Control of Local Rho GTPase Crosstalk by Abr

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Summary

Background: The Rho GTPases—Rho, Rac, and Cdc42—regulate the dynamics of F-actin (filamentous actin) and myosin-2 with considerable subcellular precision. Consistent with this ability, active Rho and Cdc42 occupy mutually exclusive zones during single-cell wound repair and asymmetric cytokinesis, suggesting the existence of mechanisms for local crosstalk, but how local Rho GTPase crosstalk is controlled is unknown.

Results: Using a candidate screen approach for Rho GTPase activators (guanine nucleotide exchange factors; GEFs) and Rho GTPase inactivators (GTPase-activating proteins; GAPs), we find that Abr, a protein with both GEF and GAP activity, regulates Rho and Cdc42 during single-cell wound repair. Abr is targeted to the Rho activity zone via active Rho. Within the Rho zone, Abr promotes local Rho activation via its GEF domain and controls local crosstalk via its GAP domain, which limits Cdc42 activity within the Rho zone. Depletion of Abr attenuates Rho activity and wound repair.

Conclusions: Abr is the first identified Rho GTPase regulator of single-cell wound healing. Its novel mode of targeting by interaction with active Rho allows Abr to rapidly amplify local increases in Rho activity using its GEF domain while its ability to inactivate Cdc42 using its GAP domain results in sharp segregation of the Rho and Cdc42 zones. Similar mechanisms of local Rho GTPase activation and segregation enforcement may be employed in other processes that exhibit local Rho GTPase crosstalk.

Introduction

Dynamic processes powered by actin filaments (F-actin) and myosin-2 such as cell migration and cell division entail a high degree of local regulation, ensuring that assembly and disassembly of F-actin and myosin-2 filaments are spatially coordinated with each other and with other events such as adhesion and deatheseth. At least some of this local coordination must be exerted at the level of the Rho GTPases—Cdc42, Rac, and Rho—which regulate F-actin and myosin-2 with considerable specificity [1]. For example, Cdc42 and Rac can promote assembly of highly dynamic branched F-actin networks, whereas Rho can stimulate myosin-2 and formation of unbranched F-actin networks [2]. The Rho GTPases in turn are activated by guanine nucleotide exchange factors (GEFs), inactivated by GTPase-activating proteins (GAPs), and held inactive in the cytoplasm by GDP-dissociation inhibitors (GDIs) [3–5].

It is now clear that at least some subcellular specificity in regulation of F-actin and myosin-2 is achieved by localized zones of Rho GTPase activity. For example, single-cell wound healing in Xenopus oocytes entails formation of concentric zones of Rho and Cdc42 activity around the wound, with the Cdc42 zone circumscribing the Rho zone [6]. The Rho zone directs local activation of myosin-2 while the Cdc42 zone directs local accumulation of dynamic F-actin [5, 7]. Similarly, during polar body emission, a form of asymmetric cell division, a disk-like zone of Cdc42 forms at the plasma membrane immediately over the meiotic spindle and is surrounded by a ring-like zone of Rho [8]; as with wound healing, each zone makes distinct contributions to the cytokinetic event [9]. The idea of localized, complementary Cdc42, Rac, and Rho zones can be extended to events at the leading edge of crawling cells, where spatially distinct, closely spaced bursts in Cdc42, Rac, and Rho are linked to different events required for cell protrusion and retraction [10].

The existence and segregation of complementary Rho GTPase activity zones is suggestive of subcellular GTPase crosstalk. That the Rho GTPases engage in crosstalk has been amply demonstrated by studies employing biochemical approaches or analyses of the morphology of fixed cells. Crosstalk may work through the Rac/Cdc42 effector PAK, which can negatively regulate Rho GEFs [11–13]. Other mechanisms include signaling via reactive oxygen species [14], phosphorylation and competitive binding of RhoGDI [15, 16], and binding of GEFs to actomyosin [17].

To date, however, a mechanism for local (i.e., subcellular) crosstalk has not been directly explored. Here, we identify Abr, a dual GEF–GAP, as a Rho GTPase regulator in single-cell wound repair and a mediator of local crosstalk between the Rho and Cdc42 activity zones.

