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The MSP Receptor Regulates α6β4 and α3β1 Integrins via 14-3-3 Proteins in Keratinocyte Migration

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Summary

Growth factors, integrins, and the extracellular matrix (ECM) are known to play key roles in epidermal wound healing, although the interplay between these proteins is not fully understood. We show that growth factor macrophage stimulating protein (MSP)- and its receptor Ron-mediated PI3K activation in keratinocytes induces phosphorylation of both Ron and α6β4 integrin at specific 14-3-3 binding sites. Consequently, a Ron/α6β4 complex formed via 14-3-3 binding displaces α6β4 from its location at hemidesmosomes (structures supporting cell adhesion) and relocalizes it to lamellipodia. Concomitant activation of α3β1 and keratinocyte spreading/migration on laminin-5 occurs. Further, MSP-dependent β4 tyrosine phosphorylation evokes p38 and NF-κB signaling required for keratinocyte wound closure. Based on these results, we propose a mechanism based on MSP-Ron-dependent phosphorylation and 14-3-3 association, whereby the function of α6β4 switches from a mechanical adhesive device into a signaling component, and might be critically involved in human epidermal wound healing.

Introduction

The complex interplay between the extracellular matrix (ECM), growth factors, and integrins is crucial for many biological processes, including skin wound healing. Wound healing is characterized by a number of overlapping phases including inflammation, reepithelization, granulation tissue formation, and tissue remodeling (Martin, 1997; Singer and Clark, 1999). The reepithelization process is tightly regulated by specific classes of integrin receptors and interacting extracellular matrix molecules. In particular, α3β1 and α6β4 integrins, as well as laminin-5, play a key role in keratinocyte migration (Fuchs et al., 1997; Giancotti and Ruoslahti, 1999; Nguyen et al., 2000). Laminin-5 is the primary ligand of adult epidermal basement membrane (BM) and is secreted by wounded keratinocytes in the provisional wound bed to promote migration (Zhang and Kramer, 1996). Laminin-5 is synthesized as a precursor heterotrimeric protein (α3,β3,γ2) that undergoes processing of α3 and γ2 subunits after being secreted (Colognato and Yurchenco, 2000). The domain interacting with integrin resides within the α3 C-terminal large globular domain, while the region for deposition in the BM resides in the γ2 subunit (Shang et al., 2001; Gagnoux-Palacios et al., 2001). Current models propose that laminin-5 mediates keratinocyte adhesion and migration via α6β4 and α3β1 integrins at distinct sites. α6β4 can be found in hemidesmosomes (HDs), which are structures linking ECM to keratin intermediate filaments thereby supporting cell adhesion (Borradori and Sonnenberg, 1999). α3β1 is in focal contacts and links ECM to the actin cytoskeleton, regulating cell spreading and migration (Kreidberg, 2000). The interplay between α3β1 and α6β4 and different domains of laminin-5 is crucial at the wound edges because it regulates leading keratinocytes by (1) disassembling HDs, (2) remodeling matrix interactions with increased deposition of laminin-5 and metalloproteases (MMPs), and (3) recruiting α3β1 at focal contacts to mediate migration over the provisional matrix (Fuchs et al., 1997; Nguyen et al., 2000; Ravanti and Kahari, 2000).

Macrophage stimulating protein (MSP) is the ligand of the Ron tyrosine kinase receptor. It is a biologically inactive soluble plasma factor activated at extravascular sites by specific serine proteases (Skeel and Leonard, 1994). The Ron receptor is selectively expressed by epithelia, including keratinocytes, and by hematopoietic cells (Gaudino et al., 1994; Wang et al., 1996b; Banu et al., 1996). MSP leads to receptor *trans*-autophosphorylation and activation of several signaling pathways (Wang et al., 1996a; Santoro et al., 1998). MSP and Ron modulate keratinocyte functions such as proliferation, survival, and migration, and, recently, a role in epidermal wound healing has been suggested (Wang et al., 1996b; Nanney et al., 1998; Danilkovitch et al., 2000; Cowin et al., 2001).

Here, we show that MSP-mediated PI3K pathway activation induces Ron serine phosphorylation at residue 1394 as well as α6β4 phosphorylation in the connecting sequence to generate 14-3-3 binding sites on both molecules. Thus, dimeric 14-3-3 proteins mediate the MSP-dependent formation of a Ron/α6β4 complex that in turn induces disassembly of HDs and α6β4 relocation at lamellipodia. Further, α3β1 integrin activation and keratinocyte spreading/migration on laminin-5 takes place. All these findings suggest a role for Ron and 14-3-3 in epidermal reepithelization processes.

Results

Akt Kinase Phosphorylates the Ron Receptor at Serine 1394

The MSP receptor, Ron, belongs to the HGF receptor tyrosine kinase family and contains the peculiar C-terminal proline/arginine-rich sequence RRPRPLS^{1394}EPPRPT (Figure 1A). S^{1394} is a potential phosphorylation site for

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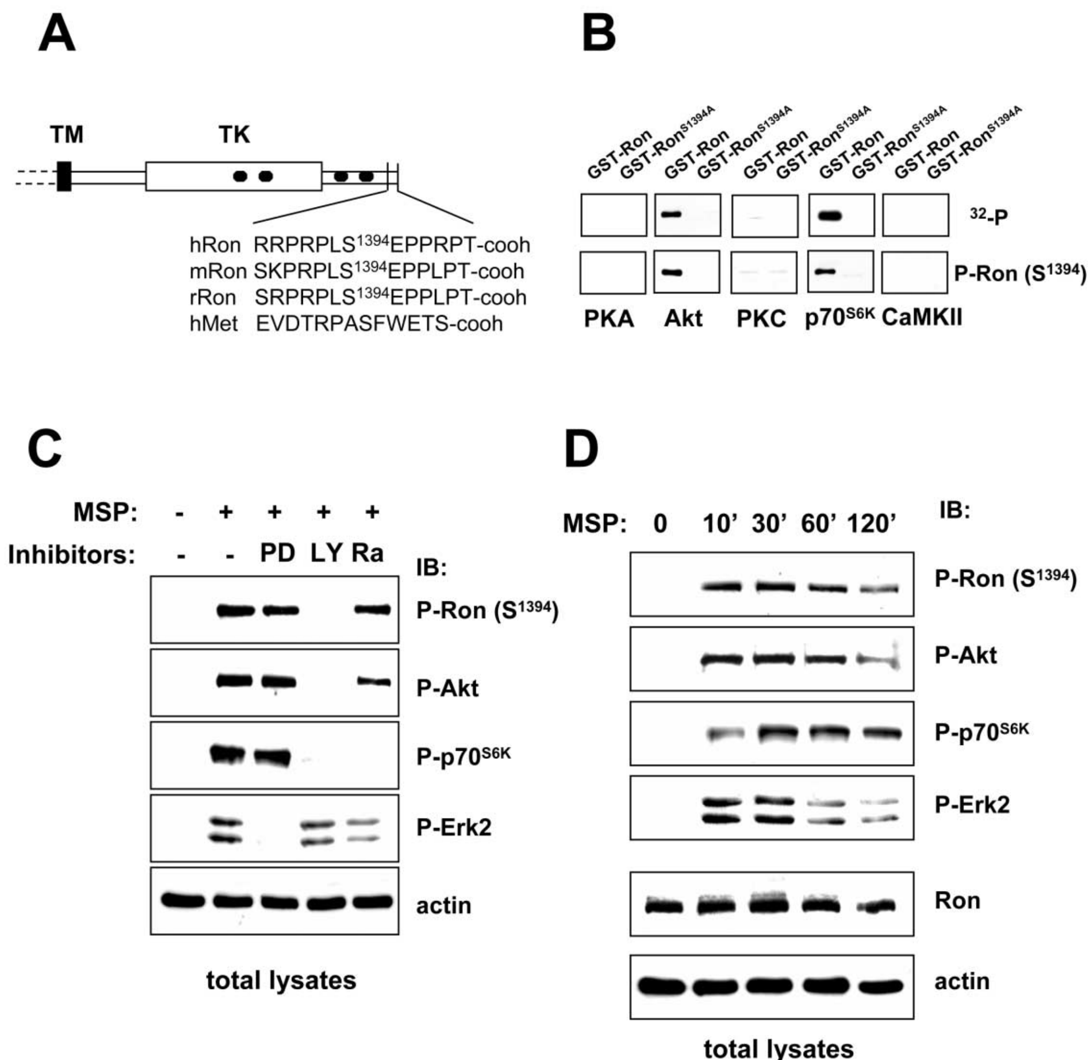

Figure 1. MSP Induces Ron Receptor S^{1394} Phosphorylation by Akt

(A) Schematic representation of Ron intracellular domain and C-terminal amino acid sequence. Black dots indicate Ron major tyrosine autophosphorylation sites responsible for receptor activation and SH2-mediated signal transduction. C-terminal amino acid sequence is characterized by a specific proline/arginine-rich amino acid sequence and a putative serine phosphorylation site (S^{1394}) that are conserved among the human, mouse, and rat homologs. The related sequence of human HGF receptor, Met, is also indicated. TM and TK indicate transmembrane and tyrosine kinase domains, respectively.

(B) Akt kinase phosphorylates Ron at serine 1394. GST-Ron and GST-Ron^{S1394A} were tested in in vitro kinase assays with recombinant active PKA, Akt, PKC, $p70^{S6K}$, and CaMKII kinases and [γ-^{32}P]ATP. Samples were subjected to autoradiography (top panel) or immunoblotting with the specific phospho-Ron (S^{1394}) antibody (bottom panel).

(C) MSP induces Ron receptor phosphorylation at S^{1394}. Serum-starved FG2 cells were preincubated with LY294002 (LY), rapamycin (Ra), or PD98059 (PD) for 30 min and then stimulated with MSP. Cell extracts were analyzed by Western blotting using phospho-Ron (S^{1394}), P-Akt, P-$p70^{S6K}$, and P-Erk2 antibodies. Membranes were probed with β-actin antibodies as control.

(D) Time course Ron S^{1394} phosphorylation by MSP. Serum-starved FG2 cells were left untreated (0′) or stimulated for 10, 30, 60, and 120 min with MSP. Cell lysates were analyzed by Western blotting with phospho-Ron (S^{1394}), P-Akt, P-$p70^{S6K}$, and P-Erk2 antibodies. The filters were probed with Ron and β-actin antibodies as controls.

