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Novel regulation and dynamics of myosin-II activation during epidermal wound responses

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Abstract

Wound healing in the skin is an important and complex process that involves 3-dimensional tissue reorganization, including matrix and chemokine-triggered cell migration, paracrine signaling, and matrix remodeling. The molecular signals and underlying mechanisms that stimulate myosin II activity during skin wound healing have not been elucidated. To begin understanding the signaling pathways involved in the activation of myosin II in this process, we have evaluated myosin II activation in migrating primary human keratinocytes in response to scratch wounding in vitro. We report here that myosin II activation and recruitment to the cytoskeleton in wounded keratinocytes is biphasic. Post-wounding, a rapid phosphorylation of myosin II regulatory light chain (RLC) occurs with resultant translocation of myosin IIA to the cell cortex, far in advance of the later polarization and cell migration. During this acute-phase of myosin II activation, pharmacological approaches reveal p38-MAP kinase and cytosolic calcium as having critical roles in the phosphorylation driving cytoskeletal assembly. Although p38-MAPK has known roles in keratinocyte migration, and known roles in leading-edge focal complex dynamics, to our knowledge this is the first report of p38-MAPK acting as an upstream activator of myosin II phosphorylation and assembly during any type of wound response.

Keywords

myosin II; cytoskeleton; wound healing; cell migration; epidermis

Introduction

Epithelial cell migration plays a fundamental role in a wide variety of physiological processes ranging from tissue and organ remodeling during embryogenesis to cancer progression and other pathological events. Epithelial cell sheet migration has a central role in skin wound healing. Earlier studies have implicated a number of signaling pathways in skin wound healing, such as signaling via mitogen-activated protein kinases (MAPKs), Protein kinase C (PKC), and via the epidermal growth factor receptor (EGFR) [1-3]. Much of this signaling is ultimately

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directed at activating migration into the wound zone, but the downstream mechanisms by which epidermal keratinocytes remodel their actomyosin cytoskeleton to activate migration remain poorly understood.

Myosin II, an abundant motor protein in nonmuscle cells, has key roles in this respect during cell migration, in posterior cell retraction, and via poorly understood mechanisms, in helping stabilize nascent focal adhesions in the anterior portion of the cell [4-6]. Multiple signaling pathways are capable of activating myosin II function in adherent cells, and myosin II-containing structures in cells appear to have diverse roles, not all of which are pro-migratory. For example, Rho kinase (also known as Rho-associated coiled-coil containing protein kinase or ROCK), is an activator of RLC phosphorylation. Rho kinase is thought to be predominantly active in the central and posterior regions of migrating fibroblasts [4]. This activity tends to favor formation of stable matrix adhesions and stress fibers, which reduce migratory activity. In contrast, myosin II activation in the leading edge lamella participates in the stabilization of nascent focal contacts rather than stress fiber formation, and this anterior role is important for normal protrusive behavior [7]. Thus multiple and sometimes opposing types of myosin II contractility are present in migratory cells.

Although the roles of myosin II have received significant attention in cells ranging from fibroblasts to cancer cells [7-9], surprisingly little is known about the regulatory mechanism of this key cytoskeletal protein in human keratinocytes. Keratinocyte migration in a wound setting is triggered after an initial mechanical trauma that damages the skin. Keratinocytes near the damage zone respond with activation of both proliferation and migration, in response to an array of signals that include matrix-derived stimuli and growth factors released from activated platelets [3,10]. As an initial step towards understanding myosin II regulation during human skin wound healing, we have established the localization and activation behavior of myosin II during human keratinocyte responses to scratch-wounding in culture. This analysis reveals behavior consistent with earlier work in other cell types, such as a role for myosin II in leading edge protrusion, but also reveals novel regulation and dynamics. In particular, in response to scratch-wounding, primary keratinocytes display a dramatic and rapid activation of myosin II concurrent with translocation to the cell cortex, a behavior that we suggest may serve a protective role in response to mechanical trauma.

