK activation in migrating fibroblasts (Cuevas et al., 2003; Pritchard et al., 2004). Interestingly, the phenotype of the MEKK-1 and of the B-Raf KO are opposite: in the MEKK-1 KO, lack of ERK activation causes defective rear-end detachment, due to the lack of calpain activation and focal adhesion turnover (Cuevas et al., 2003). This results in reduced migration. In contrast, B-Raf KO fibroblasts are more spread, and migrate faster, than WT cells, due to increased stress fibers depolymerization (Pritchard et al., 2004). Thus, ERK appears to accomplish distinct functions during migration, that can be evidenced by deleting specific upstream kinases.

A further Raf-1 target implicated in the regulation of mobility is the phosphatase MYPT1, which is inactivated by Raf-1–mediated phosphorylation (Broustas et al., 2002). MYPT1 dephosphorylates myosin light chain, and MYPT1 deregulation would ultimately lead to decreased cell contractility. The phenotype of the KO cells, therefore, is entirely inconsistent with MYPT1 deregulation. Lack of Raf-1 in this context is likely compensated by the hyperactivation of Rok-α, itself able to inhibit MYPT1 by phosphorylation (Kimura et al., 1996), and to phosphorylate myosin B light chain (Amano et al., 1996).

Finally, the discovery that lack of Raf-1 causes deregulation of Rok-α activity has had a major impact on the investigation of the anti-apoptotic function of Raf-1 and of the role of...
sections were stained with hematoxylin and eosin. Immunohistochemistry staining for Ki67 (Novocastra), Keratin 5, and Involucrin (BoBco) were performed using the ABC staining kit (Vector Laboratories) according to the manufacturer’s recommendations. TUNEL staining was performed using the in situ cell death detection kit (Roche).

**In vivo wound healing assay**

These experiments were performed in strict adherence to institutional guidelines for minimizing distress in experimental animals. Full-thickness skin excisions were made using a biopsy punch of 6-mm diam on the back of 8–10-wk-old mice anesthetized with Ketamine/Xylazine. Wound healing was monitored histologically by fixing the wounded skin after 2, 5, 8, 10, 12, and 16 d after wounding (n = 5 for each time point).

**Cell isolation, culture, infection, and transfection**

Primary mouse keratinocytes from 3-d-old mice were isolated as described previously (Hennings, 1994) and cultured in KGM (Ca^{2+}-free; CLONTECH Laboratories, Inc.) supplemented with 2% chelated FCS (Carroll et al., 1999), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin (Sigma-Aldrich), 10 ng/ml EGF, 60 μg/ml bovine pituitary extract, gentamycin (50 μg/ml), and 100 U/ml nystatin (Roche). The final concentration of Ca^{2+} of the KGM was 0.05 mM. COS-1 cells and 3T3-like fibroblast cell lines derived from E12.5 Raf-1 KO and WT embryos (Mikula et al., 2001) were cultured in DME supplemented with 10% FCS. Stable cell clones expressing KC and KD Raf-1 were obtained by infecting Raf-1 KO fibroblasts with retroviruses containing pMNC-Raf-1 (KC) and pMNC-Raf-1K375W (KD; a gift from W. Kolch, Beatson Institute for Cancer Research, Glasgow, UK; Kolch et al., 1991). Transient transfections were performed using lipofectamine according to the manufacturer’s instructions. COS-1 cells were transfected with HA-tagged Rok-α (Chen et al., 2002) in combination with full-length Raf-1, the Raf-1 NH2-terminal domain (aa 1–257, AC) or the Raf-1 COOH-terminal domain (ΔN, internal deletion of aa 26–302; Bruder et al., 1992). Fibroblasts were transfected with KD, dominant negative Rok-α (pXJ40-HA-Rok-αKD; Leung et al., 1996), and DN-Ez-Eg (a gift from R. Lamb, Institute of Cancer Research, London, UK) or the corresponding empty vectors.

**Adhesion and migration assays**

The attachment assays were performed using the Cytomatrix kit (CHEMICON International, Inc.) according to the manufacturer’s instructions. For the in vitro wounding assay, starved cells were pretreated with 10 μg/ml mitomycin C for 2 h to block proliferation and a cell-free area was created by scratching the monolayer with a 200-μl pipette tip. Cell migration into the wound area was monitored in serum-free medium or in the presence of 30 ng/ml EGF or 10% FCS. Photographs were taken using a phase-contrast microscope (DIAPHOT 300; Nikon). In selected experiments, the ROK-α inhibitor Y27632 (10 μM; Calbiochem) was added to the monolayers 20 min before in vitro wounding. For the modified Boyden chamber migration assay, equal numbers of cells suspended in serum-free medium or in the presence of 30 ng/ml EGF or 10% FCS migrated through the pores (8 μm) of a polycarbonate membrane toward a chemoattractant (KGM, EGF, KGF, or FCS) placed in the bottom chamber. Cells on the underside of the membrane were fixed, stained with 10% crystal violet and counted (5–7 random fields/membrane) by two independent investigators. In the assays using HA-Raf-α-KD–transfected fibroblasts, total cells were visualized by staining the filters with rhodamine-conjugated phallloidin (Molecular Probes), whereas transfected cells were detected by immunofluorescence using a monoclonal α-HA antibody (12CA5).

