Regulation of collective cell migration by RhoGAP myosin IXA

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Collective cell migration is a key process during epithelial morphogenesis, tissue regeneration and tumor dissemination. During collective epithelial migration, anterior-posterior polarity, apical-basal polarity and cell-cell junctions must be dynamically coordinated, but the underlying molecular mechanisms controlling this complex behavior are unclear. Rho GTPases regulate the actin cytoskeleton, in particular protrusive and contractile activities at cell-cell contacts. Recently a number of regulators—nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs)—have been identified and suggested to provide spatio-temporal control of Rho GTPases at cell-cell contacts. One of these is myosin IXA, a member of class IX, single-headed actin motors having a conserved RhoGAP domain. Using its actin-binding and motor activities, myosin IX interacts with actin filaments and moves toward filament plus ends. At the plasma membrane, myosin IX’s RhoGAP activity negatively regulates Rho to facilitate localized reorganization of the actin cytoskeleton. Here, I discuss how myosin IXA regulates Rho and the actin cytoskeleton during the assembly of nascent cell-cell contacts and how this might contribute to collective epithelial migration.

Introduction

Collective cell migration is characterized by the maintenance of a physical interaction between cells coupled with coordinated anterior-posterior polarization of individual cells within a migrating monolayer or group (Fig. 1). It has a fundamental role in embryonic development, regeneration and cancer metastasis. Collective cell migration has been studied in vitro in migrating epithelial or endothelial monolayers in response to a scratch wound, on patterned substrates, in tissue explants of cancer cells, mesoderm or mammary ducts, and in 3D. Examples of in vivo models of collective cell migration are numerous and include developing embryos of fruit fly, zebrafish, mouse and metastatic cancers in mice. The molecular mechanisms underlying such coordinated migration are, however, not well understood.

Examples of in vivo models of collective cell migration include basal anterior-posterior cell polarity manifested as unidirectional, actin-rich protrusions at the front of multiple cell rows (Fig. 1A and B). This results in the migrating group having a fish scale-like arrangement (Fig. 1C). The basal protrusions of following cells penetrate under leading cells and have a distinct cadherin distribution (Fig. 1B and underlapping) and actin cytoskeletal organization reflecting complex cell-cell interactions in this region. Highly coordinated regulation of cell-cell junctions (localization and clustering of junctional proteins) and the actin cytoskeleton associated with junctions (affecting stabilization, adhesion strength and protrusive activity) are key features of collective cell migration. Recent experiments have revealed that cell-cell adhesion strength can regulate the directionality of coordinated cell movement, as demonstrated by collective E-cadherin-mediated mesendoderm migration during zebrafish gastrulation. E-cadherin is essential for collective epithelial migration, but excess cell-cell adhesion blocks collective migration, for example in Drosophila.
A major contributor to collective cell migration is thought to be a mechanical force. Actin-myosin contractile forces regulate cell shape and the stability of cell-matrix and cell-cell junctional adhesions. The forces generated by actin-myosin contractile filament bundles associated with cell-cell junctions can also be transmitted throughout migrating cell groups to regulate collective behavior, as seen in monolayers and tissues.

Two spatially and functionally distinct actin populations have been reported at cell-cell contacts in epithelial cells: junctional or radial actin, and tangential contractile thin bundles. The collision of two protruding lamellipodia stimulates local clustering of E-cadherins (primordial junctions) accompanied by the formation of junctional actin. The junctional actin associates with and stabilizes E-cadherins and in mouse mammary epithelial cells.

The Rho family of small GTPases are major regulators of the actin cytoskeleton, with protrusive lamellipodial activity promoted by Rac, filopodia formation by Cdc42 and contractile actin-myosin activity by Rho. They also regulate cell-cell junction dynamics (adherens and tight junctions), both directly (transport and clustering) and indirectly (through the associated actin cytoskeleton). Rho GTPases are molecular switches and are themselves controlled by interconversion between active GTP-bound, and inactive GDP-bound states. When active, GTPases bind specific effector proteins to stimulate downstream signaling. Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). Some one hundred and fifty genes encode mammalian GEFs and GAPs, and most are not well characterized. It is likely that these regulators play a central role in defining the spatiotemporal activity of Rho GTPases during migration.

In a recent study, we described a role for myosin IXA, a Rho-specific GAP, in the collective migration of human bronchial epitheliocytes, 16HBE cells. These cells, which show remarkably coordinated collective migration in culture, were used in an siRNA-based screen to identify GEFs and GAPs involved in collective cell migration. We found that in the absence of myosin IXA, 16HBE cells failed to form stable adherens junctions during migration resulting in cell scattering and subsequent random migration. More careful analysis revealed that remodeling of the actin cytoskeleton at cell-cell contacts in response to cadherin-mediated adhesion was defective in myosin IXA-depleted cells.