Materials and Methods

Cell culture and reagents

Primary human keratinocytes were isolated from discarded de-identified human foreskins in compliance with federal and Cleveland Clinic Foundation guidelines, as described previously [11]. Primary keratinocytes were maintained in KSFM (keratinocyte serum free medium; Clonetechn, Mountain View, CA), and used within three passages of initial isolation.

Isoform-specific MHC IIA and IIB and actin antibodies were purchased from Sigma (St. Louis, MO). Antibody against MHC IIC was a generous gift from Dr. Robert Adelstein (NIH). Antibodies against Phospho-Myosin Regulatory Light chain 2 [P-RLC (Thr 18/ Ser 19)] (Cat No. 3674), total RLC (Cat No. 3672), Phospho-p38 MAP Kinase [P-p38 (Thr 180/ Tyr 182)] (Cat No. 9211), total p38 MAPK (Cat No. 9212), Phospho-p44/42 MAPK [P-ERK1/2 (Thr 202/ Tyr 204)] (Cat No. 9106), total ERK1/2 (Cat No. 9102), and Phospho -EGF Receptor [P-EGFR (Tyr 1173)] (Cat No. 4407), were purchased from Cell Signaling, Inc. (Beverly, MA).

Scratch wound methods for western blots and immunocytochemistry

For immunofluorescent imaging, coverslip-bottom cell culture chambers were pretreated with 20 µg/ml fibronectin (Sigma, St. Louis, MO) for 1 hour at room temperature followed by rinsing with phosphate buffered saline (PBS). Primary human keratinocytes were then plated at near-confluence in KSFM for 36–48 hours. Scratch-wounding was performed with a plastic pipette tip, and cells were fixed at indicated time points with 4% paraformaldehyde buffer (PBS carrying 2 mM MgCl₂, 2 mM EGTA). After washing with PBS cells were permeabilized by incubating in PBS carrying 2 mM MgCl₂, 2 mM EGTA and 0.5% TritonX-100. Antibody incubations, washes, and confocal imaging were performed as described [12]. For western blot analysis of scratch-wound responses, keratinocytes were plated in 10 cm tissue culture dishes at near-confluence, and grown for an additional 48–72 hours in KSFM. These confluent cultures were subjected to high-density scratch-wounding using a sterilized hair comb. Inhibitors were added 15 min prior to scratching. Scratches were performed in a uniform pattern to ensure consistent levels of stimulation between different samples. Lysates were collected at each time point using ice-cold 10% TCA, as described [12]. This TCA lysis method is critical for preserving consistent RLC phosphorylation patterns. Final protein lysates in 2× sample buffer were subjected to SDS-PAGE and Coomassie staining, and whole-lane densitometry was performed to calculate protein sample concentrations. Loading quantities for subsequent phospho-RLC westerns were normalized based on the densitometry. For the graphs presented in Results, densitometry was performed on three independent sets of western blots for each inhibitor condition. Within each independent replicate, RLC-P signals were first normalized to total RLC signal for that time point, then these values were normalized to the 0 min time point of the control sample (no inhibitor). Following this normalization, the three independent replicates for each inhibitor condition were averaged to generate the presented graphs. To generate the graph in figure 1 (n=18), all of the “control” scratch wound data sets for the inhibitor studies were pooled to all statistical analysis with meaningful n values.

For EGF stimulation experiments, keratinocyte cultures at ~70% confluency were stimulated with EGF (20 ng/ml) for 10 min, then harvested via the TCA lysis method. Inhibitors were added to cultures 10 min before stimulation with EGF.

Inhibitors used in scratch-wound and EGF stimulation studies include AG1478 (inhibits EGFR; 15 µM), ML-7 (inhibits MLCK; 10 µM), Y-27632 (inhibits ROCK; 5 µM), PD-098059 (inhibits MEKK preventing ERK1/2 activation; 20 µM), SB-203580 (inhibits p38-MAPK; 15 µM), and BAPTA to inhibit calcium fluxes (50 µM). For some p38-MAPK inhibition experiments the structurally related SB-203580 compound (15 µM) was used. Inhibitors Y-27632, blebbistatin, PD-98059, AG-1478 were purchased from Calbiochem (La Jolla, CA). ML-7, SB-202190, SB-203580, and BAPTA-AM were purchased from Sigma (St. Louis, MO).