Results

A Candidate Screen Identifies Abr as a Potential Regulator of Rho and Cdc42 during the Single-Cell Wound Response

To identify potential regulators of Rho and Cdc42 during the single-cell wound response, we employed a candidate screen approach. A small pool of Xenopus GEFs and GAPs (see Figure S1A and Movie S1 available online) were selected, cloned, tagged with eGFP, and assessed for localization to wounds, effects on Rho and Cdc42 zones following overexpression, and effects on Rho and Cdc42 zones following dominant-negative expression (Figure 1A). Active Rho was detected with eGFP-rGBD (eGFP fused to the GTPase-binding domain of the Rho effector rhotekin), whereas active Cdc42 was detected with mRFP-wGBD (mRFP fused to the GTPase-binding domain of the Cdc42 effector N-WASP) [6].
Abr, a protein with both GEF and GAP activity for the Rho GTPases [18], emerged as the only candidate to satisfy all three criteria (Figure 1; Figure S1A). Moreover, its ability to disrupt the Rho and Cdc42 zones was evident at concentrations of mRNA 20–40 times lower than those effective for the other candidates (see Figure 3; Figure S1). Abr is expressed in oocytes (Figures S1B and S1D), and endogenous Abr as well as 3eGFP-, 3eGFP-, or 3mCherry-Abr localized to wounds (Figures 1B–1D; Movie S2). To further test the observed localization pattern and the Abr antibody, we immunostained cells expressing untagged, exogenous Abr after wounding; this manipulation resulted in a significant increase in the Abr signal at the wound (Figure S1C).

Live-cell imaging revealed that Abr was rapidly recruited to the wound edge (Figures 1C and 1D; see also Figures 2C and 2D). Comparison of the distribution of Abr to Cdc42 or the Rho zone in live or fixed samples revealed that Abr consistently concentrated within the Rho zone, with a slight enrichment at the trailing edge (Figures 1B–1D). Z view comparisons to a plasma membrane marker, farnesylated eGFP, revealed that wounding resulted in Abr recruitment from the cytoplasm to the plasma membrane at regions flanking wounds (Figure 1E). In addition to Abr itself, Xenopus has a closely related homolog. The Abr homolog also localizes to the Rho zone (Figure S1E) and otherwise behaves identically to Abr (see Figure S3A). Thus, except where mentioned, the rest of the results are concerned with Abr itself.

Abr Localizes to Wounds via GEF and GAP Domain-Dependent Interaction with Active Rho

Abr consists of a DH-PH GEF domain, a C2 domain, and a GAP domain (Figure 2A). To assess which domains of Abr are important for its localization, we compared the localization of wild-type (WT) Abr-3eGFP, AbrDH-3eGFP, and AbrGAP-3eGFP (Figures 2A and 2B). Whereas the WT Abr protein localized at the wound edge, deletion of either the DH domain or the GAP domain abrogated localization (Figure 2B). Recruitment failure could not be explained by reduced expression of the mutants in that eGFP signal was clearly evident for both AbrDH-3eGFP and AbrGAP-3eGFP (Figure 2B), and immunoblotting demonstrated that AbrDH-eGFP and AbrGAP-eGFP are expressed at least as well as WTAbr-eGFP (Figure S2A). The C2 domain could potentially contribute to localization; however, it lacks the residues required for calcium binding, and an AbrC2-eGFP fusion failed to localize to the wound, whereas the C2 domain of protein kinase Cβ displays robust localization to wounds (data not shown, but see [19]).

Because the GEF and GAP GTPase-interacting domains are required for localization and because Abr colocalizes with the active Rho zone, we hypothesized that Abr is recruited to wounds by binding active Rho. Consistent with this hypothesis, kymograph analysis revealed a tight spatial and temporal correlation between active Rho recruitment and Abr recruitment (Figures 2C and 2D), but not between Abr and active Cdc42 (Figures S2B and S2C). This localization pattern cannot be explained by transport of Abr via contraction-powered cortical flow, because Abr localized normally in cells pretreated with the lectin WGA (Figure S2D), which blocks cortical flow [20].

To directly test the role of active Rho in Abr localization, we microinjected cells with C3 exotransferase, which inactivates Rho. C3 completely eliminated Abr recruitment to wounds (Figure 2E), showing directly that active Rho is necessary for Abr recruitment to wounds. To determine whether active Rho is sufficient for Abr recruitment, we assessed Abr localization after manipulation of GTPase activity in unwounded cells. In control cells, Abr was predominantly...
cytoplasmic (Figure 1E; Figure 2F), as it was following expression of constitutively active (CA) Cdc42. In contrast, expression of CA Rho caused recruitment of Abr to the plasma membrane (PM) (Figure 2F). Likewise, expression of the RhoGEF GEF-H1 elevated PM Rho activity (Figure S1A) and caused recruitment of Abr to the PM (Figure 2F). This recruitment occurred in the presence of the F-actin inhibitor latrunculin (Figure 2F), indicating that Abr is not recruited via interaction with actomyosin. These results, taken together with those showing that recruitment is dependent on the GEF and GAP domains, indicate that Abr is recruited to wounds via interaction with active Rho.