**Immunoprecipitation, kinase assays, and Western blot analysis**

Cells lysis, Western blotting, Raf-1 immunoprecipitation, and kinase assay were performed as described previously (Mikula et al., 2001). To assay Rok-α kinase activity, Raf-1 was immunoprecipitated from 150 μg of lysate using an antibody against the NH2-terminal domain of the protein (Upstate Biotechnology). The immunoprecipitates were incubated (15 min, 30°C) with 1 μg of recombinant MYPT1 (Upstate Biotechnology). The supernatant of the reactions was loaded on a gel and the phosphorylation of MYPT1 analyzed on a PhosphorImager.

**Materials and methods**

**Generation of craf-1^+/-;α^-' mice**

Mice carrying a fixed craf-1 allele were crossed to transgenic mice expressing the Cre recombinase under the control of the keratinocyte specific Keratin 5 promoter (K5Cre2; Tarutani et al., 1997) to obtain craf-1^+/-;α^-' animals. Allele-specific PCR genotyping was performed as described previously (Jesenberger et al., 2001).

**Histology, immunohistochemistry, and TUNEL assay**

Tissues from newborn or adult mice were fixed overnight in buffered 4% PFA (in PBS, pH 7) at 4°C, dehydrated, and embedded in paraffin. 5-μm sections were stained with hematoxylin and eosin. Immunohistochemistry staining for Ki67 (Novocastra), Keratin 5, and Involucrin (BoBco) were performed using the ABC staining kit (Vector Laboratories) according to the manufacturer’s recommendations. TUNEL staining was performed using the in situ cell death detection kit (Roche).

This kinase in embryonic development. We could recently show that hyperphosphorylation and deregulation of ezrin secondary to Rok-α activation are responsible for the selective up-regulation of the death receptor Fas in Raf-1 KO fibroblasts in vitro and liver cells in vivo. By genetically reducing Fas expression, we could further show that this up-regulation is responsible for fetal liver apoptosis and for the embryonic death of Raf-1 KO embryos (unpublished data). These data indicate that, whereas the presence of enzymes with similar or identical substrate specificity may enable cells to cope with the absence of the catalytic function of a specific kinase, KO experiments like ours are more likely to uncover specialized essential functions connected with protein–protein interactions.

**Figure 7. Raf-1, but not its kinase activity, is required for correct Rok-α localization.** (A and B) Rok-α is not excluded from cell protrusions in migrating Raf-1 KO fibroblasts (A) and keratinocytes (B). The defect is corrected in fibroblasts expressing KC or KD Raf-1, but not in those transfected with empty vector (V; A). Rok-α staining was visualized in permeabilized fibroblasts by confocal microscopy (A); in subconfluent keratinocytes by epifluorescence (B). The dotted lines in panel f/f represent the contours of the keratinocytes. Arrows in A and B point to Rok-α membrane staining.
Immunofluorescence

Cells plated on fibronectin (Invitrogen) were permeabilized (0.01% Triton X-100), fixed in 4% PFA, and blocked with 0.2% gelatin before incubation with primary antibodies (α-vinculin; Sigma-Aldrich; α-Raf-1 NH2-terminus and α-RosA [BD Biosciences]) and staining with the appropriate TRITC- or FITC-conjugated secondary antibodies (Molecular Probes). Rho-domain conjugated phallolidin (Molecular Probes) was used to visualize actin filaments. To visualize vimentin intermediate filaments cells were extracted/fixed in methanol containing 5 mM EDTA and permeabilized with 0.5% Triton X-100. Cells were subsequently stained with an α-vimentin antibody (Sigma-Aldrich) followed by TRITC-conjugated secondary antibodies before mounting with ProLong Antifade (Promega). Epifluorescence was performed using an Axioptan 2 microscope (Carl Zeiss Microimaging, Inc.) equipped with a Plan-Neofluar 40×/1.30 oil objective and a CCD camera (Spot2; Diagnostic Instruments, Inc.). Images were acquired using the MetaVue 5.0r6 software (Universal Imaging Corporation). Confocal microscopy was performed with an Axiovert 100M confocal microscope (Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Apochromat 63×/1.40 oil objective. The confocal images were acquired using the LSM 510 2.3 software. The pictures show Z-stacks.

Rho pull down assay

The amount of active Rho present in migrating cells was determined by affinity chromatography on Rho binding domain of rhotekin immobilized on agarose beads using the Rho activity assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. The amount of bound (active) and total Rho was determined by immunoblotting.

ADP-ribosylation of Rho

Rho ADP-ribosylation was achieved by incubating fibroblasts for 3 h with C. botulinum C2 toxin ([C2lla: 600 ng/ml] to enable uptake of the C2-like exoenzyme) plus a fusion toxin ([C2INtC3: 300 ng/ml] consisting of the NH2-terminal part of binding component of the C. botulinum C2 toxin ([C2INt2C1]) and the full-length C2-like ADP-ribosyltransferase from C. limosum lo toxin, as described previously (Barth et al., 1998; a gift from H. Barth and K. Aktories, University of Freiburg, Freiburg, Germany).

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