PKA, $p70^{S6K}$, Akt, and CaMKII cytosolic kinases (Yaffe et al., 2001). Using an in vitro kinase assay, we tested S^{1394} phosphorylation by incubating a GST-Ron fusion protein containing the last 40 amino acids of the Ron C-terminal region, with constitutively active forms of PKA, Akt, PKC, $p70^{S6K}$, and CaMKII in the presence of [γ-^{32}P]ATP (Figure 1B, top). Only Akt and $p70^{S6K}$ kinases phosphorylated GST-Ron. The phosphorylation was lost in the GST-Ron^{S1394A} mutant, where the serine is replaced by alanine. Ron S^{1394} phosphorylation was also tested using a phospho-Ron (S^{1394}) antibody recognizing the phosphorylated form of serine 1394 (Figure 1B, bottom). We conclude that Ron S^{1394} is phosphorylated by Akt and $p70^{S6K}$ in vitro.

Ron serine phosphorylation was investigated in lysates of MSP-stimulated FG2 epithelial cells using a phospho-Ron (S^{1394}) antibody (Figure 1C). MSP induced Ron S^{1394} phosphorylation that was inhibited by LY294002 (or

wortmannin; data not shown) but not by rapamycin, indicating that Akt and not $p70^{S6K}$ phosphorylates Ron in vivo. PD98059, inhibiting MEK and blocking Erk2 kinase, did not influence S^{1394} phosphorylation. In a time course experiment of MSP stimulation, Ron S^{1394} phosphorylation showed the same kinetics of Akt, but not of $p70^{S6K}$ and Erk2 activation (Figure 1D). We conclude that Ron S^{1394} phosphorylation is induced by MSP through a receptor-mediated PI3K and Akt activation.

Ron Binds to 14-3-3 Proteins via S^{1394} Phosphorylation

The Ron C-terminal sequence matches the consensus motifs for several protein-protein interaction modules (Kay et al., 2000; Yaffe and Elia, 2001). To identify protein(s) binding to this sequence, we screened a HeLa cDNA expression library by a yeast two-hybrid system. The last 50 C-terminal amino acids of Ron were fused to the LexA binding domain and used as bait. Three independent partial clones, all coding for the 14-3-3τ protein, were isolated. Full-length 14-3-3 isoforms were tested for Ron interaction in yeast cells, τ and β isoforms being preferred for binding (Figure 2A). It is noteworthy that the Ron C-terminal amino acid sequence fits with the canonical 14-3-3 consensus sequences (RXRXXpSXP; Muslin et al., 1996; Yaffe et al., 1997). A Ron^{S1394A} bait was tested for 14-3-3τ binding and it did not associate to 14-3-3τ, as expected, indicating that the integrity of S^{1394} is required for Ron/14-3-3 interaction. Moreover, deletion mutants of 14-3-3τ either at the N terminus (14-3-3$\tau\Delta$N), lacking the first two α helices involved in dimer formation, or at the C terminus (14-3-3$\tau\Delta$C), devoid of the last α helix required for target binding, were examined for Ron binding. The 14-3-3$\tau\Delta$C mutant, but not the ΔN, failed to interact with Ron (Figure 2B), indicating that the last α helix of 14-3-3τ is mandatory for Ron association.

The role of S^{1394} phosphorylation in binding to 14-3-3 was tested in pull-down experiments using GST-14-3-3τ and lysates of FG2 epithelial cells (Figure 2C). The binding of GST-14-3-3τ to Ron was direct and strictly dependent on Akt activation and was abolished by phosphatase treatment of MSP-stimulated lysates. This demonstrates that S^{1394} phosphorylation by Akt is required for Ron/14-3-3 interaction. In FG2 epithelial cells, 14-3-3 proteins were coimmunoprecipitated by Ron antibodies specific for the extracellular portion of the molecules (Ron/N), but not by other Ron antibodies (Ron/C), whose epitope corresponds to the 14-3-3 binding site. The blockade of the PI3K/Akt pathway by LY294002 abrogated Ron S^{1394} phosphorylation and 14-3-3 association (Figure 2D), but did not alter the binding of other transducers to Ron, such as Gab-1 or p85 (data not shown).

Immunofluorescence analyses in epithelial cells stimulated with MSP revealed that a significant fraction of Ron colocalized with 14-3-3τ molecules at the leading edge of migrating cells. This indicates that the Ron/14-3-3 complex take places at protruding membranes and lamellipodia (Figure 2E).

These results identify 14-3-3 as a Ron binding protein in epithelial cells, and show that Ron S^{1394} phosphorylation by Akt causes the binding in an MSP-dependent way.

Ron/14-3-3 Association Is a Prerequisite for Keratinocyte Spreading and Migration on Laminin-5

Next, we investigated the Ron/14-3-3 complex in keratinocytes, where proteins are expressed or upregulated (Olsen et al., 1995; Nanney et al., 1998; Cowin et al., 2001). Primary human foreskin keratinocytes (HEK) were stimulated with MSP. Lysates were immunoprecipitated with Ron, Met, and EGFR antibodies and immunoblotted with 14-3-3 antibodies. 14-3-3 associated with Ron but not with Met or EGFR, when stimulated by their respective ligands (Figure 3A). Notably, this shows that interaction is Ron specific and occurs between the endogenous proteins.

MSP and Ron regulate proliferation and migration of keratinocytes (Wang et al., 1996b). To test whether Ron/14-3-3 interaction modifies these processes, we transduced HEK cells with lentiviral vectors encoding for Ron, Ron^{S1394A}, and 14-3-3$\tau\Delta$C mutants, obtaining comparable levels of expression (Figure 3B, right). Pools of infected cells were stimulated with MSP, and the Ron/14-3-3 complex was analyzed by coimmunoprecipitation assays (Figure 3B, left). Expression of Ron^{S1394A} or 14-3-3$\tau\Delta$C mutants interfered with Ron/14-3-3 complex formation, showing a loss of function for these mutants in regulation of Ron/14-3-3 association. In 14-3-3$\tau\Delta$C-expressing cells association was not completely abolished, possibly because of high endogenous 14-3-3 expression. We analyzed keratinocyte migration on different ECM proteins using chemotaxis assays in the presence of mitomycin, to avoid possible influence of cell proliferation (Figure 3C). MSP dramatically increased the rate of keratinocyte migration on laminin-5 and, to a lesser extent on fibronectin, but not on vitronectin and collagen-I. Ron^{S1394A}- and 14-3-3$\tau\Delta$C-expressing cells displayed a lower cell migration on laminin-5 but migrated on fibronectin, suggesting that Ron/14-3-3 association sustained specific migration on laminin-5. No differences in proliferation rate were observed in MSP-stimulated keratinocytes expressing Ron or Ron S^{1394A} (data not shown).

Spreading assays were performed by plating quiescent HEK on purified fibronectin, vitronectin, collagen-I, and laminin-5 matrices and stimulating them with MSP (Figure 3D, panels a–d). MSP did induce cell spreading on laminin-5, but not on other substrates. The rapid spreading on laminin-5 in Ron-expressing keratinocytes was markedly decreased in Ron^{S1394A}-expressing cells (Figure 3E), supporting the role of the Ron/14-3-3 complex in MSP-induced migration.

These results show that Ron phosphorylation and/or 14-3-3 binding regulates cell spreading and migration on laminin-5 but not cell proliferation, possibly via an integrin-mediated mechanism.

β4 Integrin Is Phosphorylated by PKC and Binds 14-3-3 Proteins

To test whether integrins may interact with 14-3-3 proteins, we used β1, β2, β3, and β5 baits as well as five different β4 baits encompassing the whole cytodomain of β4 for interaction with 14-3-3 in yeast two-hybrid systems. While β1, β2, β3, and β5 did not bind 14-3-3 (data not shown), only β4 baits containing the connecting sequence (CS) were positive (Figure 4A). In pull-down assays of GST-14-3-3 fusion proteins on stimulated keratinocyte lysates, β4 bound to GST-14-3-3-τ

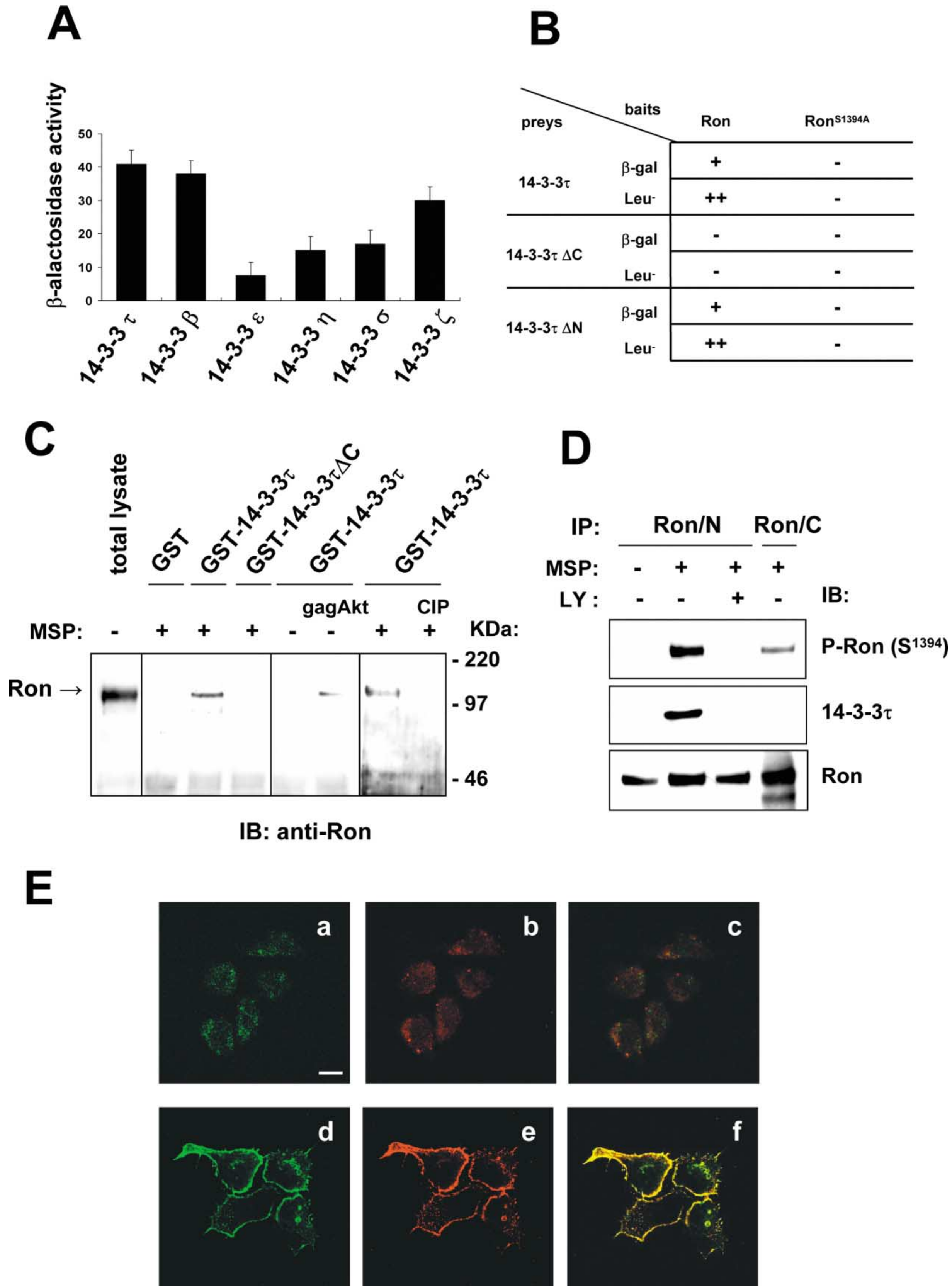

Figure 2. The Ron Receptor Binds 14-3-3 Proteins after S^{1394} Phosphorylation by Akt

(A) Interaction of Ron with 14-3-3 proteins in a yeast two-hybrid system. Yeast cells containing the LexA-Ron baits were transformed with the full-length 14-3-3 τ, β, ϵ, η, σ, and ζ isoforms under the control of the GAL promoter. Interactions were evaluated for β-galactosidase activity on GAL-containing medium.

and -β, but not to GST alone (Figure 4B), suggesting that 14-3-3 is an interactor of α6β4 integrin.