Unless indicated otherwise, all X-Y immunofluorescent images are confocal slices that represent confocal Z-section planes collected ~1µm above the base of the cell. The cell base is defined as the Z-section in the cell closest to the coverslip, where assembled F-actin could be detected across the whole cell surface. This plane is readily detected by scrolling through the Z-section image series. All images were collected on a Zeiss LSM510 confocal microscope.

Results

Acute activation and translocation of myosin II following scratch-wounding

In response to skin wounding, mammalian epidermis undergoes dramatic cellular reorganization that involves sheet-like migration, breakdown and recycling of cell-matrix adhesion, and stimulation of cell proliferation. Despite the importance of myosin II in migration and cell division in other settings, there is little published information regarding myosin II

isoform expression, roles, or regulation in skin or during skin wound healing. Mammalian myosin II isoforms include IIA, IIB, and IIC, with each corresponding myosin heavy chain (MHC) encoded by an independent gene with unique tissue and cell expression patterns [13]. We performed western blot analysis to identify myosin II isoforms expressed in primary human keratinocytes. This analysis revealed expression of MHC IIA and IIB isoforms (Fig 1A). No expression of the third nonmuscle isoform, MHC IIC, was detected (data not shown).

To assess myosin behavior in scratch-wound margin keratinocytes as they polarize and migrate into the wound zone, we first performed high density scratch-wounding with a hair comb on confluent keratinocyte cultures in conjunction with western blot analysis of RLC phosphorylation with antibodies specific to RLC phosphorylated on threonine 18/serine 19 (T18P/S19P). Consistent with a role for myosin II in polarized migration, enhanced levels of phospho-RLC were observed at later time points after scratch-wounding relative to the unscratched cultures (Fig. 1B, 240 min time point). Unexpectedly, however, we observed a much more dramatic activation of RLC phosphorylation immediately after scratch wounding. This peak was typically maximal ~1-5 min after the wound challenge (Fig 1B).

To further evaluate this acute RLC phosphorylation event, we performed confocal microscopy imaging on keratinocyte cultures fixed for immunohistochemistry at a series of time points post-scratch wounding. This analysis revealed a dramatic accumulation of phospho-RLC in the cell cortex in keratinocytes near the scratch wound margin (Fig 2). This accumulation was detectable in many cells within 1 min of the scratch wound, and typically peaked in the 1 min and 5 min time points post-wounding. At the 1 min time point the response was apparent in scattered cells along the wound margin, including cells 2-3 positions removed from the scratch itself. At later time points (Fig 2, 15 min & 120 min samples), the cortical staining for RLC-P typically became less robust, with a tendency for more of the staining to be at the leading edges of the cells immediately along the wound margin. Pseudo color heat maps of pixel intensity for these same samples (Figure S1) further support the pattern observed via western blot approaches, that there is a peak of RLC-phosphorylation in the 1-5 minute time period post-wounding. These staining patterns suggest that there is a rapid global recruitment of myosin II to the cell cortex that extends several cell layers away from the wound zone itself, and that this activation may involve distinct regulation from the later time points where RLC-P accumulation correlates with cell polarization and migration.