Abr Positively Regulates Rho While Negatively Regulating Cdc42
To test the functional role of Abr during wound healing, we monitored the effects of Abr expression on the Rho and Cdc42 activity zones. Microinjection of oocytes with Abr significantly increased the breadth of the Rho zone at the expense of the Cdc42 zone (Figure 3A; Movie S3). To determine whether these effects were concentration dependent, we microinjected Abr at 5, 10, 50, 100, and 500 μg/ml (needle concentration). As the concentration of Abr increased, the intensity of the Cdc42 zone decreased (Figures 3B and 3C), whereas the Rho zone intensity was not significantly affected (Figure S3A). However, the zone of active Rho widened with respect to controls as the concentration of Abr increased (Figure 3D). Similar results were observed with the Abr homolog (Figure S3B).

GEF-Dead Abr Blocks Rho Activity and Cdc42 Activity
Because the Abr GAP domain is not required for increased Rho zone width and because Abr locally activates Rho but not Cdc42 (Figure 3A), it follows that Abr might locally stimulate Rho activity through its GEF domain. To test this hypothesis, we made S104A and R244A mutations in the DH domain of Abr (Figure 5A). These mutations correspond to the T506A and R634A mutations in Dbl, which result in dramatic reduction of GDP/GTP exchange [22]. Abr SR/AA localized to wounds (Figure 5B) and dramatically reduced Rho activity relative to uninjected and WT Abr-expressing controls (Figures 5C and 5E; Movie S5). Cdc42 activity was also inhibited relative to controls, consistent with the role of the GAP domain in promoting Cdc42 inactivation independent of GEF activity (Figures 5D and 5E). Together, our findings indicate that Abr negatively regulates Cdc42 through its GAP activity and positively regulates Rho through its GEF activity.
The above results indicate that Abr locally activates Rho in the Rho zone while enforcing zone segregation via Cdc42 inhibition. If this model is correct, Abr depletion would be predicted to suppress local Rho activity while broadening the Cdc42 zone at the expense of the Rho zone. Efforts to deplete Abr in oocytes were unsuccessful, so we turned to Xenopus embryos, which have a robust healing response [19] and permit morpholino (MO)-mediated depletion of target proteins following fertilization [23, 24]. Consistent with results obtained in oocytes, Abr localized to the Rho zone around single-cell embryo wounds as well as to cell-cell junctions near wounds (Figures 6A and 6B), which correspond to local hot spots of Rho activity [19]. Furthermore, WT Abr expression in embryos expanded...
the Rho zone at the expense of the Cdc42 zone (Figure 6C), an effect accompanied by formation of highly developed stress folds around the wound, presumably a consequence of excess, Rho-mediated contractility.

To determine the functional role of Abr in single-cell wound healing, we used a MO approach to knock down endogenous Abr in *Xenopus* embryos. MOs designed to target the 5’ end of both Abr mRNA and Abr homolog mRNA were microinjected into embryos (see Experimental Procedures). Western blotting revealed a decrease in Abr and Abr homolog protein levels in MO-injected embryos relative to controls at 18 hr postfertilization (Figure 6D). Abr depletion resulted in a significant reduction in Rho, but not Cdc42, activity around single-cell wounds (Figures 6E and 6F). Furthermore, Abr depletion also promoted the narrowing of the Rho zone (Figure 6D), the precise opposite effect of that produced by Abr overexpression. Finally, whereas embryos injected with control MO healed properly, MO-injected embryos displayed several other phenotypes including stalled or delayed gastrulation (data not shown). Abr overexpression. Finally, whereas embryos injected with control MO healed properly, Abr MO-injected embryos did not prevent cell division but did consistently inhibit gastrulation (data not shown). Consistent with the oocyte results, Abr depletion resulted in a significant reduction in Rho, but not Cdc42, activity around single-cell wounds (Figures 6E and 6F). Furthermore, Abr depletion also promoted the narrowing of the Rho zone (Figure 6D), the precise opposite effect of that produced by Abr overexpression. Finally, whereas embryos injected with control MO healed properly, MO-injected embryos displayed several other phenotypes including stalled or delayed healing (Figure 6E; Figure S4). In some cases, wounded cells were completely unable to mount a healing response and ultimately lysed (data not shown). These results support the notion that Abr positively regulates Rho activity and reveal that Abr is required for proper wound healing.