A role for PKC kinase in β4 serine/threonine phosphorylation has been suggested (Rabinovitz et al., 1999; Alt et al., 2001). Constitutively active PKCα and δ phosphorylated in vitro the GST-fusion proteins containing the CS region of β4. These were specifically recognized by an antibody selective for phosphorylated 14-3-3 binding motifs (Figure 4C). These data show that the CS of α6β4 is the target for PKC phosphorylation and 14-3-3 binding.

Lysates of MSP-stimulated HaCat cells were analyzed for β4/14-3-3 coimmunoprecipitation and β4 phosphorylation at 14-3-3 binding sites using the phospho-14-3-3 binding motifs antibody. MSP and TPA induced α6β4 phosphorylation at the 14-3-3 binding site as well as association of 14-3-3 proteins (Figure 4D). Pretreatments with the PKCα inhibitor Gö6976 blocked both β4 phosphorylation and 14-3-3 binding, while the selective PKCδ inhibitor Rottlerin was totally inactive.

These data suggest that MSP induced α6β4/14-3-3 complex formation in keratinocytes via PKCα-mediated phosphorylation of the CS of β4 integrin.

14-3-3 Proteins Mediate Ron/β4 Association and Modulate α6β4 Integrin Activity in HDs

14-3-3 proteins act as phosphorylation-dependent scaffold molecules bridging different signaling proteins together (Aitken, 1996; Tzivion et al., 2001). Thus, we analyzed whether 14-3-3 could mediate the Ron/β4 complex in keratinocytes. Extracts of Ron, Ron^{S1394A}, and 14-3-3τΔC HEK transfectants were immunoprecipitated with α6 integrin antibody and immunoblotted with Ron and 14-3-3 antibodies (Figure 5A). α6β4 specifically associated with Ron and 14-3-3 proteins after MSP stimulation. Ron^{S1394A} or 14-3-3τΔC mutants impaired this complex, indicating that Ron is associated with α6β4 by means of 14-3-3 scaffold proteins. Reciprocal experiments of immunoprecipitation with the 14-3-3 antibody, followed by Ron and β4 immunoblotting, were performed. The Ron/14-3-3/α6β4 complex was also isolated with 14-3-3 antibodies after MSP stimulation in a PI3K- and PKC-dependent manner.

We tested whether Ron/14-3-3/α6β4 interaction modulates HDs in keratinocytes. Ron-transfected HEK cells and Ron^{S1394A} or 14-3-3τΔC transfectants were grown on laminin-5 to form HD-like structures. Cells were then stimulated with MSP and analyzed by immunofluorescence for α6β4 localization (Figure 5B). Upon MSP stimulation and Ron/14-3-3 association, α6β4 integrin relocated to lamellipodia, where it also colocalized with actin filaments (data not shown). On the contrary, in unstimulated cells as well as in MSP-stimulated Ron^{S1394A}- and 14-3-3τΔC-expressing cells, α6β4 gathered to the basal surface in HD-like structures.

HDs were studied by confocal microscopy in Ron, Ron^{S1394A}, or 14-3-3τΔC HEK transfectants (Figure 5C). In the absence of MSP, the HD marker BP180 and β4 integrin colocalize at cell-substrate contact in patches, described as HD-like structures (Ron). Localization of β4 and BP180 to HDs was significantly reduced after MSP stimulation (Ron + MSP). Furthermore, α6β4 redistribute from HDs to Triton X-100-soluble compartments (Ron + MSP + Tx). This did not occur for Ron/14-3-3 association-deficient mutants (Ron^{S1394A} and 14-3-3τΔC), indicating that this event depends on Ron/14-3-3/α6β4 complex formation.

The same transfectants were used to perform adhesion assays on laminin-5 (Figure 5D). Using function-blocking antibodies, we analyzed α6β4- and α3β1-mediated adhesion on laminin-5 after Ron activation. In MSP-treated cells, α3β1 antibodies significantly impaired cell adhesion, while anti-α6β4 was markedly less effective. On the contrary, anti-α6β4 reduced cell adhesion in Ron^{S1394A} or in 14-3-3τΔC transfectants, while anti-α3β1 was less effective. These data show that the Ron/14-3-3/α6β4 complex acts to switch laminin-5 adhesion from α6β4 to α3β1 integrin.

MSP Promotes Keratinocyte Migration in a 14-3-3-Dependent Manner

HD disassembly and integrin switching from α6β4 to α3β1 for laminin-5 adhesion occur in epidermal keratinocytes during wound reepithelization. We investigated the physiological relevance of MSP and Ron in wound reepithelization by studying the healing of a cutaneous wound experimentally made in mice. Full-thickness wounds on mice back skin were treated with exogenous MSP or with carrier alone and examined for wound closure and reepithelization. A clear difference in wound size and rate of healing was evident. Carrier-treated wounds had a 2 to 3 day delay, relative to MSP-treated lesions. Histological analysis of wound cross-sections revealed that the epithelial margins progressed more

(B) Requirement of Ron S^{1394} residue and α helix I of 14-3-3τ isoform for complex formation. Yeasts were transformed with LexA-Ron or LexA-Ron^{S1394A} in combination with vectors coding for full-length 14-3-3τ isoform, 14-3-3τΔC, and 14-3-3τΔN mutants. Specific interactions were monitored for growth on selective medium (Leu^{-}) and galactosidase activity (β-gal).

(C) Direct interaction between Ron and 14-3-3 molecules is dependent on Ron S^{1394} phosphorylation. Extracts of MSP-stimulated FG2 cells were incubated with GST-fusion proteins coding for full-length 14-3-3τ or 14-3-3τΔC. Alternatively, cells were transfected with a constitutively active form of Akt (gag-Akt), or cell lysates were treated with phosphatases (CIP). Protein complexes were analyzed by Western blotting with Ron antibody. Total cell extract was used as a positive control for Ron expression.

(D) Ron/14-3-3 association is dependent on MSP stimulation and the PI3K/Akt pathway in living cells. Lysates of MSP-stimulated FG2 cells were coimmunoprecipitated with Ron antibodies directed against the extracellular region (Ron/N) or against the C-terminal tail (Ron/C) of the Ron receptor. Extracts of cells pretreated with LY294002 were also evaluated. Samples were analyzed by Western blotting with phospho-Ron (S^{1394}), 14-3-3τ, and Ron antibodies.

(E) Ron and 14-3-3 proteins colocalized on lamellipodia of migrating epithelial cells. FG2 cells were left untreated (panels a–c) or stimulated with MSP (panels d–f). Cells were analyzed for 14-3-3τ (green) and Ron receptor (red) staining. Merge is in yellow. The scale bar represents 10 μm.

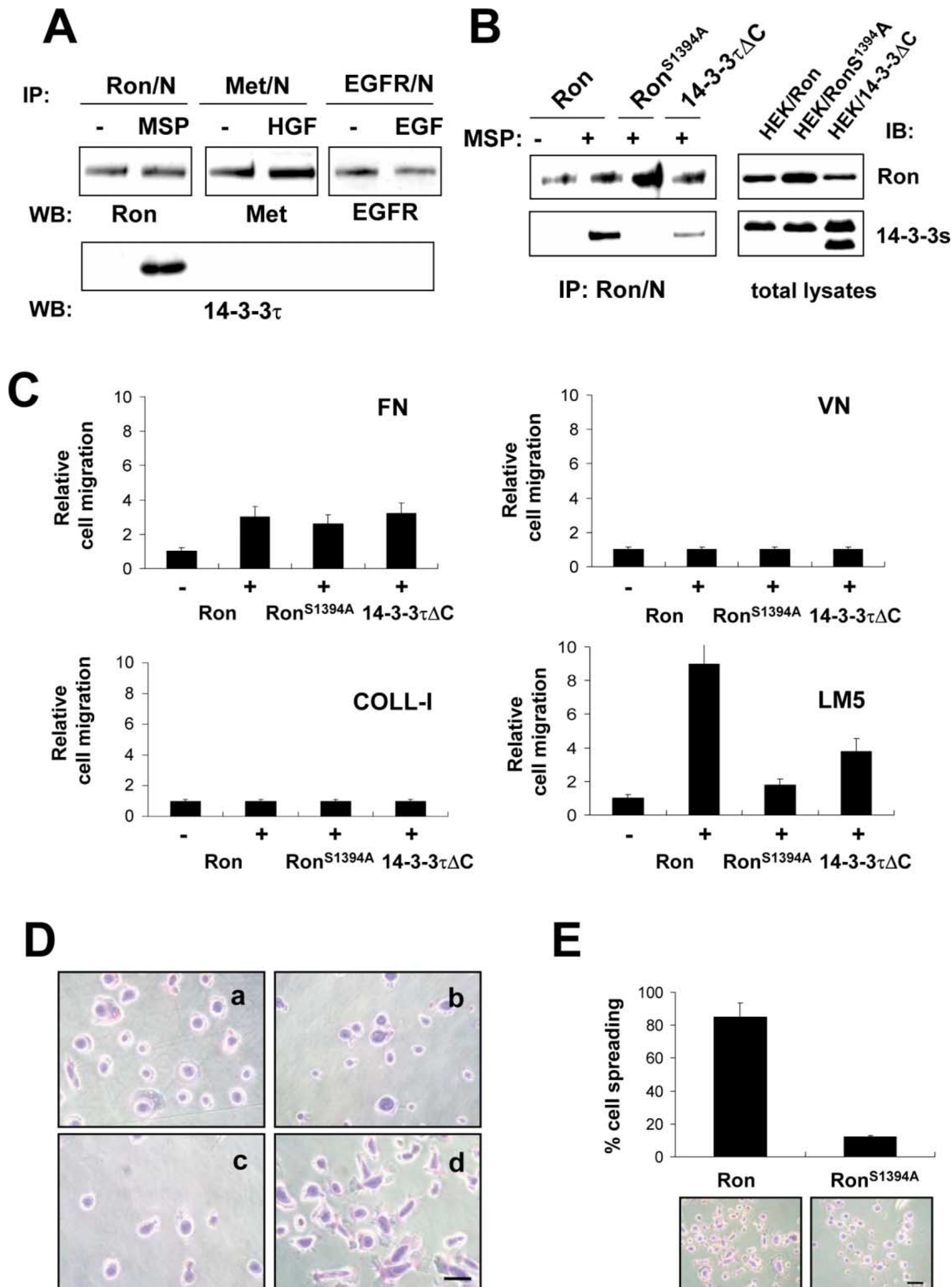

Figure 3. Ron/14-3-3 Association Is Required for MSP-Mediated HEK Migration and Spreading on Laminin-5

(A) Ron associates selectively with 14-3-3 proteins in keratinocyte cells. Primary human foreskin keratinocytes (HEK cells) were serum starved and stimulated with 5 nM of MSP, HGF, or EGF as indicated. Cell extracts were then coimmunoprecipitated with antibodies directed against the extracellular portion of the respective receptors and subjected to Western blotting with Ron, Met, or EGFR antibodies as well as anti-14-3-3τ antibodies.