Myosin IIA and IIB responses during the acute scratch-wound response

To determine whether RLC-P accumulation in the cell cortex post-scratch wounding represented recruitment of myosin IIA and/or myosin IIB, we performed isoform-specific immunohistochemical evaluation during the acute wound response. In samples stained for myosin IIA, we observed accumulation of cortical staining near wound margins that was strikingly similar to the staining observed for RLC-P (Fig. 3). Although some cortical staining could be detected in unscratched samples (Fig 3A), this staining became much more dramatic near scratch margins by 5 min post-wounding (Fig 3B). As with RLC-P, enhanced staining was detectable even in cells 2-3 cell positions away from the immediate wound margin. Confocal Z-sections revealed that the cortical accumulation of myosin IIA occurred throughout the cortex of cells near the wound margin. Although some myosin IIA could be detected throughout the cortex of unscratched samples (Fig 3C), this staining became more dramatic at the 5 min time point post-wounding (Fig 3D). This accumulation was sometimes very prominent in protrusions of cells at the immediate wound margin (Fig 3D, red arrowhead), but accumulation was also consistently observed throughout the dorsal cortex of cells up to 2-3 positions away from the immediate wound margin (red arrow). As a further method to quantify the myosin IIA recruitment post-wounding, images were analyzed via heat maps of pixel intensity. Figure S2 presents the same fields of view as in Fig 3 A & B, as heat maps. This

analysis clearly reveals strong recruitment of myosin II to the cortices of cells at and near the scratch margin.

Parallel staining for MHC IIB was performed to assess responses of the myosin IIB isoform. Staining intensities for MHC IIB were consistently much weaker as compared to MHC IIA, suggesting that this isoform is not abundantly expressed in primary keratinocytes. Although some cortical accumulation for myosin IIB could be observed in both unscratched samples (Fig 4A) and in 5 min post-wound samples (Fig 4B), we did not observe a consistent increase in cortical myosin IIB accumulation post-wounding. In some fields of view modest accumulation at the immediate wound margin was detectable (white arrowheads in Fig 4B). Confocal Z-section series (Fig 4 C & D) also failed to reveal a global cortical response comparable to the myosin IIA response. Heat maps of pixel intensity for these same images emphasize the generally low signal for MHC IIB staining in these samples, and lack of clear cortical recruitment response (Fig. S3). As a further method to quantify the myosin IIB recruitment post-wounding, images were analyzed via heat maps of pixel intensity. Figure S3 presents the same fields of view as in Fig 4 A & B, displayed as heat maps of the unprocessed images. This analysis emphasizes the weak signal for MHC IIB and very limited recruitment response.

Z-section analysis of samples stained for RLC-(T18P/S19P) was performed to examine the 3-D distribution of activated myosin II during the acute wound response. While RLC-P accumulated around the entire circumference of cells near the wound margin (Fig S4, A & B), Z-section analysis revealed that this RLC-P staining was significantly more dramatic within 1-2 μ m of the basal attached surface of the cell, with far less staining detectable in the more dorsal portions of the cell (Fig S4D, red arrowheads indicate RLC-P accumulation within one cell, at the base, flanking the central nucleus stained with DAPI). Thus although myosin IIA clearly accumulates throughout the cell cortex during this response, the most highly activated phospho-RLC appears to be accumulating near the basal portions of the cells. This organization could represent differential activation levels in different zones of the cells, or perhaps could represent an active contraction or cortical flow of the more activated myosin II towards the basal attached portions of the cell cortex.

Upstream pathways mediating acute myosin II activation

The acute myosin II activation response occurs before significant numbers of cells have formed polarized lamellar protrusions. This suggests that signaling pathway(s) independent of substrate fibronectin engagement likely mediate the acute activation response. In view of earlier reports indicating activation of ERK1/2 in response to scratch-wounding of corneal epithelial cells [14], we asked whether MAP kinase pathways were activated post scratch-wounding in the current studies. Via phospho western blots, we observed a rapid activation of both ERK1/2 and p38-MAPK, correlating closely with the peak of phospho-RLC (Fig. 5). No activation of JNK was observed (data not shown). This analysis suggests the possibility that one or both of these MAPKs could mediate acute myosin II activation in scratch-wounded keratinocytes.