**Discussion**

The results of this study show that Abr, a dual Rho GTPase GEF-GAP, is a critical regulator of Rho and Cdc42 during the single-cell wound response and provide what is, to the best of our knowledge, the first characterization of a subcellular Rho GTPase crosstalk mechanism in vivo. Specifically, the results indicate that Abr is recruited to the incipient Rho zone by interaction with active Rho, where it locally amplifies Rho activity via its GEF domain. Simultaneously, Abr locally suppresses Cdc42 activity via its GAP activity (Figure 7). We do not know whether Abr binds directly to active Rho, although the results are consistent with this possibility. This novel mechanism provides a simple explanation not only for zone segregation but also for how the initially broad and dilute distribution of active Rho [6] is rapidly converted into a tight, intense zone inside the Cdc42 zone: assuming that the concentration of active Rho is slightly higher near the wound edge than at regions distal to the wound, the initial asymmetry in active Rho and Abr would be rapidly amplified via positive feedback.

Additional features of Rho GTPase regulation are also revealed by what does not happen upon WT Abr expression: the intensity of the Rho zone does not increase even at high levels of overexpression, nor does the zone spread beyond the area normally occupied by the Cdc42 zone. This result indicates that the positive feedback is somehow antagonized even in the near total absence of active Cdc42. Although these limits could be imposed by the availability of Rho itself or some other component of the system such as the GDIs, we favor the idea that localized Rho activation is normally limited by simultaneous inactivation via Rho GAP activity, a hypothesis previously described as the “GTPase flux” model [23, 25]. If this model is correct, positive feedback between active Rho and Abr may be restrained by one or more Rho GAPs, at least one of which would be concentrated at the trailing edge of the expanded Rho zone that results from WT Abr overexpression.

Are Abr and its homolog the only GEFs activated during wound healing? After all, both the GEF-dead Abr and Abr depletion severely curtail Rho activation. However, we suspect that there is at least one additional, non-Abr Rho GEF involved that would account for the small amount of Rho activity observed even after a high level of GEF-dead Abr expression. A Rho GEF that acts immediately after wounding would serve the role of “priming” the Rho zone by providing the initial pool of active Rho needed for Abr...
recruitment. Furthermore, for a process as fundamental as cell wound repair, it makes sense that redundant mechanisms would be employed.

To what extent can the current results be extended to other systems? A general role for Abr in cellular wound repair is consistent with the fact that Abr is particularly abundant in brain and muscle tissues [26], because these contain very large cells that are especially prone to mechanical damage. More generally, a role as a regulator of local crosstalk could explain the participation of Abr in cell migration and spreading.

Figure 6. Abr Localizes with Active Rho in Embryos, and Abr Depletion Inhibits Rho and Perturbs Wound Healing
(A) Embryos expressing eGFP-rGBD and 3×mCherry-Abr. Top panel shows merge; bottom panel shows Abr.
(B) Embryos expressing Abr-3×eGFP and mCherry-UtrCH to label F-actin were wounded near a cell border. Top panel shows merge, with cell border indicated by arrowheads. Bottom panel shows Abr, with Abr accumulation at the cell border indicated by an arrow.
(C) Embryos expressing 3×mCherry-wGBD and eGFP-rGBD alone (top panel) or with WT Abr (bottom panel). Stress folds are indicated by arrowheads. Scale bars in (A)–(C) represent 20 μm.
(D) Embryos were uninjected (U), injected with 2 mM control MO, or injected with 1 mM Abr MO and 1 mM Abr homolog MO (Abr MO 1+2) and homogenized 18 hr postfertilization. Abr and tubulin were detected by immunoblotting.
(E) Embryos expressing eGFP-rGBD, mCherry-wGBD, and Wee1 with either control MO or Abr MO 1+2 and imaged 18 hr postfertilization. Top row shows control MO phenotype; middle and bottom rows show wounds from Abr MO 1+2-injected embryos.
(F) Rho and Cdc42 zone intensity from cells in (E) was quantified after 48 s (Rho: n = 25; ***p < 0.0001; Cdc42: n = 17; p = 0.2907; unpaired t test).
(G) Rho zone width was quantified from cells in (E) (n = 17; ***p < 0.001; unpaired t test). In (F) and (G), top and bottom whiskers represent maximum and minimum values, respectively.
That is, studies from mouse macrophages lacking both Abr and Bcr (the only other known dual GEF-GAP for Rho GTPases) indicate that loss of Abr and Bcr results in excessive cell spreading [21]. Similarly, in human pluripotent stem cells, Abr depletion suppresses cell rounding after dissociation of local Rho, Rac, and Cdc42 activity during cell adhesion and spreading indicating that local hot spots of Rho activity are spatially complementary to local hot spots of Cdc42 and Rac activity [10], we suggest that the deficits observed in cells lacking Abr or Abr and Bcr reflect loss of local, Abr-enforced Rho-Cdc42/Rac crosstalk.