(B) Dominant-negative effects of lentiviral vectors encoding Ron^{S1394A} and 14-3-3τΔC mutants in HEK cells. HEK cells were infected with lentiviral vectors encoding for Ron, Ron^{S1394A}, and 14-3-3τΔC mutants (right panel) and analyzed for Ron/14-3-3 binding (left panel). Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants were stimulated with MSP and subjected to coimmunoprecipitation analyses with Ron/N antibodies. Western blots with Ron and pan14-3-3 antibodies were performed.

(C) Cell migration of Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants on different ECM. Cell migration assays were performed in Transwell chambers

extensively in MSP-treated wounds than in carrier-treated ones. These data indicate that MSP-Ron promotes in vivo keratinocyte migration of cutaneous wounds. We looked at the role of Ron/14-3-3/α6β4 interaction in this process using an in vitro wound healing assay as a model for keratinocyte migration (Goldfinger et al., 1999; Nguyen et al., 2000). Scrape wounds were done in quiescent primary keratinocytes and cells were allowed to heal in the presence of MSP (Figure 6B). Upon MSP stimulation, keratinocytes healed the wound completely within 24 hr (Figure 6B, panel b), even in the presence of mitomycin (panel c). In Ron^{S1394A}- or 14-3-3τΔC-expressing cells, healing was inhibited (panels d and e). Furthermore, β4 null keratinocytes (PA-JEB cells) did not migrate upon MSP stimulation, proving that β4 is required for MSP-induced keratinocyte wound healing (panel f). We performed the assays adding function-inhibitory antibodies for α2β1, α3β1, and α6β4 integrin receptors and laminin-5 α3 and γ2 subunits to wounded monolayers (Figure 6C). Scraped cells incubated with MSP and control IgG antibody healed completely. Antibodies against α2β1 or α6β4 did not inhibit healing, but inhibition occurred with the α3β1 blocking antibodies or with a function-blocking antibody to the globular region of the α3 laminin-5 subunit. On the contrary, antibodies against the laminin-5 γ2 subunit had no effect on cell migration. These data indicate that MSP induces wound repair via Ron/14-3-3/α6β4 complex formation and mediates α3β1-dependent keratinocyte migration.

We analyzed focal contact formation and α3β1 localization in MSP-stimulated wounded keratinocytes (Figure 6D). Migrating cells formed focal contacts as shown by immunofluorescence analyses with paxillin and vinculin antibodies (Figure 6D, panels a and c). In parallel, α3β1 integrin is redistributed from the plasma membrane to focal contacts over the migrating front (panel e). These events were completely abolished in Ron^{S1394A}-expressing cells (panels b, d, and f). These data suggest a model in which MSP stimulation and 14-3-3 molecules guide the Ron receptor to bind to α6β4 integrin. This induces HD disassembly and α3β1-dependent focal contact formation required for keratinocyte migration (Figure 6E).

Ron-Mediated β4 Signaling Activates a p38- and NF-κB-Dependent Genetic Program

Finally, we investigated whether the 14-3-3-mediated Ron/α6β4 complex may modulate Ron signaling. Tyrosine phosphorylation of the β4 subunit generates several signaling pathways (Mainiero et al., 1995; Shaw et al., 1997). Ron- and Ron^{S1394A}-transduced HaCat cells, as well as HEK and β4 null keratinocytes (PA-JEB), were stimulated with MSP and analyzed for Ron and β4 tyrosine phosphorylation. Also Akt, $p70^{S6K}$, p38, and Erk2 activities were evaluated (Figure 7A). Both Ron and Ron^{S1394A} receptors were tyrosine phosphorylated and activated the Erk2 pathway. On the contrary, β4 tyrosine phosphorylation occurred only in the presence of a Ron/α6β4 complex (Ron, HEK), but not when this complex was impaired (Ron^{S1394A}, PA-JEB). This correlated with stronger activation of Akt and $p70^{S6K}$, as well as with de novo activation of p38, suggesting that β4 reinforces and modulates MSP/Ron signaling.

Akt and p38 pathways stimulate NF-κB transcriptional activity, through phosphorylation and degradation of IκBα (Romashkova and Makarov, 1999; Conejo et al., 2002). We show that MSP signaling induced IκB phosphorylation in Ron-expressing keratinocytes, but not in Ron^{S1394A} or 14-3-3τΔC transfectants or in PA-JEB cells (Figure 7B). This suggests the existence of a Ron/α6β4-dependent NF-κB transcriptional activity in keratinocytes.

In vitro wound healing assays were performed in the presence of MSP together with PD169316, a blocking agent for p38 MAP kinase and with BAY11-7082, a selective IκBα phosphorylation inhibitor (Figure 7C). Wound closure was markedly inhibited under these conditions, indicating that p38 and NF-κB signaling is required for keratinocyte migration. Thus, we analyzed the expression of matrix metalloproteases (MMPs) that are normally induced during the keratinocyte wound healing process (Ravanti and Kahari, 2000). RT-PCR analysis revealed that MMP2 and MMP3 as well as MSP mRNAs were expressed in a p38- and NF-κB-dependent way (Figure 7D), suggesting the existence of a specific genetic program triggered by Ron/α6β4 complex formation and required for wound closure.

Overall, these data indicate a functional role for α6β4 in keratinocytes linked to activation of specific genetic programs.

Discussion

Many biological processes are jointly regulated by growth factor receptors and integrins. Their cooperation, involving reciprocal *trans*-activation, modulation of their cellular compartments, and signaling crosstalk, often implies formation of membrane macromolecular complexes (Miranti and Brugge, 2002). Here we propose a mechanism of integrin regulation that is operated by growth factor receptors and 14-3-3 proteins, and served by multiple phosphorylation events. In particular, we show that MSP-mediated activation of the PI3K pathway in epithelia induces phosphorylation of the Ron receptor

previously coated with different substrates: fibronectin (FN), vitronectin (VN), collagen-I (COLL-I), and laminin-5 (LM5). Quiescent HEK cells were seeded and incubated in the presence of MSP for 12 hr and mitomycin. Migration was quantified by counting the cells that migrated through filters. Bars indicate the deviations standards of three independent experiments. Results are expressed as relative cell migration with respect to unstimulated cells.

(D) Cell spreading on different ECM by MSP. MSP-stimulated HEK cells were seeded on wells coated with fibronectin (a), vitronectin (b), collagen-I (c), and laminin-5 (d). Thirty minutes after MSP stimulation cells were fixed, stained with crystal violet, and photographed. The scale bar represents 40 μm.

(E) Ron/14-3-3 binding is required for keratinocyte spreading on laminin-5. Ron- and Ron^{S1394A}-expressing cells that spread on laminin-5 in the presence of MSP have been quantitated after three separate experiments as a percentage of spread cells out of the total adherent cells. Representative pictures are indicated. The scale bar represents 100 μm.

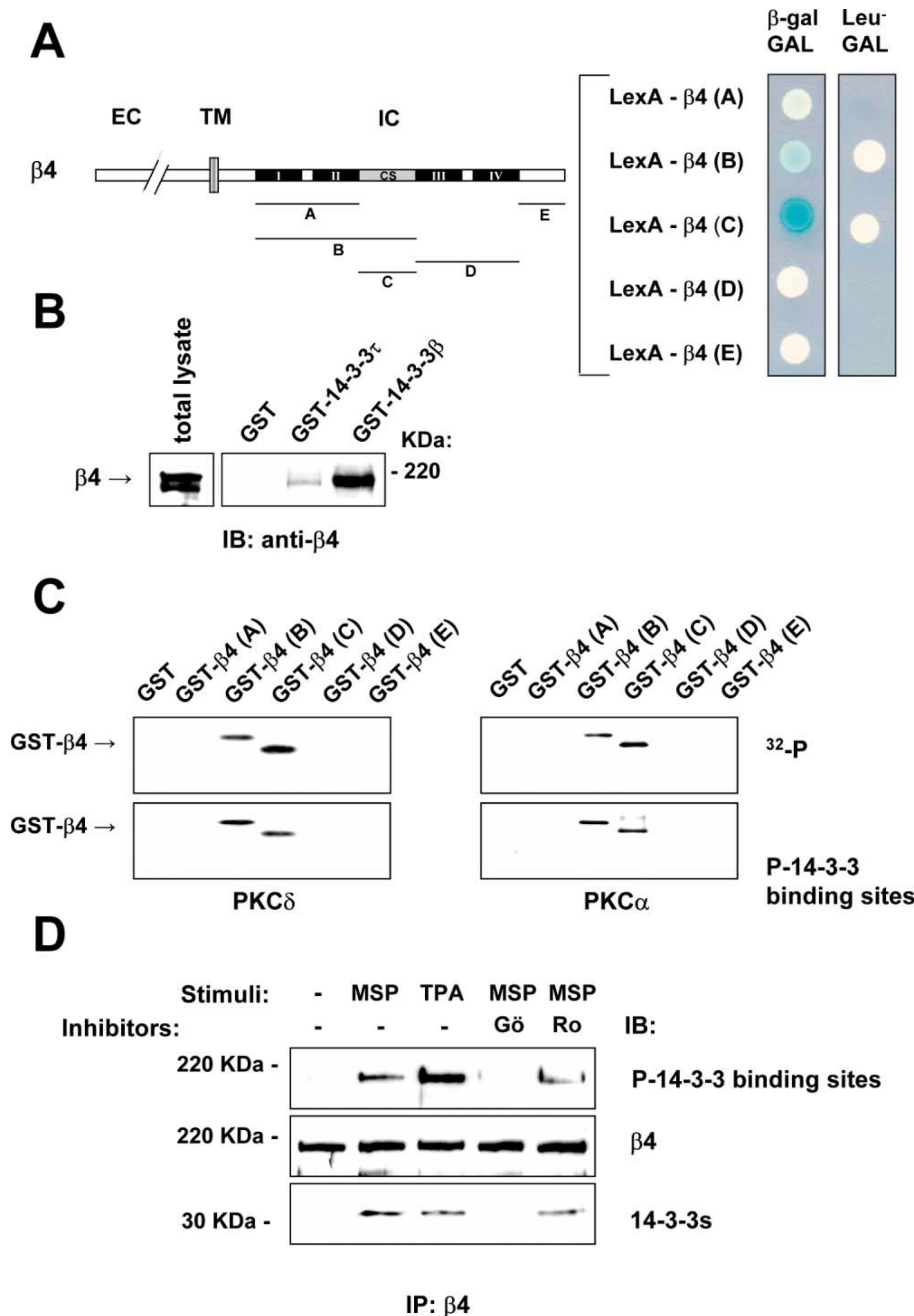

Figure 4. β4 Integrin Is Phosphorylated by Akt in the Connecting Sequence (CS) and Binds 14-3-3 Proteins