Here I will discuss our current ideas about how the regulation of Rho by myosin IXA likely contributes to effective collective migration of these epithelial cells.
and primordial junctions. During junctional maturation, actin filaments become tangential, circumferential bundles (Fig. 2). These are contractile and co-align with linear tangential E-cadherin-mediated adhesions and function to increase the height of the lateral surface of cell-cell contact. In 16HBE epitheliocytes, actin puncta and radial actin finger bundles are associated with primordial junctions, and this later reorganizes into circumferential bundles and mature junctions (Fig. 2).

Diverse mechanisms control actin-myosin contractility, but a major player, particularly during cell migration, is Rho. Activation of Rho by a variety of GEFs results in activation of the effector Rho kinase (ROCK). ROCK in turn activates myosin II directly, by phosphorylation of myosin light chain II, and probably more importantly indirectly, by inactivating myosin light chain phosphatase. Bundled filaments of active myosin II motors pull on the F-actin network to generate contractile force.

Rho is an important player in the collective migration of 16HBE cells. Overexpression of constitutively active or dominant negative forms of RhoA has been reported to alter the actin cytoskeleton and inhibit monolayer migration. A more detailed analysis revealed that the depletion of RhoA by RNAi inhibits the formation of mature cell-cell contacts and the formation of tangential actin bundles, but does not prevent the assembly of junctional actin and radial bundles associated with primordial junctions. On the contrary, it seems likely that the initial stages of cell-cell contact establishment, including the formation of radial actin fingers, are inhibited by contractile forces associated with Rho and myosin II activity. Direct evidence to support this has emerged from studies on the spatio-temporal activity of RhoA using FRET-based biosensors in various epitheliocytes. They reveal that active RhoA accumulates at the lateral expanding edges of cell-cell contacts, but not at the overlapping central area. This raises the possibility that RhoA is locally inhibited and an obvious mechanism for this would be through the recruitment of a RhoGAP.

A number of Rho family regulators including RhoGAP domain containing proteins have been characterized in the context of cell-cell contact regulation. In fibroblasts, N-cadherin-mediated cell-cell junctions are regulated by p190RhoGAP-mediated inhibition of RhoA. It was shown that Rac1 activation promoted recruitment of p190RhoGAP to adherens junctions where p190RhoGAP transiently interacted with p120catenin leading to local inhibition of RhoA. However, the exact role of p190RhoGAP in epithelial cells remains unclear. In epithelial cells, ARHGAP10 was found to localize at cell-cell contacts and interact with α-catenin. In siRNA depletion experiments ARHGAP10 was necessary for recruitment of α-catenin. Overexpression of C-terminal RhoGAP-containing fragment led to disassembly of the actin stress fibers. ARHGAP12 was recently identified and cloned as a new junctional protein localized to E-cadherin-mediated junctions of intestinal epithelia. It seems likely that the negative regulation of Rho GTPases underlies the dynamic behavior of cell-cell contacts, and it will be interesting to investigate the functional roles of these different Gaps at junctions.

Figure 2. Cell-cell contact formation in 16HBE cells is controlled by myosin IXA. Protrusive lamellipodia collide and overlap leading to accumulation of junctional actin (red dots). Myosin IXA is recruited at this time and the GAP domain reduces Rho activity at contact zones allowing the formation of radial actin bundles (red diagonal lines). Maturation of the cell-cell contact leads to tangential actin bundles. In myosin IXA depleted cells, high Rho activity prevents lamellipodial overlapping, formulation of radial actin and stabilization of cell-cell contacts. Colliding lamellipodia retract leading to cell scattering.
In our work, using an RNAi-based screen to identify Rho family GTPase regulators required for the collective migration of human bronchial epithelial cells, we identified RhoGAP myosin IXA. High-resolution time-lapse imaging and structure-function analysis of RhoGAP myosin IXA allowed us to investigate how RhoGAP activity regulates the junctional actin cytoskeleton and cell-cell adhesions in collectively migrating cells. In 16HBE epitheliocytes, FRET ratio imaging experiments revealed high levels of RhoA activity at primordial cell-cell contacts after myosin IXA depletion, resulting in destabilization of overlapping lamellipodia and inhibition of radial actin bundle formation. Experimentally reducing Rho-dependent contractility, using a ROCK inhibitor or expressing the RhoGAP domain-containing fragment of myosin IXA, increased cell-cell contact stability in myosin IXA-depleted cells.21 These data support the idea that local inhibition of Rho-dependent actin-myosin contractility is important for actin reorganization supporting cell-cell contact stability during collective epithelial migration.

