Hold on tightly
How to keep the local activation of small GTPases

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Signaling regulated by Rho small GTPases plays a pivotal role in cell migration, cell attachment to substratum or to their neighbors among other functions. Concerted efforts have focused on understanding how different GTPases are activated by specific stimuli and which regulator is responsible for the spatio-temporal control of their activity at particular intracellular sites. We have recently described the role of a scaffold protein, Ajuba, in adherens junction maintenance via direct stabilization of activated small GTPase Rac1 at cell–cell contacts. Ajuba binds to both active and inactive forms of Rac1. Upon junction formation, Rac1 activation initiates a positive feedback loop leading to Ajuba phosphorylation and Ajuba-mediated retention of activated Rac1 at junctions. Thus, cytoskeletal proteins may have a dual role to provide a scaffolding platform and dynamically modulate small GTPases function at a specific place, irrespective of their ability to interact with active and inactive forms. Here we discuss similar mechanisms via which cytoskeletal proteins can facilitate cellular processes downstream of Rho proteins by increasing their affinity to activated GTPases.

Cell–cell adhesion receptors enable the assembly of multi-protein signaling complexes to regulate tissue homeostasis and function.1 Within these signaling platforms, there are scaffolding cytoskeletal proteins that provide docking sites for a number of kinases, phosphatases, small GTPases and their regulators GDIs (GDP dissociation inhibitors), GAPs (GTPase-activating proteins), and GEFs (guanine-nucleotide exchange factors).2-4 Appropriate positioning and regulation of these signaling complexes during cell–cell adhesion is essential to modulate the strength and dynamics of adhesion sites. These signaling complexes also regulate additional junction-dependent cellular events such as epithelial cell shape, polarized intracellular trafficking and inhibition of proliferation and migration.1,3,5,6

Rho small GTPases are key regulators of cell–cell adhesion. In particular, RhoA, Rac1, and Cdc42 have been implicated in the biogenesis and maintenance of adherens junctions and tight junctions in epithelial cells.6,7 How the spatio-temporal activation of Rho small GTPases is regulated at cell–cell contacts is not completely understood.4 The current understanding is that GAPs and GEFs are positioned at specific intracellular sites via interactions with cytoskeletal filaments or associated proteins. However, more recently it has become apparent that this scenario is more complex: interactions with cytoskeletal proteins can either activate or inactivate the activity of GEFs and GAPs, thereby providing an extra level of localized control of their function.4

Examples are found during growth factor-mediated activation of the small GTPase Ras, in which ERM proteins (ezrin, radixin, and moesin), a family of actin-binding proteins located at the plasma membrane, act as a platform for the recruitment of a multiprotein complex that includes Ras and its GEF SOS1 (Son of Sevenless 1). The formation of this ERM-dependent complex is necessary to enhance the catalytic activity of...
SOS1 and thereby Ras activation. In contrast, GEF-H1, a GEF for RhoA, is held inactive at the junctions when bound directly to the cytoskeletal protein cingulin. Disruption of microtubules or tight junctions releases GEF-H1, leading to activation of RhoA.

Less understood are the interactions of small GTPases with cytoskeletal proteins and whether these can directly modulate the function of Rho proteins via protein stabilization, localization, or kinetics of GTP loading or hydrolysis. Distinct cytoskeletal proteins interact specifically with the active form of small GTPases (GTP-bound): they are known as effectors and become activated upon interaction with small GTPases to regulate distinct cellular events (Fig. 1A). Other proteins interact with inactivated GTPases (i.e., GDP-bound form) and could retain them at particular intracellular sites, thereby reducing the pool available for activation. These are referred here as “sequesters” (Fig. 1A). However, several other proteins associate with both GTP- and GDP-loaded GTPases and are thus not considered effectors or sequesters.