(A) Interactions between β4 integrin and 14-3-3 proteins by a yeast two-hybrid system. Cytodomain of β4 integrin has been divided into five LexA-based constructs: bait A contains the first two fibronectin type III domains, bait B the first two fibronectin type III domains and the connecting sequence (CS), bait C the merely connecting sequence, bait D the last two fibronectin type III domains, and bait E the C-terminal region (Biffo et al., 1997). Yeast cells were cotransformed with 14-3-3 plasmid and LexA-β4 constructs. Specific interactions among 14-3-3 and these baits were monitored on GAL-containing medium for growth on leucine-minus medium and Lac-Z reporter gene transcription. EC, TM, and IC: extracellular, transmembrane, and intracellular domains, respectively.

(B) Direct interaction between β4 integrin and 14-3-3 proteins. Lysates of MSP-stimulated HaCat cells were incubated with GST-fusion proteins encoding the 14-3-3τ and β isoforms as well as GST alone. Pull-down proteins were analyzed by Western blotting with β4 antibodies.

(C) In vitro phosphorylation of β4 connecting sequence (CS) by PKC. GST-fusion proteins were generated from the five β4 LexA baits illustrated above and used as substrates for in vitro kinase assays with human recombinant PKCα and δ and [γ-^{32}P]ATP. Samples were separated by SDS-PAGE and analyzed by autoradiography and by Western blotting with a phospho-14-3-3 binding site antibody.

(D) α6β4/14-3-3 association depends on PKC-mediated integrin phosphorylation at 14-3-3 binding sites. Serum-starved HaCat cells were pretreated with Gö6976 (Gö) or Rottlerin (Ro) inhibitors for 30 min and then stimulated with 5 nM MSP or 100 nM TPA. Cell extracts were coimmunoprecipitated with β4 antibodies directed against the extracellular domain. Immunocomplexes were analyzed by Western blotting with pan-14-3-3, β4, and phospho-14-3-3 binding site antibodies.

by Akt and of $\alpha6\beta4$ integrin by PKC, generating 14-3-3 binding sites on both proteins. Consequently, joining of 14-3-3 molecules mediates the formation of a complex between Ron and $\alpha6\beta4$. These findings, consistent with the recognized role of 14-3-3 molecules in bridging different proteins by means of phosphorylated serine/threonine residues (Aitken, 1996; Tzivion et al., 2001), suggest a molecular mechanism for growth factor receptor-integrin interaction, as well as an original association promoted by a specific PI3K-dependent signaling pathway.

14-3-3 binding modulates the biological functions of client proteins (Yaffe and Elia, 2001). Accordingly, we propose a mechanism based on serine/threonine phosphorylation and 14-3-3 association, which is responsible for switching $\alpha6\beta4$ function from a mechanical adhesive device, associated with HDs, into a signaling component, associated with F-actin in membrane protrusions and lamellipodia (Dans et al., 2001; Mercurio et al., 2001). Our data are consistent with the reports indicating that PKC phosphorylation of $\beta4$ induces integrin relocalization from HD to lamellipodia (Rabinovitz et al., 1999; Alt et al., 2001). We also show that $\alpha6\beta4$ dissociation from HD, mediated by the Ron/14-3-3 complex, correlates with MSP-induced $\beta4$ tyrosine phosphorylation. It has been reported that $\beta4$ tyrosine phosphorylation by Fyn disrupts $\alpha6\beta4$ function at HDs (Mariotti et al., 2001). Our data are in agreement with this report, and our preliminary observations reveal that Ron kinase is not responsible for direct $\beta4$ phosphorylation, but that a Src-like kinase is possibly involved (M.M.S., unpublished data).

Here, we show that $\beta4$-mediated signaling modulates cell migration by regulating transcription. By means of Ron/14-3-3 association, $\beta4$ signaling activates p38 and NF-κB pathways that induce MSP and MMP gene transcription responsible for complete keratinocyte reepithelization. The finding that three putative NF-κB/c-rel binding sites are located 713, 778, and 802 bp upstream of the transcription start site of the MSP human gene (M.M.S., unpublished data) further support that MSP gene is a target of NF-κB.

Complementary roles for $\alpha6\beta4$ and $\alpha3\beta1$ in mediating keratinocyte adhesion and motility in wound healing processes have been proposed (Goldfinger et al., 1999). The involvement of $\alpha3\beta1$ integrin in cell migration is established, but the contribution of $\alpha6\beta4$ to keratinocyte migration is still unclear. One possibility is that $\alpha6\beta4$ contributes by regulating $\alpha3\beta1$ function (Nguyen et al., 2000; Mercurio et al., 2001). The interference by $\alpha6\beta4$ integrin on $\alpha3\beta1$-mediated migration on laminin-5 has been recently described in HaCat keratinocytes (Hintermann et al., 2001). This "negative" control of one integrin by another integrin has been observed also in vivo and is known as "*trans*-dominant" inhibition (Diaz-Gonzalez et al., 1996; Hodivala-Dilke et al., 1998). We show that quiescent keratinocyte $\alpha6\beta4$ exerts a *trans*-dominant effect on $\alpha3\beta1$ and mediates cell anchorage to BM. MSP stimulation induced Ron binding to $\alpha6\beta4$ and promoted $\alpha6\beta4$ relocation from HDs to lamellipodia. Concomitantly, $\alpha3\beta1$ becomes "unlocked" and moves to focal contacts and regulates actin-myosin-driven processes required for cell spreading and migration (Figure 5E). We propose a model where Ron activation and 14-3-3 binding are responsible for a shift in integrin dominance, hence modulating keratinocyte migration during epidermal wound healing.

Wound reepithelization depends on specific chemoattractants that regulate integrin receptors, actin cytoskeleton, and metalloprotease secretion (Martin, 1997; Singer and Clark, 1999). Here we show that MSP and Ron can modulate all these events through a specific molecular mechanism involving $\alpha6\beta4$ and 14-3-3 proteins. Moreover, we propose that MSP is a cytokine involved in skin wound healing, mainly active in the early phase of keratinocyte reepithelization.

Experimental Procedures

Cell Lines and Transfectants

HaCat and FG2 as well as primary human foreskin keratinocytes (Clonetics) were cultured in an appropriate medium according to the manufacturer's protocols. Immortalized PA-JEB $\beta4$ null keratinocytes were a gift of G. Meneguzzi. Pools of stably transfected HEK and HaCat cells were obtained by lentiviral infection. Lentiviruses were produced in 293T packaging cells transfected with the pRRLsinPPTWpre.CMV vector. Transient transfections, virus production, titer, and infection were performed as described (Follenzi and Naldini, 2002).

Reagents and Antibodies

ECM proteins were from Sigma. Purified laminin-5 and anti-α3LM5 were kindly provided by R. Burgeson. Recombinant HGF, EGF, TNFα, and MSP are from R&D Systems and were used at 5 nM. Purified Ron C-terminal (Ron/C) and N-terminal (Ron/N) antibodies were described elsewhere (Gaudino et al., 1994; Wang et al., 1996a). The polyclonal phospho-Ron (S^{1394}) antibody was generated using the CNVRRPRPLpSEPPRPT phosphopeptide and cross-selected to remove non-phospho-Ron-interacting species. It specifically recognizes Ron serine 1394 in the phosphorylated form. Anti-pan14-3-3 and -τ antibodies were obtained from Santa Cruz. Met and phosphotyrosine antibodies were from UBI. EGFR antibody was a gift of Y. Yarden. P-Akt, P-p70^{S6K}, P-p38, P-14-3-3 binding motif, P-IκBα, and IκBα antibodies were purchased from Cell Signaling. P-Erk2 and β-actin antibodies were obtained from Sigma. Antibodies against $\alpha2$ (P1E6), $\alpha3$ (P1B5), $\alpha6$ (GoH3), $\beta4$ (3E1), and γ2LM5 (D4B5) and $\beta1$ (P4C10) were from Chemicon. Anti-BP180 was a generous gift of Dr. G. Meneguzzi. Paxillin and vinculin antibodies were from BD Bioscience. Chemical compounds were from Sigma and ICN. PD98059 and PD169316 were used at 25 μM, wortmannin and TPA at 100 nM, BAY11-7082 at 5 μM, LY294002 and Rottlerin at 10 μM, rapamycin at 200 nM, Gö6976 at 1 μM, and mitomycin at 10 μg/ml.

Yeast Two-Hybrid System

A LexA-based system was used for yeast two-hybrid experiments according to standard procedures. LexA-Ron bait was generated subcloning an EcoRI PCR fragment in the pEG202 vector. The LexA-Ron^{S1394A} baits were performed by PCR using specific primers. An IMAGE clone encoding full-length human HS1 protein was used to isolate the coding sequence of 14-3-3τ proteins. Modifications of the 14-3-3τ construct were performed by PCR. Briefly, the 14-3-3$\tau\Delta$N mutant was generated using the sense primer 5′-AGAATTCG GCGCCGAGCTG-3′, which deletes the first 33 amino acids (Δ1–33) of the 14-3-3τ protein and inserts an in-frame EcoRI site. The 14-3-3$\tau\Delta$C constructs were generated via the insertion of a stop codon and an XhoI site in the reverse primer 5′-AAACTCGAGTTAATTCAG TGTATC-3,′ which deletes the last 38 amino acids (Δ207–245) of the 14-3-3τ protein. Protein-protein interactions between Ron and 14-3-3 were evaluated in yeast cells by quantifying the β-galactoside activity in liquid cultures using the o-nitrophenyl-β-galactopyranoside (ONPG) substrate. Each value represents the average of three independent experiments.