Inhibitor approaches were used to test a series of candidate upstream regulators of this acute activation. Confluent keratinocyte monolayers were pretreated with a series of inhibitors and treated or untreated cultures were subjected to scratch-wounding. Comparisons of the effects of MLCK inhibition versus ROCK inhibition revealed that both of these RLC activators contributed to the acute scratch-wound phosphorylation of myosin II (Fig. 6, A & B). The exact pattern of RLC activation showed some variation between independent experiments evaluated via western blotting. However, when RLC phosphorylation responses were normalized to total RLC signal in each experiment, then averaged data from multiple independent experiments was pooled (n=3 or greater for each set), we observed that inhibition of MLCK caused a more

consistent reduction in the acute myosin II activation response as compared to Rho kinase inhibition (Fig. 6, A & B). However, the inhibitor studies clearly suggest that both MLCK and Rho kinase pathways contribute to the acute RLC phosphorylation response.

We next asked whether EGFR and MAPK pathways were involved in the acute myosin activation response. EGFR activation is a well-established response of keratinocytes and epidermal tissue to wound challenge [14,15]. Surprisingly, however, EGFR kinase inhibition did not block myosin II phosphorylation (Fig 6C). In fact a consistent rapid increase in RLC phosphorylation was observed in all tests where the EGFR kinase inhibitor AG1478 was present prior to scratch-wounding. The cause of this effect is an enigma. This paradoxical apparent activation made it difficult to draw clear conclusions regarding the role of EGFR family receptors in the acute activation of RLC-P. However, it is clear that inhibiting EGFR kinase activity did not eliminate RLC phosphorylation in these samples. Inhibition of the MEKK/ERK1/2 pathway immediately prior to scratch-wounding did not reduce the acute RLC phosphorylation response (Fig. 6D). In fact in most replicates MEKK/ERK1/2 inhibition actually enhanced the spike of RLC phosphorylation occurring 1-5 min after the wound challenge. In contrast, inhibition of p38-MAPK just prior to scratch-wounding resulted in a consistent near-complete inhibition of the acute RLC phosphorylation response (Fig. 6E). Finally, we observed that pretreatment of cultures with the cell-permeant calcium chelator BAPTA also potentially eliminated the acute RLC phosphorylation response (Fig. 6F). These studies implicate both p38-MAPK and calcium transients as key upstream mediators of this novel acute myosin II activation response to scratch-wounding, and further argue that neither EGFR kinase activity nor ERK1/2 mediate this response. In BAPTA-pretreated cultures subjected to scratch wounding, p38-MAPK still displayed robust and rapid activation at ~1-5 min (Fig. 7). This result indicates that although p38-MAPK and calcium transients are both involved in the acute myosin II activation response, calcium transients are not required for the p38-MAPK activation.

Given the strong inhibitory effect on RLC phosphorylation when either p38-MAPK or calcium fluxes were blocked, we evaluated the effects of inhibiting these pathways on translocation of myosin IIA to the cortical cytoskeleton post-wounding. Pretreatment with SB202190 to block p38-MAPK activity eliminated the rapid translocation response for myosin IIA (Fig 8). Although a modest enrichment of myosin IIA could be observed in the cell margin with this treatment, no change in intensity was observed at 5 min post-wounding (Fig 8, A vs B). Furthermore in Z-section analysis, myosin IIA staining appeared more diffuse and even throughout the cytosol (Fig 8, C & D), as compared to untreated samples (as in Fig 3 C & D). Staining for F-actin was also reduced in these samples, suggesting that p38-MAPK may have roles in stabilizing or favoring F-actin in keratinocytes. Similar behavior was observed when keratinocytes were pretreated with BAPTA prior to scratch-wounding. In many fields of view myosin IIA enrichment could be detected in lamellar protrusions into the wound gap, but a global cortical recruitment was not observed (Fig 9A & B), and in Z-section analysis the myosin IIA staining was generally more diffuse than in untreated samples (Fig 9 C & D). These results support the hypothesis that the enhanced RLC phosphorylation observed post-scratch wounding is responsible for the cytoskeletal translocation and assembly observed by immunostaining, and that p38-MAPK and calcium fluxes are necessary both for the phosphorylation event and for the correlated global assembly of myosin IIA into the cytoskeleton.