**Regulation of Rho at Cell-cell Contacts by Myosin IXA**

Myosin IXA (Myo9a) and myosin IXB (Myo9b) are the only class IX myosins in mammals.38 These two proteins share similar domains: N-terminal RA (Ras-associated) domain, the motor domain composed of two parts separated by loop 2 insertion (the head), multiple IQ motifs (a light chain binding region, the neck), an atypical C1 domain and a RhoGAP domain (the tail) (Fig. 3A). The motor domain has a unique structure allowing myosins IX to attach to actin filaments and move processively toward the plus end of F-actin.39,40 The loop 2 insertion has been shown to be required for actin filament attachment.41 The RhoGAP domain is highly conserved and shows specificity to RhoA/B/C in vitro and in vivo.42,43 The IQ motifs play a regulatory role by binding calmodulin, but the function of the RA and C1 domains is not clear.

In contrast to myosin IXB, whose biochemical and motor properties have been characterized in some detail, little is known about myosin IXA.38,44 A myosin IXA knockout mouse revealed defects in cell-cell junctions and cell morphology of brain ependymal epithelial cells, indicating the physiological importance of myosin IXA function at junctions.38,45 We found that EGFP-myosin IXA localizes to actin bundles through its motor domain, and in a punctate fashion similar to myosin II.21 These EGFP-myosin IXA puncta were able to translocate along actin bundles, as observed by TIRF microscopy in live epitheliocytes (data not published), and to move together by retrograde flow. Mutations abolishing motor activity or actin-binding (loop 2 insertion deletion) of myosin IXA led to the loss of puncta and a diffuse localization of the protein into the cytoplasm. Thus, motor and actin-binding activities are important for junctional localization of myosin IXA.

**Figure 3.** Possible mechanism of myosin IXA function at cell-cell contacts. (A) Schematic diagram of the domain organization of the human class IX myosins. The domains are RA (Ras-associated domain, purple), MOTOR (motor domain divided by loop 2 insertion, green), IQ (five IQ motifs, a light chain binding region, yellow), C1 domain (dark blue) and RhoGAP domain (blue). Sequence length is in aminoacids (red numbers). (B) Model for myosin IXA RhoGAP function at cell-cell contact site. Targeted to the cell-cell collision sites, myosin IXA maintains low Rho-GTP levels at the plasma membrane. Low Rho favors the formation of thin actin fingers associated with primordial junctions. Myosin IXA RhoGAP activity is later attenuated, perhaps by binding to actin filaments and removal by retrograde flow.
More detailed imaging analysis will be required to understand how myosin IXA is delivered to primordial cell-cell junctions. It will also be interesting to investigate the nature of EGFP-myosin IXA puncta, for example whether they have actin cross-linking activity.

Inactivation of Rhogap activity, by a point mutation in the conserved arginine finger at the catalytic site, revealed an important role for GAPI activity at cell-cell contacts in 16HBE cells.\textsuperscript{21,40} Low-level expression of the Rhogap domain alone partially rescued the cell scattering phenotype induced by myosin IXA depletion. Together with the FRET imaging data, this indicates that the GAPI domain of myosin IXA acts as a localized, negative regulator of Rh to control contractile forces during the early stages of cell-cell contact formation.\textsuperscript{21}

How might myosin IXA regulate Rho activity at cell-cell junctions? It seems likely that during lamellipodial collisions, myosin IXA is targeted to the membrane at nascent cell-cell contacts, through its actin-binding and motor properties. Low Rho/ROCK activity at junctions is required to understand how myosin IXA regulates Rho activity at cell-cell junctions. This might include RhoGAP activity at cell-cell junctions, through the FRET imaging phenotype induced by myosin IXA depletion. The removal of this RhoGAP activity could either be inactivated (perhaps through phosphorylation) or removed by retrograde flow (Fig. 3B). The removal of this Rho GAP activity would allow a build up of Rho activity, to promote subsequent maturation of junctional contacts.

**Concluding Remarks**

Collective cell migration plays a major role during morphogenetic movements in development and disease. Collective cell migration requires dynamic rearrangements of cell-cell junctions and the associated actin cytoskeleton and this likely involves the localized and coordinated action of Rho family GEFs and GAPs. In agreement with this, a number of Rho family GAP and GEF domain containing proteins have been characterized in the context of cell-cell contact regulation. The Rho GAP activity of myosin IXA negatively regulates Rho-dependent actin-myosin contractility during early stages of cell-cell contact formation, but during junctional maturation Rho activity is restored, presumably through recruitment of specific GEFs. The spatio-temporal regulation of Rho family GEFs and GAPs is likely to be a major driving force for collective epithelial cell migration.

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