Biochemical Assays

Immunoprecipitation experiments were performed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1%

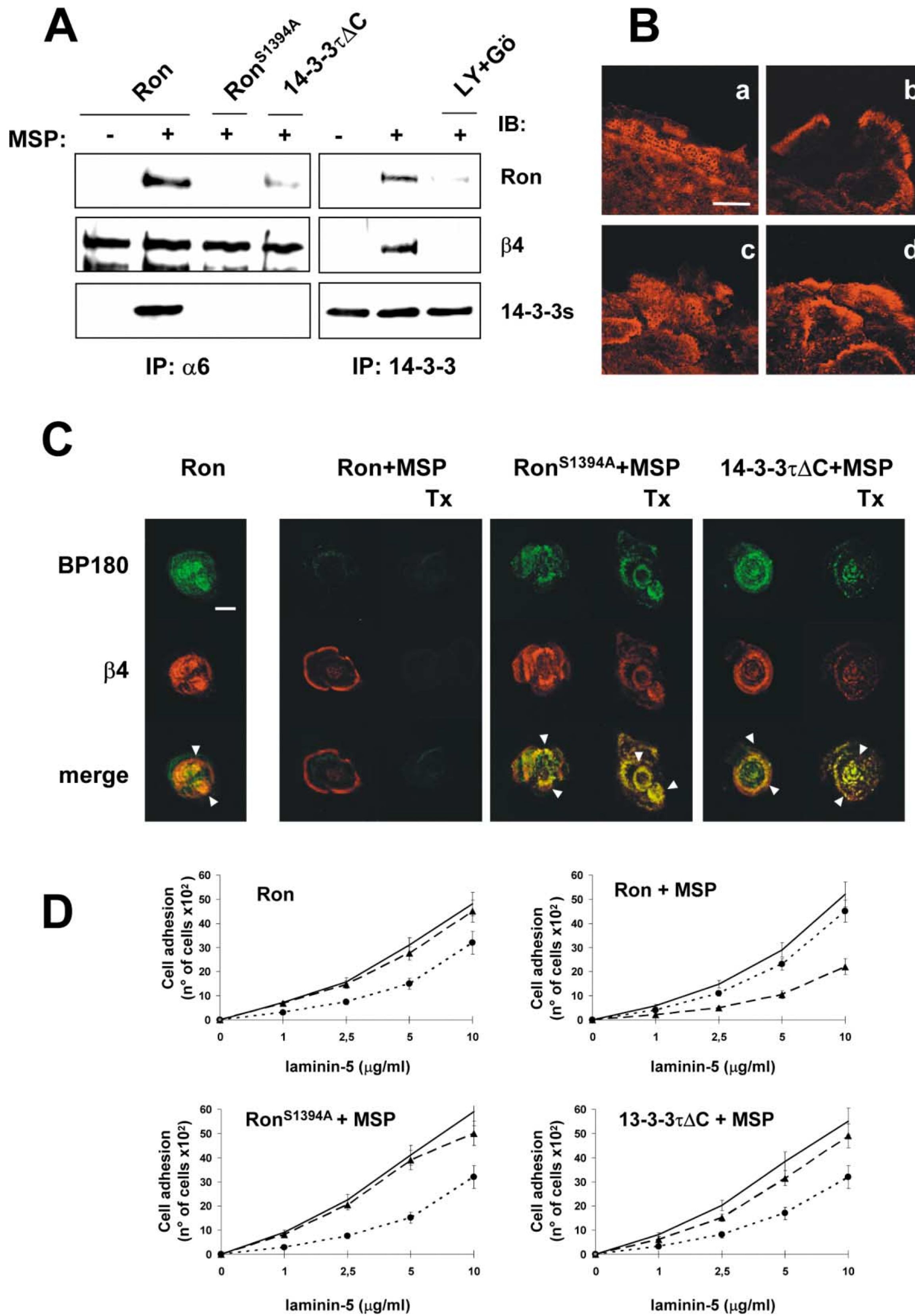

Figure 5. MSP Induces 14-3-3-Dependent Ron/α6β4 Association and α6β4 Displacement from HDs

(A) Ron associates selectively with α6β4 in an MSP- and 14-3-3-dependent manner. Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants were stimulated with MSP for 30 min and lysates were then subjected to coimmunoprecipitation experiments with α6 antibodies. In parallel, HEK cells were

Triton X-100, 20 mM β-glycerophosphate, 10% glycerol, 0.5 mM DTT, 10 mM NaPPi, 20 mM NaF, 1 mM sodium orthovanadate, and inhibitors of proteases. Pull-down assays were performed incubating 1 mg of total cell lysate with 5 μg of GST-fusion proteins for 4 hr at 4°C. Protein complexes were collected by adding glutathione-Sepharose beads and eluted after high stringency washes. After solubilization, proteins were separated on SDS-PAGE and analyzed by Western blotting using appropriate antibodies. Plasmids coding for the different GST-fusion proteins were prepared subcloning the coding regions of Ron, 14-3-3, and integrins in the pGEX4T1 plasmid using the EcoRI/XhoI sites. Expression of GST-fused proteins was performed as described by the manufacturer's protocol.

In kinase assays recombinant active Akt, PKA, $p70^{S6K}$, PKC, and CaMKII kinases were incubated at 30°C for 30 min in 30 μl of kinase buffer containing 50 μM unlabeled ATP, 5 μCi [γ-^{32}P]ATP and 2 μg of GST-fusion proteins or positive controls. Reactions were blocked by addition of boiling Laemmli buffer. Proteins were separated by SDS-PAGE and incorporation of ^{32}P into substrates was evaluated by autoradiography or Western blotting with specific antibodies. Equal amount of loaded proteins was estimated by Coomassie blue staining.

Biological Assays

Plates were coated with purified laminin-5, collagen-I, fibronectin, and vitronectin in PBS. In migration assays, 2.5×10^4 cells were seeded on Transwell filters and stimulated with MSP. Filters were maintained at 37°C for 12 hr and migrated cells were analyzed by colorimetric assays. In adhesion assays, 2.5×10^4 cells/well were seeded in coated plates. When indicated, function-blocking antibodies at 10 μg/ml were added before plating for 30 min. Attached cells were analyzed after MSP addition. Filters or wells were viewed under bright-field optics and cells were counted in eight fields from each of two filters for each condition, determining the mean number of cells counted per field. Each experiment was done at least three times and results are expressed as mean ± SD of relative cell adhesion/migration. In spreading assays, cells were seeded at a density of 10^4 cells/well and allowed to adhere for 1 hr. Cutaneous wound healing assays were performed on mice reared in a standard animal facility. Full-thickness paired wounds of 5 mm in diameter were made on the dorsum of adult female CD-1 mice using a punch biopsy instrument. One wound was treated with MSP, while the other was treated with PBS. MSP and PBS were dressed on the wound using growth factor-reduced Matrigel (BD Bioscience) unable to promote wound closure by itself (data not shown). Percent of wound closure was evaluated as $(A_o - A_t)/A_o \times 100$, where A_o is the original wound area, and A_t is the area of the wound at a specific time. Wounded areas were measured by a precision caliper. Results are representative of at least two experiments with $n = 6$. Epithelial margin length was evaluated on hematoxylin and eosin-stained tissue sections perpendicular to the wound surface using Image Pro Plus program. Data represent mean ± SD using Student's t test, with $P < 0.05$ as significant. Keratinocytes were grown to confluence and left in spent medium for 3 days. Cell monolayers were scraped with a plastic pipette tip, washed with PBS, and incubated in low trypsin at 37°C for 30 s to remove cell debris. Cells were allowed to migrate in MSP-containing medium. Function-blocking antibodies or chemical inhibitors were added to the medium before stimulation.

Reverse Transcriptase-PCR

Total RNA was extracted from keratinocytes with the guanidium thiocyanate system and used for RT-PCR analysis. The following oligonucleotides were used for amplification: 5′-CGAG GCACAGC CCCGCCAAGA-3′ and 5′-AGGGTGAGTTGCCCGGATGGCC-3′ for MSP; 5′-GCGACAAGAAGTATGGCTTC-3′ and 5′-TGCCAAGGTCAA TGTCAGGA-3′ for MMP2; 5′-GGTGAGGACACCAGCATGA-3′ and 5′-ATCCCTGGAAAGTCTTCAGC-3′ for MMP3; and 5′-GTGGGG CGCCCCAGGCACCA-3′ and 5′-TCCTTAATGTCACGCACGATTTC-3′ for β-actin.

Immunofluorescence Analyses

Cells were grown on glass coverslips and fixed in PBS (pH 7.6) containing 3% paraformaldehyde, 4% sucrose for 5 min at room temperature. Cells were then permeabilized by soaking coverslips for 5 min at 4°C in 20 mM HEPES-Triton buffer. Primary antibodies were incubated for 30 min at 37°C. After rinsing in PBS-0.2% BSA, coverslips were incubated with the appropriate rhodamine or fluorescein-tagged secondary antibodies for 30 min at 37°C. Coverslips were mounted in Mowiol 4-88 and analyzed with a Leica confocal laser scanning microscope.

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References

Aitken, A. (1996). 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends Cell Biol. *6*, 341–347.

Alt, A., Ohba, M., Li, L., Gartsbein, M., Belanger, A., Denning, M.F., Kuroki, T., Yuspa, S.H., and Tennenbaum, T. (2001). Protein kinase Cδ-mediated phosphorylation of α6β4 is associated with reduced integrin localization to the HD and decreased keratinocyte attachment. Cancer Res. *61*, 4591–4598.

Banu, N., Price, D.J., London, R., Deng, B., Mark, M., Godowski, P.J., and Avraham, H. (1996). Modulation of megakaryocytopoiesis by human macrophage-stimulating protein, the ligand for the RON receptor. J. Immunol. *156*, 2933–2940.

Biffo, S., Sanvito, F., Costa, S., Preve, L., Pignatelli, R., Spinardi, R., and Marchisio, P.C. (1997). Isolation of a novel β 4 integrin-binding protein (p27(BBP)) highly expressed in epithelial cells. J. Biol. Chem. *272*, 30314–30321.

Borradori, L., and Sonnenberg, A. (1999). Structure and function of HDs: more than simple adhesion complexes. J. Invest. Dermatol. *112*, 411–418.

Colognato, H., and Yurchenco, P.D. (2000). Form and function: the laminin family of heterotrimers. Dev. Dyn. *218*, 213–234.