**Cytoskeletal Proteins Able to Associate with Active and Inactive GTPases**

The functional implications of cytoskeletal proteins interacting with both active and inactive forms are not fully understood. Two examples, PACSIN2 (protein kinase C and casein kinase 2 substrate in neurons) and Nedd4 (neutral precursor cell expressed developmentally downregulated 4) bind to active and inactive Rac1. Yet, PACSIN2 and Nedd4 interact more efficiently to active Rac1 leading to modulation of specific Rac1-dependent cellular events. Active Rac1 modulates the localization of PACSIN2 within the cell, and in turn PACSIN2 inhibits Rac1 function by promoting its internalization from the cell membrane and subsequent inactivation. At the cellular level, PACSIN2 perturbs Rac1-dependent cell migration. In contrast, Rac1 activation promotes the ubiquitin activity of Nedd4, resulting in degradation of the adaptor protein Dvl1 (dishevelled-1). Dvl1 is a negative regulator of cell–cell adhesion, and its degradation downstream of Rac1-Nedd4 thereby stabilizes cell–cell contacts.

Another group of proteins able to interact with both GTP- and GDP-loaded Rac1 are proteins containing IMD [insulin receptor substrate of 53 kDa (IRSp53)/missing in metastasis (MIM) homology domain]. They are scaffold proteins involved in Rac1-induced intracellular trafficking and actin dynamics, including promoting filopodia-like protrusions (IRSp53), adherens junction stability (MIM), lamellipodia-like protrusions (MIM-B), or clusters of short actin bundles by the insulin receptor tyrosine kinase substrate (IRTKS). The relevance of IRSp53 and IRTKS binding to both forms of Rac1 is not clear. MIM or MIM-B expression induces Rac1 activation in vivo. In the case of MIM-B, this correlates with its binding to Rac1, but the mechanisms remain to be elucidated.

**Ajuba is a Scaffold Protein that Modulates Rac1 Function**

New mechanistic insights have been shed on the above issue by our study showing that the cytoskeletal protein Ajuba interacts with both active and inactive Rac1 (Fig. 1B). Ajuba is an actin-binding protein that belongs to the family of LIM domain-containing proteins. Ajuba localizes to focal adhesions, nucleus and cell–cell contacts, where it regulates different cellular processes such as cell determination, repression of gene transcription, cell–cell adhesion, migration, and wound healing. During wound healing in fibroblasts, Ajuba is required for appropriate levels of Rac1 activation at the lamellae and Ajuba-null fibroblasts show delayed wound closure. In this context, Ajuba indirectly facilitates Rac1 activation by facilitating the recruitment of DOCK180, an unconventional Rac1 GEF to the leading edge of motile cells.

We recently demonstrated that the functional interplay between Ajuba and Rac1 is more interesting than initially thought. In epithelial cells, both Ajuba and Rac1 are known to regulate the stability of cell–cell contacts mediated by cadherin receptors. Similar to Rac1, Ajuba depletion severely perturbs the ability of cadherin to recruit F-actin to junctions or clustered receptors on beads. However, in contrast to Rac1, which is essential for cadherin adhesion, Ajuba is important for the maintenance of pre-formed adherens junctions rather than the initial formation of cell–cell contacts.

Consistent with this role, Ajuba is required to maintain levels of active Rac1 at later, but not earlier, time points of junction assembly. Thus, Ajuba can fine-tune Rac1 retention and activity levels at adherens junctions, thereby contributing to Rac1-dependent events that stabilize pre-formed cell–cell contacts. Mechanistically, we find that Ajuba binds directly to Rac1 but not to other GTPases involved in cell–cell contact, such as RhoA or Cdc42. The fact that TRIP6, a member of the same family as Ajuba, is also able to associate with Rac1 suggests that these interactions may extend to distinct LIM domain-containing proteins.

Even though Ajuba clearly stabilizes junctions downstream of Rac1, Ajuba is not considered a Rac1 effector. It is intriguing how Ajuba is important in this regulation, considering that its interaction with Rac1 has no preference for the active or inactive form. Furthermore, Ajuba does not regulate Rac1 recruitment to cell–cell contacts. Potentially, Ajuba could facilitate the recruitment of a GEF or GAP to modulate Rac1 activity locally at cell–cell contacts, as shown for wound healing. While this possibility is feasible, we unraveled another, unexpected, mechanism for the interplay Ajuba-Rac1 at junctions.