Finally, the possibility that the role played by Abr revealed here could potentially be played by complexes of Rho GTPase GEFs and GAPs in other contexts should be considered. For example, cytokinesis is dependent on interaction of a Rho GEF (Ect2) with a Rho GAP (MgcRacGAP) [28]. If this GEF-GAP complex can localize via interaction with active Rho, both positive feedback via Ect2 and crosstalk via the GAP domain of MgcRacGAP could potentially occur.

Experimental Procedures

Plasmids

eGFP-rGBD, mRFP-wGBD, mCherry-UtrCH, and pCS2+3×eGFP were generated as described [6, 7, 23]. 3×mCherry-pCS2+ was developed by inserting three consecutive mCherry sequences between BamH I and BspE I in the pCS2+ vector. 3×mCherry-wGBD was constructed by inserting the wGBD fragment into 3×mCherry-pCS2+ with Xho I and Xba I. pCS2+eGFP were injected at 100 μg/ml, 3×mCherry-Abr, and the Abr-3×eGFP mutants, and eGFP plasmids, where R666 was changed by mutating CGT to GCT, respectively. Abr S104A and R244A were created by changing AGT to GCT and AGA to GCG. The N778A mutation was generated by changing AAT to ATT.

Embryo Injection and Morpholinos

Embryos were fertilized in vitro and injected with a 5 nl volume of mRNA at the two-cell stage. eGFP-rGBD, Abr-3×eGFP, 3×mCherry-Abr, and mCherry-UtrCH were injected at 500 μg/ml, 3×mCherry-Abr, the Abr-3×eGFP mutants, and mCherry-UtrCH were injected at 750 μg/ml needle concentration. CA Cdc42, CA Rho, and GEF-H1 were injected at 1 mg/ml each. Abr RN/AA 3×eGFP and Abr SR/AA 3×eGFP were injected at 100 μg/ml, and untagged Abr RN/AA and Abr SR/AA were injected at 500 μg/ml. Screen candidate mRNAs (XGEF, chimera, and GEF-H1, Ect2, and DN MgcRacGAP) were injected between 1 and 2 mg/ml. For western blotting, the eGFP-tagged Abr mutants were injected at 1 mg/ml each. Oocytes were incubated with 10 μM latrunculin A for 30 min to 1 hr. C3 exoenzyme was injected to a final concentration of 0.08 μg/ml 20–40 min before imaging.

Immunofluorescence and Western Blotting

Oocytes were injected with mRFP-wGBD, laser wounded after 24 hr, and immediately placed in fix buffer (10 mM EGTA, 100 mM KCl, 3 mM MgCl2, 10 mM HEPES, 150 mM sucrose [pH 7.6] with 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1% Triton X-100) and incubated overnight at room temperature. Cells were washed with 1× phosphate-buffered saline (PBS), quenched with 100 mM NaBH4 for 4 hr, washed twice with 1× PBS, bisected, and incubated in TBSN-BSA (5 mg/ml bovine serum albumin and 0.1% NP-40 in 1× Tris-buffered saline) overnight at 4°C. a-Abr (BD Biosciences) was then added at 1:200 and incubated overnight at 4°C. Cells were washed with TBSN-BSA four times for 1 hr and incubated overnight at 4°C. Oregon green goat α-mouse (Promega) was added at 1:200, and the incubation steps were repeated.

For mutant expression analysis, ten oocytes were washed three times with 1× PBS and homogenized by pipetting in homogenization buffer...
(250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 2 mM MgCl₂, 0.1% Triton X-100, 10 mM E-64, 4 mM pefabloc, 60 µg/ml chymostatin, 5 µg/ml leupeptin, 1 µg/ml pepstatin, 3.75 µg/ml apronitin). Homogenates were fractionated at 12,000 rpm at 4 °C for 2 min, and the cytoplasmic layer was extracted and spun again. Laemmli sample buffer was added to the cleared cytoplasmic fraction, and lysates were loaded on a 10% SDS-polyacrylamide gel. The gel was transferred onto nitrocellulose membrane, blocked, and incubated with a specific primary antibody. The membranes were subsequently incubated with a secondary antibody conjugated with horseradish peroxidase, followed by chemiluminescent detection with ECL reagent (Thermo Scientific). A Molecular Imager Chemidoc XRS+ (Bio-Rad) was used to detect Abr. Western blotting was performed on M Alln-injected embryos as above. Tubulin was detected with Pierce ECL reagent (Thermo Scientific). Scientific).

Supplemental Information

Additional information includes four figures and five movies and can be found with this article online at doi:10.1016/j.cub.2011.01.014.

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