Conejo, R., de Alvaro, C., Benito, M., Cuadrado, A., and Lorenzo, M. (2002). Insulin restores differentiation of Ras-transformed C2C12 myoblasts by inducing NF-κB through an AKT/P70S6K/p38-MAPK pathway. Oncogene *21*, 3739–3753.

pretreated with LY294002 (LY) and Gö6976 (Gö) inhibitors, and lysates were immunoprecipitated with 14-3-3 antibody. Immunocomplexes were resolved by SDS-PAGE and immunoblotted with Ron, β4, and pan14-3-3 antibodies.

(B) α6β4 relocation from HDs to lamellipodia in MSP-stimulated keratinocytes. Cells were cultured on purified laminin-5 and untreated (panel a) or stimulated with MSP for 12 hr (panels b–d). Panels c and d indicate Ron^{S1394A} and 14-3-3τΔC transfectants, respectively. Cells were stained with α6 antibodies and analyzed at confocal microscopy. The scale bar represents 40 μm.

(C) HD disassembly by MSP via Ron and 14-3-3 proteins. Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants were left untreated or stimulated with MSP. Cells were solubilized with a 0.5% Triton X-100 buffer (Tx) or directly fixed. Slides were subjected to immunofluorescence analyses with BP180 or β4 antibodies. Arrowheads are HD structures. The scale bar represents 40 μm.

(D) MSP induces a switch from α6β4 to α3β1 integrin for adhesion on laminin-5. Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants were plated on wells coated with increasing concentrations of laminin-5. Cells were plated alone or with function-blocking mAbs to α6β4 (●) or α3β1 (▲).

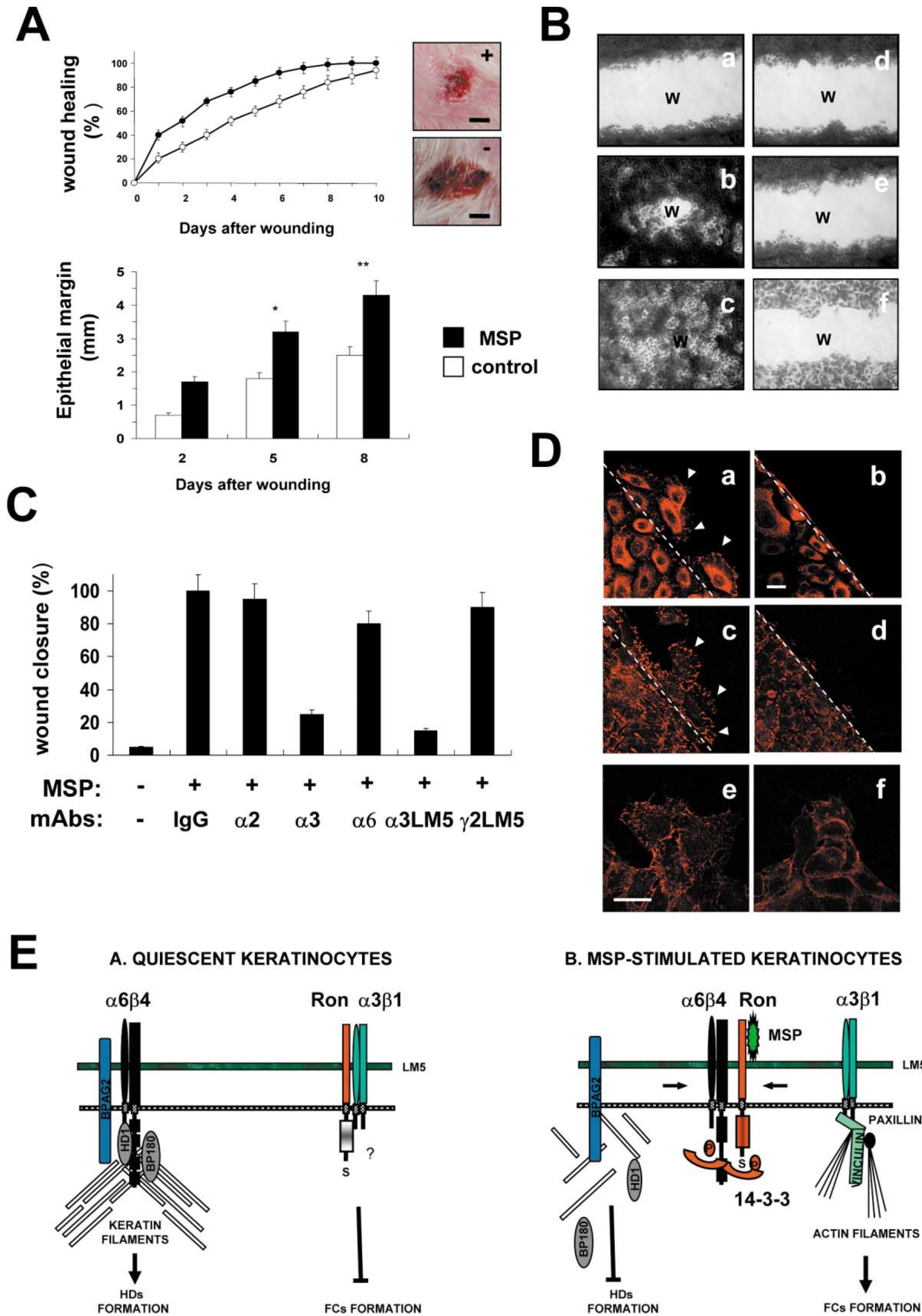

Figure 6. MSP-Ron Mediates Wound Healing through 14-3-3 Proteins

(A) MSP promotes wound healing in vivo. Top: wound closure kinetics in MSP-treated (●) and carrier-treated (○) wounds (means ± SD). Wound appearance at day 4 after + and − MSP treatment. The scale bar represents 1 mm. Bottom: wound healing is expressed as the distance covered by the new epithelium from the original wound margin at 2, 5, and 8 days after wounding. $^{*}P < 0.05$, $^{**}P < 0.005$; n = 4 using an unpaired two-group Student's t test.

Cowin, A.J., Kallincos, N., Hatzirodos, N., Robertson, J.G., Pickering, K.J., Couper, J., and Belford, D.A. (2001). Hepatocyte growth factor and macrophage-stimulating protein are upregulated during excisional wound repair in rats. Cell Tissue Res. *306*, 239–250.

Danilkovitch, A., Donley, S., Skeel, A., and Leonard, E.J. (2000). Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells. Mol. Cell. Biol. *20*, 2218–2227.

Dans, M., Gagnoux-Palacios, L., Bòaikie, P., Klein, S., Mariotti, A., and Giancotti, F.G. (2001). Tyrosine phosphorylation of the β4 integrin cytoplasmic domain mediates Shc signaling to extracellular signal-regulated kinase and antagonizes formation of HDs. J. Biol. Chem. *276*, 1494–1502.

Diaz-Gonzalez, F., Forsyth, J., Steiner, B., and Ginsberg, M.H. (1996). Trans-dominant inhibition of integrin function. Mol. Biol. Cell *7*, 1939–1951.

Follenzi, A., and Naldini, L. (2002). HIV-based vectors: preparation and use. Methods Mol. Med. *69*, 259–274.

Fuchs, E., Dowling, J., Segre, J., Lo, S.H., and Yu, Q.C. (1997). Integrators of epidermal growth and differentiation: distinct function for β1 and β4 integrins. Curr. Opin. Genet. Dev. *7*, 672–682.

Gagnoux-Palacios, L., Allegra, M., Spirito, F., Pommeret, O., Romero, C., Ortonne, J.P., and Meneguzzi, G. (2001). The short arm of the laminin γ2 chain plays a pivotal role in the incorporation of laminin-5 into the extracellular matrix and in cell adhesion. J. Cell Biol. *153*, 835–849.

Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, K.A., Godowski, P.J., and Comoglio, P.M. (1994). RON is a heterodimeric tyrosine kinase receptor activated by HGF homologue MSP. EMBO J. *13*, 3524–3532.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028–1032.

Goldfinger, L.E., Hopkinson, S.B., deHart, G.W., Collawn, S., Couchman, J.R., and Jones, J.C. (1999). The α3 laminin subunit, α6β4 and α3β1 integrin coordinately regulate wound healing in cultured epithelial cells and the skin. J. Cell Sci. *112*, 2615–2629.

Hintermann, E., Bilban, M., Sharabi, A., and Quaranta, V. (2001). Inhibitory role of α6β4-associated erbB-2 and phosphoinositide 3-kinase in keratinocyte haptotactic migration dependent on α3β1 integrin. J. Cell Biol. *153*, 465–478.

Hodivala-Dilke, K.M., DiPersio, C.M., Kreidberg, J.A., and Hynes, R.O. (1998). Novel roles for α3β1 integrin as a regulator of cytoskeletal assembly and as a trans-dominant inhibitor of integrin receptor function in mouse keratinocytes. J. Cell Biol. *142*, 1357–1369.

Kay, B.K., Williamson, M.P., and Sudol, M. (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J. *14*, 231–241.

Kreidberg, J.A. (2000). Functions of α3β1 integrin. Curr. Opin. Cell Biol. *12*, 548–553.

Mainiero, F., Pepe, A., Wary, K.K., Spinardi, L., Mohammidi, M., Schlessinger, J., and Giancotti, F.G. (1995). Signal transduction by the α6β4 integrin: distinct β4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of HDs. EMBO J. *14*, 4470–4481.

Mariotti, A., Kedeshian, P.A., Dans, M., Curatola, A.M., Gagnoux-Palacios, L., and Giancotti, F.G. (2001). EGF-R signaling through Fyn kinase disrupts the function of α6β4 at HDs: role in epithelial cell migration and carcinoma invasion. J. Cell Biol. *155*, 447–458.

Martin, P. (1997). Wound healing—aiming for perfect skin regeneration. Science *276*, 75–81.

Mercurio, A.M., Rabinovitz, I., and Shaw, L.M. (2001). The α6β4 integrin and epithelial cell migration. Curr. Opin. Cell Biol. *13*, 541–545.

Miranti, C.K., and Brugge, J. (2002). Sensing the environment: a historical perspective on integrin signal transduction. Nat. Cell Biol. *4*, 83–90.

Muslin, A.J., Tanner, J.W., Allen, P.M., and Shaw, A.S. (1996). Interaction of 14-3-3 with signaling protein is mediated by the recognition of phosphoSer. Cell *84*, 889–897.

Nanney, L.B., Skeel, A., Luan, J., Polis, S., Richmond, A., Wang, M.H., and Leonard, E.J. (1998). Proteolytic cleavage and activation of pro-macrophage-stimulating protein and upregulation of its receptor in tissue injury. J. Invest. Dermatol. *111*, 573–581.