PAK1 is a serine/threonine kinase and a known effector of Rac1 and Cdc42 that is involved in many cellular processes. PAK1 is essential for the regulation of cell motility and cytoskeletal dynamics, cell death, survival, and cell division, and its dysregulation is implicated in several pathologies, including neurodegenerative diseases and cancer. In epithelial cells, PAK1 translocates from focal complexes to cell–cell contacts in a cadherin-dependent manner and it is essential for growth inhibitory signals during wound healing. PAK1 is transiently activated by junction assembly in keratinocytes, presumably downstream of Rac1, as Cdc42 is inactivated by newly-formed contacts in these cells. Silencing of PAK1 by
RNAi leads to a similar phenotype as Ajuba depletion: perturbing pre-formed contacts and actin recruitment to cadherin complexes. These data indicate that PAK1 may modulate the cross-talk between Rac1 and Ajuba during junction maintenance. Activated PAK1 specifically phosphorylates Ajuba, which appears to place Ajuba downstream of PAK1 in the stabilization of cell–cell adhesion. As both phosphomimetic and non-phosphorylatable Ajuba mutants localize similarly to junctions and are able to bundle filaments, it is unlikely that PAK1 phosphorylation regulates these events. Instead, a surprising function for phosphorylated Ajuba is its preferential binding to Rac1-GTP (Fig. 1B), by an order of 2-fold over its binding to inactive Rac1. The functional consequence is that Ajuba can regulate local levels of Rac1 activation at junctions through its differential affinity for active or inactive Rac1, depending on its phosphorylation state. How the above processes are integrated with the in vivo regulation of a specific cellular process? Recruitment of Ajuba to newly formed cell–cell contacts initiates a positive feedback loop where E-cadherin-dependent activation of Rac1 and PAK1 results in direct phosphorylation of Ajuba by PAK1, thereby increasing the interaction between Ajuba and active Rac1 to promote junction stabilization. At the moment, we cannot exclude that Ajuba phosphorylation may also facilitate the recruitment of a GEF to cell–cell contacts, which can then activate the Rac1 pool found at the site. Alternatively, Ajuba may maintain higher Rac1-GTP levels by inactivation of a GAP...
found at junctions or by a direct insulation of activated Rac1 from its inhibitors (i.e., via steric hindrance).

**Broader Implications of Phosphorylation to Switch the Affinity for GTPases**

Other proteins have also been shown to control their binding affinity for Rho GTPases through phosphorylation. For example, IQGAP1 is an activating binding protein involved in many different cellular processes, such as cell growth and survival, motility, cytoskeletal organization and cell–cell adhesion.34 IQGAP1 was first described as an effector of Rac1 and Cdc42, but is now known to bind to Cdc42 in both its active and inactive form.35,36 Phosphorylation of IQGAP1 increases its binding affinity for activated Cdc42, helping to stabilize its active levels in cells.37,38

In contrast, phosphorylation of Rho GDP dissociation inhibitor (RhoGDI) changes its affinity to different types of GTPases.3 RhoGDI interacts with Rac, Rho or Cdc42 mostly in their inactive forms and retains them in the cytoplasm, thereby preventing their activation.2 Phosphorylation of RhoGDI on Ser101 and Ser174 by PAK1 selectively releases Rac1 to be activated, but not RhoA.39 Interestingly, protein kinase C (PKC) phosphorylates RhoGDI at a different residue (Ser34) leading to the release of RhoA, but not Rac1 or Cdc42.40 Thus, phosphorylation of interacting proteins emerges as a mechanism to modulate their affinity to Rho small GTPases and activation of specific pathways, depending on a specific stimulus.

It is increasingly evident that certain scaffold proteins that bind to both active and inactive Rho GTPases may modulate their function. Although the mechanisms via which phosphorylation of cytoskeletal proteins increases the affinity for and stabilizes active GTPases are not yet fully understood, this process is likely to participate in a wide variety of cellular events. As shown for Ajuba and IQGAP1, other scaffolding proteins may also act as “stabilizers” of the active form of the GTPases, shifting the balance toward their activation within the cells (Fig. 1A and B). The fact that a large number of proteins are able to interact with both active and inactive forms of small GTPases10,11,14,15 suggests the broader implications that such mechanism may have in the regulation of a number of adhesive events. Exciting times lie ahead to unravel which scaffolding proteins can modulate spatio-temporal signaling in a similar manner.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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