Scratch-wound activation of RLC phosphorylation involves different signaling pathways than EGF-triggered activation

The enzyme p38-MAPK is not typically considered to be upstream of myosin II activation or assembly in the literature, thus its critical role in the acute wound setting was surprising. EGF

receptor activation is known to accompany or drive an array of wound responses in vivo and in keratinocyte culture settings [15,16]. EGFR is also a known modulator of myosin II assembly in breast cancer cells [17]. In this context, it was surprising that EGFR inhibition did not seem to abrogate RLC phosphorylation in primary keratinocytes (as shown earlier in Fig 6C). To further clarify pathways that can activate RLC phosphorylation in primary human keratinocytes, we asked whether direct EGF stimulation of primary keratinocytes would activate RLC phosphorylation, and whether p38-MAPK activity was necessary for RLC phosphorylation in this setting. As expected from studies in other cell types [17], EGF induced a rapid phosphorylation of RLC (Fig 10). As has been established previously [16], we found that keratinocytes also respond to EGF stimulation with activation of both p38-MAPK and ERK1/2 pathways (Fig 10). Inhibitor analysis confirmed that in this setting of direct EGF stimulation, the EGFR is a necessary mediator of RLC phosphorylation (Fig 10). This result contrasts with what we observed when EGFR was inhibited during scratch-wounding (Fig 6C), where AG1478 did not inhibit RLC phosphorylation, arguing that the acute scratch-wound response involves other signaling intermediates than the direct EGFR-mediated pathways.

These inhibitor tests also confirm both MLCK and ROCK as contributing to RLC phosphorylation in response to direct EGF stimulation (Fig 10). However, in this setting of direct EGF stimulation, blockade of the p38-MAPK pathway did not inhibit RLC phosphorylation at all (Fig 10). This result is again in sharp contrast to the effects of p38-MAPK inhibition during scratch-wound responses, where we observed a critical role for p38-MAPK in order to activate RLC phosphorylation (Fig 6E). The obligatory role for p38-MAPK during scratch-wound activation of RLC-P, and the irrelevance of p38-MAPK during RLC-P activation with EGF stimulation, argues that the acute scratch-wound activation of myosin II phosphorylation/assembly involves distinct modes of signaling and regulation as compared to simple EGFR activation.

Discussion

Despite the recognized importance of myosin II for cell polarization and normal matrix adhesion dynamics, there is little understanding of the roles and regulation of this protein in normal human keratinocyte migration biology or skin wound healing. The studies presented here reveal a novel and rapid myosin II activation/assembly event in scratch-wounded keratinocytes that corresponds to a global assembly of myosin II filaments in the cell cortex. Although EGF receptor signaling has clear and widespread roles in skin wound healing and keratinocyte migration biology [15,16], our analysis indicates that this acute myosin II activation post scratch-wounding appears to use distinct signaling mechanisms beyond simple EGF receptor signaling. In contrast to reports that ERK1/2 mediates RLC phosphorylation in integrin-stimulated COS-7 cells [18], ERK1/2 appears to have no activating role in RLC phosphorylation in keratinocytes post scratch-wounding. Calcium flux does appear critical for the acute RLC phosphorylation response. We hypothesize that elevated cytosolic calcium occurs via either mechanical damage to the plasma membrane or via stretch-activated channels, activating MLCK (Fig 11). Given the tight cell-cell adhesion of epithelial sheets (and fully confluent keratinocyte cultures), both mechanical membrane damage and stretch-activated signaling could propagate to nearby cells not directly touching the wound edge itself. We suggest that the myosin II activation and assembly observed in our studies may be related to myosin II protective roles observed when single cells or embryos are subjected to puncture wounds, known to induce small GTPase activation and exocytic vesicle fusion at the site of membrane damage [19-21]. In parallel to the need for cytosolic calcium flux, activation of p38-MAPK has a critical role in the acute wound response. To our knowledge, a role for p38-MAPK in activating myosin II during wound responses of any sort has not been previously reported. We speculate that p38-MAPK may in some manner facilitate calcium influx. Intriguingly, a positive role for p38-MAPK in RLC phosphorylation has been reported previously in the case