Nguyen, B.P., Ryan, M.C., Gil, S.G., and Carter, W.G. (2000). Deposition of laminin 5 epidermal wounds regulates integrin signaling and adhesion. Curr. Opin. Cell Biol. *12*, 554–562.

Olsen, E., Rasmussen, H.H., and Celis, J.E. (1995). Identification of proteins that are abnormally regulated in differentiated cultured human keratinocytes. Electrophoresis *16*, 2241–2248.

Rabinovitz, I., Toker, A., and Mercurio, A.M. (1999). Protein kinase C-dependent mobilization of the α6β4 integrin from HDs and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J. Cell Biol. *146*, 1147–1160.

Ravanti, L., and Kahari, V.-M. (2000). Matrix metalloproteinases in wound repair. Int. J. Mol. Med. *6*, 391–407.

Romashkova, J.A., and Makarov, S.S. (1999). NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature *401*, 86–90.

Santoro, M.M., Penengo, L., Minetto, M., Orecchia, S., Cilli, M., and Gaudino, G. (1998). Point mutations in the tyrosine kinase domain release the oncogenic and metastatic potential of the Ron receptor. Oncogene *17*, 741–749.

Shang, M., Koshikawa, N., Schenk, S., and Quaranta, V. (2001). The LG3 modules of laminin-5 harbors a binding site for integrin α3β1 that promotes cell adhesion, spreading and migration. J. Biol. Chem. *276*, 33045–33053.

Shaw, L.M., Rabinovitz, I., Wang, H.H., Toker, A., and Mercurio, A.M. (1997). Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. Cell *91*, 949–960.

Singer, A.J., and Clark, R.A. (1999). Cutaneous wound healing. N. Engl. J. Med. *341*, 738–746.

Skeel, A., and Leonard, E.J. (1994). Action and target cell specificity of human macrophage-stimulating protein (MSP). J. Immunol. *152*, 4618–4623.

(B) MSP-Ron induces in vitro wound healing of human primary keratinocytes. Scrape wounds (W) were made in quiescent monolayers of HEK cells (panels a–c), Ron^{S1394A}-transfectants (d), 14-3-3τΔC transfectants (e), and β4 null primary keratinocytes (f). Wounded cells were allowed to heal in serum-free medium (a) or in the presence of MSP (b–f). In (c), the cells were also incubated with mitomycin. After 24 hr, cell monolayers were fixed, stained, and photographed. Representative fields are indicated.

(C) MSP drives keratinocyte migration via α3β1 integrin activation. In vitro wound assays were performed in the presence of MSP and function-inhibitory antibodies against α2 (P1E6), α3 (P1B5), and α6 (GoH3) integrins as well as α3 (BM4) or γ2 (D4B5) laminin-5 subunits, as indicated. Normal IgG antibodies were used as control. The average extent of wound closure was quantitated by multiple measurements of the width of the wound space for each of these cases. Thirty measurements of two separate trials were made for all conditions.

(D) The Ron/14-3-3/α6β4 complex induced focal contact formation and α3β1 integrin activation. Ron (panels a, c, and e) and Ron^{S1394A}-transfectants (b, d, and f) were wounded (dot line) and then incubated with MSP. Cells were fixed and analyzed for focal contact formation (arrowheads) by paxillin (a and b), vinculin (c and d), and α3β1 integrin (e and f) staining and confocal microscopy analysis. The scale bar represents 20 μm.

(E) Schematic model of Ron switching from α3β1 to α6β4 via 14-3-3 proteins. MSP stimulation and 14-3-3 proteins drive the Ron receptor to associate with α6β4 integrin inducing HD disassembly and α3β1-dependent focal contact formation.

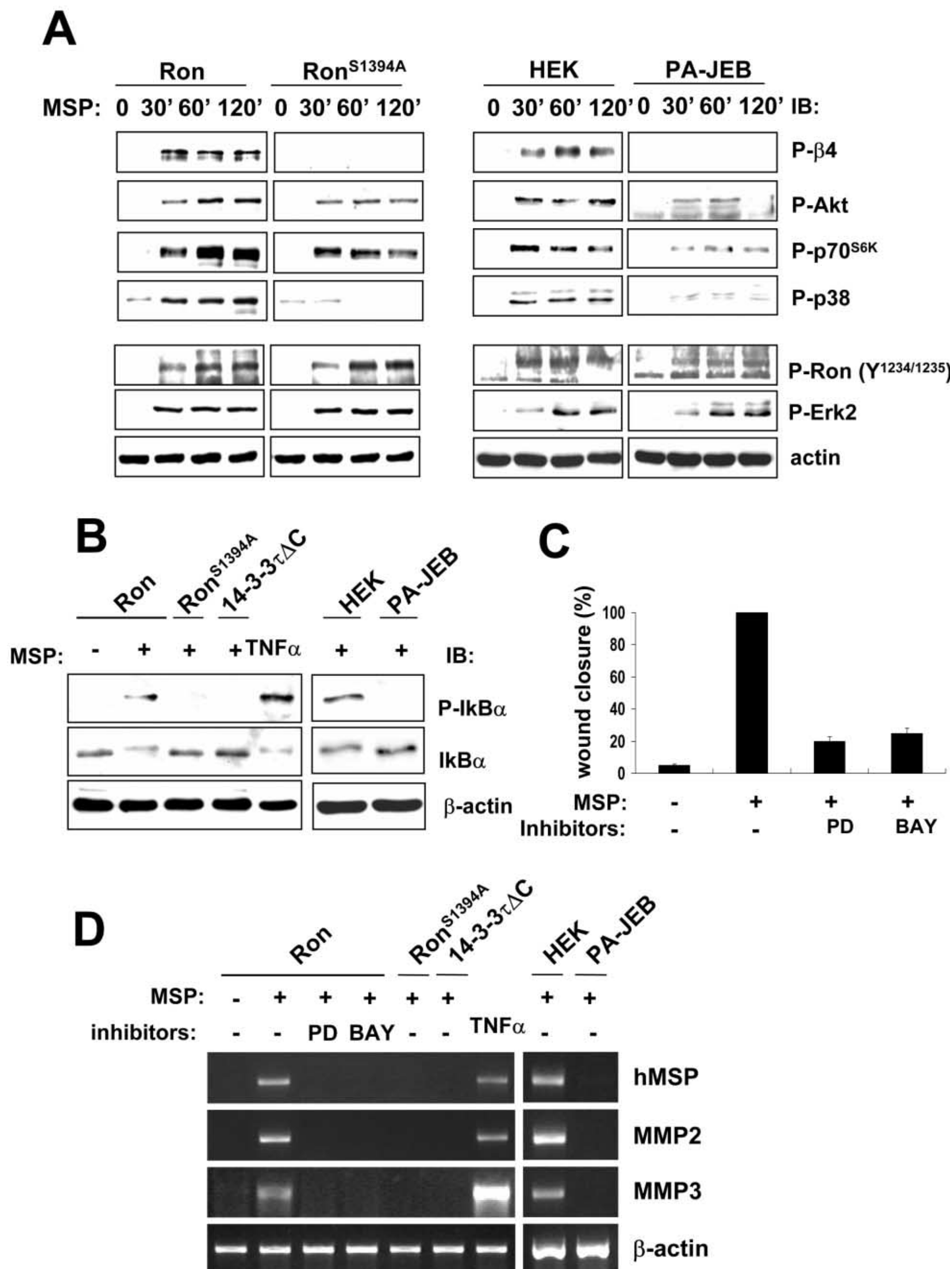

Figure 7. MSP-Mediated β4 Tyrosine Phosphorylation Elicits a p38- and NF-κB-Dependent Signaling Required for Keratinocyte Migration

(A) Ron/α6β4 association modulates MSP signaling. Ron- and Ron^{S1394A}-expressing HaCat as well as HEK and PA-JEB cells were stimulated with MSP for the indicated time. Total cell lysates were analyzed by Western blotting with phosphotyrosine antibody to evaluate the level of β4 tyrosine phosphorylation (P-β4). Samples were also analyzed with antibodies against active Akt, p70^{S6K}, and p38 proteins to observe differences in Ron signaling. Afterwards, the membranes were also probed with phospho-Ron(Y$^{1234/1235}$), active Erk2, and β-actin antibodies.

(B) IκBα phosphorylation and degradation are mediated by Ron/α6β4-mediated signaling. Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants as well as HEK and PA-JEB cells were stimulated with MSP. Total cell extracts were analyzed for IκBα phosphorylation and protein degradation using specific phospho-IκBα and IκBα antibodies. TNFα-stimulated cells were used as control.

(C) p38- and NF-κB-dependent signaling is required for MSP-mediated keratinocyte migration. Monolayers of quiescent HEK cells were scraped, treated with the PD169316 and BAY11-7082 inhibitors, and then stimulated with MSP. Wound closure was evaluated through 30 measurements of two separate trials.

(D) p38- and NF-κB-dependent transcriptional activity promotes MSP as well as MMP2 and MMP3 expression. Ron, Ron^{S1394A}, and 14-3-3τΔC transfectants as well as HEK and PA-JEB cells were pretreated with PD169316 (PD) or BAY 11-7082 (BAY) before MSP stimulation. Total RNA were isolated and analyzed by RT-PCR for MSP as well as MMP2 and MMP3 gene expression using specific oligonucleotides. TNFα-stimulated cells were used as control.

Tzivion, G., Shen, Y.H., and Zhu, J. (2001). 14-3-3 proteins, bringing new definitions to scaffolding. Oncogene *20*, 6331–6338.

Wang, M.H., Montero-Julian, F.A., Dauny, I., and Leonard, E.J. (1996a). Requirement of phosphatidylinositol-3 kinase for epithelial cell migration activated by human macrophage stimulating protein. Oncogene *13*, 2167–2175.

Wang, M.H., Dlugosz, A.A., Sun, Y., Suda, T., Skeel, A., and Leonard, E.J. (1996b). Macrophage-stimulating protein induces proliferation and migration of murine keratinocytes. Exp. Cell Res. *226*, 39–46.

Yaffe, M.B., and Elia, A.E. (2001). Phosphoserine/threonine-binding domains. Curr. Opin. Cell Biol. *13*, 131–138.

Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J., and Cantley, L.C. (1997). The structural basis for 14-3-3:phosphopeptide binding specificity. Cell *91*, 961–971.

Yaffe, M.B., Leparc, G.G., Lai, J., Obata, T., Volinia, S., and Cantley, L.C. (2001). A motif-based profile scanning approach for genome-wide prediction of signaling pathways. Nat. Biotechnol. *19*, 348–353.

Zhang, K., and Kramer, R.H. (1996). Laminin 5 deposition promotes keratinocyte motility. Exp. Cell Res. *227*, 309–322.