of endothelial cells stimulated with TGF β 1. In that study, p38-MAPK blockade eliminated TGF β 1-induced RLC phosphorylation, while ERK1/2 blockade did not [22], results very reminiscent of what we report here. Further studies are clearly needed to establish the roles of p38-MAPK, and mechanisms of action of this kinase, with respect to activation of myosin II functions in nonmuscle cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Rapid activation of RLC phosphorylation in scratch-wounded primary human keratinocytes

(A). Western blot analysis was performed with total cell lysates from HeLa-Clontech and COS-7 cells, as positive controls for expression of MHC IIA and IIB. Primary human keratinocytes collected from sub-confluent tissue culture plates display expression of both MHC IIA and MHC IIB. (B). Low passage human keratinocytes were plated at confluence on fibronectin, cultured for 36-48 hours, then dishes were subjected to high density scratch-wounding. Cell lysates were collected at indicated time points for western blot analysis. Densitometry was used to quantify RLC phosphorylation levels from western blots, illustrating strong activation at ~5 min, and a weaker and more variable activation in the 4 hr time points.

Phosphorylation was detected using an antibody specific to RLC-(T18P/S19P). Graph represents data pooled from 18 independent scratch wound experiments using keratinocytes derived from 3 different foreskin samples. Error bars represent S.E.M.

Figure 2. RLC phosphorylation is activated rapidly and extends several cell diameters away from the scratch-wound margin

Confluent primary human keratinocyte cultures were scratch-wounded then fixed at the indicated time points, followed by staining for F-actin or RLC-(T18P/S19P), with DAPI staining of nuclei. Elevated RLC-P near the wound margin is detectable in some cells by 1 min post-wounding, and is detectable in many cells by 5'. Unless indicated otherwise, all immunofluorescent images in this figure and later figures were processed with identical image adjustments to insure faithful and accurate intensity differences between samples. Bar, 50 μm .

Figure 3. Myosin IIA displays strong cortical recruitment concurrent with RLC phosphorylation (A & B). Keratinocyte cultures were scratched and processed as in the previous figure, but stained for MHC IIA. Strong recruitment of myosin IIA to the cell cortex was observed near wound margins by 5 min. Yellow dotted lines in (A) and (B) indicate position of Z-section that was used for Z-projections presented in panels C & D, respectively. (C & D). Z-section confocal profiles of unscratched samples (C), or of samples 5 min post-wounding (D) reveal recruitment of myosin IIA both to lamellar edges of marginal cells (red arrowhead) as well as to the entire cortex of nearby cells (red arrow). In panels (C) and (D) of this figure and later figures, the vertical dimension of this Z-axis profile has been stretched 2× for clarity. Bar, 50 μm.

Figure 4. Myosin IIB displays lower signal and minimal cortical recruitment in response to wounding

(A & B). Images of keratinocyte monolayers unscratched or 5 min post-wounding, immunostained for MHC IIB. Weak marginal recruitment of myosin IIB was observed in some fields of view (as in panel B, arrowheads), but this recruitment was neither consistent nor strong. Overall, cortical MHC IIB signals were significantly weaker than for MHC IIA (note that for panels A and B in this figure, fluorescent signal intensity was increased significantly for the MHC IIB images during image preparation, relative to all other figures in this paper, to allow visualization of the weak MHC IIB signal). Yellow dotted lines indicate positions of Z-slices that are presented in panels C & D below. (C & D). Z-section confocal profiles unscratched (C) or 5 min post-wounding (D). Bar, 50 μm. For panels C & D, the green channel signal was increased significantly in this figure, relative to other figures in this paper, to allow signal to be visible.

Figure 5. ERK1/2 and p38-MAPK are activated with kinetics similar to RLC phosphorylation
Keratinocyte lysates were prepared as in figure 1, and western blot samples were probed for activated ERK1/2 (phospho-Thr202/Tyr204) or activated p38-MAPK (Thr 180/ Tyr 182).

Figure 6. Signaling pathways important for acute myosin II activation in response to scratch-wounding

Keratinocytes were allowed to form confluent monolayers, then subjected to scratch wounding. Parallel samples were harvested at each time point either with no inhibitor (black lines), or with (A) the MLCK inhibitor ML-7, or with (B) the ROCK inhibitor Y-27632, indicated with red lines. Sample western blots are presented; graphs represent average of three independent experiments for each series. (C-F) Upstream pathways involved in acute myosin II activation in response to scratch-wounding. Parallel samples were harvested at each time point either with no inhibitor (black lines), or with (C) the EGFR kinase inhibitor AG-1478, or with (D) the ERK1/2 inhibitor PD-098059, (E) the p38-MAPK inhibitor SB-202190, or (F) the cytosolic

calcium chelator BAPTA-AM. Sample western blots are presented, graphs represent average of three independent experiments for each series.

Figure 7. Acute activation of p38-MAPK does not require calcium flux

Confluent keratinocyte monolayers were subjected to scratch-wounding with or without BAPTA-AM preincubation, followed by phospho-western blotting analysis. In two independent experiments a robust and rapid phosphorylation of p38-MAPK was still observed.

Figure 8. p38-MAPK activity is necessary for rapid myosin IIA recruitment to the cell cortex
 Confluent keratinocyte cultures were scratch-wounded and fixed for immunostaining as in earlier figures. SB202190 was added 15 min prior to wounding to inhibit p38-MAPK. Under these conditions recruitment of myosin IIA to the cell cortex was largely eliminated at 5 min post-wounding. (A & B) unscratched and 5 min post-scratch samples. (C & D) Z-section profiles of unscratched and 5 min post-scratch samples. Bar, 50 μ m. Yellow dotted lines in (A) and (B) indicate position of Z-section that was used for Z-projections presented in panels C & D, respectively.

Figure 9. Calcium flux is necessary for rapid myosin IIA recruitment to the cell cortex

Confluent keratinocyte cultures were scratch-wounded and fixed for immunostaining as in earlier figures. BAPTA was added 15 min prior to wounding to buffer cytosolic calcium flux. Accumulation of cortical myosin IIA was severely reduced under these conditions. (A & B) unscratched and 5 min post-scratch samples. (C & D) Z-section profiles of unscratched and 5 min post-scratch samples. Bar, 50 μm . Yellow dotted lines in (A) and (B) indicate position of Z-section that was used for Z-projections presented in panels C & D, respectively.

Figure 10. Myosin II activation in response to EGF receptor stimulation involves different pathways than activation by scratch-wounding

(A) Near confluent cultures were EGF-starved overnight in serum free medium, then stimulated for indicated times with EGF, followed by lysate collection and western blot analysis. (B) Inhibitor analysis to identify pathways mediating myosin II activation in response to EGF stimulation. Western blot analysis of human keratinocytes stimulated with EGF for 10 min reveals pronounced activation of myosin RLC phosphorylation (left two lanes). Parallel samples were stimulated with EGF in the presence of indicated inhibitors of EGFR kinase activity, MLCK, ROCK, ERK1/2, or p38-MAPK. Both MLCK and ROCK appear important

for this activation, but neither ERK1/2 nor p38-MAPK activity are necessary for the activation of RLC (T18/S19) phosphorylation when cells are stimulated with EGF.

Figure 11. Model for pathways mediating rapid myosin II activation during keratinocyte wound responses

Acute activation of RLC phosphorylation involves cytosolic calcium flux (influx in this model, although release from stores could be responsible), and requires activation of p38-MAPK via a calcium-independent mechanism. Although ERK1/2 is activated with similar kinetics, its activity is not needed